THE PREPARATION OF GELATINE.

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INTRODUCTION.

In spite of the large number of published papers describing the properties of gelatine, there are relatively few devoted specially to its preparation. A variety of reasons probably conspired to produce this result. The earliest workers on gelatine were interested in its properties as an emulsoid colloid, of which group of substances it became the prototype. Then the tanners, foiled by the inertness of collagen which formed their raw material, turned to gelatine, a closely related and more tractible substitute. While it is true that by so doing, knowledge was gained as to the mechanism of various tanning processes, analogies were frequently pushed too far. Further, as stress was mostly laid on the importance of behaviour from the tanners point of view only, the value of such work was somewhat lessened. Until recently the importance of knowledge which could be gained by a study of the conversion of collagen into gelatine was not appreciated. It is probable that much information of an unsystematic nature was in possession of those charged with the preparation of gelatine on a large scale, but circumstances did not permit of this being published.

Earlier papers on the extraction of gelatine dealt with the more obvious and less involved aspects of the process. For example, Manning & Schryver (Biochem. J. 1921, 15, 523) found that the rate of extraction of gelatine from osseine was independent of the state of subdivision of the osseine. This might have been expected from the porous nature of the substance, which retains the structure of the bone from which it is made. It can, however, be shown that large pieces of osseine are more resistant to the action of lime during the soaking process than small pieces. Bogue (J.I.E.C., 1923, 15, 1154) provided a study of the conditions affecting the hydrolysis of collagen to gelatine. On the whole this is a good paper but certain points invite criticism. The time of extraction used in the experiments was unnecessarily long for the best results to be obtained with carefully soaked material. Again, the range of Ph at which extractions were carried out was so extended that much of the gelatine obtained was of very poor quality, owing to hydrolysis of the gelatine itself. Nevertheless, the general conclusions which cover the practical aspects of the problem are on the whole sound.

A paper which is of special interest was published by Knaggs & Schryver in 1924 (Biochem. J., 1924, 18, 1095). In this they stated that gelatines differed in their Hausmann numbers, that is, the proportion of the total nitrogen present in gelatine, occurring in different forms, varied according to the treatment previously applied to the precursors. Gelatine extracted from a precursor which had been treated with acid had a lower value for di-amino nitrogen than gelatine from a precursor treated with alkali. They also stated that the rate of extraction when acid was used was greater than when alkali was used. It is doubtful if this is correct, but the point is not of first importance. Knaggs and Schryver were of the opinion that even the purest gelatine did not behave as a chemical entity. This is a point of far greater import, and one which is bound to give rise to much argument, but it must be admitted that in many ways purified gelatine of known type does behave as a definite substance.

The suggestion that gelatine could exist in more than one form was taken up by Briefer & Cohen (J.I.E.C., 1928, <u>20</u>, 408) and by Briefer (J.I.E C., 1929, <u>21</u>, 266). In these papers it was shown that the method of preparation was the factor which determined the type of gelatine obtained. Gelatine prepared by an acid process was found to have an iso-electric point in the region of Ph 8, while gelatine made in any way from material conditioned by lime became iso-electric in the region of Ph 5. Although not immediately developed or expanded, this discovery was of great importance. Nevertheless its nature and interest does not appear to have been appreciated by other investigators who were still confused as to the two positions of the iso-electric point of gelatine. No other aspects of the preparation of gelatine were dealt with by Briefer & Cohen - indeed, the main part of the second paper consisted of a thorough discussion of the validity of the opalescence method of finding the iso-electric point.

An entirely different aspect of the matter was studied by Wohlisch (Biochem. Z., 1932, 247, 329). He stated that the conversion of collagen into gelatine was an endothermic reaction, which occurs above a sharply defined transformation temperature. The transformation temperature varies with the material used and its physical pondition. The existence of collagen in more than one form is also implied in this paper. The transformation temperature of collagen is not likely to be ascertainable with any degree of certainty owing to the difficulty of preventing the action of bacteria at low temperatures, that is about blood heat, and it is in this neighbourhood that one would expect the limiting temperature to lie.

A number of interesting papers appeared in 1937 in which attempts were made to explain the conversion of collagen into gelatine. Cherbuliez Jeannerat & Meyer (Arch. Sci. Phys. Nat., 1937, V19, Suppl. 122) studied the transformation of the fibres of calves feet tendons into gelatine. This takes place in two stages. The first stage, brought about by solvents, is the loss of the crystalline structure of the native fibre, described as collagen A, and is reversible. The fibre after the change is still resistant to trypsin hydrolysis. The second stage consists of hydrolysis by boiling the material with water, whereby gelatine is produced. Collagen B is obtained from collagen A by partial hydrolysis and is partly attacked by trypsin. Perhaps the most interesting point about this paper is the appreciation of the existence of collagen in more than one form, although the authors do not appear to have understood the proper relation between the two varieties. In a second paper (Z. Phys. Chem., 1938, 255, 241) the same authors confirmed their previous results. In these experiments they investigated the effects of 2% caustic soda, hot and cold water, formamide, ammonium thiocyanate, ether, and solid carbon dioxide on pig's tendon, in a stretched and unstretched condition. The first stage of the conversion leads to the production of amorphous rubber-like material resistant to the action of trypsin, and the second to the destruction of the three dimensional structure and of the power to resist trypsin. This is followed by dissolution and progressive decrease in viscosity.

Almost at the same time Cherbuliez & Meyer (Compt. Rend. Trav. Lab. Carlsberg, 1938, 22, 118) published another paper on the conversion of collagen into collagen II, these varieties being apparently the same as collagen A and B mentioned above. Pig's tendon was again used in these experiments. When the fibres were immersed for 45 seconds in water at 62°C. a contraction took place. They said that the fibres changed from a substance containing crystallites into an amorphous body designated as collagen II which was attacked by trypsin. If the amorphous fibres are carefully stretched in a mixture of ice and water the fibres revert to their original length and recover their power of resisting trypsin. Formamide at 37°C. has a similar action to water at 62°C. When the tendons are placed in 2% caustic soda for several weeks, marked swelling takes place without elongation, and the resulting product is rapidly attacked by trypsin. Heating the swollen fibres to between 50° and 60°C. causes rapid contraction and dissolution to form gelatine. While there is some inconsistency in the reported action of trypsin on the two forms of collagen, there can be no doubt that the existence of two forms was definitely recognised. The shrinkage of collagen on heating was also considered to be of some significance.

An entirely different aspect of the conversion of collagen into gelatine was noted by Bate Smith (J.S.C.I., 1938, 57, 82). He found that small quantities of phosphate and arsenate increased the rate of dissolution. There was no evidence of the phosphate or arsenate being combined, so that their influence on the reaction was presumably catalytic. It should be noted that the experiments were carried out under rather drastic conditions in an autoclave at 121°C. and that no effort was made to obtain gelatine in this way. The same effect might not be noticeable under conditions which would render possible the recovery of the dissolved nitrogen as gelatine of good quality.

Another paper dealing with the structural aspect of the conversion of collagen into gelatine was published by Kuntzel (Stiasnyfestzchr., 1937, 191). In his experiments, tendons from rats tails were used and an attempt made to describe the changes in structure which occurred when collagen was heated and gelatine produced.

A paper deserving the closest study, and perhaps after Bogue's early work the most outstanding, entitled "The Function of Liming in the Conversion of Collagen into Gelatine", was published in 1938 by Kuntzel & Koepff (Collegium 1938, 433). In this paper it was shown that the period of liming could be shortened by raising the temperature, or by the addition of various neutral salts. The methods used, although open to criticism, were very thorough; probably in a number of cases unnecessary trouble was taken to secure accuracy. The soaking was carried out in a lime paste in order to be able to determine the loss of hide substance. It appears doubtful if the loss in hide substance is of sufficient importance to warrant this method of soaking which prevents the samples being kept under observation. Then a standard time of extraction was used. (Actually this was carried out in a thermostat, quite an unnecessary refinement). This would result in the figures showing the type of gelatine produced, from being of very much value. The figures chosen to characterise the gelatine obtained, the viscosity and melting-point, are in any case not as valuable as jelly strength figures, which can now be expressed in standard units universally comparable. The formo-titration figures to some extent compensate for the unsatisfactory figures chosen to show the nature of the gelatine, as they indicate the degree of degradation or degree of hydrolysis. The chief criticism against the paper is that the figures do not clearly exhibit the progressive nature of the liming process.

Previous to the publication of this recent group of papers the present author undertook a series of investigations into the properties of gelatine as influenced by the method of preparation, the results of which were communicated in an earlier paper (Thesis, Edinburgh 1941). The work involved in these experiments provided an account of the preparation of gelatine, but numerous questions arose which could only be answered by further investigation. The present paper is an account of work undertaken in an endeavour to fill the gaps in our knowledge, and the two papers together are intended to provide a complete picture of the preparation of gelatine. Such a task would be justifiable, even if it consisted in merely bringing together information scattered throughout chemical literature, but in addition, many new facts have been brought to light, and knowledge hitherto regarded from the point of view of tanning, applied to the extraction of gelatine.

On first considering the matter, it appeared difficult to arrange an account of the varied types of experiments in any sort of logical order, but the inter-relations of apparently disjointed facts later became apparent. The loss of ammonia which was found to occur when collagen is converted into gelatine, together with attempts to identify its point of origin, provide the main themes connecting different parts of the work. Following a description of swelling experiments necessary to establish the most suitable working conditions, a large portion of the paper is concerned with progressive soaking experiments using caustic soda, lime, and various combinations of both, particular attention being paid to the effect of temperature. Consideration of the influence of temperature on soaking leads naturally to an investigation of the maximum temperature which may be safely used, and to the shrinkage temperature of swollen collagen. Possible methods of accelerating the reaction taking place during soaking, and for prevention of the excessive swelling caused by caustic soda, are then considered. Estimations of the nitrogen content of acid-prepared and alkaline-prepared gelatine suggest consideration of the iso-electric point of hide and sinew before and after soaking in lime. This further implies an investigation into the amount and origin of the ammonia obtained duringthe extraction of gelatine. The variable iso-electric point necessitates the construction of curves showing the relationship between acidity and Ph for different types of gelatine. The stability of the different types of gelatine and some consideration of deaminised collagen concludes the practical part of the paper. In the discussion which follows, an effort has been made to show what bearing the experimental facts have on the structure of collagen.

CAUSTIC SODA As A SOAKING AGENT.

Preliminary Experiments.

When preparing gelatine by the alkaline process it is necessery, in order to obtain the best results, to soak the precursor for a relatively long period in milk of lime. During the soaking period unwanted impurities are removed and the precursor becomes more readily hydrolysable. At the same time the iso-electric point of the gelatine obtained moves to a higher acidity as the soaking process proceeds. As the time required in lime water may extend to as much as twelve weeks or even more, some method of accelerating the soaking process was very desirable. Now it was known that caustic soda either by itself or in combination with lime had been used for this purpose; caustic soda was generally thought to shorten the process of soaking, as might have been anticipated from the very much greater concentrations possible with the highly soluble alkali. Accordingly, during the previous investigations, numerous experiments were carried out with caustic soda. For convenience these results have been collected together and are shown in Table I.

The material used for all the experiments except the last one was shredded rabbit skin, because it was not so difficult to handle as other materials. Collagen in any other form swelled to such an extent in caustic

TABLE I.

Material used: 200 grms. Shredded Rabbit Skin.

Treatment.	Time of Extraction	Yield	Jelly Strength	Iso-Electric Point Acidity
Soaked in excess of caustic soda and washed.	1½ Hrs.	15·5%	71	·46%
Soaked in excess of caustic soda overnight and washed.	1½ Hrs.	17·5%	207	·36%
Soaked in 2% caustic soda soln. for 3 days and washed.	1 Hr.	18·5%	165	·73%
Soaked in 2 changes of water each containing 8 grms. NaOH (Total soaking time 2 weeks) and washed.	1½ Hrs.	14·0%	263	·56%
Soaked in 4 changes of water each containing 8 grms. NaOH (Total soaking time 4 weeks) and washed.	1½ Hrs.	17·1%	270	·75%
Two soaks in 210 c.c.'s $\frac{N}{5}$ NaOH, washed after each soak. Process required 3 days.	1¼ Hrs.	11·9%	>255	·03%

TABLE I. (cont.)

Treatment	Time of Extraction	Yield	Jelly Strength	Iso-Electric Point Acidity.
Three soaks in 210 c.c.'s N/5 NaOH, washed after each soak. Process required 4 days.	2 Hrs.	12·7%	>255	·12%
Two soaks in 420 c.c.'s N/5 NaOH, washed after each soak. Then soaked in 180 c.c.'s N/5 NaOH. Process required 3 days.	1 Hr.	23·4%	213	·76%
Material used: 1 kilo Salted Ox Hide.				
Soaked in 2 lots of 8 grs. and 2 lots of 16 grms. caustic soda and washed. Process required 23 days.	4 Hrs.	4%	152	·6%

soda that subsequent operations on the swollen material could only be carried out with difficulty. For other reasons the choice of rabbit skin was unfortunate. The jelly strength of gelatine made from this material by almost any treatment was invariably good, so that jelly strength gave no indication of the value of any particular method. Nor could too much reliance be placed on the yield, or the rate of extraction, which for shredded rabbit skin bore little relation to the pre-treatment. However, for this group of experiments, the gelatines having their iso-electric point at the highest acidity showed the greatest rate of extraction. These were the gelatines which resulted from the most energetic caustic soda treatment, which showed that caustic soda caused the iso-electric point to move in the same direction as lime. In no case, however, did the position of the iso-electric point reach the value which would have been attained by the use of lime. Two conclusions were thus possible: either caustic soda produced gelatine with a lower iso-electric point than lime, or too much had been expected from the quantities of caustic used and the short soaking times. The last experiment on ox-hide was designed to give an indication as to which was the correct explanation. This it failed to do, as the results only indicated that the material had received insufficient treatment, which was in itself a satisfactory explanation of the low iso-electric point. At the same time it did throw doubt upon the value of caustic soda as a soaking reagent at low concentrations. Thus the earlier experiments failed to demonstrate clearly what effects were produced by a soak in caustic soda, and a thorough investigation was rendered necessary.

TABLE II.

Treatment	Time of Extraction	Yield	Jelly Strength	Iso-Electric Point Acidity.
Material used: 200 grs. Shredded Rabbit Skin.				
Soaked in 5 lots of 1 gm. and 7 lots of 2 gms. caustic soda for 8 weeks & washed.	1½ Hrs.	15·8%	207	·71%
Soaked in 8 lots of 8 gms. NaOH for 6 weeks and washed.	2 Hrs.	14·5%	255	1·06%
Soaked in 16 grms caustic soda for 9 weeks. Caustic soda changed as required. Then washed.	1 Hr.	14·75%	> 255	1·55%
Soaked in 32 grms. caustic soda for one night and washed.	2 Hrs.	16·0%	> 255	·38%
Soaked in 2 litres 3% NaOH soln. overnight, then in 1 litre 3% NaOH soln. for 2 days and washed.	¾ Hr.	23·23%	> 255	·96%
Soaked in 2 litres 5% NaOH soln. overnight and washed	½ Hr.	19·5%	178	·95%

TABLE II. (cont.)

Treatment	Time of Extraction	Yield	Jelly Strength	Iso-Electric Point Acidity
Material used: 200 grms. Osseine.				
Soaked in 4 lots of 16 grms. NaOH in 1 litre water for 7 days & washed.	3 Hrs.	11·7%	-	1·33%
Material used: 200 grs. Sinews.				
Soaked in 9 lots of 8 grms. NaOH for 10 weeks and washed.	4 Hrs.	16·6%	132	·78%
Material used: 1 kilo. Salted Ox Hide.				
Soaked in 10 lots 8 grms. NaOH for 9 weeks and washed.	2 Hrs.	3·5%	162	·5%
Soaked overnight in 2 litres 5% NaOH soln. and washed. Then for 4 days in two 2-litre quantities of 5% NaOH, and washed.	$\frac{1}{4}$ Hr. (up to 60°C.)	9·5%	-	·55%
Soaked 1 day in 2 litres 3% NaOH. Soaked 5 days in 2 lots 3 litres 3% NaOH and washed for 3 days.	1 Hr.	13·65%	238	1·45%

TABLE II. (cont.)

Treatment	Time of Extraction	Yield	Jelly Strength	Iso-Electric Point Acidity.
Soaked in 2 lots of 3 litres 1% NaOH for 5 days and washed.	5 Hrs.	11·7%	105	·86%
Soaked in 3 lots of 3 litres 1% NaOH for 15 days and washed.	4 Hrs.	4·5%	184	·90%

A second series of experiments was carried out using rabbit skin, ox-hide, sinews and osseine. The period of soak and the concentrations of caustic soda used were on the whole greater than those in previous experiments. These results are shown in Table II. The results enabled it to be stated definitely that the iso-electric point of gelatine made by caustic soda treatment was not lower than when lime was used. In the case of three different materials, iso-electric points were obtained which were at the same acidity as if lime had been the soaking agent. It became evident during the course of these experiments that the proportion of caustic soda to the original weight of material was of no importance, but that what mattered was the concentration of caustic soda in solution.

For the caustic soda to be effective, particularly with a short time of soak, very large quantities of caustic soda had to be used relative to the amount of material. Failure to realise this point, coupled with the knowledge that caustic soda was used to speed up the soaking process, was largely responsible for the confused results shown both in Table I and Table II. The second series of experiments also suggested that caustic soda was only more efficient than lime because of its superior solubility. The concentrations used in experiments where the iso-electric point was raised by the same amount as by lime were very much greater. The results further emphasised the need for a more systematic investigation. In all the experiments hitherto undertaken two factors were altering at the same time - concentration and time of soak. What was clearly required was to obtain results for fixed concentrations of caustic soda with varying periods of soak.

When using lime, the concentration is automatically fixed at ·117%, the solubility of calcium hydroxide in water. Theoretically the concentration of caustic soda which can be used is unlimited, but in practice it is restricted at the upper end by the fact that strong alkali hydrolyses collagen in the cold completely, in other words destroys collagen, while with low concentrations there is a definite tendency for the material to be attacked by bacteria, which does not happen when lime is used. The first systematic experiments were designed to exhibit generally the action of caustic soda on skin, with the particular intention of finding out suitable concentrations to work with. This was done by means of swelling experiments.

Swelling of Precursors in Alkaline Soaks.

Owing to the interest of the tanners in the plumping of hides, many results are on record of swelling experiments carried out in a variety of ways, although these were not of use for the particular purpose in view, as it was only the initial plumping taking place in relatively short periods of time which was considered important in tanning. McLaughlin (J. Amer. Leather Chem. Assoc., 1921, 16, 295), working on fresh hides, stated that alkali swelling depended on the character of the alkali and its concentration. Read (J. Amer. Leather Chem. Assoc., 1922, 17, 460) investigated the swelling of hide powder in various acids and caustic soda, by soaking the powder in the reagent for 20 hours and observing the increase in weight. Wilson & Gallun (J.I.E.C., 1923, 15, 71) obtained curves showing the plumping of hides over a range of Ph values, by measuring the thickness of the skin by means of a thickness gauge. This was not a new method of examining the behaviour of skin, for it had been used many years before by Wood, Sand & Law (J.S.C.I., 1913, <u>33</u>, 398) to investigate the shrinkage or falling of skin after bating. This method was also used by Page & Page (J.I.E.C., 1927, <u>19</u>, 1264) to measure the repressive effects of salts on the acid and alkaline swelling of bated skin. Kuntzel & Phillip (Collegium, 1932, 267) stated that the swelling of collagen in caustic soda was different in type from the swelling in hydrochloric acid. The effects of caustic soda and lime on collagen were fundamentally different. Kuntzel & Koepff (Collegium, 1938, 433) in the course of their experiments on the preparation of gelatine, measured the amount of swelling by weighing the raw material before and after soaking. Kubelka and Knodel (Collegium, 1938, 49), working on hide powder, stated that the effect of saturated lime water was greater than an equivalent concentration of NaOH.

Lloyd & Shore (Chemistry of Proteins, 1938, p.390) provided a bibliography of papers relating to the swelling of tissues including tendons, skin, and hide powder, from which it was evident that the subject had been widely studied.

There was thus available a choice of methods which had been used for swelling experiments, but it was decided that a simple displacement method would be sufficiently accurate. An advantage of this apparently crude method of measuring the swelling of hide was that practically no previous treatment of the raw material was necessary. In many of the experiments noted above, the hide had to be limed and bated before it was possible to work with it. It was assumed that the total volume of skin and liquor remained the same. This was not strictly true, for Theis & Neville (J.I.E.C. 1930, 22, 64 & 66) showed that hydration caused an alteration in total volume, but the alteration was very small relative to the swelling, and so it could be neglected.

A piece of salted ox-hide was washed to remove the salt, and the hair scraped off. Seven strips of hide were then cut, of approximately the same length and breadth (one inch wide and seven inches long). The volume of each strip was measured by displacement using a 250 c.c. graduated cylinder. (In the first experiment the strips varied in volume from 25 to 33 c.c.'s when suspended in water). The strips were then suspended by means of thin copper wire in milk of lime, and various concentrations of caustic soda, contained in 250 c.c. graduated cylinders. The volume of the skin was ascertained from time to time by removing the skins, and measuring the fall in the surface of the liquor after the skin had time to drip.

In order to eliminate variations arising from the use of a natural material, six experiments were done. I, II and III were carried out with lime alone and concentrations of caustic soda running from ·5% up to 5%. Two experiments, IV and V, were devoted to combinations of lime, caustic soda, and soda-ash, with the necessary controls. The last experiment, No. VI, was particularly designed to show the effect of temperature. All the experiments should have been carried out under constant temperature conditions but this was not possible to arrange. In the tables which show the results, the original volume of the skin was taken to be 100, and the volume as measured at any given time calculated proportionately. In comparing the results, as there was variation between different experiments, the figures for the lime soak were taken as a reference. This was possible in every experiment except No. III. The results are shown in Tables III to VI.

Lime.

In milk of lime, swelling takes place rapidly during the first day's soak. Thereafter there is either no change in volume or a very slight increase. At higher temperatures the initial change in volume is greater, and swelling goes on as long as the material is soaking. Thus a greater degree of swelling is secured at high temperatures than at low temperatures. The results obtained in Experiment VI when the temperature was carefully controlled shows that variations between the other experiments can be accounted for by temperature differences.

TABLE III.

(Volume of material as percentages)

Time of Soak.	Lime		·5% NaOH			1% NaOH		
Days	I	II	I	II	III	I	II	III
Nil	100	100	100	100	100	100	100	100
1	120	112	138	143	177	136	143	128
2	127	112	146	157	177	136	143	131
3	-	112	-	157	177	-	143	131
4	127	112	146	157	177	136	143	133
5	127	-	146	157	-	136	143	-
6	127	125	146	-	185	136	-	139
7	127	125	146	157	185	136	143	139
8	127	125	146	157	185	136	143	139
9	127	125	146	157	185	136	143	139
10	-	125	-	157	185	-	143	144
11	127	125	146	157	185	136	143	144
12	127	-	146	157	-	136	143	-
13	127	-	146	-	192	136	-	144
14	127	125	146	157	-	136	143	-
15	127	125	146	157	-	136	143	-
16	-	125	-	157	-	-	157	-
17	-	125	-	157	-	-	157	-
18	127	-	146	-	-	136	-	-
19	-	125	-	157	-	-	157	-
20	-	-	-	-	-	-	-	-
21	-	125	-	157	-	-	157	-
42	-	137	-	-	-	-	-	-
49	-	137	-	157	-	-	157	-
57	-	137	-	157	-	-	157	-

•5% Caustic Soda.

As with lime, swelling takes place rapidly during the first day. It is evident that the swelling is to a large extent completed in a very short time, although the process may go on slowly during the remainder of the soaking period. The amount of swelling in •5% caustic soda solution is greater than in milk of lime. In comparing the behaviour of skin in •5% and 1% caustic soda solutions, certain inconsistencies are visible. In two out of four experiments, more swelling was observed in the less concentrated solution. In one experiment the amount of swelling was the same, while in the remaining experiment, skin suspended in 1% solution exhibited more swelling than skin in •5% solution. Comparing the initial swelling, however, which is the greater part of the total, in all cases it is slightly greater in •5% solution than in 1% solution. It appears then that skin swells more in the dilute solution than in the concentrated solution. The inconsistencies observed may be due to lack of control of temperature or to variations in the material. In no case did a •5% solution of caustic soda damage the material, even after somewhat lengthy periods of immersion.

1% Caustic Soda.

This has the same effect as milk of lime, except that the amount of swelling is greater. Most of the swelling takes place at the beginning. Thereafter the increase is very slight, although there is evidence that raising the temperature induces the swelling to continue. There were no signs of the material being attacked, even after 57 days soaking.

2% Caustic Soda.

At the beginning of the soaking period, this produces the same amount of swelling as a 1% solution. Thereafter the amount of swelling goes on increasing steadily, so that the final swelling is far greater than in 1% solution, and hence very much more than is produced by milk of lime. Temperature appears to have a marked effect in speeding the action up. Two out of the three samples examined were destroyed after soaking for 21 days and 12 days - relatively short periods of time.

3% Caustic Soda.

At the start of the soaking period the skin does not swell to any greater extent than in 1% solution, but subsequently the rate of swelling increases markedly. The total swelling is three times what it would be in milk of lime. Destruction of the material takes place after a short time in the solution. One sample was destroyed in 5 days, and the others in 13 days, so that temperature probably plays an important part in varying the activity of solutions of this concentration.

TABLE IV.

(Volume of Material as percentage)

Time of Soak Days.	2% NaOH			3%NaOH			4% NaOH		
	I	II	III	I	II	III	I	II	III
Nil	100	100	100	100	100	100	100	100	100
1	128	137	133	133	128	160	127	150	169
2	128	150	139	150	133	190	151	164	204
3	-	150	150	-	139	225	-	186	231
4	136	150	155	167	144	290	197	214	270
5	144	163	-	183	166	-	226	257	-
6	144	-	178	216	-	-	286	-	-
7	144	175	189	234	200	-	318	358	-
8	152	175	203	250	222	-	334	-	-
9	152	187	222	284	244				
10	-	187	250	-	277				
11	160	200	278	334	300				
12	160	212	-	350	334				
13	168	-							
14	168	225							
15	168	238							
16	-	250							
17	-	262							
18	184	-							
19	-	288							
21	-	-							

TABLE IV. (cont.)

Time of Soak Days.	5% NaOH I	II	III
Nil	100	100	100
1	117	150	196
2	150	175	286
3	-	206	
4	226	250	
5	268	312	
6	268		

4% Caustic Soda.

Provided the temperature is low, skin does not at first swell any more in this concentration of caustic soda than it does in milk of lime. With higher temperatures, swelling proceeds very rapidly from the moment of immersion. In every case the skin continues to swell at a great rate, and in 5 to 8 days reaches a maximum after which it is destroyed. The maximum amount of swelling is approximately three times what was observed in milk of lime.

5% Caustic Soda.

Skin behaves in the same way in solution of this concentration, as in 4% solution. Destruction of the skin is, however, so rapid that it never reaches the same degree of swelling as in a 4% solution. Complete destruction occurs in from 3 to 7 days.

These last three experiments show that any of the concentrations of caustic soda examined cause more swelling of the skin than lime. The greatest amount of swelling was obtained with 3% and 4% solutions. It appears that of the caustic soda solutions investigated 1% produces the least amount of swelling. Concentrations below 1% appear to cause more swelling than 1% solutions. In other words, the swelling of skin in solutions of caustic soda is least when the concentration is 1%. Although there was no control of temperature in these experiments, it is clear that raising the temperature considerably increases the activity of caustic soda. Every concentration above 1% ultimately brought about destruction of the material, so that a limit was set for subsequent investigations. It was thus of no moment to consider the action of higher concentrations in the preparation of gelatine.

The degree of swelling of material is one of the factors commonly used to determine the readiness of material for extraction. As far as hide is concerned, the present series of experiments shows that the two properties are not related. Most of the swelling takes place at the beginning of the soaking period, and thereafter increases slowly. Thus, while hide which is ready for extraction will always be well swollen, owing to the length of time it has been soaked, a piece of hide may be fairly well swollen without possessing the property of readily yielding gelatine. It is particularly important to keep this in mind when caustic soda is used as the soaking reagent. Caustic soda immediately induces a degree of swelling comparable with the effect of a long period in lime, so that the hide appears to be in a condition suitable for extraction. It is shown in later experiments with dried sinews that a precursor can be greatly swollen by caustic soda and yet difficult to extract. Consequently, when caustic soda is used for soaking either dried or undried material, swelling gives no indication of the ease with which the extraction of gelatine can be carried out. In the case of dried material soaked in lime, swelling will at least be an indication of proper penetration of the liquor, and in this way provides a rough means of knowing whether the material is ready for extraction or not.

The greater magnitude of the swelling brought about by caustic soda introduces a difficulty in its practical use. Washing the soaked material becomes extremely difficult. Since there is more swelling in dilute solutions, as the washing proceeds the swelling increases for a time, thus rendering the material difficult to manipulate and less easy to wash. Considering that the swelling in caustic soda may be as much as three times that in milk of lime, the difficulties likely to be encountered in washing will be of no slight order.

The next two swelling experiments (Nos. IV and V) carried out were concerned with combinations of lime and caustic soda, or soda-ash. An experiment was done with lime to serve as a control for No. IV, and experiment No. V was provided with ·5% caustic soda and 1% caustic soda controls in addition to a lime control. The soda-ash had an alkalinity of 68% caustic soda, and the quantities used in the experiments were approximately equivalent to the quantities of caustic soda used. The reason for the use of caustic soda and lime together is that lime prevents bacterial decomposition which very readily takes place when caustic soda is used alone. Soda-ash is generally to be preferred as a source of caustic on account of its cheapness, and its use is obviously called for when the presence of lime is necessary for another purpose. The results of the swelling experiments are shown in Table V.

Lime and ·5% Caustic Soda Solution.

The skin as in previous experiments swells very rapidly at the start, and then swelling proceeds continuously and gradually. In experiment IV, the total swelling produced is less than if the two substances were acting separately, whereas in experiment V the total swelling is greater. There is no indication that this combination of chemicals destroys the skin.

Lime and ·7% Soda-ash.

In experiment IV the carbonate combination produces more swelling than the lime and caustic soda; in experiment V the reverse is the case. In the one case the swelling is rather greater than each reagent would produce separately, and in the other less. These inconsistencies may arise from lack of temperature control. From the behaviour of the skin in milk of lime, it is likely that during experiment V the temperature was lower than during experiment IV. There is again no evidence that the reagents are attacking the skin.

TABLE V.

(Volume of material as percentage)

Time of Soak Days.	Lime		·5% NaOH	1% NaOH	Lime & ·5% NaOH	
	IV	V	V	V	IV	V
Nil	100	100	100	100	100	100
1	131	121	140	140	153	147
2	138	121	147	145	160	160
3	146	121	147	150	167	160
4	146	121	153	150	167	160
5	154	131	153	155	167	167
7	162	137	153	155	167	167
8	162	137	153	160	174	167
9	162	137	153	160	174	167
10	162	142	153	165	180	167
11	169	142	153	165	180	174
12	169	142	153	165	187	174
14	177	142	153	165	187	180
15	177	142	153	165	193	180
16	177	142	153	165	193	180
17	185	142	153	165	200	180
18	185	142	153	170	200	180
19	185	142	153	170	200	180
21	-	142	153	175	-	186
22	-	142	153	175	-	186
23	-	142	153	175	-	186

TABLE V. (cont.)

(Volume of material as percentage)

Time of Soak Days.	Lime		·5% NaOH	1% NaOH	Lime & ·5% NaOH	
	IV	V	V	V	IV	V
24	-	142	153	175	-	186
25	-	142	153	175	-	193
26	-	142	153	175	-	193
28	-	148	153	175	-	200
30	-	148	153	175	-	200
32	-	148	153	175	-	207
35	-	148	153	180	-	214
37	-	148	153	180	-	220

Time of Soak Days	Lime & ·7% Soda Ash		Lime & 1% NaOH		Lime & 1·4% Soda Ash.	
	IV	V	IV	V	IV	V
Nil	100	100	100	100	100	100
1	190	144	146	155	136	147
2	200	150	161	165	150	152
3	200	155	161	170	157	157
4	200	155	161	175	157	166
5	200	155	169	175	157	166
7	200	155	169	185	165	171
8	210	161	176	185	172	176

TABLE V. (cont.)

(Volume of material as percentage)

Time of Soak Days.	Lime & ·7% Soda Ash		Lime & 1% NaOH		Lime & 1·4% Soda Ash.	
	IV	V	IV	V	IV	V
9	210	161	176	190	186	176
10	210	161	185	195	186	181
11	220	161	185	200	193	181
12	220	161	169	204	200	190
14	230	167	146	214	193	200
15	230	167	146	175	193	200
16	240	167	138	175	178	204
17	240	167	138	175	165	210
18	240	172	138	180	165	214
19	250	172	138	180	157	218
21	-	178	-	180	-	224
22	-	178	-	175	-	224
23	-	178	-	165	-	224
24	-	178	-	155	-	224
25	-	183	-	150	-	228
26	-	183	-	150	-	224
28	-	183	-	150	-	224
30	-	189	-	150	-	218
32	-	189	-	145	-	218
35	-	200	-	145	-	204
37	-	200	-	140	-	195

Lime and 1% Caustic Soda.

The rate of swelling is greater than with the lower concentrations. After 11 days soak in the one experiment, and 14 days in the other, the volume of the skin diminishes, indicating a destructive attack by the chemicals. Because of this the maximum swelling attained tends to be less than with the lower concentrations of caustic and soda.

Lime and 1·4% Soda Ash.

The rate of swelling is of the same order as if caustic soda had been used in place of soda ash. The volume of the skin continues to increase in the one experiment for 12 days, and in the other experiment for 25 days, after which the volume diminishes, again pointing to destruction of the skin. The maximum swelling attained is greater than was observed in the case of the corresponding caustic soda combination. As the quantity of soda used was equivalent to the amount of caustic soda, it is clear that the formation of caustic soda in the soaking bath may lead to more energetic action than by adding caustic soda itself. These experiments indicated that when combinations of milk of lime and caustic soda or soda ash are used to treat skin, the effects on the skin are additive. Consequently, the maximum safe concentrations which can be used along with milk of lime are ·5% caustic soda

TABLE VI.

Soaking Expt. No. VI.

(Volume of material as percentage)

Time of Soak Days	Lime		Lime + 1% NaOH		Lime + 1·4% Soda Ash.	
	2°C	20°C	2°C	20°C	2°C	20°C
Nil	100	100	100	100	100	100
1	120	175	160	180	170	146
2	120	175	170	190	190	164
3	120	175	180	200	190	164
4	130	187	180	200	190	164
5	130	187	180	200	190	164
7	130	187	180	210	190	164
9	130	187	190	220	200	173
10	130	187	190	220	200	180
12	130	200	190	220	200	180
14	130	200	190	220	210	190
16	130	200	190	240	210	200
18	130	200	190	250	210	200
19	130	200	190	260	210	210
21	140	200	190	260	210	218
23	140	212	200	280	210	228
25	140	212	200	290	210	236
26	140	212	200	300	210	246
28	140	212	200	310	210	254

TABLE VI. (cont.)

Soaking Expt. No. VI.

(Volume of material as percentage)

Time of Soak Days.	Lime		Lime + 1% NaOH		Lime + 1·4% Soda Ash.	
	2°C	20°C	2°C	20°C	2°C	20°C
30	140	225	200	320	220	264
32	140	225	200	330	220	264
33	140	225	200	340	220	282
36	140	225	200	360	220	300
39	140	225	200	380	220	316
40	140	225	200	380	220	316
42	140	225	200	370*	220	324
43	140	225	200	360	220	346
45	-	225	-	360	-	346
47	140	225	200	360	220	338 x
49	-	225	-	360	-	324
52	140	238	200	340	220	-

* Destruction of material begins.

solution, and ·7% soda ash solution.

As difficulties had been created by the lack of consistency in repetitions of the various swelling experiments, and as it was clear from occasional observations of temperature that variable temperatures were largely to blame for this, an experiment was designed to show the effect of temperature on the swelling of skin by different reagents. The results which are shown in Table VI proved to be of considerable interest.

At the low temperature, lime causes a sudden small amount of swelling which increases slowly and remains constant during the second half of the soaking period. At the higher temperature, the initial swelling is far greater, and then increases steadily. The maximum swelling at 20°C. is three and a half times that at 2°C. It is slightly more than the maximum swelling produced in the cold by the combinations of reagents. There is no sign of the skin breaking up, either in the hot or in the cold.

It is interesting to note that the swelling at 2°C. agrees with the figure given by Kuntzel & Koepff (loc. cit) for 0°C. On the other hand, the swelling at 20°C. is much greater than the figures given by them for 15°C. and 30°C. This is in agreement with experiments carried out subsequently, which showed that 20°C. represented approximately the maximum temperature at which soaking could be carried out. Up to 20°C. the amount of swelling increases as the temperature rises, so that the maximum swelling at 20°C. is greater than at 15°C. At 30°C., however, a lime soak appears to cause less swelling, and in addition there are losses due to disintegration which made the results obtained by Kuntzel & Koepff even lower than they might have been.

In the case of milk of lime and caustic soda, the difference in swelling at high and low temperatures, though marked, is not as great as with milk of lime alone. In the cold, after a large increase in volume at the start, the rate of swelling becomes very small and ceases after 21 days. At the high temperature the swelling continues to increase steadily up to 40 days, when the volume begins to diminish, indicating destruction of the material. No attack on the skin was observed in the case of the low temperature soak.

Exactly similar results are obtained with the mixture of milk of lime, and soda ash, except that it is slightly less active. Again the skin is not destroyed in the cold but begins to break up after 45 days soak at the higher temperature.

This experiment demonstrates the effect of temperature on swelling in a very marked way. The results suggest that the inactivity of lime, when the soaking is done at a low temperature, may be compensated by the addition of caustic soda or soda ash. It is evident that rise in temperature has a greater effect in increasing the activity of lime alone than increasing the activity of the mixtures, in consequence of which, soda lime mixtures might conceivably be more useful in the cold, and milk of lime when the soaking temperature was high. These matters are fully dealt with in later experiments. The experiment also confirms that when milk of lime is used, the concentration of caustic/or soda ash cannot be allowed to exceed ·5% and ·7% without danger to the material.

Influence of the Period of Soak in Caustic Soda on the Extraction and Properties of Gelatine.

Experimental Methods.

The methods used in all the progressive soaking experiments were the same as had been proved to be useful in work described in a previous communication.

Acidity. Instead of measuring Ph, the acidity of the gelatine was estimated as grms. sulphuric acid per 100 grms. air-dried gelatine. Acidities were determined by titrating 6% solutions of gelatine with N/5 caustic soda, using phenolphthalein as indicator and working at a temperature of 40°C. When the gelatine was alkaline its alkalinity was expressed as a minus acidity. In any determination where the acidity was of importance, boiled distilled water was used. Criticism might reasonably be directed against the use of this system, in place of the universally used Ph scale. However, the use of acidity can be defended on two grounds. Firstly, the determination is more easily carried out than a Ph measurement. Secondly, as will be shown later from the nature of the titration curves of acid and alkaline gelatine, acidities tend to show bigger differences in the position of the iso-electric points of the two types of gelatine than is shown by Ph figures. During the last period of a soaking experiment the Ph at which the gelatine is iso-electric alters very little, whereas there is a considerable difference in the corresponding acidity figures. For this reason the behaviour of material during soaking can be more closely followed by means of the acidity of the iso-electric point.

Ph Value. In certain cases Ph values have been shown in addition to acidity figures. These were obtained using a glass electrode and a Cambridge valve potentiometer. It was found necessary to use 1% or 2% solutions to obtain results which could be repeated, and as some of the gelatine made strong jellies, a temperature of 20°C. was adopted to keep the solutions from setting.

Iso-Electric Points. These were found by making use of the well known property of gelatine that dilute solutions when cooled and allowed to set to a jelly become very opalescent at their iso-electric point. This method has been carefully studied by Briefer (J.I.E.C., 1929, 21, 266) who also used it in his investigations. When the gelatine has been purified by washing, a very sharp determination of the iso-electric point is possible by the opalescence method. Hunter & Turner (Trans. Faraday Soc., 1940, 36, 835) showed that the results obtained by the opalescence method agreed with determinations made in a number of other ways. The gelatine to be examined was made up in boiling tubes in the form of 2% solutions. The acidity of the gelatine having been determined previously, a number of tubes were prepared exhibiting a range of acidities by replacing so much of the water required for the solution by appropriate quantities of N/50 caustic soda or N/50 sulphuric acid. Each tube differed from the next by ·2% acidity as sulphuric acid, and as a rule five tubes were sufficient to fix the iso-electric point. After the gelatine was softened by soaking, it was melted up and cooled in ice. The tube showing most opalescence indicated the acidity at which the gelatine was at its iso-electric point.

Jelly Strength. The strength or firmness of the jelly which a known concentration makes with water under strictly regulated conditions has for long been accepted as the best measure of the quality of gelatine and is referred to as its jelly strength. Unfortunately no entirely satisfactory method has been evolved for measuring this property of jellies. Nevertheless, a standard machine, the Bloom Gelometer, is now available for carrying out jelly strength tests (B.S.I., No.757-1937), and the figures given in the Tables for this property are Bloom Gelometer jelly strengths for a concentration of $6\frac{2}{3}\%$. The apparatus actually used for jelly strength tests was somewhat similar to the Bloom Gelometer but differed in that the load measured was that required to break the jelly, the amount of depression of the plunger being variable. In the Bloom Gelometer the depression of the plunger is kept constant. Jellies were made with 3% or 4% solutions, and tested at 4°C. To eliminate irregularities arising from possible differences in the history of the jellies, each batch of tests included samples of a standard gelatine, which served as a reference. The jelly strength figures obtained were converted into Bloom strengths by means of conversion tables previously prepared. The method has been found by experience to give accurate results.

Viscosity. Although viscosity has not been used in these experiments as an indication of the quality of the gelatine, on account of the arbitrary results frequently obtained, it was thought that the behaviour of viscosity might throw some light on the mechanism of the soaking process. Accordingly, viscosity determinations were carried out using an Ostwald Viscosimeter. The gelatine was made up at 20% and the tests carried out at 40°C. in a water bath fitted with a mechanical stirrer and thermo-regulator. The results were converted into absolute units by means of a factor.

Method of Extraction. Undoubtedly the best way of measuring the readiness of a precursor for extraction would be in every case to heat for the same period of time, under identical conditions of temperature and Ph, with a constant ratio of water to material. The yield of gelatine obtained, or more correctly the extracted nitrogen, would under these conditions give an exact record of the progress of the reaction taking place during a soak in lime. Unfortunately, except for constant temperature and time of extraction, such conditions could only be approximately realised in practice. The Ph of extraction was not a figure which could be kept absolutely constant, even with careful washing, and the ratio of water to material depended on the degree of swelling of the precursor, which varied from the start to the finish of a soaking experiment.

Another reason why a constant time of extraction could not be adopted was the necessity of obtaining gelatine which could be dried off and used for various determinations. If a constant extraction time had been selected, which would have enabled dry gelatine to be prepared from a precursor at the beginning of a soaking experment, the gelatine obtained at later periods would have been so spoilt by long exposure to heat, as to be difficult or impossible to dry off. Conversely, an extraction time suitable for a well-soaked precursor would have resulted in no extraction from material at the beginning of a soak. For these reasons the time of extraction was varied so as to be just long enough for the extraction of sufficient gelatine to form a reasonably firm jelly. This method enabled gelatine to be obtained from the precursor at every stage in the soaking process. There appeared to be no objection to carrying out the extractions in this manner, provided the time of extraction, yield, and jelly strength, were all considered in assessing the results.

The gelatine was extracted by heating the precursor in a water bath with or without the addition of water, depending on the degree of plumpness of the material. The temperature was kept about 80°C., use of a thermostat being considered unnecessary. When sufficient gelatine had been extracted to set to a fairly stiff jelly in ice, the extract was strained off by pouring through coarse fabric, and the volume measured. This, together with the concentration which was then found by means of a specially graduated hydrometer, enabled the yield of gelatine to be calculated. Any more elaborate method of obtaining yield figures was not considered justifiable, as other factors rendered only big variations in yield of importance. After slight acidification to reduce bacterial activity, the solution was filtered, first through filter pulp, and then through a layer of kieselguhr on well boiled pulp. The clear solution was poured into plates and chilled, so as to make a jelly. The slabs of jelly were spread on netting, and dried off in a current of hot air. The dried cakes of gelatine were washed by soaking them in a number of changes of distilled water, and redried. The gelatine then contained 12% to 15% moisture, depending on atmospheric conditions. Beyond careful filtration and washing of the dried cake, nothing further was done to purify the gelatine, because of the practical difficulty of making large enough quantities, and because additional purification was thought to be unnecessary. It may be noted that most of the gelatine made was fairly pure, with a low ash content, and a small proportion of degradation products.

The swelling experiments carried out on skin in caustic soda solutions left no doubt that the maximum concentrations which could be used without destruction of the material was of the order of 1% or less. This narrowed the field of profitable investigation sufficiently for larger scale experiments to be carried out, in which gelatine was made from various materials at every stage of the soaking process. In the previous paper, one such experiment was described in which ox-hide was soaked in milk of lime. In the present instance, three different kinds of material were selected, the solution used being 1% caustic soda.

Shredded Rabbit Skin.

The first material chosen for these experiments was rabbit skin. This was the most convenient material to use because it was easily manipulated. Moreover, previous attempts to examine its behaviour in caustic soda had produced results of such inconsistency that much curiosity had been aroused as to what was really happening.

Nine lots of 200 grms. shredded rabbit pelts were soaked overnight in 2 litres 1% caustic soda solution. Next day the dirty liquor was strained off, the skin pressed and put back into 1½ litres caustic soda. On the third day the liquour was again strained off, and the skin pressed. These operations got rid of much of the superficial dirt and impurity. The skin was then replaced in one litre of caustic soda. Thereafter the caustic soda solution was changed weekly. Before extraction, the skin was washed until just faintly alkaline (bluish-green to B.D.H. Universal Indicator). One lot of skin was extracted each week, and the gelatine subjected to various tests, the results of which are shown in Table VII.

TABLE VII.

Time of Soak	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point	
					Acidity	Ph
1 week	3 Hrs.	19%	223	206	1·05%	4·88
2 weeks	2½ Hrs.	16·5%	175	175	1·35%	4·78
3 "	1 Hr.	17·4%	>255	334	1·22%	4·81
4 "	1 Hr.	16·0%	255	315	1·33%	4·78
5 "	1 Hr.	21·0%	230	266	1·53%	4·75
6 "	1 Hr.	18·0%	233	322	1·53%	4·75
7 "	¾ Hr.	18·2%	230	305	1·40%	4·77
8 "	¾ Hr.	22·8%	238	662	1·40%	4·77
9 "	½ Hr.	15·2%	>255	575	1·40%	4·77

When the extractions were made, the gelatine was found to be fairly clear, and there was only slight precipitation on acidification of the solutions. This slight precipitation disappeared entirely after the skins had been soaked for two or three weeks. It will be recollected that dullness gave rise to considerable difficulty in earlier experiments, so that the precautions taken to clean the skins at the start of the experiment were effective. The clarity of the finished samples of gelatine improved with length of soak, an indication that the substances causing dullness were removed during the soaking period.

The jelly strength and the yield remained constant throughout the experiment. This is a characteristic of rabbit skin, and makes it less useful than other materials for an experiment of this kind. In the one case where the strength was poor, some decomposition of the gelatine must have taken place during manipulation.

The time of extraction, however, gradually fell from start to finish, so that there was no doubt that the material gradually became more easily hydrolysed.

After 5 weeks, the iso-electric point reached its maximum at the same acidity as would have been obtained with a lime-soak, and thereafter did not appear to rise. Caustic soda was thus acting in the same way as lime, but rather faster. Considering that the concentration of the caustic soda was very much greater, the acceleration of the soaking process was much less than would have been anticipated.

Although the viscosity of the various gelatines fluctuated, there was a well marked tendency for the viscosity to rise as the time of soak increased. Actually, the viscosities obtained were all high compared with gelatine as generally obtained commercially.

Dried Sinews.

The next material to be examined was dried sinew. This was interesting because it consisted more entirely of collagen than skin. The sinews were crushed before the experiment, in order to enable them to be soaked more easily. Nine lots of 200 grms. of sinews were soaked in 4 litres each of 1% caustic soda solution. The solution was changed weekly. Each week, one lot was removed and extracted after being washed for two days in water. The results are shown in Table VIII.

TABLE VIII.

Time of Soak	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point	
					Acidity	Ph
		%			%	
1 week	4 Hrs.	17·5	175	211	1·13	4·85
2 weeks	5 Hrs.	23·0	156	147	1·28	4·80
3 "	2 Hrs.	10·0	223	625	1·38	4·78
4 "	5 Hrs.	25·1	190	268	1·45	4·76
5 "	3 Hrs.	33·7	255	536	1·53	4·75
6 "	1 Hr.	15·0	217	420	1·48	4·76
7 "	1 Hr.	10·0	>255	1150	1·51	4·75
8 "	4 Hrs.	33·7	220	658	1·66	-
9 "	1½ Hrs.	30·6	204	341	1·76	-

Gelatine prepared after a soak of two weeks showed a precipitate on acidification, and the dried cakes when melted up again in water gave rather dull solutions. This lack of clarity was not observed in the other samples, so that a soak of over two weeks cleansed the sinews thoroughly.

The swelling of sinews in caustic soda was very great, probably more than was observed to take place with skin, so that washing and extraction of the sinews was attended with considerable difficulty. As regards washing, the difficulty could have been surmounted by the cautious use of acid (HCl), but it was not known at the time that the small quantities required would not interfere with the position of the iso-electric point; consequently acid was not used in the washing process. In all the experiments, except for the sinews extracted after 2 weeks and 4 weeks soaking, the sinews were shrunk by heat before extraction, and the expelled water discarded. Had this not been done the gelatine solution would have been too dilute to dry off.

Although the yield appeared to increase as the time of soak increased, the large amount of swelling and the consequent need for discarding some of the surplus water caused rather more variation than usual in the ratio of water to material, which in turn caused irregular variations in the yield. The result was that the gradual increase in yield tended to be obscured by the irregular results.

For the same reason the extraction time showed irregular variations, but these were insufficient to conceal the general tendency for the time of extraction to fall as the time of soak increased.

The jelly strength reached a high value after three weeks soak, and was consistently good at any subsequent time.

The viscosity was subject to very irregular movements, but there seemed to be a tendency for this quantity to increase as the time of soak lengthened.

The iso-electric point rose fairly steadily from start to finish. After four weeks it had risen to 1·45%. It was not expected that it would continue to rise, but the acidity at which the gelatine was at its iso-electric point gradually rose until after 9 weeks soak it had reached 1·76%. This was contrary to numerous other experiments, thus an additional lot of sinews was soaked for ten weeks in 1% caustic soda. These produced gelatine with an iso-electric point of 1·85% acidity (Ph 4·75).

There could be no doubt, after examining the results of this experiment, that 1% caustic soda was behaving in exactly the same way as lime, but the process was taking place faster. The high iso-electric point obtained with long soaking obviously called for elucidation. If this happened generally when caustic soda was used, it pointed to a difference in the behaviour of caustic soda and lime, at least as regards the final end point, and was rather upsetting to any theory of gelatine of definite composition.

The difficulty of washing the much swollen sinews was thought to be the reason for the abnormally high acidity figures obtained in this experiment. These figures were also abnormal in the sense that they were higher than the Ph, as determined by the glass electrode, would have led one to expect. The gelatine showing the highest acidity for its iso-electric point had a normal iso-electric point, as shown by a Ph determination - namely, at Ph 4·75. Three additional experiments were carried out with 100 grm. lots of uncrushed sinews. These were soaked for relatively long periods of time in 1% caustic soda which was changed weekly. Use of a smaller quantity of material, and leaving the sinews whole, was intended to facilitate washing operations. At the end of the soaking period the sinews were washed, soaked carefully in small quantities of hydrochloric acid to bring them to their shrinkage point, and washed again. They were then extracted with the following results:-

Time of Soak	Time of Extraction	Yield %	Jelly Strength	Visc. Centipoises 20% at 40°C	Iso-Electric Point Acidity %
8 weeks	$\frac{3}{4}$ Hr.	30·0	247	489	1·40
11 weeks	$\frac{3}{4}$ Hr.	39·6	242	269	1·54
16 weeks	$\frac{3}{4}$ Hr.	27·9	251	179	1·58

Although in two of these experiments the time of soak was longer than in any of the previous experiments with 1% caustic soda, the acidity of the iso-electric point was at a normal value and showed no tendency to keep on rising. This bore out the necessity for careful washing of the material.

The strength of the gelatine was high and very uniform. The viscosity appeared to drop as the time of soak increased, but it happened that the acidity at which the gelatine was extracted was ·6%, ·74%, and ·98%. That is, the viscosity fell as the acidity of extraction rose. This was an interesting illustration of the sensitiveness of viscosity to small alterations in conditions, and gave a clue to some of the irregularities previously observed.

An effort was made to trace the source of the occasional errors in acidity, but without success. The most likely causes of trouble, carbonate or ammonia, were found to be eliminated during the washing process to which the gelatine was subjected. All that can be said, therefore, is that the acidity figure can be made reasonably reliable by sufficient attention to washing the precursor.

Wet Salted Ox Hide.

The third material on which a large-scale soaking experiment was carried out was wet salted ox-hide. One kilo lots of skin were washed to remove the salt and dirt. They were then soaked in lime for a day or so to enable the hair to be scraped off. The skin was cut up into shreds, about a quarter of an inch broad. It was then washed and soaked in 1% caustic soda solution. During the period of soak, the liquor was changed weekly. At weekly intervals, a lot of skin was removed, washed for two days and extracted. The results are shown in Table IX.

TABLE IX.

Time of soak	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point	
					Acidity	Ph
		%			%	
1 week	1½ Hrs.	4·0	242	320	1·00	4·91
2 weeks	1½ Hrs.	7·7	184	179	1·36	4·78
3 "	¾ Hr.	4·5	240	979	1·30	4·79
4 "	1 Hr.	7·2	220	183	1·46	4·76
5 "	1 Hr.	5·8	>255	217	1·51	4·75
6 "	½ Hr.	5·8	234	276	1·71	-
7 "	1 Hr.	6·5	251	284	1·68	-
8 "	½ Hr.	6·5	181	1152	1·45	4·76
9 "	½ Hr.	7·6	225	279	1·41	4·77

Examination of the results showed that this material was not very satisfactory for the purpose in view, probably because of the large amount of substances other than collagen present in variable amounts in the different samples. Whatever the reason, the properties of the gelatine obtained were found to diverge from the regular behaviour which other experiments had shown it was reasonable to expect.

The gelatine tended to be rather dull and cloudy on the whole, becoming clearer as the time of soak increased. However, this was one of the properties which exhibited irregularities. For example, the skins soaked for 7 weeks and 9 weeks looked very clean before removal from the caustic solution, and yielded clear gelatine after washing, while skins soaked for 6 weeks and 8 weeks showed a white suspension in the caustic liquour and yielded extremely dull gelatine on extraction. After soaking for 8 weeks, clear gelatine might have been expected, but the gelatine was so dull that it had to be clarified with sodium aluminate. This was also done with the gelatine prepared from skin soaked for three weeks, but at the earlier stages of the process, with this type of material, clear gelatine was not expected. The dullness appeared to be worse when caustic soda was used than when lime was used, and it may be that the taut condition produced by the much greater amount of swelling, allowed more impurity to remain in the skin in an emulsified condition. The irregular variations could only be due to the presence of varying amounts of foreign matter in the different pieces of skin.

The time of extraction fell regularly from start to finish of the experiment. It was noted, however, that even at the beginning the extraction time was short. In this respect the wet salted ox-hide differs from both dry rabbit skin and dried sinew. Possibly the fact that the ox-hide has never been properly dried may be the reason for this. Although no explanation can be given, it is known that material which has been dried is not so readily brought into suitable condition for extraction as material which has never been dried. The extraction time when 1% caustic soda was used was less than when lime was used.

The yield of gelatine remained practically the same throughout the experiment. It was better than on a previous occasion when the same material was treated with lime. There was no sign that the yield increased with length of treatment, except that the two lowest yields fell at the beginning of the experiment.

The jelly strength was high and did not give any indication of progressive increase as the time of soak lengthened, in this respect following the behaviour of dried rabbit skin. The same thing was noticed when ox-hide was soaked in lime, but it required a soak of three weeks for a high jelly strength to be attained. Although the jelly strength was higher when caustic soda was used than when lime was used, the difference was such as might be expected from a slight alteration in the conditions of extraction. In this case the determining factor was probably the more rapid rate of extraction which caustic soda apparently rendered possible.

The iso-electric point reached its normal position in four weeks, and thereafter did not rise. As had previously been shown, the iso-electric point reached its maximum acidity some time after the gelatine had reached its maximum strength.

Nothing was deduced from the viscosity figures, for these did not vary in any regular manner. Again, the use of sodium aluminate may have masked the real viscosity of the two samples of gelatine in which it was incorporated. These viscosities were very much higher than the others, which appeared to place the cause beyond doubt.

Summary.

The three experiments, the results of which have been discussed in detail above, indicated that caustic soda acted upon gelatine-producing material in the same manner as lime. In the concentration used (1%), the normal position of the iso-electric point was found for gelatine soaked for four weeks, as against six to nine weeks for lime (p. 78 , Table XIII; and Thesis, Edin., 1941, p. 30, Table VII). This did not show caustic soda to be a particularly active substance, considering the relatively high concentration used.

Another point which the experiments made clear was that neither rabbit skin nor ox-hide were very suitable materials to use, as they did not exhibit the progressive nature of the soaking process clearly, except with regard to the position of the iso-electric point. For this reason, subsequent work was carried out with dried sinew which, although more difficult to handle, gave more uniform results.

Comparison of Caustic Soda and Lime as Soaking Agents.

In order to compare more closely the effectiveness of caustic soda as a soaking agent against lime, it was decided to carry through an experiment with a lower concentration of caustic which might be expected to act at about the same speed as lime. For this purpose it was thought that a concentration of ·5% would be effective.

Accordingly, ten lots of 200 grms. sinew were each soaked in 6 litres ·5% caustic soda solution. The solution was renewed weekly, and except for a break in the middle of the experiment where conditions remained stationary for some time, a sample was removed each week , washed, and extracted. The results are shown in Table X.

TABLE X.

Time of Soak	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point	
					Acidity	Ph
		%			%	
1 week	6 Hrs.	12·7	152	133	·81	5·06
2 weeks	5 Hrs.	13·8	181	168	1·06	4·88
3 weeks	3 Hrs.	12·7	173	168	1·06	4·88
4 weeks	4 Hrs.	14·2	182	174	1·25	4·81
5 weeks	4 Hrs.	17·7	198	263	1·38	4·78
9 weeks	3 Hrs.	21·3	198	248	1·35	4·78
10 weeks	3 Hrs.	15·4	234	346	1·53	4·75
11 weeks	4 Hrs.	15·1	194	182	1·36	4·78
12 weeks	4 Hrs.	21·4	181	222	1·50	4·75

The gelatine obtained from the first extraction went dull on acidification. The gelatine from the second lot gave a very slight precipitate on acidification. Thereafter the gelatine obtained was reasonably clear and showed no further improvement during the course of the experiment.

The time of extraction was high to begin with and never fell very much. Actually, the time of extraction at any stage of the soaking process was much longer than was later found necessary for the same material when soaked in lime.

The yield, although subject to a certain amount of variability, gradually increased as the soaking proceeded.

The jelly strength did not reach a high level until the material had been soaked for five weeks. A comparison of the values for jelly strength with those obtained later from the same material soaked in lime showed that the advantage lay with lime. This could be accounted for by greater degradation of the precursor during the longer time of extraction. As, however, the method of extraction was designed to obtain gelatine of the best attainable jelly-strength, no matter what the conditions, the relatively lower jelly-strengths exhibited by the samples prepared by the use of ·5% caustic soda suggested that the concentration used was below what was necessary to produce good results. Thus the concentrations which could be used in practice fell within very narrow limits, being restricted to below 1% in order not to destroy the material, and to above ·5% in order to produce results at all.

The viscosity figures, while subject to certain irregular variations, showed a definite tendency to rise with increasing time of soak. In magnitude these viscosities were much lower than corresponding values obtained from a series of gelatines prepared by the use of lime.

The iso-electric point at the start of the experiment rose quickly to a value of about 1% acidity. At this stage the movement towards the acid end of the scale was faster than would be produced by lime. It required five weeks soaking for the iso-electric point to reach its final normal position. In the later experiment with lime, this was accomplished in six weeks.

When the results of this experiment were considered as a whole, particularly when they were compared with an exactly similar experiment with lime, it became clear that a ·5% solution of caustic soda was less effective as a preparatory reagent in the making of gelatine than lime. In other words, the value of caustic soda lay in the possibility of increasing the concentration of dissolved alkali. When using lime, the effectiveness of the alkali was restricted by the very small amount in solution. Clearly when the two substances were considered at similar concentrations, caustic soda was much inferior to lime. In making this comparison, due allowance was made for the fact that as the lime in solution was used up, undissolved lime was always present to take its place. In the case of caustic soda solutions, the caustic soda was only at its full strength at the start of each week. The drop in concentration was not, however, large, and the unused caustic soda was still in greater concentration than was possible with lime.

The conclusions reached with regard to the efficiency of caustic soda as a preparatory reagent for the extraction of gelatine are supported by previous observations. Kuntzel & Philips (Collegium 1933, 193, 207, 213) stated that the conversion of collagen to gelatine is more readily effected by the use of lime than by caustic soda. Lime is also a better solvent for proteins other than collagen. This is a possible explanation of the difficulty of obtaining clear gelatine encountered when using caustic soda, although clarity can generally be attained by frequent changes of the soaking reagent and by extension of the period of soak. Kubelka & Knodel (Collegium 1938, 49) state that hide powder is hydrolysed by alkalis according to their concentration and duration of action. The effect of saturated lime water is greater than the equivalent concentration of caustic soda. They also note that lime has a greater sterilising effect than caustic soda in low concentrations, in preventing bacterial decomposition of the material. The maximum concentration of caustic soda which can be used without loss of material is given as ·4 N - that is, 1·6%. The figure suggested as a result of the swelling experiments described in the earlier part of the present paper was 1%, and as the concentrations studied moved up by jumps of 1%, it is quite possible that 1·6% would be a safe figure, but it must be very close to the limit.

The Use of Mixtures of Lime and Soda Ash in the Preparation of Gelatine.

As was pointed out in connection with swelling experiments, it is not advisable to use caustic soda alone, as it does not keep down the growth of bacteria in the same way as lime. Also, in order to carry out the operation of soaking faster than with lime, considerable quantities of caustic would be required. Swelling experiments having given an indication that the use of caustic soda along with lime was additive where swelling was concerned, it appeared likely that the combination of the two substances would accelerate in the same manner the preparation of material for extraction of gelatine. The caustic soda was applied in the form of soda ash for two reasons. Firstly, soda ash is much less expensive than caustic soda. Secondly, there was always the possibility that caustic soda at the moment of formation from lime and sodium carbonate might be more potent than a solution made from the dissolved alkali. By using milk of lime, an excess of lime was assured at all stages of the soaking process.

Dried Sinew in 1% Soda Ash Solutions & Lime.

Eight quantities of 200 grms. crushed sinews were each soaked in 6 litres of 1% soda ash solutions containing a suspension of lime. The temperature at which this experiment was carried out was kept between 20°C. and 22°C. The liquor was as far as possible changed each week but this rapidly became impossible owing to the magnitude of the swelling. A lot of sinews was washed at the end of each week, and gelatine extracted. At the end of three weeks the material had become very soft, and washing was extremely difficult; at the end of four weeks the material was breaking up and the experiment had to be discontinued. The results obtained are shown in Table XI

TABLE XI.

Time of Soak	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point Acidity	Iso-Electric Point Ph
		%			%	
1 week	2½ Hrs.	17·0	204	144	1·1	4·86
2 weeks	1¼ Hrs.	16·5	>255	661	1·26	4·80
3 weeks	½ Hr.	14·7	255	84	1·58	4·75
4 weeks	1½ Hrs.	27·0	>255	533	1·58	4·75

The gelatine obtained from the first extraction was dull, owing to lack of purification. After two weeks the clarity of the gelatine was better but it became poor again after three and four weeks, owing to the impossibility of washing the swollen and partly-degraded material. This was noted as a point of considerable importance. In this experiment the concentration of chemicals used and the temperature of soak were such as to enable gelatine to be extracted after a short soaking period but it appeared difficult, if not impossible, to separate the gelatine-yielding material from the emulsified impurities.

During the first three weeks, the time of extraction rapidly fell and the rate of extraction increased. The reason for the break in this sequence in the fourth week was a practical one. At this stage the sinews were very much swollen, and even after washing still held a large quantity of water. This resulted in an abnormallly large quantity of water being used for the last extraction. Hence, in order to obtain a jelly which would be firm enough to dry, it was necessary to extract more gelatine, which required a longer time. The rate of extraction was less with the larger amount of water.

For the same reason the last extraction had to be considered abnormal as regards yield. In the other three extractions the yield was rather lower than might have been expected. (20% to 25% was taken as a normal value for the method of extraction used). The low yield, however, might have arisen from the exceptional strength of the gelatine, which enabled the time of extraction to be short.

The jelly strength was good, even after a soak of only one week, and was outstanding for the other three extractions. Even in this greatly accelerated soak, the jelly strength reached a high figure before the iso-electric point had reached its normal value. This was another instance of a more rapid approach to high jelly strength, which was noted in the original experiment carried out with lime.

Nothing can be said about the viscosity figures, which varied with no regularity. It was possible that this was due to the varying alkalinity at which the extractions were carried out. Owing to washing difficulties the alkalinity of the sinews at the time of extraction would vary very considerably.

The iso-electric point moved to its normal position rapidly. It was already fairly high after one week's soak, and reached its highest position in three weeks. Lime at the same temperature required six weeks, so that the addition of the 1% soda ash solution shortened the time of soak by 50% as measured by the time required for the iso-electric point of the gelatine to reach its normal value.

The rapid action of the mixture of soda-ash and lime in this experiment was unexpected. In particular, the destruction of the material, after a relatively short period of soak, had not been previously observed with this concentration of soda ash and lime, which as a result of the preliminary swelling experiments had been supposed to fall within safe limits. It was concluded that the reason for the increased reactivity was the rather high temperature at which the experiment was carried out. This illustrated the need for experiments to be carried out in which the influence of temperature upon soaking was investigated.

INFLUENCE Of TEMPERATURE In The PREPARATION Of MATERIALS For The EXTRACTION Of GELATINE.

The experiments hitherto carried out on soaking had not, on account of practical difficulties, been carried out at a controlled temperature, except for the one just described. It was fully realised and known from large-scale manufacturing operations that temperature did influence the rate at which material was brought into suitable condition for the extraction of gelatine. This became evident during a series of long cold winters, when it was noticed that material soaking in positions exposed to the weather required a very much longer time to become ready for extraction. During hot weather, material soaking outside showed a tendency to break up if left soaking too long. The differences, however, during most years when the weather was neither abnormally hot nor unusually cold passed unnoticed.

References in literature to the effect of temperature on the soaking of skins show that this factor was appreciated by the tanners although the purpose of their soaking was restricted to depilation and the initial plumping brought about by lime. Coombs Swinbourne & Gabb in a paper on fell mongering and tanning of sheepskin (J.S.C.I., 1916, 35, 227) mention that while sheepskins are ordinarily left in lime for four to eight days after removal of the wool, they are left in one day extra when the weather is cool. McLaughlin & Theiss (J. Amer. Leather Chem. Assoc., 1925, 20, 246) state that the use of warm water pools after liming reduces the difficulty in unhairing, and produces a smooth grain.

Merrill & Fleming (J.I.E.C., 1928, 20, 21) studied the relative rates of hydrolysis of hair and skin at different temperatures, again with a view to obtaining more exact knowledge of the process of depilation. At temperatures below 35°C. the hair was more hydrolysed than the skin after a three days soak. At higher temperatures the reverse was the case. The temperatures used in the experiments were rather high to be of interest for the preparation of gelatine, but there can be no doubt from the results that between 30°C. and 45°C., the rate of the action of lime on skin increased very much.

More valuable and interesting information is furnished in a series of papers by Please dealing with the influence of temperature on the liming of oxhides and goatskins (J. Soc. Leather Trades Chemists, 1937, <u>21</u>, 89, 208, 308, 476). Please shows that the water absorption and loss of nitrogen by the hide increase as the temperature is raised from 0°C. to 25°C. A larger period of soak, namely 26 days, renders these results more definitely applicable to the preparation of gelatine. Moreover, the loss of nitrogen, as will be shown later, is an alternative way of following the progress of the reaction with lime during the soaking period. The water absorption which is measured by increase in weight is another means of following the swelling of the hide, which is thus also shown to be dependent on temperature.

Kuntzel & Koepff (Collegium 1938, 433) actually made gelatine from hide soaked in lime at 0°C., 15°C. and 30°C. Their results show that the soaking process proceeds more quickly at the higher temperatures, although the properties used to characterise the gelatine obtained, and their method of soak, make it difficult to assess the influence of temperature, except in a general way. There are indications that soaking at 30°C. does not give satisfactory results, the action on the hide being too vigorous.

While certain information was thus available as to the influence of temperature on the preliminary lime soak, it was decided that more useful knowledge might be obtained by extending our investigations to soaking at controlled temperatures.

Soaking Experiments at 2°C. and 20°C.

The effect of temperature during the soaking period upon the quality of the gelatine produced was demonstrated by two simple experiments on osseine. Two lots of osseine were soaked in milk of lime for ten weeks, one lot being kept at a temperature of 15°C. - 18°C., the other at 2°C. After soaking, both lots were washed and gelatine extracted by heating on a water bath at 80°C. The experiment was repeated on two other lots, and the following results were obtained:-

Temp. of Soak	Time of Extraction	Yield	Jelly Strength
15°C. - 18°C.	½ Hr.	4½%	210
2°C.	1 Hr.	3%	182
15°C. - 18°C.	½ Hr.	12½%	234
2°C.	1 Hr.	13%	181

In the first experiment, the osseine prepared in the cold required a longer time of extraction, gave a lower yield and a gelatine of lower jelly strength. In the second experiment (which must have been carried out with a different lot of osseine), the same differences were evident, except that the yield was the same from both lots of material. This simple experiment, although carried out before the more complete technique of following the position of the iso-electric point had been adopted, showed quite definitely that the reactions involved in soaking did not proceed as well in the cold as under warmer conditions.

Dried Sinew in 1% Soda Ash and Lime in the Cold.

The importance of temperature was shown in a striking manner by repeating the experiment carried out with sinews at 20°C. - 22°C., at a temperature of 2°C. Eight lots of 200 grms. crushed sinews were soaked in 6 litres of 1% soda ash solution to which was added an excess of lime. During the soaking period the containers were kept in an ice store, the temperature of which remained at 2°C. The liquor was changed each week. At appropriate times a lot of sinews was washed, and gelatine extracted. Owing to the large amount of swelling caused by the soda, it was necessary in every case to shrink the sinews by heat before extraction, discarding the water expelled. The results are shown in Table XII.

TABLE XII.

Time of Soak	Time of Extraction	Yield	Jelly Strength	Visc. centipoises 20% at 40°C.	Iso-Electric Point Acidity	Iso-Electric Point Ph
		%			%	
1 week	5 Hrs.	11·2	108	136	·70	5·22
2 weeks	5 Hrs.	21·2	146	111	·73	5·17
3 "	4 Hrs.	18·4	144	183	·85	5·01
5 "	2 Hrs.	17·1	176	272	1·03	4·89
6 "	1½ Hrs.	23·3	200	494	1·15	4·84
7 "	1½ Hrs.	16·4	220	463	1·11	4·86
10 "	1 Hr.	24·3	227	371	1·23	4·81
11 "	1 Hr.	22·0	>255	403	1·36	4·78

No trouble was experienced at any stage of the experiment with dullness in the gelatine. It seems as if the gentle nature of the action induced by the low temperature prevented the emulsification of impurities which caused the dullness so often noted when caustic soda was used, and particularly in this same experiment when carried out at a higher temperature. Possibly that is also the reason why opalescent precipitates are hardly ever obtained when lime is used. It may simply be that the gentle action of lime does not emulsify the impurities to the same extent, and consequently they can be more completely washed out.

The time of extraction gradually diminished as the length of soak increased, to a final value of the same order as was reached when the chemicals acted at a higher temperature. Thus it was evident that the sinews were being reacted upon in the cold, although much more slowly.

The yield also showed the usual tendency to increase with lengthening soak, although this figure, as in other cases, exhibited irregularities.

The jelly strength was poor in the early stages of the experiment, and it required six weeks to attain a reasonably high value, compared with one week at the higher temperature. Subsequent strengths were all very good. This experiment provided another example of the way in which good strength may be attained before the iso-electric point has reached its normal position.

The iso-electric point had not reached its proper value at the end of eleven weeks soaking, although it gradually rose all through the experiment. With the temperature at 20°C. - 22°C., this happened after three weeks.

The viscosity figures were rather more regular than usual and indicated a probable steady rise as the time of soak lengthened.

The importance of this experiment lay in its illustration of the important part played by temperature in the soaking of material for the preparation of gelatine. A mixture of lime and soda ash, which enabled gelatine of good quality to be extracted after the very short soaking time of only a week at a temperature of 20°C., required six weeks at 2°C. Again, at 20°C. the normal position of the iso-electric point was reached in three weeks, whereas at 2°C. it was not reached at all during the period of observation which extended to eleven weeks. Moreover, at the higher temperature the material was destroyed in a little over four weeks.

Dried Sinew in Milk of Lime.

At this stage of the experiments it was thought that the behaviour of sinews in milk of lime alone, at a temperature of 20°C. - 22°C., would provide a very convenient basis of comparison. Accordingly eight lots of 200 grms. crushed sinews were soaked in milk of lime. The liquor was changed weekly. At appropriate intervals, a particular lot of sinews was removed, washed for two days and extracted. From the fourth week onwards the sinews were so swollen that they had to be shrunk by heat before extraction, and the water expelled discarded. The last three lots were, in addition, shrunk during washing by careful use of acid. It was known that use of acid in this way did not interfere with the final results. These are shown in Table XIII.

TABLE XIII.

Time of Soak	Time of Extraction	Yield	Jelly Strength	Visc. centipoises 20% at 40°C.	Iso-Electric Point	
					Acidity	Ph
		%			%	
1 week	5 Hrs.	11·4	116	117	·61	5·38
2 weeks	5 Hrs.	14·3	166	159	·87	5·00
4 "	2 Hrs.	12·8	204	224	1·18	4·83
5 "	1 Hr.	15·6	207	401	1·16	4·84
8 "	1¼ Hrs.	28·4	232	287	1·43	4·76
9 "	1 Hr.	22·5	255	280	1·40	4·77
10 "	1 Hr.	24·0	255	659	1·43	4·76
11 "	1 Hr.	27·8	218	49	1·57	4·75

This experiment provided a good demonstration of the progressive nature of the preparatory soaking process for the extraction of gelatine. All the characteristics noted in the numerous experiments of this kind were clearly marked.

At no stage of the soak in lime was there any tendency for dullness to appear in the gelatine, which might have been expected at the higher temperature at which the soaking was carried out. This differentiated lime from the mixture of lime and soda, which produced dull gelatines at every stage at the higher soaking temperature.

The time of extraction dropped as the soak progressed. It reached its minimum fairly rapidly before the gelatine had attained a normal iso-electric point. This had also been found to be the case when skin was soaked in lime (see Table VII, Thesis, Edin. 1941).

The jelly strength reached a reasonably high figure after four weeks soak and thereafter was consistently good. Once again this provided confirmation that a gelatine of good quality can be extracted although of abnormally low iso-electric point, for a good jelly strength was attained several weeks before the gelatine showed an iso-electric point in the normal position.

The results for yield, jelly strength, and the position of the iso-electric point may be interpreted in another way. When curves are drawn showing strength, rate of extraction, and the acidity of the iso-electric point, against time of soak, they take the form of two straight lines the intersection of which represents the time at which the values become constant. When this was carried out for the present series of results it was found that the maximum strength and the normal position of the iso-electric point were attained in six weeks, and the maximum rate of extraction in eight weeks: from which it would appear that the different values follow each other more closely than might have been supposed. Use of this method of interpretation does not invalidate the main conclusions arrived at by consideration of individual results, although it does introduce differences in detail.

The viscosity figures showed, as previous results had taught us to expect, a certain amount of variability. Neglecting the last result which was sufficiently different from the remainder to indicate some sort of bacterial attack, it appeared that viscosity tended to rise as the time of soak increased.

The iso-electric point reached its maximum position after eight weeks (six weeks using a curve), and thereafter remained constant. In the previous experiment with skin the time required was nine weeks. When the results were compared with those in Table XII, it was evident that lime by itself was more efficient at 20°C. than the mixture of lime and 1% soda at 2°C. On the other hand, at 20°C. the lime and 1% soda mixture destroyed the sinews rapidly. This was a clear illustration of the very great importance of temperature in the preparation of material for the extraction of gelatine. The mixture of lime and 1% soda was barely satisfactory at a temperature of 2°C. Even assuming that the difference between this mixture and lime was less at low than at high temperatures, it was evident that lime would produce very poor results at low temperatures. In actual fact it would hardly act at all. This had been shown to be the case in the early experiments on osseine. In the preparation of gelatine on a large scale this temperature effect is one of very great importance, and it will be necessary to revert to it after a description of one more experiment with lime and soda ash.

Dried Sinews in ½% Soda Ash Solution and Milk of Lime.

In the previous experiment at 20°C. on a mixture of soda ash and lime, a concentration of 1% soda ash was found to be too great, with the result that the material was rapidly destroyed, and comparison with the behaviour in lime alone at the same temperature was thereby prevented. It was therefore decided to try a mixture of ½% soda ash solution and milk of lime at 20°C. in order to see how this compared with lime alone, and to decide if that would be a safe mixture to use at such a relatively high temperature.

Accordingly, nine lots of crushed dried sinews were each soaked in 5 litres of milk of lime containing 25 grms. soda ash. The liquor was changed weekly until this became too difficult, owing to the breaking up of the material. At weekly intervals, a lot of sinews was removed, washed for two days, and extracted. The last four lots could only be washed with the aid of small quantities of hydrochloric acid to bring about shrinkage. Heat shrinkage could not be used, as most of the gelatine would have been discarded in the shrinkage water. The gelatine made from the seventh lot was lost in drying. The eighth and ninth lots were combined, as the loss in washing was very considerable, and what remained of the separate lots would hardly have provided sufficient gelatine for the necessary determinations. The swelling produced by this combination of soda ash and lime was very great, and towards the end of the experiment the material became very broken up. The results obtained are shown in Table XIV.

TABLE XIV.

Time of Soak	Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point Acidity	 Ph
		%			% H_2SO_4	
1 week	4 Hrs.	20·2	149	164	·86	5·01
2 weeks	2 Hrs.	15·0	178	196	1·03	4·89
3 "	1½ Hrs.	18·3	230	485	1·25	4·81
4 "	1½ Hrs.	17·5	238	455	1·38	4·77
5 "	1½ Hrs.	28·7	255	458	1·37	4·77
6 "	1½ Hrs.	15·8	>255	281	1·50	4·75
8 "	Heated to 70·C.	5·7	>255	888	1·47	4·75

The gelatine obtained from this series of extractions was not so clear as that obtained when lime was used alone under the same conditions of temperature. The clarity was best after 4 and 5 weeks soak and was at its worst after 6 and 8 weeks soak. This was due to the difficulty of washing the broken up and soft material. With a combination of 1% soda ash and lime at 2°C., the gelatine obtained was as clear as that obtained by the use of lime alone, so it must be concluded that even moderate rises in temperature will prevent clear gelatine being obtained when soda ash is used during the preliminary soaking of the material.

The time of extraction dropped very rapidly as the soak progressed, and reached a minimum, except for the last extraction, after 3 weeks soak. The last lot of gelatine was extracted by merely raising the temperature to 70°C., the material being excessively soft. In the case of lime alone, 5 weeks soak was required for the extraction time to reach a minimum, so that there was a definite sign of the soda ash quickening the soaking process.

The jelly strength steadily increased with length of soaking, reaching a high figure after the short period of three weeks. This was some time before the iso-electric point had reached its final position. There was distinct evidence that use of soda ash along with lime had more effect on the rapid attainment of a good strength than on the speed with which the iso-electric point reached its final value.

The viscosity figures, although subject to irregular variations, showed definite signs of a rise as the time of soak increased. The difference in this experiment between the start and the finish is very marked.

The iso-electric point rose rapidly at the start, reaching a value of 1·25% acidity in three weeks; thereafter the progress to the final value was slower. This was reached in five or six weeks. Lime alone required eight weeks. By the time the iso-electric point had reached its maximum the material was very swollen and broken up.

This experiment showed the effect of soda ash on the rate of soaking. The actual shortening of the soaking time was not so very much, and smaller concentrations of soda ash would probably make very little difference. The behaviour of the relatively high concentration of soda ash used in this experiment showed how earlier experiments with very low concentrations were bound to fail. Two disadvantages of the use of soda ash at higher temperatures were very evident. These were the large amount of swelling and the dullness in the gelatine extracted. In addition, it could hardly be said that soda ash in combination with lime was safe to use at the temperature of the experiment, on account of the rapidity with which the material broke up - almost before the iso-electric point reached its final fixed position.

Summary of Soaking Experiments on Sinews.

It is difficult to appreciate the striking nature of the figures obtained from the six soaking experiments on sinews, without having certain of the results shown together in condensed form. Accordingly, in Table XV, the iso-electric points and jelly strengths are shown for all the experiments. In every case a good jelly strength is obtained before the maximum position of the iso-electric point is reached. This is in agreement with the results of the first progressive soaking experiment described in the previous paper. Although the two properties behave in this way, their variation takes place,as it were, on parallel curves. That is, if any particular method of soaking produces a quick rise in jelly strength it will also produce a quick rise in the position of the iso-electric point. The maximum jelly strength is generally attained at the same time as the iso-electric point reaches its final position. The only exception to this rule is the mixture of 1% soda ash and lime, which gives high jelly strength before the final iso-electric point is reached. It does this both in the hot and the cold. In other words, using this combination of chemicals for soaking, more effect is produced on the jelly strength than is the case with other chemicals.

TABLE XV.

Time of Soak (Weeks)	1% Caustic Soda	·5% Caustic Soda	Lime 1% Soda Ash 20-22°C.	Lime 1% Soda Ash 2°C.	Lime ·5% Soda Ash 20-22°C.	Lime 20-22°C.
	Iso-Electric Points % Sulphuric Acid.					
1	1·13	·81	1·1	·70	·86	·61
2	1·28	1·06	1·26	·73	1·03	·87
3	1·38	1·06	1·58	·85	1·25	-
4	1·45	1·25	1·58	-	1·38	1·18
5	1·53	1·38	-	1·03	1·37	1·16
6	1·48	-	-	1·15	1·50	-
7	1·51	-	-	1·11	-	-
8	1·66	-	-	-	1·47	1·43
9	1·76	1·35	-	-	-	1·40
10	-	1·53	_	1·23	-	1·43
11	-	1·36	-	1·36	-	1·57
12	-	1·50	-	-	-	-
	Jelly Strength.					
1	175	152	204	108	158	116
2	156	181	255	146	176	166
3	223	173	255	144	207	-
4	190	182	255	-	212	204
5	255	198	-	176	220	207
6	217	-	-	200	228	-
7	255	-	-	220	-	-
8	220	-	-	-	221	232
9	204	198	-	-	-	255
10	-	234	-	227	-	255
11	-	194	-	255	-	218
12	-	181	-	-	-	-

The results also show that caustic soda by itself or in the latent form as produced by the interaction of lime and soda ash has exactly the same effect upon gelatine-producing materials as lime. A 1% caustic soda solution is faster than lime, whereas a ·5% solution is slower. The general belief that caustic soda can be used to quicken up the soaking process is thus quite correct, but its truth depends on the high concentration of alkali obtainable and not upon any special virtue of caustic soda. As far as can be seen from our earlier experiments and from this series, in low concentration caustic soda is less efficient than lime.

The important influence of temperature is very clearly demonstrated by these experiments. At a temperature not much above average room temperature, lime and 1% soda ash cause the soaking to proceed at such a rate as to be almost uncontrollable. At 2°C., the same combination does not succeed in raising the iso-electric point to its normal position in eleven weeks, and six weeks is required for the attainment of a reasonably good jelly strength. As lime alone causes the reaction to proceed much more slowly at the higher temperature, at a temperature of 2°C. lime will accomplish practically nothing at all.

The practical use of caustic soda alone or in combination with lime is greatly restricted by two factors which are not deducible from the figures shown in the table. The first of these is the much greater amount of swelling produced in the precursor, and the second is dullness in the gelatine. Both factors are greatly exaggerated with rise in temperature. For this reason it is certain that caustic soda can not be used at anything but low temperatures, and it is somewhat doubtful if it could be made thoroughly successful even then. However, it has been ascertained definitely that there is less swelling in the cold than at higher temperatures, and it has also been shown that clearer gelatine can be obtained from material soaked at a lower temperature. The cause of the dullness appears to arise from emulsification of impurities in the precursor by caustic soda. Lime either does not do this,or to a much lesser extent. The swollen condition of the material prevents the emulsified materials from being washed out. In fact, the washing process, as it reduces the concentration of alkali, may cause precipitation in the material itself. During the extraction, more precipitation takes place, and again when the gelatine is acidified. The resultant precipitate can not be properly filtered out without using chemical means of coagulation such as sodium aluminate which reduces the strength of the gelatine and raises the ash content.

Instead of using caustic soda, another alternative method of shortening the time of soak is to raise the temperature. Use of lime alone at 20°C. results in a fairly quick soak without the disadvantages of caustic soda. The use of even higher temperatures is possible but requires further investigation. No doubt the maximum temperature will be limited by the point at which heat would bring about shrinkage of the swollen material, as this no doubt represents some other and possibly indeterminable change.

Lime Soaking at Higher Temperatures.

The soaking experiments which could be carried out at temperatures above 20°C. were restricted in scope by the necessity of using an incubator of limited capacity. Accordingly it was decided to fix upon 30°C. and 40°C. as being the most suitable temperatures to try. The material used was dried cattle sinews from the same lot as had been used to carry out the other experiments. The results obtained are shown in Table XVI.

TABLE XVI.

Temp.	Time of Soak	Time of Extraction	Yield	Jelly Strength	Iso-Electric Point Acidity %
			%		
30°C.	7 days	6½ Hrs.	31·5	-	1·08
30°C.	24 days	1½ Hrs.	11·5	207	1·50
30°C.	24 days	2½ Hrs.	9·3	160	1·50
40°C.	7 days	6 Hrs.	8·0	-	1·31
40°C.	16 days	4½ Hrs.	7·1	-	1·44

Three experiments were carried out at 30°C. It was observed that at this temperature practically no swelling took place. The sinews also disintegrated on the surface, which was undesirable, as material which would otherwise have been capable of yielding good gelatine was being completely hydrolysed during the initial lime soak, and lost. After a short soak of seven days the length of the extraction time showed that the material was not ready for extraction. The yield in this experiment was good because there had not been time for the lime to damage the sinews. The iso-electric point had moved up to a position not much below normal. In the first experiment with 24 days soaking, gelatine of good quality was obtained and the iso-electric point had reached the normal position; in the second 24 day experiment, gelatine of poor quality resulted, although the position of the iso-electric point was normal. The yield in both cases was rather low. All the gelatine made after soaking at 30°C. was dull. Thus the soaking and subsequent washing had failed to remove the organic impurities which were the source of the dullness.

The sinews soaked at 40°C. produced even less promising results. As far as could be seen, there was no swelling but the material was almost at once attacked on the surface. At the end of the longer soaking period the sinews were very badly broken up, large quantities of collagen having dissolved in the liquor. In spite of the disintegration of the sinews a relatively long time of extraction was required. In both experiments, gelatine was obtained with iso-electric points at the normal position, but the jelly strength was poor. The yield, as might have been anticipated from the condition of the precursor after soaking, was also low. Both gelatines were very dull.

It was concluded from these experiments that higher temperatures were not satisfactory for the preliminary lime soak, which was best carried out at temperatures not exceeding 22°C. This conclusion had not been expected. As the temperature was raised from 0°C. to 20°C., the rate of swelling and the speed with which the precursor became suitable for extraction gradually increased. Accordingly, it was assumed that as the temperature was raised still further the process would continue to accelerate, and the only limit would be when the reaction became so fast as to be uncontrollable. The behaviour of sinew in milk of lime at 30°C. showed that between 20°C. and 30°C. there was a break in continuity of behaviour. The sinews disintegrated without swelling, and although the gelatine obtained had a normal iso-electric point, it tended to have a poor jelly strength. The action of lime on collagen at these higher temperatures was therefore different from what happened at temperatures up to 20°C., and was not what was required for the preparation of gelatine. It almost seemed as if a stage in the process was missed, and the lime hydrolysed the sinew directly into less complex forms than gelatine.

A certain amount of loss of collagen did take place during a long soak in milk of lime at lower temperatures, but the mechanism of this loss appeared to be different. Sinew became very swollen and in time slimy on the surface. Under these conditions the slimy parts of the sinew were gradually passing into solution. At temperatures of 30°C. and 40°C., the sinew disintegrated without swelling, and with no surface slimyness. They tended rather to become brittle. Thus the visible effects of hydrolysis confirmed the view that a different reaction took place at temperatures above a certain limit.

Heat Shrinkage of Lime Soaked Collagen.

A property of hide or sinew which might have suggested the inadvisability of carrying out the preparatory liming process above a certain temperature is the shrinkage which takes place on exposure to heat. This is also of interest during the actual extraction when working with well plumped material, for the water which is exuded during the shrinkage is frequently an embarrassment, as it dilutes the gelatine extracted, and in certain experiments it was found necessary to discard a portion of the shrinkage water and start the extraction afresh. Powarnin & Aggeew (Collegium 1924, 198) investigated this phenomenon with a view to its use in controlling tanning operations. They attached strips of hide to the bulb of a thermometer immersed in water and noted the temperature when shrinkage took place. They found that raw ox-hide showed shrinkage between 62°C. and 68°C. Subjecting the hide to the action of lime caused the shrinkage temperature to fall, until after 21 days it was down to 42°C. It was thought that the shrinkage temperature of fully limed sinew or hide might be even lower, and in that case swelling during liming at temperatures of 30°C. and 40°C. would be inhibited. Moreover, it would then be clear that a new and distinct effect was taking place which did not happen at lower temperatures, in which heat attacked at least the physical structure of the material. Accordingly, a number of shrinkage experiments were carried out with fully limed sinew and ox-hide.

A piece of fully limed sinew of suitable size (approximately 2" in diameter and 2" long) was cut from a complete sinew. This was carefully washed until the Ph was between 7 and 8, in order to prevent excessive loss by hydrolysis at higher temperatures. The washed sinew was suspended by a thin wire in water contained in a litre measure, which enabled the volume of the sinew to be measured at any time by displacement. The cylinder containing the sinew was placed in a water bath fitted with a thermostatic control. A similar experiment was carried out with pieces of fully limed dehaired ox-hide (approximately 2" by 6"). The material was kept at a given temperature for as long as shrinkage was observed, and readings taken at intervals. The results are shown in Table XVII.

In every case a small amount of gelatine went into solution during the heating, but not in sufficient quantity to interfere materially with the results. Sinew heated at 60°C. shrank almost instantaneously; at 50°C. and 40°C. the shrinkage took longer to reach its maximum. At 35°C. the shrinkage after 6 hours heating, though definite, was very much less. No shrinkage could be detected at 30°C.

Limed ox-hide showed similar behaviour, except

TABLE XVII.

Temp. °C.	Time	Shrinkage as %age of original volume	Temp. °C.	Time	Shrinkage as %age of original volume
Sinew.					
60	10 mins.	44	40	1 Hr.	36
60	10 mins.	60		2 Hrs.	43
50	5 mins.	23		3 Hrs.	50
	10 mins.	31	40	30 mins.	33
	30 mins.	39		1 Hr.	42
	45 mins.	46		1½ Hrs.	50
50	5 mins.	42	35	1 Hr.	Nil.
	10 mins.	58		6 Hrs.	16
			30	7 Hrs.	Nil.
Ox-Hide.					
60	5 mins.	Nil.	50	15 mins.	16
	10 mins.	33		30 mins.	33
	20 mins.	50		45 mins.	33
				60 mins.	33
40	7 Hrs.	Nil.			

that no shrinkage could be detected at 40°C., but it was rapid at 50°C., results which agreed with Powarnin (loc. cit.). The difference between the minimum shrinkage temperature of the sinew and ox-hide may have been due to the one material being more fully limed than the other, or may represent a difference in behaviour.

Although no shrinkage could be detected at 30°C., this temperature is clearly near the point at which heat will cause limed sinew to shrink, and 40°C. is well above it. Consequently, when sinews are limed, no swelling would be expected at 40°C. and probably very little at 30°C. Also it clearly represents some alternation in the internal structure of the collagen, which in turn may be detrimental to the extraction of gelatine. Gelatine is extracted by heating limed collagen with water, but from these experiments it does not appear that heat can be applied during liming or, by inference, before lime treatment, without reducing the efficiency of the reaction.

Application of Heat before Soaking.

The effect of heat sufficient to bring about shrinkage of the precursor before liming has been studied by Kuntzel and Koepff (Collegium 1938, 433), but their results do not appear to be consistent. They found, using temperatures of 30°C., that the soaking process was speeded up, but the gelatine obtained was of inferior quality. On the other hand, after shrinking the material by plunging it into water at 90°C. for 30 secs., they obtained gelatine of good quality after a very short soak in lime. The experiments described in the previous sections indicated a possible connection between the shrinkage of the raw material by heat and the unsuitability of higher temperatures for soaking. It did not therefore appear likely that shrunk material could be made to yield good gelatine after soaking.

A number of experiments were carried out to throw further light on this question of the application of heat prior to liming. A 200 grms. lot of dry sinews was soaked in water for several days to bring about softening. They were then heated in water at 60°C. for 3 hours. It might have been objected that this period of time was too long, for 15 minutes would have been sufficient to bring about shrinkage. On the other hand, it was necessary to make certain that heat was was applied for long enough to produce the maximum effect. After the preliminary heating, the sinews were soaked in lime water for 4 weeks. After a week, the sinews were beginning to go soft and broken up at the ends. At the end of the soaking period the sinews were breaking up all over the surface and showed no signs of swelling. They were washed and extracted, with the following results:-

Time of Extraction	Yield	Jelly Strength	Iso-Electric Point Acidity
1¼ Hrs.	7·9%	192	1·45%

The yield was poor, not only because of loss of material but because what was left did not appear very easily extracted. The jelly strength was not as high as would have been expected from gelatine with a normal iso-electric point, but was not low enough to indicate gelatine of poor quality. Apart from the poor yield, the large amount of organic impurity still remaining in the extracted gelatine was sufficient to make this method an impossible one. The behaviour of the pre-heated sinews in lime was the same as when they were subjected to a lime soak at 40°C.

A number of experiments were also carried out after subjecting the sinews to heating in air. A quantity (200 grms.) of dried sinews were heated at 60°C. in air for 48 hours, after which they were soaked in lime water for 9 weeks. At the start they appeared to soak more slowly than sinews which had not been thus treated, but at the finish they appeared to be in good condition and were plumped at the ends. When washed and extracted the following results were obtained:-

Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C	Iso-Electric Point Acidity
2 Hrs.	12·8%	217	226	1·45%

From the rather low yield and not very rapid rate of extraction it was concluded that the effect of the preliminary heating had been to slow the rate of soaking down by drying the sinews. This type of preheating, however, had no detrimental effect either on the material itself or the resulting gelatine, but neither did it contribute any recognisable benefit to the process.

Another lot of sinews (200 gms.) was heated in air at 100°C. for 72 hours and allowed to cool. They were then soaked in lime water for 3 weeks. After 6 days soaking, the material started to break up. At the finish of the soak, the outer skin of the sinew was tough and the inside mushy. No swelling was observed. These sinews were washed and extracted with the following results:-

Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-electric Point	
				Acidity	Ph
2 Hrs.	18·6%	107	59	1·2%	4·9

In this case a fairly good yield was obtained, but of inferior gelatine whose iso-electric point has not attained its maximum value. It should be noted that if a complete extraction had been carried out, the total yield would have been very low owing to loss of material during soaking.

An attempt was also made to extract another lot of sinews which had been heated in air for 3 hours. at 100°C. After 7 hours extraction no appreciable amount of gelatine was found in solution, so that it was concluded that the use of an alkaline soak could not be dispensed with, even after energetic heat treatment.

Another experiment was carried out in which sinews were shrunk at 60°C. before soaking: in this case, the time of heating was just sufficient to shrink the sinews had they been in a swollen condition. A lot of sinews (200 grms.) was soaked in water for two days to soften them, after which they were plunged into water at 60°C. for 15 minutes. They were then chilled in cold water and soaked in milk of lime for four weeks. During the soaking period the sinews exhibited signs of breaking up on the surface. Very little swelling took place. After washing, the sinews were extracted with the following results:-

Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20%at 40°C.	Iso-Electric Point.	
				Acidity	Ph
6 Hrs.	15·7%	135	124	1·21%	4·9

Although gelatine of rather better quality was obtained in this way, it was dull, and the iso-electric point was not at its maximum point.

As none of our experiments had given satisfactory results, it was resolved to repeat one of those carried out by Kuntzel & Koepff. Some sinews (200 grms.) were soaked in water for two days to soften them. They were then plunged into water at 90°C. and kept there for 30 seconds. This caused them to shrink and curl up. After cooling, the sinews were put into milk of lime and left for 12 days. They then appeared to be broken up on the surface and there was no swelling. After washing, the sinews were extracted, which gave the following results:-

Time of Extraction	Yield	Jelly Strength	Visc. Centi-poises 20% at 40°C.	Iso-Electric Point	
				Acidity	Ph
6 Hrs.	18·5%	101	76	·73%	4·9

The gelatine obtained was dull and of very poor quality, and altogether the experiment showed that something of an undesirable nature had happened to the material. The iso-electric point had not reached the normal position.

It was concluded from these experiments that any form of heat prior to soaking was thoroughly bad. Sinews thus treated behaved in the same way as they did when soaked at temperatures above 25°C - that is, they rapidly broke up in lime liquor and did not swell. It is not clear how Kuntzel & Koepff claimed that this was a good method of extracting gelatine, but they did not keep the material under observation during the soaking period, and the figures they used to characterise the gelatine did not include jelly strength which is undoubtedly the best physical property for the purpose. What was shown with certainty was that heating sinews before liming (or, as shown previously, carrying out the lime soak at temperatures above 25°C.) had a different effect upon collagen, and moreover not what was required for the conversion of collagen into gelatine. There were indications that heating before soaking did accelerate the move of the iso-electric point to its normal position, but the advantage of this was more than offset by other effects of a different nature which must be presumed to arise from subjection of the material to even moderately high temperatures for short periods of time.

THE EFFECT Of NEUTRAL SALTS On ALKALINE SOAKING.

This section is a report of work carried out to check up a statement by Kuntzel & Koepff (Collegium 1938, 433) that the duration of liming can be shortened, and a better gelatine produced by the addition of calcium chloride, and also to investigate the possibility of reducing the swelling caused by caustic soda by the addition of neutral salt.

As the tanners make use of neutral salts to control the swelling of hide on the acid side, their effect in reducing acid swelling has been fully investigated. Some experiments have also been carried out with alkali. Reid (J. Amer. Leather Chem. Assoc., 1922, 17, 460) studied the repressive action of salts on the acid swelling of hide powder but also included caustic soda. Page & Page (J.I.E.C., 1927, 19, 1264) investigated the influence of calcium chloride, common salt, and sodium sulphate at Ph values ranging from 8 to 11. In alkaline solution, common salt had practically no effect on swelling, but sodium sulphate had a marked repressive effect. No results were obtained for calcium chloride above Ph 8, where it seemed to increase the swelling. Kuntzel & Phillips (Collegium 1932, 267) compared the swelling of collagen in acid and alkali. Alkaline swelling was only slightly repressed by neutral salts. In the case of sodium chloride, the swelling was actually increased by the formation of NaOH. The action of salt during a lime soak was confirmed by Please (J.Soc. Leather Trades Chemists, 1937, 21, 89, 208) who found swelling increased by the addition of sodium chloride, and also an increased rate of dissolution of nitrogeneous matter.

To examine the behaviour of calcium chloride, two lots of 200 grms. were soaked in lime liquor . One lot of sinews was given in addition to lime, 110 grms.

per litre of calcium chloride. Both lots were allowed to soak for four weeks. At the end of that time the sinews treated with calcium chloride showed slight swelling and were starting to break up. Both lots were washed and extracted with the following results:-

Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point	
				Acidity	Ph
		Calcium Chloride + Lime.			
2½ Hrs.	9·3%	170	213	1·25%	4·89
		Lime Alone.			
4 Hrs.	37%	112	82	1·35%	4·87

In neither case was gelatine of good quality obtained, the reason being that the time of soak was kept short in order to allow for any acceleration produced by the calcium chloride. The iso-electric points were both below the normal position as regards acidity. What seemed to be happening was that the calcium chloride caused the lime to attack the surface of the sinews only, so that the outside parts became broken up, while the inside was not ready for extraction. There was no evidence of any general speeding up of the soaking process. As regards quality, the calcium chloride gelatine was no better than would have been expected from the use of lime alone. Unfortunately the gelatine obtained from the control was poorer than it should have been (probably owing to spoilage in drying), so that the comparison was in favour of calcium chloride treatment as regards quality of gelatine. However, in a previous experiment a four weeks soak in lime resulted in gelatine with a jelly strength of 204, so that it was concluded that the claim as to the improvement brought about by the use of calcium chloride was probably unfounded. The gelatine made from the sinews soaked in calcium chloride and lime was also duller than that made from the control, which suggested less purification during soaking.

The unsatisfactory nature of this experiment and the investigations of Thomas & Foster (J.I.E.C., 1925, <u>17</u>, 1162), which showed that calcium chloride catalysed the hydrolysis of hide substance in the cold, led to the conclusion that calcium chloride was not a desirable substance to have present during a lime soak, and that the conclusions reached by Kuntzel & Koepff were incorrect.

The experiments with sodium sulphate and caustic soda were carried out in order to discover if there was any way of reducing the swelling produced by use of caustic, which gave rise to much difficulty during the washing process. It appeared from previous investigations that sodium sulphate suppressed alkaline swelling to a remarkable degree.

Two lots of dried sinews, each of 200 grms., were soaked in 1% caustic soda solution containing 32 grms.

anhydrous sodium sulphate per litre. The liquor was changed from time to time. One lot was soaked for 10 weeks, a very long period for this concentration of caustic soda, and the other lot was soaked for 5 weeks. During the soaking period, practically no swelling took place. When the sinews were washed, however, they swelled just as badly as if no sodium sulphate had been added, and those soaked for the longer period could only be handled with very great difficulty. The lot left in caustic soda for 10 weeks had been soaked too long, as they were completely extracted by raising the temperature to 65°C. The results obtained were as follows:-

	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point	
					Acidity	Ph
10 weeks soak.	½ Hr.	5·1%	184	25	1·55%	4·13
5 weeks soak.	6 Hrs.	15·0%	150	87	1·28%	4·76
5 weeks soak. 1% caustic alone.	3 Hrs.	33%	255	536	1·53%	4·75

The fact that the sinews swelled as much during the washing operation rendered the addition of sodium sulphate of no value. The results obtained, after a five weeks soak, also suggested that prevention of swelling during soaking slowed the reaction with caustic soda down, because the figures previously obtained from 1% caustic soda alone were very much better. The Ph values obtained for the iso-electric points were rather low, but the acidity figure for the five week soak indicated that the iso-electric point had not reached its final position. These experiments showed that the objectionable features associated with the use of caustic soda were not to be eliminated by the addition of sodium sulphate.

According to Thomas & Foster (loc. cit.), sodium sulphate, as compared with calcium chloride, has hardly any hydrolytic effect on hide substance in the cold, so that the poor results were more likely to have arisen from the slowing down of the caustic soda reaction. The possibility of making use of the hydrolytic properties of neutral salts for the preparation of gelatine might perhaps be worth further investigation.

USE Of TRYPSIN In The EXTRACTION Of GELATINE.

Trypsin has for long been known as the active principle of tanners bate, in conjunction with ammonium chloride. Bating serves the two-fold purpose of deplumping the skins prior to tanning and of rendering the hide pliable after tanning, but what the bating process actually does to the hide has never been completely explained, in spite of much investigation.

Moeller (Collegium 1918, 105) appears to have been the first to suggest that trypsin opened the hide up by removing the elastin fibres without attacking the collagen. It was considered inadvisable to damage the collagen fibres. Wilson (J.I.E.C., 1920, 12, 1087) favoured this elastin theory which, however, was denied by Marriott (J. Soc. Leather Trades Chem., 1921, 5,280) who suggested the removal of the cementing substance as one of the essential features of bating. Again, Wilson & Daub (J.I.E.C., 1921, 13, 1137) produced further support for the elastin removal theory of bating, by means of a microscopical study of hide during bating. Marriott (J. Soc. Leather Trades Chem., 1926, 10, 132) introduced a new theme into current theories of the bating process. It was pointed out that skin could be limed until only traces of elastin remained, and the skin shrunk,and yet it could not be considered to be properly bated, although got by other means into what was apparently the same condition as bated skin. This difference was accounted for by the presence of lime-degraded collagen in the hide which was removed by trypsin. Thus bating consisted in the reduction of plumping, separation of the fibres by removal of cementing proteins, and removal of degraded collagen. That trypsin had any function in altering the collagen was undoubtedly a new idea. McLaughlin and others (J. Amer. Leath. Chem. Assoc., 1929, 24, 339) considered that the removal of coagulated protein from the skin was the most important function of bating. They also attributed the removal of keratose from the grain-layer to the agency of trypsin. Page & Page (J.I.E.C., 1930, 22, 545) concluded that trypsin had a wider action on collagen than mere solution. Besides removing a certain amount of hide proteins in the process of bating, trypsin altered the collagen in such a way that its degree of swelling was less at a given Ph. From their experiments it appeared that the amount of degraded collagen removed by trypsin was small.

An entirely new claim was made for trypsin by Gavrilov & Simskaya (Biochem. Z., 1931, 238, 44): that it moved the iso-electric point of collagen to the acid side. It was claimed to have reached Ph 3·4. Swelling experiments reported elsewhere in this paper, carried out on bated hide before and after lime treatment, failed to substantiate this claim; but the statement illustrates the tendency to expect some modification of the collagen itself by the trypsin. Kuntzel & Pototsching (Collegium 1932, 480, 559, 571) devoted much attention to the behaviour of individual fibres of collagen during bating. They appeared to favour the theory which postulated some alteration in collagen by trypsin. This was, however, different from the effect of lime. Lloyd & Robertson (Nature, 1934, 133, 102) noted that proteolytic enzymes attacked collagen fibres with ease at a cut end, but omly with difficulty at an undamaged surface.

From the information available about the bating process, it appeared likely that trypsin brought about some change in collagen, and it was desirable to know if this led to the ready extraction of gelatine. Further, it was of interest to discover if the action of trypsin was similar to that of lime, which could be done by noting its influence on the position of the iso-electric point. A number of experiments were therefore carried out to find out if trypsin could be made to play a part in the preparation of gelatine.

A first attempt was made with sinews. They were soaked in water for two days to soften them and put into phosphate buffer solution at Ph 8, to which had been added ·2 grm. per litre of trypsin. They were then left overnight in an incubator at 30°C., and thereafter washed. Trypsin produced no visible effect on the sinews, which failed to yield any appreciable amount of gelatine after 7 hours heating.

As it was thought that a short treatment with lime might enable trypsin to attack the sinews, two lots of 200 grms. sinews were soaked in lime for 5 days and 10 days respectively, at 30°C. The sinews were washed and left overnight in water containing ammonium chloride. They were then put into phosphate buffer solution at

Ph 8, the five day soak given ·2 grm. trypsin per litre, and the ten day soak ·4 grm. trypsin per litre. The sinews soaked in lime for five days were left overnight in an incubator at 30°C., while those soaked for ten days were left for three days at 30°C. The second experiment might thus be expected to show the effect of trypsin if there was anything to observe at all. Having been incubated, the sinews were washed and extracted with the following results:-

	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point Acidity
5 days in lime. ·2 grm. trypsin.	7 Hrs.	10%	113	-	·81%
10 days in lime. ·4 grm. trypsin.	1st Extract.				
	$1\frac{1}{4}$ Hrs.	16%	197	48	1·25%
	2nd Extract.				
	3 Hrs.	7·5%	-	124	1·15%

After treatment with trypsin, the sinews soaked for the shorter period exhibited no visible signs of attack. The second lot, which had been subjected to the action of a large amount of trypsin for a long time, showed signs of attack at the ends and at places on the surface. No effect was produced on the extraction of gelatine. What was obtained was exactly the same as would have been extracted from sinews soaked at 30°C. in lime without the addition of trypsin. The only variation which could be attributed to the action of trypsin was that the first extract from the sinews which did exhibit signs of attack at the surface and at the ends had an unusually low viscosity. There was no indication of any move in the position of the iso-electric point. It was also noticed that trypsin, under the conditions of these experiments, did not remove all protein other than collagen, for the gelatine obtained was very cloudy. From these experiments it was concluded that untreated sinews or lightly limed sinews were not readily attacked by trypsin, and there was no indication of pre-treatment with trypsin facilitating the conversion of sinews into gelatine.

A series of experiments were carried out on ox-hide. This was in the form of split hide which had been lightly limed by the tanners. Except for variation in the amount of trypsin used, and the time of incubation, the procedure adopted was the same in every case. One kilo of hide was given a rough wash and was left standing overnight in water containing 4 grms. of ammonium chloride per litre. After rinsing, the hide was put into phosphate buffer solution at Ph 8 containing trypsin, and incubated as a rule for 24 hours. In the case of the lightly limed hide, the ammonium chloride caused the skin to shrink and become quite flaccid. After incubation, the hide was washed and extracted. To provide a control, one lot of hide was extracted after washing, without any treatment with trypsin.

Two experiments were carried out with similar split hides which had been fully limed. In the first experiment, extraction was so rapid and the effect of trypsin on the gelatine so marked that no gelatine could be obtained for tests, as what was extracted would not set to a jelly. In the second experiment, after digestion with trypsin the hides were washed and gradually made acid with hydrochloric acid. In this way any trypsin left in the hides was rendered inactive without any alteration in the type of gelatine produced. It was noted that the fully limed hide did not shrink when immersed in ammonium chloride, nor did its appearance seem to be altered by digestion with trypsin. The results of the experiments are shown in Table XVIII.

Considering the lightly limed hide, it is clear that trypsin has in some way altered the course of extraction. Hide becomes more easily extracted. The gelatine obtained is, however, poorer in jelly-forming power, and very much lower in viscosity. There is no sign of any shift of the iso-electric point to the acid side; it falls in the same region as that of the control. Thus trypsin makes hide more easily hydrolysable without removal of ammonia, and its action is different

TABLE XVIII.

Treatment	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Iso-Electric Point	
					Acidity	Ph
		%			%	
Lightly limed; no trypsin.	4 Hrs.	4·5	172	138	·65	5·31
Lightly limed; ·1 gm. trypsin.	2 Hrs.	4·4	160	37	·76	5·51
Lightly limed; ·2 gm. trypsin; 2 days incubation.	1 Hr.	4·5	147	81	-	-
Lightly limed; ·4 gm. trypsin; 2 days incubation.	2 Hrs.	6·0%	144	46	·74	5·45
Fully limed; ·1 gm. trypsin.	1½ Hrs.	2·5	225	33	1·38	4·86
Lightly limed; ·1 gm. trypsin 1st Extract.	½ Hr.	7·0	232	50	·68	5·24
2nd Extract.	¾ Hr.	6·7	217	44	·56	5·54
3rd Extract.	¾ Hr.	4·6	217	82	·50	5·27

from that of lime.

The effect of trypsin on fully limed hide appears to be the same but is much more violent, since in order to obtain gelatine which will form a jelly, special precautions have to be taken. When this is done, gelatine may be prepared with no diminution in jelly strength, but of very low viscosity. There is again no sign of trypsin moving the iso-electric point to the acid side.

The last group of figures in the table deserve very special attention, for they record an experiment which so far has defied numerous attempts at repetition. It was carried out in exactly the same manner and should have given results similar to those obtained for other lots of lightly limed hide. The only points which this experiment has in common with the other is that the iso-electric point of the gelatine produced is exactly where it ought to be: that is, there is no sign of any move to the acid side, and the viscosity of the gelatine produced is very low. On the other hand, in three rapid extractions, almost the whole of the hide has been converted into gelatine of good quality as measured by jelly strength. The results of this experiment agree generally with those obtained from the other experiments; the difference lies in the completeness and speed of the hydrolysis. Ten additional experiments were carried out in an endeavour to repeat this experiment, and all that can be said meantime is that enzyme activity is prone to unforeseen fluctuation. It may be added that if trypsin could be made to function with certainty in the manner of the last experiment, it would provide a very elegant method for the preparation of gelatine, where high viscosity was not a necessity.

It may be concluded from these experiments that digestion with trypsin alters collagen, both before and after liming, in such a manner as to make it more easily hydrolysed. The alteration is not similar to that brought about by alkali, as there is no move in the position of the iso-electric point of the resulting gelatine. With more knowledge at our disposal regarding the control of enzyme reaction, pancreatin or trypsin might be applied to provide a new method for the preparation of gelatine.

THE NATURE Of The PROCESSES Involved In The EXTRACTION Of GELATINE.

The Nitrogen Content of Acid Extracted and Alkaline Extracted Gelatine.

It was shown in the previous paper that gelatine prepared by the acid method differed from gelatine prepared by the alkaline method in having its iso-electric point at a lower acidity or a higher Ph. From this it was argued that acid-prepared gelatine contained more basic groups than the other variety. As the basic groups formed the nitrogeneous part of the substance, it followed that the nitrogen content of acid -prepared gelatine was greater than that of alkaline-prepared gelatine. If this were possible of demonstration, it would bear out the accuracy of the previous observations, and provide additional evidence for the existence of different types of gelatine.

At first sight it would appear a perfectly simple matter to find the difference in nitrogen content if it existed. However, as the expected difference was of the order of ·5% , it was clear from the outset that every precaution would require to be taken in order to attain a sufficient degree of accuracy. The gelatine itself, as obtained in all previous experiments, had a variable moisture content. There was no way of controlling this except to eliminate moisture altogether, and accordingly the estimations were carried out on specially dried moisture-free gelatine. The ash content of the gelatine also varied. In the samples used for the estimations the ash was reduced to negligible proportions by bringing the gelatine to its iso-electric point and washing with distilled water. The method used for the nitrogen estimations was a modification of the Kjeldahl method. This can also be a fruitful source of errors, but every precaution was taken to eliminate those. The distillation apparatus was carefully overhauled and made gas-tight. Six blank determinations were carried out on the chemicals used, so that a correction could be made for any nitrogen introduced in course of the estimation. Check estimations of nitrogen were then carried out on ferrous ammonium sulphate and urea, which showed that the error arising from the actual Kjeldahl estimation was not likely to exceed one part in five hundred either way. As a further precaution, three nitrogen determinations on each type of gelatine were carried out at the same time, the distillation apparatus being designed to distil six samples at once. In this way it was hoped that even if there was an error in the absolute value of the results, the difference would still be evident. When the estimations were actually carried out, it was found that this precaution was unnecessary.

Preparation of Alkaline Gelatine.

Alakline gelatine was prepared for the estimations in the following manner. A commercial powdered gelatine of good quality was obtained, which had an acidity of 1·00% and an iso-electric point of 1·22% acidity. To bring this gelatine to its iso-electric point, 100 grms. were melted up in distilled water containing 20 c.c. N/5 H_2SO_4. The gelatine was cast in the form of thin cakes, washed, and dried. Its acidity was then ·71%, its iso-electric point 1·1%, and its ash ·35%. The sample had too high an ash content and was considered to be too far away from its iso-electric point. The remainder, amounting to 65 grms., was melted up in distilled water containing 25 c.c. N/5 H_2SO_4. It was again cast into the form of thin cakes which were washed in distilled water and dried. The gelatine was now in a satisfactory condition for use, the acidity being 1·13%, the iso-electric point 1·13%, and the ash ·10%.

A second sample of commercial alkaline gelatine was obtained, whose iso-electric point was 1·42% acidity and acidity ·82%. To bring this to its iso-electric point, and to reduce the ash, 100 grms. were melted up with distilled water containing 60 c.c. N/5 H_2SO_4. The gelatine was cast in the form of thin cakes, dried, washed in distilled water and dried again. Its ash was then ·12%, its iso-electric point 1·41%, and acidity 1·31%. It was thought possible to improve on those figures, so the remainder of the gelatine, 80 grms., was melted up with 9 c.c. N/5 H_2SO_4, cast into thin cakes, dried, washed in distilled water, and dried again. Its acidity was then 1·48%, iso-electric point 1·48%, and ash ·07%, so that the gelatine was then in good condition for the purpose of the experiment.

The third sample of commercial alkaline gelatine examined had an acidity of ·87%, and its iso-electric point was 1·45% acidity. To bring this to its iso-electric point, 100 grms. were melted up in water containing 69 c.c. N/5 H_2SO_4. The gelatine was cast into thin slabs, dried, washed in distilled water, and dried again. Its acidity was then 1·48%, its iso-electric point 1·48%, and its ash ·06%, which was again satisfactory for the purpose in view.

All three samples of gelatine prepared by the alkaline method had been extracted from a mixture of dried sinew and wet ox-hide. The type of precursor could have no influence on the results obtained.

Preparation of Acid Gelatine.

The samples of acid gelatine were prepared in the laboratory, which made certain that the whole history of the sample was known, as was the case with the commercial samples of alkaline gelatine. The first sample was made from dried sinew. Three lots of 200 grms. crushed sinews were soaked for several days in 3 litres of 2% conc. HCl solution. Each lot of sinews was washed for one day and extracted separately. The gelatine obtained had an average acidity of 1·69% and its iso-electric point was assumed to be at -·2% acidity. The three lots, making 140 grms. in all, were soaked in water containing 265 c.c. N/5 NaOH to bring the gelatine to its iso-electric point. The gelatine was melted up, cast in the form of thin cakes, and dried. It was then washed in distilled water, and dried again. This resulted in gelatine whose acidity was ·11%, iso-electric point ·11%, and ash ·08%, so that the sample had the properties essential for the purpose in view.

A second sample of acid gelatine was prepared from crushed sinew in the same way. After extraction, the gelatine had an acidity of 1·06% and its iso-electric point was -·14% acidity. To bring this gelatine to its iso-electric point, 145 grms. were soaked in water containing 190 c.c. N/5 caustic soda. After washing this gelatine it was found to be at ·12% acidity. Its iso-electric point was ·12% acidity, and its ash ·26%. As the ash was considered rather high, the gelatine was washed again, when the ash was reduced to ·1%. The final acidity and iso-electric point was ·18%.

The third sample of acid gelatine was prepared from shredded rabbit skin. Four extractions were carried out, each on 200 grms. rabbit skin. The rabbit skin was soaked in water, and washed to remove the dirt. Each lot of 200 grms. was then soaked overnight in water containing 2 grms. caustic soda. This cleansed the skin still further. The skin was then carefully washed. The skins, after wringing out, were put into water containing 1·5 c.c. conc. sulphuric acid. After soaking overnight they were extracted with the same acid water. In this way 104 grms. of gelatine were obtained with an average acidity of 1·0%. The iso-electric point was assumed to be at -·2% acidity. To bring this to its iso-electric point, 104 grms. were soaked in sufficient water to make a 10% solution containing 125 c.c. N/5 caustic soda. The gelatine was melted up and cast into thin cakes and dried. It was then washed in distilled water, and again dried. The gelatine had an acidity of -·07%, its iso-electric point was at -·27%, and the ash ·06%. An endeavour was made to bring the acidity of the gelatine closer to the iso-electric point. This was done by soaking 54 grms. of the gelatine in water to make a 10% solution, to which 7 c.c. N/5 caustic soda had been added. After drying and washing, the final figures on the gelatine were: acidity -·08%, iso-electric point -·08%, ash ·06%. This was considered very satisfactory for obtaining an accurate nitrogen content.

Nitrogen Determinations.

All six samples were powdered, and a certain degree of uniformity of particle size assured by putting the powder through a sieve. The coarse particles were rejected. The powdered gelatine was spread in a thin layer on petri dishes and dried in an air oven at 110°C. The temperature at which the oven was maintained was carefully controlled, for over-heating was known to cause decomposition with loss of ammonia or other nitrogeneous compounds, and drying below 110°C. was likely to be incomplete. When the gelatine appeared to be dry, as evinced by constancy in weight, it was left standing for several days over phosphorous pentoxide before use.

Six nitrogen determinations were carried out on each sample. Three estimations on an acid gelatine and three on an alkaline gelatine were done at the same time, in order to assist in the comparison of the two types. The results are as shown in Table XIX.

TABLE XIX.

Alkaline Gelatine		Acid Gelatine	
Iso-Electric Point Acidity %	Nitrogen %	Iso-Electric Point Acidity %	Nitrogen%
1·13	17·53	·11	17·98
	17·42		17·87
	17·53		17·87
	17·64		17·92
	17·47		17·97
	17·42		17·81
Average	17·50	Average	17·90
1·48	17·35	·18	17·91
	17·29		17·91
	17·35		17·91
	17·35		17·91
	17·29		17·85
	17·41		17·85
Average	17·34	Average	17·89
1·48	17·54	-·08	17·93
	17·42		17·93
	17·42		17·93
	17·42		17·93
	17·42		17·87
	17·42		17·87
Average	17·44	Average	17·91

Difference in Acidity % of Iso-Electric Point.	Difference in Nitrogen Content.	Calculated Difference in Nitrogen %
1·02	0·40	0·34
1·30	0·55	0·44
1·56	0·47	0·52

The calculated difference in nitrogen content was arrived at by estimating the amount of nitrogen equivalent to the difference in acidity between the iso-electric points. As the acidity determinations had been carried out on air-dried gelatine, allowance had to be made for an average moisture content of 15% in order that the corresponding amount of nitrogen could be calculated for absolutely moisture-free gelatine.

In every case, the nitrogen content of gelatine prepared by acid extraction was found to be higher than that of the alkaline variety. The differences shown between the two types agreed closely with the calculated differences, and it was noted with interest that the alkaline gelatine whose iso-electric point was at a lower acidity had the highest nitrogen content. The nitrogen content of the acid-extracted gelatine showed more constancy than that of the alkaline variety. Bogue has given the nitrogen content of gelatine as 17·5% (Chem. Met. Eng., <u>23</u>, 1920, 105), and Smith as 17·53% (J.A.C.S., <u>43</u>, 1921, 1350). Our average figure for alkaline gelatine of 17·42% nitrogen is a little lower than this. Acid-prepared gelatine with a nitrogen content of 17·90% thus contains ·48% more nitrogen.

These experiments on the amount of nitrogen present in the two types of gelatine fully confirmed the deductions made from earlier experiments of quite a different nature, and acid-extracted gelatine must therefore be considered to be a different compound from alkaline-prepared gelatine.

The nitrogen lost when pure collagen is treated with a suspension of lime was investigated by Marriott (The Swelling of Proteins, Int. Soc. Leather Trades. Ch. 1933, pp. 53 & 54) who measured the amount of ammonia found in solution after the soak, and found it to be ·46% of the collagen. This is in very close agreement with the figure given above for the difference in nitrogen content for the two types of gelatine. As the methods of arriving at the nitrogen loss were entirely different, the presumption that the figure is very close to the true value is greatly increased.

At the same time Marriott measured the ammonia liberated when collagen was soaked in N/20 caustic soda, and found it to be ·2%, from which it was assumed that lime evolved slightly more than twice the amount of ammonia as caustic soda. It is quite clear from numerous soaking experiments described in the earlier part of the present paper that caustic soda, if sufficiently concentrated, behaves in exactly the same way as lime, and the amount of ammonia liberated from collagen should therefore be the same. It must also be accepted as certain that dilute solutions of caustic soda have practically no action on collagen. To obtain a satisfactory result for the evolution of ammonia from collagen by caustic soda, a fifth normal solution would probably be required. The low result obtained by

Marriott for the ammonia liberated from collagen by caustic soda must therefore be attributed entirely to the use of too dilute solution.

The Effect of Lime on the Iso-Electric Point of Skin and Sinew.

The nitrogen loss during the preparation of gelatine by an alkaline process can take place during the soaking period, during the extraction, or during both operations. If, however, any appreciable amount of nitrogen is lost during the soak in lime, the iso-electric point of the precursor would be expected to move to a lower Ph value. In this way, untreated skin or sinew would have a different iso-electric point from limed skin or sinew. As ammonia is known to be evolved during the soaking operation, it appeared almost certain that a change in the position of the iso-electric point would be observed when skin or sinew are exposed to the action of lime. Collagen must then exist in two forms, one of which would be less basic than the other. Apart from those highly important theoretical considerations, the point of minimum swelling of gelatine raw material is of practical importance in the washing operation. It has been frequently observed in the course of our experiments that skin or sinew were difficult to wash out when fully swollen. The process can be made easier by altering the Ph so that the material is brought to its shrinkage point.

Failure to understand the action of lime, and the existence of more than one form of collagen, led to confusion in scientific literature dealing with this matter, a confusion not made any less by difficulties arising from the inert nature of the substance. Procter & Burton (J.S.C.I., 1916, 35, 404) built up an osmotic pressure theory to account for the swelling of gelatine in acid. They extended their theory to include hide, and deduced that the point of minimum swelling of hide should be slightly on the alkaline side of neutrality. This was an erroneous conclusion. Porter (J. Soc. Leather Trades Chem., 1921, 5, 259) gave an account of the swelling of hide powder in buffer solutions, and found a point of minimum swelling at Ph 4·8. Thomas & Kelly (J.A.C.S., 1922, 44, 194) investigated the iso-electric point of hide substance by careful swelling experiments and by use of acid and basic dyestuffs. They worked with hide powder, and in spite of much perseverance failed to obtain sharp end points. They concluded, therefore, that hide substance, generally referred to as collagen, was a mixture of proteins with an iso-electric point in the region of Ph 5.

Wilson & Gallun (J.I.E.C., 1923, 15, 71) made use of a different method. A thickness gauge was used to measure the compressibility of calf-skin at Ph values which ranged from 4 to 11. The calf-skin had been limed to dehair it, and bated. They found two points of minimum swelling - one at Ph 5·1 and the other at Ph 7·6. As far as can be judged from the account given of the preparation of the calf-skin, an iso-electric point might have been expected in the region of Ph 7·6, but not at Ph 5·1. In washing skins on a large scale, when the approximate position of the shrinkage point can be readily observed, limed skin appears to have only one point of shrinkage, which is on the acid side of neutrality.

Meunier, Chambard & Jamet (J. Soc. Leather Trades Chem., 1925, 9, 200) found the Ph of fresh calf-pelt, from which all mineral matter had been removed, to be 5·5, and this was also the point of minimum swelling. Subsequently, working with carefully prepared hide, Meunier & Chambard (Rev. Gen. Colloid, 1926, 4, 161) found the iso-electric point to be at Ph 5·4 to 5·6. Gustavson (J. Soc. Leather Trades Chemists, 1926, 10, 203) found the iso-electric point of hide powder to be at Ph 5·0. This work was done by treating the hide powder with complex chromium salts and watching the behaviour of the Ph of the liquour. There was evidence that the position of the iso-electric point depended on the nature of the pretreatment of the hide.

An observation of a different nature was made by

Gavrilov & Simskaya (Biochem. Z., 1931, 238, 44) who determined the iso-electric point by measuring the Ph at which collagen had the lowest calcium content. It was not constant but depended on the treatment to which the collagen had been subjected. Trypsin shifted the iso-electric point from Ph 4·64 to Ph 3·7. Then Shinidzu (Collegium 1932, 794) found the iso-electric point of collagen to be at Ph 4·69 by means of iron and gold sols. Lloyd (The Swelling of Proteins, Int. Soc. Leather Trades Chem., 1933, p. 77) provided a swelling curve for fresh collagen fibres in solutions of hydrochloric acid and caustic soda over a range from Ph 1 to Ph 12. The curve was very flat over a long range of Ph values in the neighbourhood of the point of minimum swelling, which indicated that the exact position of the iso-electric point would be difficult to fix when a swelling method was used. Another method of approach was used by Elod & Siegmund (Collegium 1934, 281) who prepared iso-electric collagen by means of an electro-dialyser from bated and non-bated calf-skins. It had a Ph of 5·7. The iso-electric point of calf-skin moved to a lower Ph as the time of liming was prolonged. Highberger (J. Amer. Leather Chem. Assoc., 1936, 31, 345) had difficulty in obtaining sharp iso-electric points for collagen; nevertheless he confirmed the existence of the two points postulated by Wilson & Gallun, and added a third at Ph 12. In a subsequent paper (J.A.C.S., 1939, 61, 2302) the same author, using an electrophoresis method, found the iso-electric point of collagen, prepared from steer-hide with a minimum alkaline treatment, to be at Ph 7·8. He suggested that the shift to Ph 4·7 was due to some structural change in the protein caused by alkaline treatment. It thus appeared, from a perusal of the literature of the subject, that untreated collagen would have an iso-electric point near neutrality, while that of limed collagen would be at some lower Ph.

Rabbit Skin.

In a previous communication, the material chosen for experiments on the point of maximum shrinkage was dried rabbit skin. This was not the best material to have chosen, as its previous history was unknown, but it was the only material which could readily be got into a suitable form for swelling experiments. The iso-electric point of rabbit skin was found to be at ·7% acidity as sulphuric acid, corresponding to approximately Ph 6. The swelling properties of this material were now investigated after a lime soak.

A quantity of shredded rabbit skin was washed, soaked in caustic soda to clean it, and washed again. It was then left in milk of lime for 11 weeks, the liquor being changed weekly. When the skins were removed from the milk of lime, they were washed, soaked in dilute HCl, to remove all the lime, and washed again. The skin having been spread in a thin layer, was dried in hot air. When dry, the skin was cut up as fine as possible. Estimations of the acidity of the skin were carried out by suspending 10 grms. quantities in boiled distilled water and titrating with N/5 caustic soda, using phenolphthalein as indicator. The acidity was found to be ·27% as sulphuric acid. A range of acidities was set up by suspending 5 grms. of skin in 100 c.c. water in stoppered measuring cylinders. After the skin had time to reach equilibrium (72 hours), the following results were obtained:-

Acidity %	Vol. of Skin (c.c.)	Vol. of Skin after washing by Decantation (c.c.)
·27	90	93
·77	65	71
1·17	61	65
1·57	70	70
1·77	73	82
1·97	71	82
2·17	76	93

The point of least swelling was at 1·17% acidity - that is, rather higher than that of untreated rabbit skin. Actually, the rise was less than was expected, although quite definite. Taking the middle of the range obtained after washing the skin by decantation, the minimum swelling was reached at 1·27% acidity.

The experiment was repeated using 2½ grm. quantities in the measuring cylinders. This was done in order to avoid any difficulty with the skin packing too tightly. The results obtained were as follows:-

Acidity % Sulphuric Acid	Vol. of Skin (c.c.)
·27	48
·67	37
1·07	30
1·47	32
1·87	38
2·27	48

The point of least swelling was found to be at 1·07% or 1·27% if the middle of the range was taken. This result was exactly the same as the previous one.

Another experiment was carried out with rabbit skin which had been soaked in lime for 9 weeks. Additional precautions were taken to get rid of lime and carbonate. After washing, the skin was cautiously and gradually neutralised with HCl during the course of a day, and washed overnight. It was then left for a day and a night in dilute HCl, and washed for a day. The skin was again left for a night in dilute HCl and finished off with another wash. When dried, the acidity of the skin was ·35%.

Two swelling experiments were carried out with 2½ grm. quantities of this rabbit skin, the results of which were as follows:-

First Experiment

Acidity % Sulphuric Acid	Volume of Skin (c.c.)
·35	64
·55	55
·75	55
·95	42
1·15	40
1·35	48
1·55	45
1·75	45

Second Experiment

Acidity % Sulphuric Acid	Volume of Skin (c.c.)
·35	58
·95	45
1·55	37
2·15	47
2·75	68

The first range of tubes was very flat and showed a disinclination for swelling to increase much as the acidity rose, so the second set of tubes was made up. The lowest reading in the first experiment was at 1·15% acidity. The lowest reading in the second experiment was 1·55%, and considering the whole range, the point of least swelling would be about 1·35% acidity.

It may be concluded with certainty from those experiments that the action of lime on the skins caused the point of least swelling to move from an acidity of ·7% to 1·2% approximately.

Sinew.

A similar experiment was carried out with dried sinew. This was only accomplished with considerable difficulty, as the success of the experiment depended on the sinew being reduced to a state of fine division. A quantity of dried sinews was soaked in water until soft, which required several days. The sinews were then split up as far as possible into threads - it was not possible to separate the individual fibres. The sinews were crushed, dried, and cut up into as small pieces as possible. An acidity was determined on 10 grms. of the cut up sinew by suspending it in boiled distilled water until it was properly soaked. It was found to be •35% as sulphuric acid. A range of graduated stoppered measuring cylinders was prepared, showing various acidities, and 2½ grm. lots of sinew put into each. After sufficient time had been allowed for the sinews to reach equilibrium, readings were taken of the volume occupied by the sinews. The results were as follows:-

Acidity % Sulphuric Acid	Volume of Sinew
-•85	85 c.c.
-•45	45 c.c.
-•05	35 c.c.
•35	22 c.c.
•75	22 c.c.
1•15	42 c.c.
1•55	89 c.c.
1•95	99 c.c.

Two cylinders showed the minimum swelling. As one of these was the untreated sinew with no acid or alkali added, this was taken to be the point of minimum swelling - that is, at ·35% acidity. This result was expected, as it was merely a demonstration of the well recognised fact that sinew swelled in either acid or alkali, but the experiment was necessary to provide a basis of comparison for the sinews treated with lime.

The remainder of the thread-like lengths of sinew were soaked in milk of lime for 6 weeks, with occasional changes of liquor as required. The sinews were then washed, treated with hydrochloric acid to remove traces of lime, washed, dried, and cut up as fine as possible. The acidity of the sinews was determined by suspending the finely-divided material in water and titrating slowly with N/5 caustic soda, using phenolphthalein as indicator. As in the case of the previous titration of sinews, care was taken that the material was properly soaked before the titration was carried out. The acidity was found to be ·4% as sulphuric acid.

A swelling experiment was then carried out on the sinews by suspending 2½ grm. lots in graduated stoppered measuring cylinders with various quantities of acid and alkali to give a range of acidities. After the tubes had time to reach equilibrium, readings were taken of the volume of the material. The results were as follows:-

Acidity % Sulphuric Acid	Volume of Skin (c.c.)
·40	72
·80	39
1·0	33
1·1	35
1·2	38
1·4	41
1·6	42
2·0	63

In this case, the point of minimum swelling was at an acidity of 1·0%. It was noteworthy that at the point of minimum swelling of untreated sinew, ·40% acidity, the lime-treated sinews were very well swollen, so that the experiment demonstrated clearly that the effect of the lime soak was to move the point of minimum swelling to the acid side. The result of soaking sinew in milk of lime is thus similar to the result obtained with rabbit skin.

Two features of the results of the swelling experiments on treated and untreated sinews were disappointing: the swelling curve was flat, and the movement of the point of least swelling brought about by the action of lime was less than had been anticipated. It was thought that better results might be obtained if the sinews, after treatment, were reduced to a finer state of division. Accordingly, some dry sinews were rasped to make a fine powder. This was found by titration to have an acidity of ·02% as sulphuric acid. Two swelling experiments were then carried out, using 2½ gm. portions of the powder, which gave the following results:-

Acidity % H_2SO_4	Expt. I. Vol. c.c.	Expt. II. Vol. c.c.
2·22	-	77
1·82	-	73
1·22	-	57
·82	47	50
·62	46	48
·42	44	-
·22	43	45
·02	43	-
-·18	53	52

The figures agreed with those obtained before, but again the swelling curve was very flat. It was noted that the sinews swelled more in alkali than in acid, but this may have arisen from specific properties of the acid and alkali used. The point of least swelling was at ·22% acidity, just on the acid side of neutrality to phenolphthalein, corresponding to a probable Ph of 6·6. On the whole, it was found that use of the sinews in the powder form gave more uniform results.

A similar experiment was carried out with sinews which had been subjected to a long soak in lime. They were washed, treated with small quantities of hydrochloric acid to bring them to their shrinkage point, washed again, and dried. The sinews were then converted into a fine powder by rasping, when it was noticed that they were much more brittle than untreated sinews. The powder was found to be at ·30% acidity, which indicated that too little acid had been used during the washing process, thus leaving more impurity in the material than had been intended. From two swelling experiments on the powder, the following results were obtained:-

Acidity % H_2SO_4	Expt. I. Vol. c.c.	Expt. II. Vol. c.c.
1·9	-	63
1·7	66	62
1·5	61	56
1·3	65	59
1·1	70	61
·9	72	63

The point of minimum swelling was found to be at an acidity of 1·5% - that is, it was at the same position as that of alkaline-prepared gelatine. In this experiment, use of the powder resulted in the iso-electric point being sharply defined. As the limed sinew, after careful washing, was in a state of considerable purity, a sharp swelling curve was to be expected.

Ox-Hide.

The results obtained from powdered sinews were so good that it was decided to attempt the same experiment with ox-hide. It was desirable to test the behaviour of several different sources of collagen, but practical difficulties had hitherto stood in the way of any experiments with thick hide.

Some pieces of dry salted unhaired ox-hide were soaked in water and washed to remove the salt. The back of the skin was carefully fleshed but the hair was left on, as its removal would have entailed chemical treatment which it was desired to avoid if possible. The skin was then dried and rasped down to powder. A good deal of hair got into the powder but this was removed by sieving. The acidity of the powder having been ascertained to be ·22% as sulphuric acid, a swelling experiment was carried out which gave the following results:-

Acidity % H_2SO_4	Volume c.c.
·62	49
·42	47
·22	47
·02	42
-·18	48

A sharp difference was obtained at the iso-electric point which was practically at the neutral point of phenolphthalein. Thus untreated ox-hide behaved in the same manner as sinews.

Two swelling experiments were attempted with the same material after it was fully limed, but any differences in swelling were too small to permit of the results being reliable. As the most likely reason for this was the amount of impurity, that is proteins other than collagen, still remaining in the skin, it was decided to work with split hide which had been soaked for a long period in lime and which, being only part of the thickness of the hide, was thinner and therefore more likely to be thoroughly purified during the lime treatment. The splits were washed, cut into narrow strips, treated gradually with HCl, washed with distilled water and dried. After being reduced to powder their acidity was ·58%.

Two swelling experiments were carried out with this powder, but in neither case could the results be considered satisfactory.

Acidity $\%H_2SO_4$	Expt. I. Vol. c.c.	Expt. II. Vol. c.c.	Expt. III. Vol. c.c.
3·0	-	59	63
2·6	-	57	57
2·2	-	56	53
1·8	47	58	49
1·6	47	-	-
1·4	48	60	49
1·2	47	-	-
1·0	51	61	51
·8	54	-	-
·6	61	63	55

In the first experiment, while the material showed swelling on the alkaline side, it did not swell at all at the acid end of the range. In the second experiment, by extending the range on the acid side, a point of minimum swelling was obtained at 2·2% acidity. This value was much higher than any other previously obtained. It was thought possible that the reluctance of the hide powder to swell in acid might be due to the presence of sulphate which is known to inhibit swelling. Accordingly, another experiment was carried out with the same powder, in which the acidification was carried out with hydrochloric acid in place of sulphuric acid. The results obtained in this manner (shown as Expt. 3) were rather better, and a point of minimum swelling was found between 1·8% and 1·4% acidity as sulphuric acid.

The unsatisfactory nature of the results obtained from hide as against those obtained from sinew was thought to be due to differences in the materials. Sinew is a relatively pure form of collagen, whereas the complete skin of any animal, or even a section thereof, contains much protein matter other than collagen. It is, however, possible to remove most of the other proteins from hide and leave the collagen, this actually taking place in the tanners bating process. The reason a method such as this was not adopted at the outset was in order to limit the treatment to which the hide was subjected. The effect of every step in the treatment on the hide had to be known. However, as it can be shown that the bating process - that is, the action of trypsin - does not alter the iso-electric point of the gelatine produced from bated skin, it was decided to carry out a final swelling experiment on ox-hide, using bated hide.

The material used was splits of ox-hide which had been lightly limed by the tanner. This liming was known to be so short that the position of the iso-electric point of the skin remained practically at its original value. One kilo of this hide was washed. It was then soaked overnight in water containing ammonium chloride (one or two grms. per litre). The hide was transferred to 1 litre of phosphate buffer solution at Ph 8 containing ·2 grm. trypsin, and incubated for 48 hours at 30°C. After a preliminary wash in running water, the hide was washed in distilled water, then dried and reduced to powder. The acidity of the powdered hide was ·16% as sulphuric acid. A swelling experiment with this powder in which hydrochloric acid was used for acidification gave the results shown below. Although the differences in volume were small, the readings were very definite and showed an iso-electric point at ·36% acidity as sulphuric acid. This lot of powder showed the same characteristics as had been noted previously, a rather long range with very little swelling, and a greater tendency to swell on the alkaline side.

A similar experiment was carried out on the same material after it had been subjected to a thorough lime soak. After the skins had been bated, they were washed and then acidified cautiously with hydrochloric acid until they reached their shrinkage point. They were then washed in running water and finished in distilled water. The acidity of the powder was found to be 1·13%. The swelling experiment gave very good results.

Swelling Experiments on Bated Hide.

Before Treatment		After Treatment	
Acidity % H_2SO_4	Volume c.c.	Acidity % H_2SO_4	Volume c.c.
·76	45	1·73	76
·56	41	1·53	71
·36	40	1·33	61
·16	41	1·13	52
-·04	46	·93	55
-·24	52	·73	70

There was a definite and well marked minimum at 1·13% acidity as sulphuric acid. This is lower than would have been expected, but the important point is that the lime soak has moved the point of minimum swelling from ·36% acidity towards a greater acidity. There was also no doubt that the purification of the hide enabled very sharp results to be attained.

Although the experimental method used in these experiments is open to criticism, in that it was assumed that the material absorbed ~~in~~ all the acid from the liquid, which is certainly not the case, the conclusions reached are established beyond doubt, as the treated and untreated materials were examined in exactly the same way. Thus, although the acidities at which the various samples exhibited the least amount of swelling may not represent exactly the acidity of the skin or sinew under examination, there can be no doubt about the difference between treated and untreated material.

It is now clear, therefore, that collagen in the form of skin and sinew has a different shrinkage point after treatment with lime from what it has in the natural condition. This clears up much of the confusion in published data for the iso-electric point of collagen, for it is evident that the pretreatment of the sample of collagen examined will determine the result obtained, which may fall anywhere between Ph 7 and Ph 5.

The move in the iso-electric point has been shown in the case of gelatine to be an indication of loss of nitrogen in the form of ammonia. The fact that this change has now been shown to take place during the soaking of collagen in alkali means that the collagen is losing ammonia. Hence, collagen which has been treated with lime is different chemically from collagen as it exists in natural tissue. It would for this reason be advisable to distinguish between the two varieties. The terms Collagen I and Collagen II have already appeared in literature (Wolisch, Biochem. Z., 1932, 247, 329; and Cherbuliez & Meyer, Compt. Rend. Trav. Lab. Carlsberg, 1938, 22, 118) although not applied to the particular forms now under discussion.

The difference between the two forms of collagen which has proved so difficult to establish with accuracy is very readily observed in two manufacturing processes. When preparing skins for tanning, they are soaked in lime for a short time for the dual purpose of removing the hair and plumping the skins. As a part of the bating process they are then caused to fall or shrink by immersion in ammonium chloride or alkaline bate at an approximately Ph 8. Now the skins shrink most violently at this point, which shows that the lightly limed skins must be near their iso-electric point. When skins which have been limed for a longer period are put into bating liquours at Ph 8 they do not shrink, showing that the skins are no longer at their iso-electric point. This observation may have been missed, as the raw material for tanning is for most purposes natural collagen unaltered by lime, except for the initial plumping. On the other hand, the raw material for the preparation of gelatine is the altered form of collagen. Consequently, when limed sinews are being washed for the preparation of gelatine, if after the addition of acid they are allowed to become sufficiently acid, they shrink to a fraction of their original size. The change is sharp and sudden although, as has been seen, difficult to demonstrate accurately in the laboratory. This shows that the iso-electric point of the limed sinews must lie considerably on the acid side of neutrality.

These observations, drawn from experience in handling hide and sinew on a large scale, also illustrate the practical bearing of the two iso-electric points of collagen. In order to wash out the alkali from swollen limed material it is important that the material should shrink, for in so doing a considerable quantity of alkaline liquor containing impurity comes out of the skin or sinew. This can be accomplished by making the material faintly acid. The alkalinity of the material can then be adjusted to a suitable Ph for extraction, after washing. In the same way, if unlimed or lightly limed material is to be extracted by an acid process, the washing should be carried out at or near neutrality to phenolphthalein, for in this condition the skins will be in a shrunk condition.

The Loss of Ammonia during Alkaline Extraction.

It has always been assumed that some nitrogen was lost in the form of ammonia during an alkaline extraction. Bogue (J.I.E.C., 1923, 15, 1154) showed how this nitrogen loss varied with Ph for an extraction of eight hours. Even with excessive alkalinity the loss was only ·25% of the total quantity of nitrogen involved and was only ·05% at Ph 8, a normal alkalinity for extraction. The material used was fully limed ox-hide. The problem which has to be solved is whether or not this nitrogen loss is adventitious, or is an essential product, when lime-treated collagen is hydrolysed to make gelatine. It has been shown that the liming process is a progressive one - that is to say, the collagen loses ammonia gradually up to a fixed amount. The experimental work is not sufficiently accurate for it to be claimed with absolute certainty that the final iso-electric point reached by the collagen coincides with that of gelatine, but it does appear to be extremely probable. Consequently, no further loss of ammonia would be expected during extraction. A further argument against this evolution of ammonia being an integral part of the reaction involved in extraction is that the iso-electric point of gelatine does rise progressively during liming. If the ammonia loss could take place either during liming or during extraction, then all gelatine would have a constant iso-electric point, for the material which had only been soaked for a short time would lose more ammonia when extracted. Again, the quantity lost during heating is very much smaller than that lost during soaking (·05% against 2·8%). For these reasons, it appeared possible that ammonia losses during extraction originated from impurities present in the collagen, and had no bearing on the main reaction. An attempt was made to decide this question.

An apparatus was arranged so that air which had been purified by passing through sulphuric acid and caustic soda was heated by passing through a water bottle immersed in a heating bath. The air was then drawn through a large flask which could be kept at 80°C. by means of the water bath, and finally through a measured volume of N/5 sulphuric acid. The material to be examined was placed in the large flask with about a litre of water, and heated for 7 hours. At the end of each experiment, the total volume of gelatine solution obtained was measured, and the nitrogen content found by means of a Kjeldahl estimation on a known volume of solution. The ammonia lost during the extraction was measured by titrating the sulphuric acid. Three experiments were carried out on dried sinews softened by soaking for a day in water. In this case it was unnecessary to worry about Ph, as the sinews had not been treated in any way and would be faintly acid to phenolphthalein. Two experiments were carried out on sinews which had been soaked for a long period in lime. The sinews were washed, soaked in very small quantities of dilute hydrochloric acid, and washing continued until they were green to B.D.H. Universal Indicator (approx. Ph 8). After heating for one hour, the gelatine solution was removed and a fresh lot of water added. A second extraction was completed during another period of one hour. During a last extraction of 2 hours duration, practically all the material went into solution. The total heating time was thus 4 hours. In the case of the second experiment, all the material went into solution in two extractions of one hour each. This method was adopted for the limed sinews, on account of the speed with which they went into solution. Had a single extraction period of 7 hours been used, the ammonia loss would largely have arisen from heating gelatine in solution.

In order to observe what proportion of the ammonia arose from the extracted gelatine in solution, two experiments were carried out with 1500 c.c. of 0·5% gelatine solution, and two experiments with 1000 c.c. 4% gelatine solution. These were subjected to continuous heating for 7 hours, and the solutions were adjusted to Ph 8. The results obtained were as follows:-

Material	Gelatine Extracted grms.	Nitrogen loss grms.	Nitrogen Loss as %age of total N Extracted.
Untreated Sinews	21·4	·0154	·47%
"	27·7	·0244	·55%
"	21·3	·0151	·46%
Limed Sinews	74·5	·0025	·023%
"	55·5	·00084	·010%
·5% Gelatine Solution	7·5	·0014	·12%
"	7·5	·0025	·22%
4% Gelatine Solution	40	·00448	·076%
"	40	·00504	·085%

The nitrogen loss when limed sinews are heated is of the same order as reported by Bogue for hide (loc. cit.). It can be entirely accounted for by ammonia lost from the extracted gelatine, and it is therefore unnecessary to assume that this loss of ammonia has any bearing on the conversion of collagen into gelatine. The amount of nitrogen lost when gelatine is heated is small and might be attributed to nitrogeneous impurities. On the other hand, the loss from the more dilute solution is greater, which points to hydrolysis of the gelatine itself as being the source of ammonia.

Considering the supposed structure of gelatine, any breakdown capable of giving rise to ammonia would have to be of a very fundamental character, which perhaps accounts for the small quantity of ammonia actually given off. Actually, the ammonia obtainable in small quantity from proteins is generally attributed to amide nitrogen existing in the residues of dibasic amino acids (Osborne, Leavenworth & Brautlecht , Am. J. Physiol., 1908-9, <u>23</u>, 180).

The nitrogen loss from untreated sinew is larger - probably too big to be attributed to further breakdown of the gelatine or to proteins other than collagen. The present experiment by itself does not provide a definite answer to the question as to the origin of this nitrogen lost from untreated sinews, but it is extremely probable that the loss in this case does arise from hydrolysis of collagen. The quantity of nitrogen lost is one fifth of the amount lost during a lime soak. If this loss occurs owing to hydrolysis of collagen, then one would expect the iso-electric point of such gelatine to fall at a higher acidity than that of acid-extracted gelatine, but at a lower acidity than alkaline-extracted gelatine, and that is exactly what has been found. It will be seen later that this conclusion as to the origin of the ammonia lost, when extracting gelatine with water alone, is confirmed by the position of the titration curve of this type of gelatine.

It may be concluded from those experiments that with material which has been properly soaked, only traces of ammonia will be lost during extraction. The quantity will, of course, increase with short soaking periods but will never approach the magnitude of the loss during soaking. Consequently, while it might be worth while recovering the nitrogen lost in lime liquors, the loss of nitrogen during extraction may be neglected. As extraction with water alone has merely a theoretical interest, the loss of nitrogen when this is done is of no practical importance.

The Effect of Acid after Lime Treatment on the Position of the Iso-electric Point.

In a previous communication it was shown for pigskin and for osseine that an acid extraction following lime treatment resulted in a gelatine whose iso-electric point was at a lower acidity. Although the original experiments were carried out with care and repeated, the differences upon which a decision had to be made were small, and small deviations in acidity were liable to arise unless the washing of the precursor was carefully attended to. The importance of very thorough washing of the precursor was not, in fact, realised until the later work described in the present paper was put in hand. For this reason there had always been some doubt about the effect of acid after lime. This was accentuated whenever it became clear that there was no alteration in the position of the iso-electric point at the time of extraction, which the results of the present series of investigations seemed to make certain. It did not appear possible that there could be any alteration of the iso-electric point when an acid extraction was used. Further, Briefer (J.I.E.C., 1929, 21, 266), who examined these matters closely, stated that the iso-electric point of gelatine extracted from limed material was at the same Ph whether it was extracted in an acid or alkaline condition.

For those reasons it was decided to investigate this point carefully. The materials originally used, wet pigskin and osseine, were no longer available, and in any case it was desired to examine the behaviour of other materials, so the experiments were carried out on dried sinew and dried shredded rabbit skin. The procedure adopted was to soak two lots of the material for the same time in milk of lime. One lot was washed and extracted in an alkaline condition, while the other lot was washed and made definitely acid before extraction. The results are shown, along with the previous figures obtained for pigskin and osseine, in Table XX.

TABLE XX.

Material	Time of Soak	Type of Extraction	Iso-electric Point Acidity as H_2SO_4
Pigskin	8 Weeks	Alkaline	1·45%
"	8 "	Acid	1·01%
"	12 "	Alkaline	1·48%
"	12 "	Acid	1·35%
"	16 "	Alkaline	1·50%
"	16 "	Acid	1·16%
Osseine	11 Weeks	Alkaline	1·45%
"	11 "	Acid	1·50%
"	10 "	Alkaline	1·46%
"	10 "	Acid	1·15%
Sinews	10 Weeks	Alkaline	1·33%
"	10 "	Acid	1·28%
"	8 "	Alkaline	1·55%
"	8 "	Acid	1·56%
"	11 "	Alkaline	1·63%
"	11 "	Acid	1·43%
Rabbit	8 Weeks	Alkaline	1·41%
"	8 "	Acid	1·40%
"	10 "	Alkaline	1·50%
"	10 "	Acid	1·56%
"	11 "	Alkaline	1·48%
"	11 "	Acid	1·55%

Considering the results from the four materials together, the weight of evidence is definitely against any lowering of the acidity of the iso-electric point by the use of acid after lime treatment, thus bringing our results into line with Briefer's. Pigskin showed a drop in two experiments out of three, osseine in one out of two, sinews and rabbit no drop at all out of three experiments on each. Why there should have been any difference shown at all, still remains a mystery. It must, however, be admitted that the later experiments on sinew and rabbit skin were carried out with a better knowledge of the importance of thorough washing of the material.

Extraction at the Iso-electric Point of Limed Collagen.

In a previous communication an experiment was described in which rabbit skin was extracted at its iso-electric point. Probably owing to the peculiar properties of rabbit skin, the results obtained concealed the real effects of an extraction carried out with collagen in its shrunk condition. The experiment now described was carried out with fully limed sinews.

Two lots of 200 grms. of dried sinews were soaked in milk of lime for 12 weeks, with regular changes of lime liquor.. At the end of that time, one lot was washed with water and extracted at a Ph of approximately 8. The second lot was washed, treated carefully with hydrochloric acid until the sinews were definitely acid, and in a shrunk condition. The sinews were washed again and extracted in a shrunk condition - that is, at their iso-electric point. The Ph of the sinews was approximately 5 as ascertained by means of B.D.H. Universal Indicator. The results of the extraction were as follows:-

	Time of Extraction	Yield	Jelly Strength	Visc. Centipoises 20% at 40°C.	Ash
Extraction at Ph 8	1½ Hrs.	36·5%	>255	280	1·56%
Extraction at Ph 5	2 Hrs.	29·4%	212	262	·46%

The behaviour of the two lots of sinews during extraction was very different. The sinews extracted at Ph 8 were well plumped before heating started. Whenever heating started they shrank and curled up, remaining firm gelatinous masses. The sinews extracted at their iso-electric point were already in a shrunk condition (but it may be noted in a shrunk condition of a different type), and on heating they expanded into a slimy mass lacking the firmness of the alkaline sinews. It might have been expected from the soft nature of this gelatinous mass that it would have gone into solution more easily than the firmer sinews but this was not so, and it also proved difficult to separate the soft slimy material from the solution of gelatine. The figures show that sinews at their iso-electric point extracted more slowly. For a longer time of extraction a smaller yield was obtained. Gelatine of high quality resulted from both extractions, but extraction at the iso-electric point of the precursor produced a poorer gelatine. As was anticipated, the ash content of the alkaline extract was higher. This is simply a good illustration of the advantage of washing material in a shrunk condition.

It may be concluded from this experiment that collagen at its iso-electric point is less easily hydrolysed than in any other condition. It is obvious that gelatine may be extracted at any Ph sufficiently far removed from the iso-electric point but not sufficiently acid or alkaline to cause rapid hydrolysis of the gelatine produced. Thus the neutral point of phenolphthalein has no particular significance in the extraction of gelatine, except that it happens to represent a Ph on the alkaline side, not too close to the iso-electric point, and yet not sufficiently alkaline to damage the extracted gelatine. This is contrary to our previous findings which arose from the use of rabbit skin which can apparently be extracted in any condition.

THE RELATION Of ACIDITY And Ph.

It was only after most of the other experimental work had been completed that apparatus became available which enabled Ph determinations to be carried out. Hence the only means possible of obtaining Ph values corresponding to acidity figures previously determined was to draw up a table showing how Ph varied with change of acidity. This involved an investigation into the Ph of the different types of gelatine, as the acidity altered.

Accordingly, a quantity of alkaline-prepared gelatine whose iso-electric point was at 1·40% acidity was brought to this acidity, washed carefully with distilled water, and dried. Its ash content was then ·08%, so that interference with the Ph value from unknown neutral salts was likely to be absent. Ph determinations were carried out on 2% solutions of this gelatine, adjusted to acidities running from 2·2% to -1·0% by steps of ·2%. The adjustments were made by adding appropriate quantities of N/50 H_2SO_4 or N/50 NaOH. The acid or alkali was added just before the readings were taken. The whole experiment was carried out in duplicate, as this was found to give more reliable results. It was found that while results could be obtained with 1% solutions, greater accuracy was secured by using a concentration of 2%. This, however, necessitated the solutions being kept at 20°C. to prevent setting. A similar set of readings was obtained for another alkaline-prepared gelatine with its iso-electric point at 1·18%, and an ash of ·05% after washing. In order to see if reliable results could be obtained from commercial alkaline-prepared gelatines without washing, two sets of readings were got from unwashed gelatines of this type. Table XXI gives the Ph figures for the four alkaline gelatines which have been distinguished by showing the acidity corresponding to their iso-electric points at the head of the Ph columns for each gelatine.

TABLE XXI.

Acidity % H_2SO_4	Standard Alkaline Gelatine 1·40% Ph	Alkaline Gelatine 1·18% Ph	Commercial Alkaline Gelatine (1) 1·42% Ph	Commercial Alkaline Gelatine (2) 1·48% Ph
2·2	4·23	4·24		
2·1	4·30	4·29		
2·0	4·37	4·35		
1·9	4·44	4·42		
1·8	4·52	4·48		
1·7	4·59	4·54		4·66
1·6	4·67	4·62	4·73	4·73
1·5	4·75	4·68	4·77	4·80
1·4	4·83	4·76	4·83	4·87
1·3	4·92	4·83	4·88	4·95
1·2	5·02	4·93	4·95	5·02
1·1	5·12	5·02	5·03	5·09
1·0	5·22	5·10	5·10	5·18
·9	5·34	5·21	5·21	5·28
·8	5·43	5·33	5·33	5·38
·7	5·63	5·45	5·46	5·50
·6	5·81	5·60	5·61	5·65
·5	6·02	5·78	5·84	5·82
·4	6·25	6·00	6·10	6·04

TABLE XXI (cont.)

Acidity % H_2SO_4	Standard Alkaline Gelatine 1·40% Ph	Alkaline Gelatine 1·18% Ph	Commercial Alkaline Gelatine (1) 1·42% Ph	Commercial Alkaline Gelatine (2) 1·48% Ph	
·3	6·55	6·23	6·43	6·34	x
·2	6·92	6·53	6·85	6·75	
·1	7·42	6·90	7·35	7·18	
0	8·00	7·40	7·90	7·73	
-·1	8·50	8·00	8·32	8·20	
-·2	8·85	8·48	8·67	8·60	
-·3	9·15	8·84	8·98	8·93	x
-·4	9·38	9·13	9·23	9·20	
-·5	9·58	9·38			
-·6	9·74	9·58			
-·7	9·88	9·75			
-·8	10'03	9·92			
-·9	10·16	10·05			
-1·0	10·28	10·17			

x Region of large errors

The figures showed good agreement amongst themselves and formed smooth titration curves. The two commercial samples gave Ph values which agreed with those of the washed gelatine, showing that it was possible to rely on results obtained from gelatine containing the small amounts of inorganic salts usually present. It is doubtful if much closer agreement could be expected because a small error in estimating the original acidity of the gelatine would shift all the Ph figures one way or the other, and absolute accuracy in the titration is complicated to a certain extent by the colour of the gelatine. There is, however, no reason to suppose that the Ph determinations are in themselves subject to other than very small errors, for the apparatus was checked against known buffer solutions at both ends of the range. The part of the table near neutrality to phenolphthalein can not be claimed to be accurate because in that region a small alteration in acidity gives rise to a big alteration in Ph. This, however, is of little importance for gelatine prepared by an alkaline process. The figures for the 1·40% washed gelatine were taken as standard figures for alkaline gelatine, as the iso-electric point was very close to its normal position.

The washed gelatine whose iso-electric point fell at 1·18% acidity showed a consistently lower Ph value corresponding to a given acidity. This arose from the iso-electric point not having reached its normal position. As far as could be seen by comparing the results for this gelatine with those obtained later for acid-prepared gelatine and the water-extracted variety, its titration curve fell into a position which corresponded exactly with the value for its iso-electric point relative to these of the other gelatines.

It was necessary to investigate the behaviour of acid-prepared gelatine in the same way. Two series of Ph readings were therefore obtained for two samples of acid-prepared gelatine, one made from rabbit skin, and the other from sinew, both samples having been brought to their iso-electric point and washed. These results were checked by readings from two other rabbit gelatines made by acid extractions, which had been washed without bringing them to their iso-electric point. The readings are shown in Table XXII, along with figures taken from a curve combining all the readings, which have been adopted as standard for gelatine of this type.

Except for the region round neutrality, where the errors are large, the results agreed very well. Owing to the difference between this type of gelatine and the alkaline variety, there was a considerable difference in the Ph values corresponding to any acidity. This was more marked on the acid side than on the alkaline side. The result of this behaviour is that for any given acidity, acid-prepared gelatine is at a lower Ph than alkaline-prepared gelatine. Unfortunately the iso-electric point of the acid variety of gelatine lies in the region where it is extremely difficult to measure Ph accurately.

TABLE XXII.

Acidity % H_2SO_4	Acid Rabbit Gelatine -·08% Ph	Acid Sinews Gelatine ·18% Ph	Acid Rabbit Gelatine ·75% Ph	Acid Rabbit Gelatine ·52% Ph	Acid Gelatine Standard Ph
2·2				3·87	3·92
2·1				3·92	3·95
2·0		4·00		3·97	3·99
1·9		4·05	4·06	4·02	4·03
1·8	4·03	4·10	4·10	4·07	4·08
1·7	4·10	4·15	4·15	4·12	4·13
1·6	4·17	4·21	4·20	4·18	4·19
1·5	4·23	4·27	4·26	4·25	4·25
1·4	4·29	4·34	4·32	4·32	4·32
1·3	4·37	4·42	4·38	4·40	4·38
1·2	4·44	4·50	4·45	4·50	4·45
1·1	4·52	4·58	4·53	4·60	4·56
1·0	4·61	4·67	4·65	4·70	4·66
·9	4·72	4·78	4·73	4·82	4·75
·8	4·83	4·89	4·83	4·98	4·87
·7	4·95	5·02	5·00	5·17	5·00
·6	5·10	5·18	5·15	5·40	5·16
·5	5·26	5·35	5·36	5·68	5·36
·4	5·45	5·58	5·63	6·00	5·56

TABLE XXII (cont.)

Acidity % H_2SO_4	Acid Rabbit Gelatine -·08% Ph	Acid Sinews Gelatine ·18% Ph	Acid Rabbit Gelatine ·75% Ph	Acid Rabbit Gelatine ·52% Ph	Acid Gelatine Standard Ph
·3	5·65	5·87	5·94	6·35	5·82 x
·2	5·90	6·25	6·40	6·77	6·15
·1	6·20	6·85	7·00	7·23	6·65
0	6·75	7·60	7·90	7·45	7·35
-·1	7·40	8·25	8·40	8·13	8·13
-·2	8·15	8·65	8·77	8·52	8·57
-·3	8·77	8·90	9·06	8·83	8·87 x
-·4	9·10	9·12	9·30	9·17	9·12
-·5	9·32	9·30	9·52	9·40	9·33
-·6	9·53	9·44		9·58	9·52
-·7	9·68	9·57		9·73	9·57
-·8	9·83	9·68		9·86	9·82
-·9	9·95			10·06	9·98

x Region of large errors.

The existence of the rather large difference in the position of the titration curves of gelatine with an iso-electric point at neutrality and of gelatine with an iso-electric point at an acidity of 1·45% complicated the problem of supplying Ph values corresponding to the acidity figures obtained during a progressive soak. As progressive soaking experiments started with gelatine which was to all intents and purposes extracted with water alone, it was necessary to find out what was the relation between acidity and Ph for this type of gelatine. Accordingly, some gelatine of this type was prepared from sinews, washed, and a range of Ph values measured. The results are shown in Table XXIII, along with the standard figures for gelatine of the acid type and alkaline type already obtained.

TABLE XXIII.

		Sample I			Sample II
Acidity % H_2SO_4	Acid Gelatine Ph	Water Gelatine Ph	Water Gelatine after storage Ph	Alkaline Gelatine Ph	Water Gelatine Ph
2·0	3·99	3·95	4·29	4·37	4·07
1·9	4·03	4·02		4·44	4·13
1·8	4·08	4·08	4·46	4·52	4·20
1·7	4·13	4·15		4·59	4·27
1·6	4·19	4·23	4·64	4·67	4·34
1·5	4·25	4·29		4·75	4·41
1·4	4·32	4·37	4·83	4·83	4·48
1·3	4·38	4·46		4·92	4·56
1·2	4·45	4·54	5·08	5·02	4·65
1·1	4·56	4·65		5·12	4·73
1·0	4·66	4·75	5·28	5·22	4·83
·9	4·75	4·87		5·34	4·95
·8	4·87	5·00	5·71	5·43	5·06
·7	5·00	5·13		5·63	5·22
·6	5·16	5·38	6·36	5·81	5·38
·5	5·36	5·63		6·02	5·60
·4	5·56	5·90		6·25	5·88
·3	5·82	6·25		6·55	6·28

TABLE XXIII (cont.)

		Sample I			Sample II
Acidity % H_2SO_4	Acid Gelatine Ph	Water Gelatine Ph	Water Gelatine after storage Ph	Alkaline Gelatine Ph	Water Gelatine Ph
·2	6·15	6·66		6·92	6·75
·1	6·65	7·32		7·42	7·45
0	7·35	7·85		8·00	8·10
-·1	8·13	8·38		8·50	8·62
-·2	8·57	8·70		8·85	9·00
-·3	8·87	9·00		9·15	9·30
-·4	9·12	9·23		9·38	9·53
-·5	9·33	9·43		9·58	9·73
-·6	9·52	9·62		9·74	9·92
-·7	9·57	9·76		9·88	10·08
-·8	9·82	9·92		10·03	10·22
-·9	9·98			10·16	10·33
-1·0				10·28	10·45

The Ph values practically coincided with those for acid gelatine at the acid end of the range, but for the most part were a shade higher: that is, the Ph acidity curve for water extracted gelatine lay in between that for acid and alkaline gelatine, near to the acid gelatine curve. On account of the importance of this result, some months later it was decided to repeat the observations on the water-extracted gelatine, using the same sample as before. When this was done, the Ph values found did not agree with the previous result and were similar to those obtained for an alkaline gelatine (the figures are shown in Table XXIII). This interesting but annoying behaviour undoubtedly arose from the gelatine deteriorating in storage. The original iso-electric point of this sample had not been determined, but it could not have been above ·5% acidity. It was found after storage to be at 1·0% acidity (Ph 5·28). Its jelly strength was 35, and viscosity 2·2 centipoises - values much lower than the gelatine must have possessed originally, for with such low physical properties it could not have been dried. This gelatine had thus broken down during storage, during which it lost ammonia, with the result that the iso-electric point had moved to the acid side. The gelatine then behaved as alkaline gelatine as regards the relationship between Ph and acidity. This phenomenon illustrated the difficulties with which the experimenter is faced in handling natural substances subject to the action of bacteria and enzymes.

Another lot of water-extracted gelatine was prepared with great care and, after drying, was brought to its iso-electric point and washed with distilled water. This gelatine had its iso-electric point at

·5% acidity (Ph 5·60), an ash content of ·3%, a jelly strength of 97 and a viscosity of 70 centipoises: normal physical properties for this method of preparation. A range of Ph values was then obtained for different acidities. This was repeated several times, as the results were not exactly what had been expected. The final results are shown in Table XXIII. On the acid side of neutrality, the Ph values were slightly higher than those for acid gelatine, and considerably lower than the figures for alkaline gelatine. Near neutrality, however, the Ph values approached those for alkaline gelatine, and below neutrality they were actually higher. As the difference in the position of the titration curves of acid and alkaline gelatines can only be due to loss of basicity during soaking, it appears reasonable to conclude that the behaviour of the titration curve of water-extracted gelatine on the alkaline side of neutrality must be due to loss of ammonia. In other words, water-extracted gelatine is not stable in alkaline solution. This will, however, require confirmation by further investigation. The fact that on the acid side of neutrality the Ph values for water-extracted gelatine are higher than those of acid-extracted gelatine, at the same acidity, points to a loss of ammonia during extraction, the loss however being on a smaller scale than occurs during a soak in alkali. The position of the titration curve of gelatine extracted with water thus fits in with the magnitude of the loss of ammonia previously observed to take place when untreated sinews are heated with water.

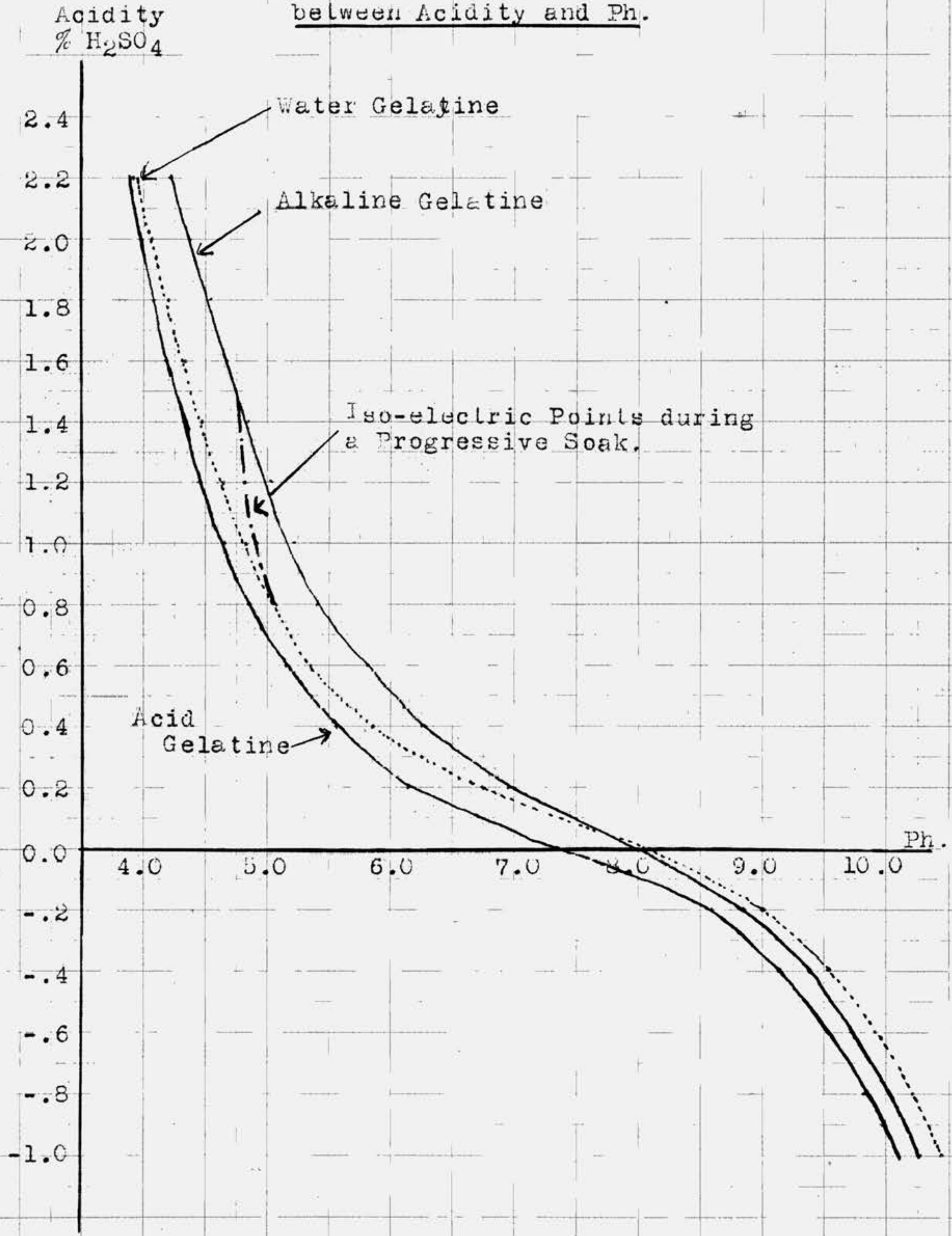
It was now possible to consider how the Ph values of iso-electric points obtained during a progressive soak would behave. Starting on the curve for water-extracted gelatine, as the soak in alkali proceeded, the Ph value corresponding to any given acidity of the gelatine produced would increase until it reached the value for alkaline-extracted gelatine. The standard figure taken for alkaline gelatine was Ph 4·75 corresponding to an acidity of 1·50%. To fix the point where the acidity-Ph relationship ceased to be the same as that of water gelatine, and also the behaviour during the transition period, use was made of two series of samples from progressive soaking experiments. Each gelatine was brought to its iso-electric point and the Ph determined. The results are shown in Table XXIV, along with figures giving the Ph corresponding to any given iso-electric point during a progressive soak. These were obtained from a curve combining the two sets of observations and have been taken as Standard. They have been used to interpolate Ph values corresponding to the acidity figures given for iso-electric points in the soaking experiments.

TABLE XXIV.

Progressive Soaking				Standard figures for Iso-electric points during Progressive soak	
Lime only		Lime/Soda Ash			
Acidity %	Ph	Acidity %	Ph	Acidity %	Ph
·61	5·31	·71	5·23	1·5	4·75
·87	4·90	·73	5·16	1·4	4·77
1·18	4·83	·85	4·96	1·3	4·79
1·16	4·87	1·03	4·86	1·2	4·82
1·43	4·67	1·23	4·76	1·1	4·86
1·40	4·71	1·36	4·69	1·0	4·91
1·43	4·75			·9	4·97
1·57	4·65			·8	5·06
				·7	5·22
				·6	5·38
				·5	5·60

Up to an acidity of about ·9% the Ph values follow those of water-extracted gelatine. Thereafter, the titration curves of the gelatines gradually move towards that of alkaline gelatine as the soak proceeds. The advantage of using acidities as against Ph values is very apparent. Between an acidity of ·9% and 1·5%, the Ph value only alters by ·22, and only by ·11 between 1·5% and 1·1%. If Ph values alone had been

Curves showing the Relation between Acidity and Ph.



used, this alteration in Ph is so small that quite a different idea might have been formed as to the speed with which the lime soak progressed. The reaction might have appeared to be complete when the iso-electric point reached an acidity of 1%, as the change in Ph after that is small.

Having thus investigated the relation between acidity and Ph for the different forms of gelatine, the results obtained provide a rather elegant means of showing how a particular sample of gelatine has been prepared. This can, of course, be done by finding the iso-electric point of the sample concerned, but an easier method is to find the acidity of the gelatine and its Ph - two simple determinations. The method of preparation is then determined at once by comparing the results with Ph/acidity tables for acid and alkaline gelatine.

Although gelatine extracted by water is not in itself of any importance, its uncertain behaviour noted in the above experiments may prove to be of considerable interest in relation to the preparation of gelatine by the alkaline method. Extraction by water may be considered an extreme case of under-soaking. Consequently, if it is later confirmed that water gelatine is unstable, it is also likely that soaking in lime for a sufficiently long period for the reaction to reach completion is necessary to ensure stability.

It may be found that gelatine made from material which has not been fully soaked is less resistant to heat and to alkali. These appeared to be profitable matters for further investigation.

THE STABILITY Of The VARIOUS TYPES Of GELATINE.

As the difficulties encountered when examining the titration curve of gelatine extracted with water indicated a lack of constancy in the basicity of the substance, which in turn probably arose from liability to lose ammonia, it was decided to examine the stability of gelatine prepared by different methods.

To accomplish this, use was made of the method previously adopted for measuring the nitrogen loss during the extraction of gelatine. A flask containing 800 c.c. of 5% gelatine solution was heated for 7 hours at 90°C. Any ammonia evolved was carried over by a current of purified air and absorbed by 50 c.c. N/5 H_2SO_4. The loss of nitrogen was then ascertained by means of a back titration. A number of experiments were carried out with gelatine made neutral to phenolphthalein. Even after 7 hours heating, the loss of ammonia was so small that it was considered desirable to make the gelatine alkaline. Accordingly, in the next series of experiments, a measured amount of caustic soda was added, which brought the acidity of the gelatine to -·3% as sulphuric acid - that is, to approximately Ph 9. This resulted in an increase in the amount of ammonia lost, but in the case of the stable form of gelatine, the ammonia involved was still of small magnitude. In the hope of obtaining a larger amount, a single experiment was done with gelatine at an acidity of -·6% (Ph 9·74). This, however, showed no marked improvement on the others.

The method finally adopted, that of steam distillation, was considered to be the best, as it resulted in the evolution of larger amounts of ammonia and gave very consistent results. A preliminary experiment was carried out by steam distilling 40 grms. of gelatine dissolved in 230 c.c. distilled water. The gelatine was made neutral to phenolphthalein, and 24 c.c. N/5 NaOH added, which brought its acidity to -·6% (Ph 9·74 in the case of alkaline gelatine). The distillate was collected in 50 c.c. N/5 H_2SO_4 , and four separate litre quantities were collected. Before carrying out the back titration, the bulk of each distillate was reduced by evaporation on the steam bath. It was found that the greater part of the ammonia came off during the distillation of the first litre. Thereafter, it continued to come off at a regular but much slower rate. Similar behaviour has been noted in connection with the acid hydrolysis of proteins, when there is rapid production of ammonia for the first few hours, and thereafter a slow production which continues over an indefinite period (LLoyd & Shore, Chemistry of the Proteins, 1938, p.119). In the final experiments, therefore, the gelatines were distilled into 50 c.c. N/5 H_2SO_4 until the volume reached 1100 c.c. This method was found to give very satisfactory results. The figures for all the experiments are shown in Table XXV.

In the first lot of experiments, in which the gelatine was neutral, and heated at 90°C. for 7 hours, the treatment was evidently too mild, for the amount of ammonia produced was low. It was noted, however, that water-extracted gelatine gave rise to a larger amount of ammonia. In the next series of experiments, in which the gelatine was made alkaline (Ph 9, approx.), the results were more satisfactory. Water-extracted gelatine evolved three times as much ammonia as any of the other samples examined. Acid-extracted gelatine behaved in the same way as gelatine made by the alkaline process. A sample of low-grade commercial gelatine which had in course of manufacture been subjected to longer heating and might thus be

TABLE XXV.

Description of Gelatine	C.c. N/5 H_2SO_4 neutralised by ammonia	Nitrogen Loss gms.per 100 gms. Gelatine
HEATING At 90°C. FOR 7 HOURS 800 c.c. 5% SOLUTION		
Alkaline-extracted gelatine made neutral to phenolphthalein.	1·5 1·6	·010 ·011
Water-extracted gelatine made neutral to phenolphthalein.	5·8 5·9	·040 ·041
Alkaline-extracted gelatine. Long lime soak, made neutral to phenolphthalein.	2·4	·017

Alkaline-extracted gelatine made neutral to phenolphthalein and 12 c.c. N/5 NaOH added. (-·3% acidity Ph 9·15).	5·3 5·4	·037 ·038
Water-extracted gelatine. (-·3% acidity Ph 9·30).	15·4 16·8	·108 ·117
Alkaline-extracted low-grade gelatine. (-·3% acidity Ph 9·15).	4·5 5·0	·031 ·035

Alkaline-extracted gelatine made neutral to phenolphthalein and 24 c.c. N/5 NaOH added. (-·6% acidity Ph 9·74).	6·7 6·9	·047 ·048

Acid-extracted gelatine (-·3% acidity Ph 8·87)	5·0 5·2	·035 ·036

TABLE XXV. (cont.)

STEAM DISTILLATION.

40 grams gelatine in 230 c.c. distilled water made neutral to phenolphthalein and 24 c.c. N/5 NaOH added (-·6% acidity).

Description of Gelatine		C.c. $N/5H_2SO_4$ neutralised by ammonia.	Nitrogen Loss grms. per 100 grms Gelatine
Alkaline-extracted gelatine (Ph 9·74)	1st Litre of distillate.	7·8	·054
	2nd Litre	1·5	·070
	3rd Litre	1·0	·007
	4th Litre	1·5	·010
Alkaline-extracted gelatine (Ph 9·74)	1100 c.c. distillate	6·8	·047
		6·8	·047
Acid-extracted gelatine (Ph 9·52)	1100 c.c. distillate	14·2	·099
		14·7	·103
Water-extracted gelatine (Ph 9·92)	1100 c.c. distillate.	27·7	·194
		27·9	·195

expected to contain more degraded products showed no greater readiness to split off ammonia than the high-grade variety. The one experiment carried out on alkaline-extracted gelatine by heating for 7 hours at Ph 9·74 gave results which agreed closely with those obtained by steam distilling. The quantities of ammonia produced by the different types of gelatine, when steam distilled in alkaline solution, were remarkable in their regularity. Acid-extracted gelatine evolved twice the amount obtained from the alkaline variety. Water-extracted gelatine produced four times the amount of ammonia which was obtainable from alkaline gelatine. As regards water-extracted gelatine, all the experiments led to the same conclusion, that it was definitely less stable. The amount of nitrogen which it was capable of losing as ammonia was of considerable magnitude, amounting to nearly half that lost when collagen was soaked in lime. Acid-prepared gelatine, although it lost more ammonia under the somewhat severe conditions of steam distillation, was considered to be relatively stable. No other experimental evidence had previously been obtained to show that it lost nitrogen readily, and this in spite of the fact that of all gelatines it contained the greatest amount of nitrogen. However, from the results obtained from the steam distillation, it was evident that the most stable gelatine was that prepared in the alkaline way, followed by gelatine prepared by use of acid, and lastly gelatine extracted by use of water alone, which might be described as unstable.

Apart from theoretical considerations which are dealt with in the general discussion, two interesting practical conclusions may be drawn from the results.

When gelatine is made from precursors which have been soaked for too short a period in lime, it will tend to possess the unstable properties of water-extracted gelatine. That is to say, its physical properties will tend to be more affected by heat, and possibly storage. This is an additional reason for paying particular attention to the length of the period of soak.

The second conclusion relates to the concentrating of dilute gelatine solutions. When an acid-extracted dilute solution of gelatine has its concentration raised by evaporation, it may be neutralised to minimise the damage by heat. After concentration, the solution will be found to be slightly acid. This, in view of the results obtained above, must be brought about by loss of ammonia during evaporation.

DEAMINISED COLLAGEN.

Having established that a small amount of nitrogen is lost when collagen is soaked in lime, resulting in a shift of the iso-electric point to a lower Ph, together with greater readiness to hydrolyse, it was considered possible that other means of removing small quantities of nitrogen from the protein molecule might lead to interesting results. When a protein is treated with nitrous acid, free or end NH_2 groups are replaced by OH groups, nitrogen is given off, and the protein is then said to be deaminised. This process has been widely studied as applied to soluble proteins such as gelatine, but little has been done with collagen. Thomas & Foster (J. Amer. Chem. Soc., 1926, 48, 489), working with standard hide powder, found that its nitrogen content was reduced by ·49% after treatment with nitrous acid. In the case of calf-skin, the loss on deaminising was found to be ·6% nitrogen. These figures were sufficiently close to the magnitude of the loss of nitrogen during a lime soak for the assumption to be made that the nitrogen in both cases came from the same source. On the other hand, both samples of collagen used in the experiments had been partially lime treated and would already have lost some nitrogen, so that the amount of nitrogen lost on deaminising was rather high for the assumption to be correct. Thomas & Foster also investigated the position of the iso-electric point of deaminised collagen and found that it had been moved to the acid side (between Ph 3·5 and Ph 4). This move in the position of the iso-electric point was more recently confirmed by Atkin (Stiasnyfestschrift Darmstadt, 1937, p. 13) who found that collagen on deaminisation showed a shift from Ph 5·3 to Ph 4·6. No information was, however, available as to the effect of deaminising on hydrolysis and it was therefore decided to examine this, and at the same time find how much nitrogen was lost from collagen which had not been treated with lime.

A preliminary experiment was carried out with 100 grms. of crushed dried sinew. After soaking in water until they were soft, the sinews were suspended in a litre of water and treated with 500 c.c. of a solution containing 100 grms. sodium nitrite and 67 c.c. glacial acetic acid. The reaction was allowed to proceed for 24 hours to make certain that it reached completion. The quantities of chemicals used were those recommended by Thomas & Foster. The reaction appeared to proceed rapidly, for the sinews soon turned canary yellow and there was a brisk evolution of gas. At the finish, the sinews were washed carefully, although they could not be made alkaline in the centre, even by use of caustic soda. This was due to the shift in the position of the isoelectric point. During washing, the sinews turned brown on the surface; alkali and heat also caused darkening. When heated in water the sinews became very leathery and no gelatine was extracted.

A second experiment was carried out in exactly the same way. Again there was no visible extraction of gelatine on heating the deaminised sinews with water. However, by taking the extract down to dryness on a steam bath, a small quantity of gelatine was seen to have been formed. This was just sufficient to form a small quantity of very weak jelly. From those two experiments it was evident that deaminising collagen had made it less easily hydrolysed on heating with water. The reaction had, indeed, produced a condition closely approaching insolubility.

Another experiment was carried out in which 100 grms. of sinews were deaminised. When the reaction was over, they were carefully washed and soaked in 2% HCl solution. The sinews at once swelled and turned reddish-orange in colour. After a further wash and the addition of a small quantity of hydrochloric acid, they were extracted. After 3 hours, 15 grms. of rather poor brown-coloured gelatine was obtained. The iso-electric point of this was found to be Ph 5·04 or 1·1% acidity.

It thus appeared that deaminised collagen could be extracted by the use of acid, although extraction was slower and more difficult. This was confirmed by a fourth experiment in which the quantity of chemicals was altered to amounts which had been found to give good results. A quantity (200 grms.) of crushed sinews was soaked until soft. They were then put into a litre of water containing 60 grms. sodium nitrite and 60 c.c. glacial acetic acid, and left for 24 hours. The deaminised sinews were washed, soaked in 2%

hydrochloric acid solution, washed again for three hours and extracted. Two extractions, extending over 7½ hours in all, resulted in 56 grms. of brown-coloured gelatine. From its behaviour in the jelly form, the gelatine was poor in strength and of low viscosity. The slowness of extraction was once again confirmed by this experiment. The iso-electric point of the gelatine was at Ph 4·97 (acidity 1·1%), so that the deaminisation has brought about a shift from Ph 8 to Ph 5 in the case of the acid type. The remainder of the material was brought to its iso-electric point, dried, washed with distilled water, and dried again. It was powdered carefully, dried at 110°C., and finally over P_2O_5 to get rid of all moisture. Its ash was then ·25%. Nitrogen determinations were carried out on the powder, which showed the nitrogen content to be 16·98%. The nitrogen content of acid-extracted gelatine was found to be 17·90%, so that the loss of nitrogen on deaminisation was practically ·9%. This was higher than either of the figures given by Thomas & Foster. From the magnitude of the result, it was clear that the nitrogen removed on deaminisation could not be the same as that which was removed during a lime soak. Another interesting feature which led to the same conclusion was that the magnitude of the shift of the iso-electric point was less than would have been expected from the relatively large loss of nitrogen. The titration curve of the deaminised gelatine in the neighbourhood of the iso-electric point fell between the curve for acid gelatine and that for alkaline gelatine. Judging from the lower nitrogen content, it should have fallen above the curve for alkaline gelatine. The actual figures were as follows:

Acidity % H_2SO_4	Ph	Acidity % H_2SO_4	Ph
1·8	4·30	1·2	4·85
1·6	4·46	1·0	5·09
1·4	4·65	0·8	5·33

It was concluded from those experiments on sinews treated with nitrous acid that the treatment did not render the sinews completely insoluble, but that they could ~~not~~ be extracted, although more slowly, by use of acid. The question of alkaline extraction had still to be settled.

Another lot of crushed sinews (200 grms.) were deaminised and, after washing, were soaked in lime for 8 weeks, the liquor being changed as required. The sinews were then washed and extracted for 6 hours at 80°C. A yield of 7·5% was obtained of very weak gelatine, which was too poor to dry off. Thus, no further determinations could be carried out on the sample. The experiment showed, however, beyond any doubt, that deaminising collagen increased its resistance to hydrolysis, from which it may be argued that the nitrogen taking part in the reaction is not the same as is lost during a lime soak for the preparation of gelatine.

It was hoped to include the results of estimations of the nitrogen evolved during the deaminisation of collagen. Unfortunately the figures obtained were low, amounting to ·38% amino-nitrogen. As this was perhaps due to the reaction being incomplete or to defects in the method, additional experiments will have to be undertaken before they can be recorded.

GENERAL DISCUSSION.

As a result of investigations reported in the present paper, it is possible to give a description of the extraction of gelatine based on observations established by experiment. Some speculation may also be permitted as to the bearing of those facts on the relation of collagen and gelatine.

There are three ways of preparing gelatine. It may be extracted from the precursor by heating with water - not a practical method although the old-fashioned one; it may be extracted in the presence of acid, or it may be extracted after soaking the precursor in alkali for a considerable period of time. It is true that acid may be used for the extraction of material which has been soaked in alkali, but this may be considered to involve merely an alteration of the Ph at which the extraction is carried out, and not as a special method. The resulting gelatine is the same as if the use of acid had been omitted.

The three methods of preparation give rise to three definite types of gelatine. In a previous communication it had been suggested that there were four kinds of gelatine. The variety made by extracting limed material in the presence of acid has since been shown to be the same as that prepared by alkaline extraction. Thus the four types postulated in the earlier paper can be reduced to three.

When a collagen-containing precursor is soaked in alkali, swelling takes place, most of the other proteins are removed, and the iso-electric point or point of minimum swelling of the collagen moves from the region of Ph 8 to Ph 5. This can only be due to a loss of basic groups from collagen, which takes place in the form of ammonia. During the soaking period, collagen becomes gradually more susceptible to hydrolysis with hot water. Even after a period of three months soaking in saturated calcium hydroxide solution, heat is required to effect the change from collagen to gelatine. Soaking alone only brings about one stage of the transformation.

The next stage is to heat the prepared collagen with water. Collagen first of all shrinks and then dissolves in the water as gelatine, the rate of solution depending on the length of the time of soak. Under similar conditions of Ph, the jelly-strength of the extracted gelatine is greatest with the shortest time of extraction, and hence improves as the time of soak lengthens. The reason for this may partly be attributed to the detrimental effect of continued heating on the gelatine which is first extracted. The main reason, however, must be that heat has also a detrimental effect on collagen, and it is the unextracted collagen which is suffering damage. During the early period of a long extraction, there is practically no gelatine in solution; most of the extraction takes place towards the finish, so that the extracted gelatine has not been exposed to heat for very much longer than it would have been during a short extraction. Lime soaked collagen does not give rise to more than traces of ammonia during the conversion to gelatine, which is in accordance with the observed positions of iso-electric points of the precursor after lime treatment and the gelatine it produces.

The gelatine obtained differs from acid-extracted gelatine in having its iso-electric point at Ph 4·7 against Ph 8, resulting in different titration curves. For a given acidity estimated as sulphuric acid, gelatine of the acid type is at a lower Ph than the alkaline-prepared variety. The nitrogen content of gelatine prepared by an alkaline method is 0·48% lower than that of gelatine extracted with acid. In addition, it also has a lower melting point when compared with gelatine of the acid type of the same jelly strength.

The difference in the extraction of gelatine by acid is that the precursor does not require any preparatory soaking beyond what is sufficient to enable the acid to penetrate the physical structure of the material. Provided that an excessive quantity of acid is not present, collagen hydrolyses rapidly on heating with water to give gelatine of good jelly strength. When a similar experiment is attempted with alkaline solution, collagen is completely hydrolysed to simpler non jelly-forming proteins. This is probably an indication that hot alkaline solutions have a more destructive effect on collagen than hot acid solutions. Jordan Lloyd (Biochem. J., 1920, 14, 147), in carrying out swelling experiments on gelatine, noted that caustic soda appeared to destroy the gelatine, whereas from acid solutions the gelatine could be recovered unchanged. The relative activity of hot acid and alkali on collagen is probably similar.

Gelatine prepared by heating untreated collagen with water alone, although of no practical importance, is of considerable theoretical interest. When collagen, without previous soaking except for a short period in water, is heated, it shrinks. Heating may be continued for some time without any visible sign of gelatine in solution, but ammonia is evolved. During the last two hours of a six hour period of extraction, gelatine appears in solution. The ammonia lost during the extraction is one-fifth of the amount lost during a soak in lime. The iso-electric point and titration curves of this gelatine occupy positions with respect to those of the other types of gelatine which are in accordance with the ammonia loss during extraction. An interesting property which is peculiar to this type of gelatine is its instability, particularly in alkaline solution, and on keeping. It appears to have a tendency to turn into the alkaline variety of gelatine, presumably by loss of ammonia.

The numerous soaking experiments described in the present paper indicate that when collagen is soaked in alkali, a slow chemical reaction takes place, resulting in a loss of nitrogen in the form of ammonia, with a consequent shift in the position of the iso-electric point. Although the reaction was studied by observing the change in the gelatine produced, it was clearly established that the chemical change took place in the collagen. As a chemical reaction the soaking of collagen in alkali is not easy to understand, for it is not a case of a change from collagen A to collagen B, with a gradual increase in the quantity of collagen B present, and a diminishing amount of collagen A. Owing probably to the ammonia being released in stages, and possibly also to the physical structure of the precursors used, gelatine may be obtained which varies in composition from that of the water-extracted variety to the final alkaline variety of fixed composition. A reaction is thus taking place where there is a gradual transition from one substance of definite composition to another of definite composition, with the possibility of gradual variation in between. There has always been some doubt as to whether or not gelatine is a specific substance (Krishnamurti & Svedberg, J.A.C.S., 1930, 52, 2897; Schryver, Biochem. J., 1923, 17, 487; Kunitz & Northup, J. Gen. Physiol., 1929, 12, 379). It is evident that alkaline gelatine, unless prepared from a well soaked precursor, may well be a mixture, and in any case will have variable characteristics. Nevertheless, if the reaction which takes place during an alkaline soak is given time to reach completion, the gelatine then obtainable is of definite composition and properties.

Careful examination of the results of the soaking experiments shows that the ease of extraction or, in other words, the readiness of collagen to hydrolyse, is exactly parallel to the course of the chemical reaction. That is, the rate of extraction, the ability of the gelatine to form firm jellies, and the position of the iso-electric point, all reach their maximum values at the same time during the soaking period. While this parallelism does not necessarily imply that loss of ammonia is essential before collagen can be easily hydrolysed, it does mean that a definite relationship is highly probable. This probability becomes almost a certainty when it is considered that the extraction of gelatine by water alone from unprepared collagen is accompanied by the evolution of ammonia. It appears that unless acid is used, collagen will not break down into gelatine without loss of nitrogen. In the preparation of alkaline gelatine, the nitrogen loss takes place during the preparatory soak; in the case of extraction with water alone it is lost during heating which has to be rather prolonged. The amount lost is only one-fifth of the quantity lost during a soak, but that does not invalidate the argument, for there are sound reasons why the nitrogen might be expected to be evolved in stages. If our deductions as to the importance of the nitrogen loss are sound, then for a proper understanding of the relation between collagen and gelatine it is necessary to know from what portion of the collagen molecule the ammonia originates. Unfortunately this is a problem which has been something of a puzzle for many years as reference to the literature of protein chemistry will show.

The difficulty arises in understanding how ammonia can be evolved from a substance consisting of a chain of amino-acid residues united by peptide bonds, without assuming a further condensation of two free NH_2 groups. Nasse (J.C.S., 1873, <u>11</u>, 760) was the first to suggest that the nitrogen contained in the ammonia must be present in the protein molecule in a state of combination different from that which is subsequently found in amino-acids. Subsequent to the work of Osborne, Leavenworth & Brautlecht (Am. J. Physiol., 1908-9, <u>23</u>, 180) it was accepted that this nitrogen existed in the amide form in the protein molecule as amides of aspartic and glutamic acids.

The relationship between gelatine and collagen is another matter which was not well understood and gave rise to a diversity of opinion. Hofmeister (Z. physiol. Chem., 1878, 2, 299) considered the conversion of collagen to gelatine to be a hydrolysis, and that it was reversible in heating gelatine to 130°C. This latter part of the statement is most certainly not correct, for when gelatine is heated to 130°C. it loses a small amount of ammonia and the small amount of moisture generally present, and becomes insoluble. Emmett & Gies (J. Biol. Chem., 1907, 3, 33) contraverted the claim that the formation of gelatine from collagen was reversible. They showed that ammonia is evolved during the hydrolysis of collagen to gelatine in hot water. When gelatine itself is boiled, no ammonia is liberated. Bogue (J.I.E.C., 1923, 15, 1154)stated that collagen was not a simple anhydride of gelatine but a polymerised complex produced by chemical condensation. The ammonia evolved during alkaline hydrolysis was thought by Bogue to be due to a further break down of the gelatine.

An interesting study was made by Aitken & Thompson (J. Soc. Leather Trades Chem., 1927, 11, 300) of the causes of the variable properties of hide powder. In their view, hide powder is collagen modified by liming which had opened up the -CO-NH-linkages forming free carboxyl and amino groups. The iso-electric points of different hide powders vary according to the number of free amino and carboxyl groups present. This theory fails to account for the loss of nitrogen during lime treatment, and also does not give a very satisfactory account of the variation in the iso-electric point. As each broken peptide linkage gives rise to a COOH group and an NH_2 group, it is difficult to see how additional free radicals would alter the position of the iso-electric point.

Merrill & Fleming (J.I.E.C., 1928, 20, 21), as a result of examining the hydrolysis of skin at temperatures between 5°C. and 45°C., concluded that secondary valency bonds in the collagen molecule were disrupted first, and then the component aggregates were hydrolysed into progressively simpler molecules. There was no mention of loss of ammonia or of its origin. The curves given by Merrill & Fleming showing the rate of hydrolysis of skin at different temperatures are interesting in that the discontinuity between 25°C. and 30°C., observed during experiments reported in the present paper, is clearly evident.

The origin of the ammonia found in lime liquors was investigated by Marriott (J. Soc. Leather Trades Chem., 1931, <u>15</u>, 25). He showed that when collagen was treated with lime, all the ammonia in the liquor was derived from acid amide groups of the protein. Spiers (The Swelling of Proteins, 1933, Int. Soc. Leather Trades Chem., p. 66) gave an account of the plumping of collagen based on the work of Marriott and others. The collagen molecule is made up of polypeptide chains which include 22 molecules of dicarboxylic acids and 30 molecules of diamino acids. There are then 23 carboxyl and 31 amino groups, allowing an extra one of each for the ends of the chains. In unlimed collagen, 19 of the carboxyl groups are present as acid amide groups. The remaining 4 carboxyl groups may be utilised in forming cross salt linkages binding the polypeptide chains together. Treatment with acid or alkali ruptures these salt links and permits the chains to be separated. There also appears to be a possibility of the formation of cross amide linkages.

The order of magnitude of the loss of nitrogen when hide powder is deaminised is similar to the loss during a lime soak, which suggested the possibility of a common source. As this nitrogen is definitely evolved from the free amino groups, the inference would be that they were also responsible for the nitrogen lost during soaking. Thomas & Foster (J. Amer. Chem. Soc., 1926, 48, 489) found that hide powder lost ·49% nitrogen on deaminisation. Calf-skin, similarly treated, showed a loss of ·6% nitrogen. Both the hide powder and the calf-skin had already been subjected to a certain amount of lime treatment, so that they would have already lost some nitrogen. This makes the nitrogen loss in the deaminising experiments rather high for the nitrogen to have come from the same source. There is nevertheless sufficient parallelism between the results of the two reactions to make a thorough investigation of the hydrolysis of deaminised collagen a matter of considerable interest.

Another aspect of the effect of calcium hydroxide on collagen was studied by Theis & Jacoby (J. Amer. Leather Chem. Assoc., 1941, 36, 375) who find that the basic amino acids, particularly histidine and lysine, are reduced by increased time of liming. The amount of formaldehyde fixed by limed collagen decreases with increasing time of liming, which may be taken to indicate a reduction in the number of free NH_2 groups, as they represent the point of attachment of formalin. The nitrogen loss on liming would accordingly arise from the elimination of amino groups. This was not the finding of Highberger & Stecker (J. Amer. Leather Chem. Assoc., 1941, 36, 368) who attributed the nitrogen loss during liming to hydrolysis of the amide groups. Urea and ammonia are formed during the treatment of collagen with lime, or with mixtures of lime and caustic soda. The amounts produced increase to a maximum with increasing time and alkalinity of the solution.

It would appear then that the balance of evidence favours the conclusion that the nitrogen evolved during the hydrolysis of collagen arises from the amides of the residues of aspartic and glutamic acids. Now if, as has been shown in the present paper, the removal of the nitrogen must take place before gelatine can be formed, these amides can hardly exist in the collagen molecule in the free form. It is difficult to understand how the removal of a small amount of amide nitrogen in side chains could in any way influence the break up of the main structure. The amides must therefore form cross linkages which may be the strongest links binding the separate polypeptide chains together. The possibility of cross amide linkages has been mentioned (Spiers, loc. cit.). If it is supposed that two glutamic acid residues share an amide group between them, a linkage of the form CO-NH-CO results, which on hydrolysis yields ammonia and leaves the acid residues unchanged. Besides the cross amide linkage, separate polypeptide chains may be connected by salt linkages and polypeptide bonds.

When collagen is soaked in lime the salt linkages are broken, probably rapidly. The cross amide linkages take time to break and give rise to the gradual evolution of ammonia. Polypeptide bonds are probably not broken during a lime soak of reasonable length, for collagen, though it may be much swollen, is still insoluble. When heat is applied after a lime soak, a complete separation of chains occurs, resulting in the formation of gelatine. The change which takes place on the application of heat is so considerable that it must indicate at least a spatial rearrangement of the molecules. There are thus two stages in the transformation of collagen into gelatine - the soaking period during which certain cross linkages are broken, and the application of heat during which the chains separate and internal rearrangement takes place. If both stages are carried out with care, a substance of definite composition and properties is obtained.

In order to account for the preparation of gelatine by the acid method, where no long preparation soak is necessary and there is no loss of ammonia, it must be assumed that the amide linkages are more easily attacked by acid.

One or two observations at the moment do not appear to fit in with this theory for the origin of the ammonia: for example, the decrease in the amount of formalin fixed by limed collagen as the length of soak increases (Theis & Jacoby, loc. cit.), and the reduction in the amount of basic amino acids. These, however, may be ancillary effects which have no bearing on the breakdown of collagen to form gelatine. Again deaminising collagen appears to render the extraction of gelatine extremely difficult. As nitrous acid only attacks free NH_2 groups, no marked difference would be expected in the behaviour of collagen after deaminising, unless the amino groups played a part in the mechanism of the transformation. This is a matter requiring further investigation.

If it is assumed that the ammonia released during a lime soak has its origin in amino groups, condensation with ring formation must take place during the reaction with lime, as two NH_2 groups are required for the formation of ammonia. This is contrary to the view generally held that the swelling which takes place during soaking is an indication of the breakdown of cross linkages (Jordan Lloyd, The Swelling of Proteins, 1933, Int. Soc. Leather Trades, Chem. p. 74). This theory also fails to explain the difficulty of hydrolysing deaminised collagen which might be supposed to be more readily attacked since the free NH_2 groups had been eliminated, although by another method. These are additional arguments against the supposition that the ammonia evolved during hydrolysis is derived from the amino groups.

Gelatine prepared by heating with water alone is of no practical importance but merits consideration on account of the simplicity of the method of extraction and its relation to other types of gelatine. When collagen is heated with water, gelatine is not obtained until a certain amount of ammonia is lost, the quantity being one-fifth of that evolved during an alkaline soak. This may be taken to imply that if a proportion of the amide links are broken, hydrolysis can take place. A peculiar property of this kind of gelatine appears to be that it very readily loses the remainder of its ammonia and becomes indistinguishable from alkaline gelatine, except in regard to jelly-forming power. It may be that once the breakdown of the amide linkages has been started, the remainder become more easily broken.

The instability of water gelatine may, however, arise in another way. The preparation of gelatine from collagen seems to take place in two stages. In the first stage - the soaking process - the amide linkages are broken with loss of ammonia. In the second stage, heat causes a rearrangement of the molecule which produces the visible result of the solution of collagen as gelatine. Now it does not appear possible to reverse the order of the two operations without altering the course of the reaction. The effect of temperature on soaking has been carefully investigated. As the temperature rises from 0°C. to 25°C., the rate at which lime and caustic soda acts increases with rise in temperature. The increase is of the order that would be expected for a chemical action. Between 25°C. and 30°C. there is a break in continuity, and any further rise in temperature, instead of accelerating the reaction, produces an altogether different type of reaction. This is visibly demonstrable, as collagen under these conditions breaks down instead of swelling. Gelatine can still be obtained from the broken down material but the amount obtained is less than would be expected, and its jelly-forming power is diminished. Similar results are visible if collagen is subjected in any way to heat before soaking in alkali, even if the soak is carried out at low temperature. The same effect is partly responsible for the type of gelatine obtained by extraction with water.

What these observations seem to indicate is that if the soaking stage is carried out first and sufficient time is allowed for elimination of ammonia, the second stage - that is, the rearrangement of the molecule by heat - results in the production of gelatine which is relatively stable. That is to say, the complete hydrolysis of collagen to amino acids has been arrested at a definite point. If, however, the order of the two stages is reversed and the heat rearrangement is allowed to take place first, then hydrolysis tends to proceed continuously so that the gelatine obtained contains a proportion of degradation products. It is possible that when the normal procedure is used, only certain linkages are broken. When the order is reversed, heat being applied first, linkages in polypeptide chains also break down, the two types of hydrolysis taking place at the same time. The degree of hydrolysis of gelatine is generally investigated by means of formo-titrations which are actually a measure of the free COOH groups. On account of restrictions imposed by the quantity of material available, not many of these were carried out during the present investigations (and the results have not been shown), but from the small variations observed, it appeared that there was little difference in the degree of hydrolysis of any of the samples examined. A systematic experiment on the progress of hydrolysis during a lime soak as shown by formo-titrations, using the methods employed in the progressive soaking experiments, might yield results of interest.

Although in the course of the experiments numerous ways were tried of improving on the lime soak as a means of making good gelatine, it must be carefully emphasised that nothing has been found superior to a lime soak of proper length. The main objection is the time the process takes, but this can be reduced by careful attention to temperature. Every effort should be made to have soaking carried out at a controlled temperature between 20°C. and 25°C. The importance of temperature is already well appreciated for certain operations in tanning, and it is even more important in the preparation of gelatine.

It is quite true that the soaking time can be reduced by the addition of caustic soda or soda ash, but the resulting gelatine is less likely to be clear, and unless the soaking temperature is low, care must be taken that the reaction does not go too far. Concentrations of caustic soda alone, sufficiently high to shorten the soaking period, cause loss of material, give rise to an inconvenient degree of swelling, and tend to produce dull gelatine. For those reasons caustic soda is probably less desirable in use than is generally believed.

It is quite clear that the acid process has certain advantages. Provided the precursor used has not been treated with lime, a gelatine will be obtained of definite composition and constant iso-electric point.

It will, as a rule, have a lower ash content, but may contain more organic impurity of an acid soluble nature. The impurities are of a kind which give rise to colour and, generally speaking, acid-extracted gelatine is darker in colour. There does not appear to be any theoretical advantage in extracting fully limed or partially limed material in an acid condition, although practical considerations may make this desirable. When this is done, gelatine will be obtained of variable nitrogen content.

SUMMARY.

The results of these investigations may be stated briefly as follows:-

1. Swelling experiments on raw hide show that at all concentrations caustic soda causes more swelling than lime. A 1% concentration of caustic soda produces the least amount of swelling. Concentrations greater than 1% destroy hide.

2. Combinations of lime and caustic soda or soda ash produce additive swelling effects on hide. Concentrations greater than ·5% caustic soda or ·7% soda ash, when used along with lime, destroy hide.

3. Rise of temperature between 0°C. and 20°C. brings about a rapid increase in the rate and amount of swelling.

4. Progressive soaking experiments show that caustic soda reacts with skin and sinew in the same manner as lime. It is not, however, as effective, except that owing to its solubility higher concentrations may be used.

5. Caustic soda has two marked disadvantages: (a) the amount of swelling produced renders washing extremely difficult; (b) its use results in the production of dull gelatine, particularly at temperatures between 15°C. and 20°C.

6. The influence of temperature on the chemical reaction taking place during soaking is marked. At 0°C. it hardly takes place at all; at 20°C. it goes very rapidly.

7. When soaking is carried out at temperatures above 25°C., the results obtained show that a different reaction is taking place from that which happens at lower temperatures.

8. The minimum shrinkage temperature of limed sinew is just above 30°C., and that of limed ox-hide just above 40°C. It is suggested that this has a bearing on the failure to obtain satisfactory results after soaking at temperatures above 25°C.

9. The application of heat prior to soaking has a detrimental effect on the quality of the gelatine produced. The type of reaction during the soak then appears to be the same as if the soak had been carried out at temperatures above 25°C.

10. Calcium chloride has a detrimental effect on gelatine obtained from precursors treated with it during the soaking period.

11. Sodium sulphate inhibits swelling during a caustic soda soak but slows down the reaction. Swelling occurs whenever the material is washed.

12. Trypsin alters collagen both before and after liming in such a way as to make it more easily hydrolysed without, however, altering the position of the iso-electric point of the collagen or of the gelatine produced. As a rule, the gelatine obtained has a low viscosity but it may have a good jelly strength.

13. The nitrogen content of gelatine prepared by alkaline extraction is 0·48% less than that prepared by acid extraction.

14. Collagen, before treatment with alkali, has its iso-electric point at Ph 7 as measured by its shrinkage point; after treatment with alkali, at Ph 5. This move in the iso-electric point corresponds to a loss of nitrogen in the form of ammonia.

15. The ammonia loss during extraction of fully limed material is of such small magnitude that it may be attributed to further break down of the gelatine. That this must be so may also be deduced from the results of progressive soaking experiments.

16. Acid extraction after alkaline soaking has no effect on the type of gelatine produced, which is the alkaline type

17. Alkaline-treated collagen should not be extracted at its iso-electric point; extraction is difficult and the resulting gelatine suffers in respect of jelly strength.

18. There are three types of gelatine:

(a) Alkaline-extracted gelatine whose iso-electric point is at Ph 4·75.

(b) Acid-extracted gelatine whose iso-electric point is at Ph 8.

(c) Water-extracted gelatine whose iso-electric point is at Ph 5·60.

19. As a result of the difference in the iso-electric point, the titration curve of alkaline prepared gelatine differs from that of acid-prepared gelatine. Water-extracted gelatine has a curve which on the acid side of neutrality lies between the other two curves.

20. Water-extracted gelatine is definitely unstable. There is some doubt about the stability of the acid-prepared variety. Alkaline gelatine is definitely the most stable of the three types.

21. Acidity is a useful value to use to follow the behaviour of the iso-electric point during a soak, as it shows bigger differences in the region of the iso-electric point than Ph values.

22. The method of preparation of a sample of gelatine can be ascertained by carrying out an acidity determination and a Ph determination.

23. It is suggested that when collagen is soaked in alkali, cross amide links binding together polypeptide chains are broken, with loss of ammonia, thus facilitating the subsequent transformation of the collagen by heat into gelatine.

24. The extraction of gelatine by water alone, and the type of gelatine obtained at different periods of a soak, show that the loss of ammonia can take place in stages.

25. Deaminisation of collagen appears to render it less easily hydrolysed. It is confirmed that deaminisation causes a shift in the iso-electric point.

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