CCXXXVI. ELECTROPHORESIS OF PEPSIN

By A. TISELIUS, G. E. HENSCHEN, and H. SVENSSON

From the Institute of Physical Chemistry, University of Upsala

THE first investigation of the behaviour of pepsin in an electric field was published by Michaelis & Davidsohn [1910]. They attempted to determine the sign of the charge on commercial pepsin ("purissimum Grübler") in the pH range 1·0–4·3, using the well-known Michaelis transference apparatus. After passing current through the apparatus, qualitative tests were made for pepsin in the compartments above the stopcocks. They found that pepsin is negatively charged at $pH > 3·7$, positively charged at $pH < 2·0$, and within this interval shows migration towards both sides. This latter phenomenon was also observed at very acid reaction (0·1 N HCl). The isoelectric point, obtained by taking the average of the hydrogen ion concentrations corresponding to the pH limits mentioned, is given as cH^+ 5·5 × 10^{-3}, or $pH = 2·25$.

Since, according to later investigations of the electrophoresis of proteins, mobility is a linear function of pH and not of cH^+ in the neighbourhood of the isoelectric point, it would seem preferable to take the average of the pH values, which would give pH 2·85 for the isoelectric point.

Ringer [1915], using a similar apparatus but a purified pepsin preparation, obtained somewhat different results: the enzyme showed anodic migration even in the most acid solutions and consequently had no isoelectric point. Addition of protein decomposition products (pepton Grübler), however, caused a partial cathodic migration in acid solution, which was interpreted as due to partial combination with the enzyme. On the other hand the electrochemical properties of crystalline pepsin, first prepared by Northrop, again indicate an isoelectric point of 2·8 (solubility minimum, titration curve, electrophoresis of particles of finely ground crystals) according to observations by Northrop himself [1930]. Ågren & Hammarsten [1937] in a recent paper made some interesting measurements on the electrophoresis of solutions of a crystalline pepsin preparation in the apparatus of H. Theorell. At pH 2·27 and 2·30 they found positive migration, at 3·34 negative, which would also indicate an isoelectric point around 2·8. A certain separation of peptic activity and total nitrogen was observed at pH 3·34, but at more acid reaction both migrated together. However, in recent measurements with a different material Ågren found only negative migration for pepsin (private communication).

The question of the electrophoretic homogeneity of pepsin was dealt with in considerable detail already by Ringer in his work referred to above, which is interesting because it also seems to be the first attempt to use electrophoretic methods for the study of enzymic homogeneity and for purification of enzymes (ptyalin and pepsin). Ringer worked with pepsin prepared, according to Pekelharing, from dog's gastric juice, by dialysis and by precipitation with ammonium sulphate. To explain why the pepsin showed no isoelectric point, but yet had a marked pH-solubility minimum, Ringer assumed that the enzyme

was partially combined with protein, which would show positive charge at acid reactions. Electrophoresis in 0·025 N HCl gave, in fact, an anodic portion of high activity, whereas the cathodic portion was inactive but nevertheless contained much protein. At pH 4·1 no such separation was observed. As regards the dependence of the separation on pH Ringer's results are therefore contrary to Hammarsten's and Ågren's.

The following is an account of some experiments on the electrophoretic behaviour of solutions of crystalline pepsin. The apparatus and procedure earlier described by one of us [Tiselius, 1937, 1] were used. This method has several advantages, e.g. diminished risk of heat convection, possibility of simultaneous analytical and optical observation of the migration, and of more effective separation by applying higher voltage and the "compensation" device for balancing the migration.

A number of different crystalline pepsin preparations were investigated, which will be designated below by I, II, IIIa and IIIb. Pepsin I had been prepared according to Philpot's modification of Northrop's procedure [Philpot, 1935; Philpot & Small, 1938] and formed beautiful crystals about 0·5 mm. in length. Pepsin II was a glycerol solution of crystals (twice crystallized Cudahy Pepsin 12-7-3) prepared in Dr Northrop's laboratory, according to the procedure developed there. Philpot & Small (unpublished) have found that pepsin can be fractionated by salt precipitation to give an easily soluble crystalline fraction, and a less soluble non-crystalline fraction with a solubility minimum at pH 3·8, the latter having a somewhat lower specific activity (private communication of the authors). Samples of these two preparations dissolved in acetate buffer were kindly provided by Dr Philpot, and will be designated below as IIIa and IIIb respectively. The authors wish to express their thanks to Drs Northrop, Philpot and Small for generously placing material and information at their disposal.

The crystals were dissolved in the buffer solution to be used; the other preparations were first dialysed to remove glycerol. Before filling the apparatus all samples were dialysed overnight against the buffer to be used as a supernatant. Nitrogen was determined by the micro-Kjeldahl method, peptic activity by the haemoglobin method [Anson & Mirsky, 1932]. Like Philpot & Small [1938] we have found that the absolute values of specific activities by this method are somewhat dependent upon the preparation of haemoglobin used. Throughout this work, therefore, a single haemoglobin preparation was used.

Preliminary electrophoresis measurements showed that the various preparations contained one definite protein component of fairly homogeneous migration, with only small differences in migration velocity. Besides the sharp boundary given by this component, a very diffuse boundary could be observed, at least in concentrated solutions (more than 0·5 %), indicating the presence of inhomogeneous material.

The final experiments involved optical determinations of the mobility of the main component at different pH (Table I, Fig. 1); in a number of experiments the optical observation was supplemented by analysis of the different compartments for nitrogen and peptic activity (Table II, Fig. 1), in order not only to correlate the activity migration with the migration of the main component but also to see how far the specific activity could be raised by electrophoretic separation. As the "analytical" experiments for this latter purpose were usually run for a much longer time, a higher buffer concentration (ionic strength 0·1) was used than in the "optical" series (ionic strength 0·02). In prolonged electrophoresis experiments disturbances due to electrolyte transport occur much more easily in dilute salt media than in more concentrated solutions.
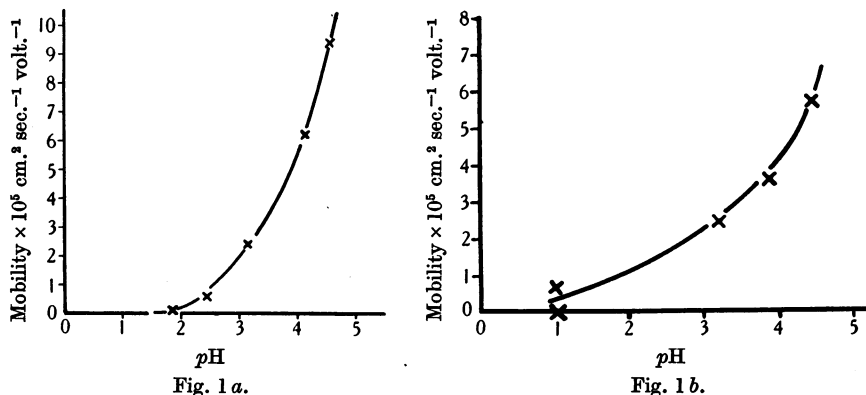
Fig. 1 a.　　　　　　　　　　Fig. 1 b.

Fig. 1. Electrophoretic mobilities of the active component in pepsin
at 0° and ionic strengths (a) 0·02 and (b) 0·1.

Table I. *Electrophoretic migration velocity of main
component in solutions of crystalline pepsin*

Optical observation. Buffer ionic strength 0·02. Pepsin conc. 0·2–0·3 %. Temp. 0°.

Preparation of pepsin	Buffer	pH	Mobility $\times 10^5$ cm.^{2} volt^{-1} sec.$^{-1}$
I	Acetate	4·57	− 9·4
IIIa, II	Acetate	4·15	− 6·2
I	Citrate, HCl	3·16	− 2·4
II	Citrate, HCl	2·47	− 0·6
II	Citrate, HCl	1·87	− 0·1

Determinations in 0·1 N HCl, pH 1·08 gave values for the mobility varying
between 0·0 and −0·4 × 10^{-5}. The variation is probably due to the fact that the
experimental error in this medium is much greater (too low buffering action).
No positive migration could be observed in any of the preparations.

Table II. *Electrophoretic migration and fractionation
of solutions of crystalline pepsin*

Optical and analytical methods used simultaneously. Ionic strength of buffer = 0·1. Temp. 0°.

Prepara-tion of pepsin	Buffer	pH	Potential gradient volt/cm.	Time min.	Peptic activity	Total nitrogen	Main com-partment (optical ob-servation)	Original solution	Top + com-partment at end of exp.
I	Citrate HCl	3·19	7·64	195	− 2·68	− 1·38	− 2·42	0·162	0·273
I	HCl	1·08	3·06	315	±0·0	+ 0·6	±0·0	0·177	0·246
II	Acetate	4·41	7·65	595	—	—	− 5·65	0·214	0·344
II	Citrate HCl	3·19	9·30	515	—	—	− 2·40	0·213	0·324
IIIa	Citrate HCl	3·19	9·04	597	—	—	− 2·50	0·240	0·314
IIIb	Citrate HCl	3·19	9·39	493	—	—	− 2·32	0·175	0·250

Columns "Mobility × 10⁵ cm.² volt⁻¹ sec.⁻¹" span Peptic activity, Total nitrogen, Main compartment; "Specific activity [P.U.] $\frac{\text{Hb}}{\text{mg. total N}}$" spans Original solution, Top + compartment.

The first two experiments in Table II were made with the purpose of measuring
migration velocities by the three different methods simultaneously, whereas
in the others we tried to obtain an appreciable separation by prolonged migration
(the main protein component migrated from 64 to 154 mm.) in combination
with compensation [Tiselius, 1937, 2]. Under these circumstances a change in the

composition of the bottom compartment was unavoidable and calculations of mobilities from analytical measurements in the usual way became impossible.

The results obtained in the experiments described in Tables I–II and Fig. 1 *a*, *b* may be summarized as follows. Solutions of crystalline pepsin, prepared by various methods, were in no case found to be quite homogeneous in electrophoresis, but contained some inactive nitrogen which could be removed by electrophoretic separation. In this way the specific activities of the various samples could be raised by 31–69 %. From the agreement between the mobilities (Table II, 6th and 8th columns) it is concluded that the peptic activity is associated with a protein component, which shows fairly homogeneous migration (does not give rise to more than one "schlieren" band in the optical observation arrangement). This protein in the *p*H range investigated (*p*H 1·08–4·57) carries a negative charge and shows no isoelectric point and thus behaves electrophoretically like an acid. Even a pepsin preparation which showed a solubility minimum at *p*H 3·8 (last experiment in Table II) behaved in principle in the same way.

Our experiments thus to a certain extent give further support to the view expressed by Northrop that the enzyme pepsin is a protein, but it is quite clear that pepsin crystals contain considerable amounts of inactive material, which may be removed by electrophoresis, and it seems likely that this material influences some of the properties of pepsin, such as solubility and acid-base combining capacity, in a marked way. The specific activity of crystalline pepsin according to Anson & Mirsky is 0·184 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. protein N}}$, but in a recent publication by Herriot [1938] a higher value is given (0·26). We have preferred to refer our values to total nitrogen instead of protein nitrogen and (as seen from Table II) we obtained activities ranging from 0·162 to 0·240 for our starting material, but as high a value as 0·344 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. total N}}$ for our most active fraction. In comparing these values with those obtained by other authors one must make the reservation that the haemoglobin preparations were not quite identical [see also Philpot & Small, 1938].

As regards the question of the homogeneity of pepsin it should be observed that the electrophoretic procedure is a very gentle separation method and could not break down a loose association complex, unless this were appreciably dissociated into its components. The homogeneity of the migration of the active component as found in the experiments described above is not therefore sufficient evidence *per se* for the chemical individuality of this enzyme, especially as the most active fractions obtained from different materials did not show the same specific activity (Table II). More experiments over a wider *p*H range and with systematically varied preparation methods are needed to clarify this question.

The somewhat surprising result that pepsin is not positively charged even in the most acid solutions investigated (*p*H 1·08) may possibly be due to an unusual tendency of the pepsin to combine with anions of the electrolyte medium. Such an effect has been previously found [Tiselius, 1930] for egg albumin, although not to as marked an extent as one would have to assume for pepsin to account for the above results. This would explain why, in the most acid solutions, the negative charge is larger when the ionic strength increases from 0·02 to 0·1 (see Fig. 1), contrary to the usually observed decrease in electrophoretic mobility with increasing salt concentration.

SUMMARY

Electrophoretic measurements show that the enzymic activity of solutions of crystalline pepsin, prepared by different methods, migrates as a homogeneous protein component with a negative charge and with no isoelectric point. Some inactive, inhomogeneous protein or protein breakdown material is left behind, and the specific activity may thus be raised by 31–69 %.

REFERENCES

Ågren & Hammarsten (1937). *Enzymologia,* **4**, 49.
Anson & Mirsky (1932). *J. gen. Physiol.* **16**, 59.
Herriot (1938). *J. gen. Physiol.* **21**, 501.
Michaelis & Davidsohn (1910). *Biochem. Z.* **28**, 1.
Northrop (1930). *J. gen. Physiol.* **13**, 739.
Philpot (1935). *Biochem. J.* **29**, 2458.
—— & Eriksson-Quensel (1933). *Nature, Lond.,* **132**, 932.
—— & Small (1938). *Biochem. J.* **32**, 542.
Ringer (1915). *Hoppe-Seyl. Z.* **95**, 195.
Tiselius (1930). *Nova Acta Reg. Soc. Scient. Upsal.* **7**, 4 (Dissertation.)
—— (1937, 1). *Trans. Faraday Soc.* **33**, 524.
—— (1937, 2). *Biochem. J.* **31**, 1464.
—— (1938). *Svensk Kem. Tidskr.* **50**, 58 (in English).