CCCXL.—*The Isoelectric Condition of Gelatin.*

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ACCORDING to the theory of amphoteric electrolytes as put forward by Michaelis it is readily shown that at the isoelectric point the concentration, C_H , of hydrion is given by the relation

$$C_H = \sqrt{(K_a K_w / K_b)}$$

where K_a and K_b are the acid and basic dissociation constants of the amphoteric electrolyte and K_w is the ionic product of water (compare Pauli's "Colloid Chemistry of the Proteins," p. 41). Consequently, the hydrion concentration at the isoelectric point should, at a given temperature, be a constant, quite independent of the dilution of the amphoteric electrolyte. Pauli and Samec's experiments (Pauli, *op. cit.*, p. 38) on the precipitation of serum-albumin by alcohol from solutions buffered by sodium acetate-acetic acid mixtures do actually show that the hydrion concentration of the point of maximum precipitation is independent of the concentration of the protein over a considerable range (0.2—1.0 per cent. of albumin). Similar results have been obtained by Michaelis and by Pauli and Samec in electrophoresis experiments (Pauli, *op. cit.*, p. 41).

Theory further shows that at the isoelectric point the undissociated fraction of the ampholyte, that is, the ratio of the number of electrically neutral particles to the total concentration, is at a maximum (Pauli, *op. cit.*, p. 31). The results of numerous experiments indicate that the occurrence of a maximum number of neutral particles will be expressed by a minimum (or maximum *)

* In the case of precipitation by alcohol, the amount of precipitate is a maximum at the isoelectric point.

in the curve showing the relation between viscosity (or some other physical property) and hydrion concentration. Pauli and Matula (*Trans. Faraday Soc.*, 1913, **9**, 34; *Kolloid Z.*, 1913, **12**, 222) found that for a 1 per cent. gelatin solution at 35°, buffered with sodium acetate-acetic acid mixtures, this minimum occurs at $p_H = 4.7$, which agrees with the hydrion concentration of the isoelectric point of gelatin as determined by electrophoresis, also in buffered solutions (Michaelis and Grineff, *Biochem. Z.*, 1921, **41**, 373).

This value of $p_H = 4.7$ for the point where solutions of gelatin show a minimum or maximum with respect to some particular physical property has often been confirmed in recent years, a number of different acids having been used to impart definite hydrion concentration to the various solutions. For example, Loeb states: that for 0.5, 1.0, and 2.0 per cent. solutions of gelatin hydrochloride the relative viscosity is a minimum at the isoelectric point, $p_H = 4.7$ (*J. Gen. Physiol.*, 1921, **3**, 827); that the viscosity and swelling curves of 1 per cent. solutions of gelatin show a minimum at $p_H = 4.7$ (*ibid.*, 1918—19, **1**, 363); that the swelling is a minimum at the isoelectric point, $p_H = 4.7$, for 1 per cent. gelatin solutions containing nitric, hydrochloric, trichloroacetic, phosphoric, oxalic, sulphuric, tartaric, and citric acids respectively (*ibid.*, 1920, **3**, 247). In fact, all through his many papers, Loeb states repeatedly that the various physical properties of gelatin solutions show a minimum value at $p_H = 4.7$. Similarly Miss Lloyd's results for the swelling of gelatin in hydrochloric acid solution (*Biochem. J.*, 1920, **14**, 147) indicate a minimum at $p_H = 4.7$ (compare Atkin, *J. Soc. Leather Trades Chem.*, 1920, **4**, 248). Other evidence has been adduced by Bogue (*J. Amer. Chem. Soc.*, 1922, **44**, 1343; *J. Ind. Eng. Chem.*, 1922, **14**, 22; *J. Franklin Inst.*, 1922, **193**, 795), etc.

Since the same minimum point is obtained irrespective of the acid used, one would conclude that gelatin behaves in the same way towards both strong and weak acids, at all events in the low acid concentrations which are necessary to bring the solution to, or near to, the isoelectric point. The results of Wintgen and Krüger (*Kolloid Z.*, 1921, **28**, 81) and Wintgen and Vogel (*ibid.*, 1922, **30**, 45) indicate also that towards hydrochloric acid gelatin acts as a mono-acid base (compare also Lloyd and Mayes, *Proc. Roy. Soc.*, 1922, [B], **93**, 69).

In practically all the experiments where single acids were used instead of buffer mixtures, attention has been directed only to the hydrion concentration produced in the protein solution and to the corresponding value of a particular physical property, and, as seen from the results already quoted, the minimum point in, for example, the viscosity curves of gelatin occurs at the same $p_H (= 4.7)$ for

different concentrations of the proteins, thus agreeing with the results obtained in buffered solutions. In these experiments with non-buffered solutions, no account seems to have been taken of the quantity of acid which was necessary to produce, for example, a minimum viscosity at various concentrations of gelatin, it apparently being assumed, in accordance with theory, that this quantity is independent of the concentration of the ampholyte (compare Pauli, *op. cit.*, p. 41). Pauli and Samec (*ibid.*, pp. 42—43) have investigated this point by studying the viscosity curves for gelatin and the alcohol precipitation of serum-albumin. The results obtained for the viscosities of gelatin in the presence of sulphuric acid at 35° are given in the following table, where t and t_0 are the times of flow of the solution and of an equal volume of water respectively through the viscometer, that is, t/t_0 is the relative viscosity.

TABLE I.

Concentration of acid.	1.09% Gelatin. t/t_0 .	0.659% Gelatin. t/t_0 .	0.33% Gelatin. t/t_0 .
0	1.485	1.248	1.113
5×10^{-6}	—	1.241	1.111
1×10^{-4}	1.453	1.237	1.109
2×10^{-4}	1.445	1.233	1.111
2.5×10^{-4}	1.443	1.234	1.113
4×10^{-4}	1.448	1.241	1.118
5×10^{-4}	1.453	1.245	

The minimum viscosities are printed in italics, and, assuming that the measurements are to be trusted to the third place of decimals, it will be seen that as the concentration of the gelatin increases the concentration of acid necessary to produce a minimum viscosity also increases. Similar results were obtained in the precipitation of albumin by alcohol. If these points of minimum viscosity have the same p_H , agreeing with that of the isoelectric point, then the results are not in accord with theory. Pauli and Samec give no record of the p_H measurements, so we have repeated their experiments, at the same time measuring the p_H of solutions of minimum viscosity. Our results (see experimental part) are in agreement with theirs, and, in addition, it is found that the p_H is the same ($= 4.7$) for all solutions of minimum viscosity. Experiments made with acetic acid in place of sulphuric acid gave similar results.

Because of the discrepancy between theory and experiment the question was reconsidered from the point of view of Sir James Walker's theory of amphoteric electrolytes (*Proc. Roy. Soc.*, 1904, [A], 73, 155). For the sake of simplicity, the external acid added to the gelatin solution is supposed to be a strong, monobasic acid.

If gelatin be considered as a simple amphoteric electrolyte, it

may be represented by the formula $HXOH$. Let an external acid, denoted by the formula HA , be added to the solution. The individuals in the solution will then be :

H^+ , OH^- , XOH , XH^+ , $HXOH$, X , A^- , HA at the respective concentrations a , b , c , d , e , f , g , h .

A salt of gelatin, HXA , with the acid will also be formed, but it has been shown by Manabe and Matula (*Biochem. Z.*, 1913, **52**, 369) that when hydrochloric acid up to a concentration of $0.05N$ is added to a 1 per cent. solution of gelatin, the salt formed may be considered as completely dissociated, and in this discussion of the effect of adding acid to gelatin the concentration of the undissociated salt is neglected.

The conditions governing the equilibrium in the solution are expressed by the following equations :

$$ab = K_w \quad . \quad . \quad . \quad (1) \quad \text{whence } b = \frac{K_w}{a}$$

$$ac = K_a e \quad . \quad . \quad . \quad (2) \quad c = \frac{K_a e}{a}$$

$$bd = K_b e \quad . \quad . \quad . \quad (3) \quad d = \frac{K_b e a}{K_w}$$

$$cd = Q f^2 \quad . \quad . \quad . \quad (4) \quad f = \sqrt{\frac{cd}{Q}} = e \sqrt{\frac{K_a K_b}{K_w Q}}$$

$$ag = Kh \quad . \quad . \quad . \quad (5) \quad h = \frac{ag}{K}$$

$$a + d = b + c + g \quad . \quad . \quad . \quad (6) \quad \text{for electrical neutrality}$$

where K_w is the ionic product for water, K_a the acidic dissociation constant for the amphoteric electrolyte, K_b the basic dissociation constant for the amphoteric electrolyte, K the dissociation constant of the acid added, and Q is a constant.

Substituting in equation (6) for b , c , and d , we have

$$a + \frac{K_b e a}{K_w} = \frac{K_w}{a} + \frac{K_a e}{a} + g$$

$$\text{whence} \quad g = a - \frac{K_w}{a} + e \left(\frac{K_b a}{K_w} - \frac{K_a}{a} \right),$$

that is to say, g is a straight-line function of e for any particular value of a , and therefore $(g + h)$, which expresses the total amount of external acid present per litre, is also a straight-line function of e , since $h = ag/K$.

At the isoelectric point, c becomes equal to d (according to definition) and the expression within the bracket becomes zero, that is, the amount of external acid to be added in order to bring the solution to the isoelectric point is independent of any factor connected with the concentration of the amphotolyte.

If the concentration of hydron, a , is less than that which obtains at the isoelectric point, the expression within the bracket will be negative, that is to say, the amount of external acid to be added in order to make the value of a less than that which holds at the isoelectric point will be smaller as e increases. On the other hand, for values of a greater than that of the isoelectric point, $(g + h)$ increases as e increases.

In the above deductions e may be replaced by the total concentration of the ampholyte for any given value of a , since e is then

FIG. 1.

proportional to the total concentration, as may be seen from the following :

The total concentration of ampholyte is

$$\begin{aligned} c + d + e + f &= K_a e / a + a K_b e / K_w + e + e \sqrt{(K_a K_b / K_w Q)} \\ &= \text{const.} \times e \text{ for a given value of } a. \end{aligned}$$

It follows that for solutions of an ampholyte, acting simply as a monoacid base and a monobasic acid, to which an external acid is added, if we plot total concentration of acid present against the hydron concentration of the solution for various concentrations of ampholyte, a series of curves should be obtained which intersect at a value of a corresponding with the isoelectric point of the

ampholyte. In the diagram,* Fig. 1, the curves are numbered 1, 2, and 3 to indicate increasing concentration of ampholyte. To obtain a hydrion concentration, a_1 , more acid is required for solution 1 than for solutions 2 and 3. The value a_2 corresponds with the isoelectric point, and at this point the amount of acid is independent of the concentration of ampholyte. For C_H of a_3 the curves have crossed, and it requires more acid for a greater concentration of ampholyte.

This appears to be the principle of Sørensen's method (*Z. Physiol. Chem.*, 1918, **103**, 192; compare Pauli, *op. cit.*, p. 52) of determining the isoelectric points, but his method of deduction is much more complicated than that given here. It should also hold for a dibasic acid such as sulphuric acid and also for weak acids; in fact, Sørensen uses it for determining the isoelectric point of egg albumin in the presence of a mixture of sulphuric acid and ammonium sulphate, which he considers to be mixtures of the acid $H(NH_4SO_4)$ with the salt $NH_4(NH_4SO_4)$.

The experimental results obtained with gelatin and sulphuric and acetic acids do not agree with this theory, since the amount of acid necessary to give minimum viscosity and a p_H of 4.7 increases as the concentration of the gelatin increases. It remains to find an explanation for this.

Pauli (*op. cit.*, pp. 45–46) states that in gelatin–hydrochloric acid mixtures the hydrion concentration at which non-preferential migration of the protein occurs in electrophoresis experiments is far below that at which the same condition occurs with regulator (buffer) mixtures. From this and other evidence, he concludes that strong acids behave differently from weak acids towards proteins. Examination of his results shows, however, that he really finds that strong acids differ from regulator mixtures, since the action of weak acids alone, that is, not in the presence of their sodium salts, was not investigated. Our results show that acetic acid gives the same anomalies as sulphuric acid. Also the many results of Loeb and others show that when strong and weak acids which dissociate into two ions are compared at the same p_H they have the same effect and that dibasic and monobasic acids have similar effects, although the quantitative results are different.

Pauli explains the anomalies to which he directs attention by assuming that in the presence of strong acids certain basic groupings in the protein molecule are brought into action which are not affected by weak acids or by regulator mixtures. It is plausible that such a difference may exist between the action of strong acids

* The curves may not be straight lines actually, but the diagram is only meant to illustrate the particular point at issue.

and regulator mixtures, owing to the presence of the neutral salt in the latter, but it is difficult to see why there should be a difference in kind rather than in degree between the action of strong and weak acids. If this difference of degree is accepted, the observed behaviour of sulphuric and acetic acids can be explained; * more of the sulphuric acid than of the acetic acid will be used up in making active the extra basic groupings, but owing to the smaller dissociation of the acetic acid more of it will be necessary than of the sulphuric acid to produce the effect which leads to the isoelectric condition. The actual quantities of sulphuric and acetic acids necessary to give minimum viscosity and the same p_H may therefore conceivably be approximately the same, as in our experiments.

In working with gelatin, it must be remembered that it is very probable that we are dealing, not with a pure substance, but with a mixture of a protein and its degradation products. Suppose we have a mixture of two ampholytes, the one of which has its isoelectric point at 4.7 and the other at 8.0, and that the amount present of the former is greatly in excess of the latter. It seems probable that when submitted to electrophoresis such a mixture will show equal and opposite electro-diffusion (as measured by Kjeldahl estimations †) at a p_H , not of 4.7, but of some higher value (lower hydrion concentration). In this case, the isoelectric point, as determined by electrophoresis, would be of a p_H intermediate between the isoelectric point of the ingredients. This would explain Pauli's results with gelatin-hydrochloric acid mixtures, especially since he used gelatin which had been dialysed for a very considerable time, and in which degradation products would be present, but it would not explain an isoelectric point of 4.7 with regulator mixtures.

Consider now the case of minimum viscosity in our hypothetical mixture. The minimum viscosity is supposed to occur when the maximum number of neutral particles is present and this condition with a simple ampholyte occurs at its isoelectric point.

The state of affairs existing in our mixture may be indicated diagrammatically (Fig. 2).

The concentration of neutral particles due to each ampholyte is plotted against p_H and the sum, shown by the thick curve, gives the total concentration of neutral particles. Again the maximum occurs between the isoelectric points. Von Schroeder (*Z. physikal. Chem.*, 1903, **45**, 75) has shown that the viscosity of the degradation

* Pauli and Hirschfeld (*Biochem. Z.*, 1914, **62**, 245) found that for a given concentration of acid more sulphuric than acetic acid is bound by albumin.

† Total nitrogen estimations alone do not in any way tell us the nature of the diffusing material.

products of gelatin is much lower than that of undegraded gelatin and therefore, in the case of gelatin, it is reasonable to assume that the viscosity will be more affected by the neutral particles of the unhydrolysed protein than by even an equal concentration of such particles of the degradation products. The viscosity minimum of the mixture would therefore most likely occur much nearer to the isoelectric point of the unhydrolysed protein than to that of the degradation products; it may practically coincide with the isoelectric point of the undegraded gelatin, as is actually found to be the case. The non-coincidence of the point of equal and opposite electrophoresis with that of minimum viscosity in the case of strong acids may thus be explained.

FIG. 2.

EXPERIMENTAL.

The gelatin used throughout these experiments was very kindly supplied by Drs. Mees and Sheppard of the Kodak Research Laboratory. It had been prepared by electrophoresis methods as described by Sheppard, Sweet, and Benedict (*J. Amer. Chem. Soc.*, 1922, **44**, 1858) and was stated to contain not more than 0.1 per cent. of ash; * our analyses confirmed this statement.

The solutions of which the viscosities were to be measured were made as follows. The amount of gelatin required to give 100 c.c.

* Gelatin which is practically ash-free, and which is often prepared by methods depending on first obtaining an isoelectric solution of gelatin, seems to be commonly spoken of as *isoelectric* gelatin. This is a misnomer, since theoretically, isoelectric gelatin can only exist in a solution containing extra hydron supplied by an external acid. Such gelatins should always be spoken of as ash-less or ash-free gelatins.

of a solution of definite strength (calculated as moisture-free gelatin) was weighed out into a 100 c.c. flask. About 75 c.c. of freshly boiled and cooled distilled water were added and the whole was then heated in a water-bath from room temperature to 75° in twenty minutes (compare Davis, Oakes, and Brown, *J. Amer. Chem. Soc.*, 1921, **43**, 1526). In the cases where acid was added, the addition was made at this point in the procedure. The volume of the solution was then made up to 100 c.c. with water at 75° and the solution quickly filtered by the aid of the pump, using hardened filter-paper. The filtered solution was placed in a flask in the thermostat at 35·4° and a sample pipetted into the viscometer held ready in the same bath.

For measuring the viscosities, a capillary viscometer of the Ubbelohde type, working under a constant head of pressure, was used (compare Hatschek, "Laboratory Manual of Elementary Colloid Chemistry," pp. 100—103; Hess, *Kolloid Z.*, 1920, **27**, 154). The viscometers were carefully calibrated with water at 35·4°. In the case of two of them, the times of flow with water were 64 and 82·2 seconds, respectively, using a head of pressure of 34·9 cm. of water. Experiments made with a different head of pressure, using the quicker-running instrument, showed that errors due to eddy currents did not occur, and therefore were much less likely to occur with gelatin solutions. For example, with a head of pressure of 20 cm. of water the product of pressure and time of flow was the same as that obtained for a pressure of 34·9 cm. In order to eliminate errors as far as possible, the same viscometer was used for the whole of any one series of measurements. Unfortunately, owing to breakages, which readily occur during cleaning, it was not possible to use the same viscometer for all the series.

A temperature in the neighbourhood of 35° was chosen for the measurements in order that the viscosity of the solutions should not alter on keeping for some time in the thermostat, and also for comparison with Pauli and Samec's results. In this way it was possible to make several observations of the time of flow with the same solution. For example, the same 1 per cent. gelatin solution at 12.30, 1.35, and 1.57 p.m. gave times of flow of 106·1, 106·2, and 106·2 seconds, respectively. Bogue's figures (*J. Amer. Chem. Soc.*, 1922, **44**, 1319) for a 2 per cent. solution at 35° also show that for a range of p_H values such as those used in this investigation the viscosity values do not vary over an interval of two or three hours.

The results obtained for 1·5, 1·0, 0·66, and 0·33 per cent. solutions, containing sulphuric acid, are given in Table II, the viscosities being expressed in centipoises. The curves showing the relation between

the viscosity and the concentration of acid in the solution are given in Fig. 3.

In the neighbourhood of the minimum point, the viscosity changes very little with alterations in the amount of acid added to the solution. The experimental method used does not permit of a greater accuracy than about 0.3 per cent., so that in repetition measurements it is possible to obtain figures which indicate either a minimum or else a region of practically non-varying viscosity. If such variations did occur, however, it was found that any minimum indicated in one experiment always occurred in the region

FIG. 3.

of non-varying viscosity obtained in repetition experiments. By comparison of the various results, the positions of the minima have been fixed, it is believed, within an accuracy of about 1 c.c. of $N/100$ -acid. The minima are indicated by being printed in italics.

The results obtained are thus very similar in type to those of Pauli and Samec, although, if they are plotted as in Fig. 4, it will be seen that they do not agree, probably because different kinds of gelatin were used. Our results further indicated that the amount of acid necessary to give a solution with minimum viscosity varies directly with the concentration of the gelatin, but it would be premature to take this relation as proved.

In contradistinction to Pauli and Samec, measurements of the

hydron concentration of solutions showing a minimum viscosity and also of some other solutions have been made. Hydrogen electrodes of the type employed by Lloyd and Mayes (*loc. cit.*) were used, and in every case determinations were made with two different and freshly prepared electrodes. The point only of each electrode was allowed to touch the solution, and measurements were made as soon as possible after contact. It will be seen (Table II) that in

FIG. 4.

Points represented by circles are values taken from Table II; those represented by crosses are values obtained by Pauli and Samec, Table I.

each case the solution showing minimum viscosity has a p_H of 4.6–4.7, that is, the isoelectric point is independent of the concentration of the gelatin. Loeb's measurements were generally made at 20° or 24°, and in all cases he found 4.7 as the p_H of the isoelectric point. The value seems to be the same at 35°, but it would only be safe to say that within the limits of error of experiment the value remains the same over the temperature interval indicated. Davis and Oakes (*J. Amer. Chem. Soc.*, 1922, **44**, 478) find that at 40° the minimum value of the viscosity for 1 per cent,

TABLE II.

Concentration of acid.	Concentration of gelatin.						pH.
	1.5%.		1.0%.		0.66%.		
	1st.	2nd.	1st.	2nd.	1st.	2nd.	
0							
9.7 × 10 ⁻⁵	1.248	1.032	0.921	0.838	0.830	0.801	1.5%.
1.45 × 10 ⁻⁴		1.032	0.921			0.785	0.66%.
1.94 × 10 ⁻⁴	1.263	1.035	0.919				1.0%.
2.42 × 10 ⁻⁴							4.8
2.91 × 10 ⁻⁴							4.8
3.88 × 10 ⁻⁴	1.228	1.032	0.914	0.838	0.830	0.789	4.6
4.85 × 10 ⁻⁴		1.029		0.895			
5.82 × 10 ⁻⁴	1.235		1.003	0.895			4.65
6.79 × 10 ⁻⁴				0.896			
6.89 × 10 ⁻⁴		1.023					
7.76 × 10 ⁻⁴	1.217	1.002	0.926	0.843			4.7
8.73 × 10 ⁻⁴		1.010					4.7
9.70 × 10 ⁻⁴	1.220		1.012	0.928			
10.67 × 10 ⁻⁴		1.025					4.6
1.164 × 10 ⁻³	1.227						
1.358 × 10 ⁻³	1.214						
1.455 × 10 ⁻³		1.037					
1.552 × 10 ⁻³	1.216						
1.746 × 10 ⁻³	1.220						
1.940 × 10 ⁻³	1.255	1.069					4.3

solutions is at p_H 8.0, but this anomaly requires further investigation.

The results obtained for the viscosities of gelatin solutions were repeatedly checked, but it was thought advisable to carry out a new series of measurements, making up the solutions in a different way. A stock solution of 2 per cent. strength was made by dispersing the gelatin in water at 70° , and, after filtering, was placed in the thermostat at 35.4° . Fifty c.c. were then pipetted into a 100 c.c. graduated flask and diluted to the mark with water and the required amount of dilute acid. The diluted solution was then heated to 70° , in order rapidly to attain equilibrium after diluting (compare Arisz, *Koll. Chem. Beihefte*, 1915, 7, 1), and again placed in the thermostat, the viscosity then being measured when the temperature had reached 35.4° . Solutions of 0.66 and 0.33 per cent. strength were similarly prepared. This method of preparation will give slightly weaker solutions than those of the corresponding strength made by the first method, so that the viscosities will be somewhat lower. This is of no importance, however, since the object of the experiments was simply to find the connexion between the hydron concentration and the minimum viscosity; the method has the advantage of using the same sample of gelatin for each series of measurements, and it is well known that gelatin is a very difficult substance to sample satisfactorily.

The results are given in Table II under the heading "Method 2" and it will be seen that they agree with those found by the first method.

Table III gives the results of measurements made when acetic acid was used, and it will be seen that the quantities of acid necessary to produce a minimum viscosity are approximately the same as those of sulphuric acid.

TABLE III.
Acetic acid and gelatin.

Concentration of acid.	Viscosities (first method only).		
	1% gelatin.	0.66%.	0.33%.
0	1.042	0.942	0.8375
2×10^{-4}	1.020	0.933	0.830
4×10^{-4}	1.005	0.933	0.838
6×10^{-4}	1.005	0.942	0.840
8×10^{-4}	1.002	0.946	0.843
10×10^{-4}	1.002		
12×10^{-4}	1.006		
14×10^{-4}	1.008		

In 1 per cent. solution in which the concentration of acetic acid was 9×10^{-4} the p_H was 4.65.

In accordance with theory, it was further found that when a solution of gelatin, made isoelectric ($p_H = 4.7$) either with acetic or sulphuric acid, was diluted to twice or three times its volume its p_H remained constant within the errors of measurement.

Summary.

1. The minimum viscosity of gelatin sols occurs at a p_H of 4.7 at 35.4° with both strong and weak acids.
2. The amount of acid required to produce the minimum viscosity in a given volume of gelatin sol is not independent of the concentration of gelatin, but appears to be a straight-line function of it.
3. The amount of sulphuric acid required to produce minimum viscosity is approximately the same as the corresponding amount of acetic acid.
4. The fact that in some cases the point of non-preferential wandering of protein under the action of an imposed *E.M.F.* occurs at a p_H above that at which minimum viscosity occurs may be due to the presence of basic degradation products of the protein.

The authors are indebted to Dr. T. Slater Price, at whose suggestion this work was undertaken, for much valuable criticism and advice.

LABORATORIES OF THE BRITISH PHOTOGRAPHIC RESEARCH ASSOCIATION.

[Received, November 4th, 1922.]