A CRITICAL STUDY OF THE USE OF PAPER ELECTROPHORESIS FOR SEPARATING PROTEINS AND MEASURING THEIR ISOELECTRIC POINTS

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Summary

A study has been made of the factors affecting the migration of proteins during electrophoresis on paper strips held between glass plates. A major difficulty in interpreting results is caused by movement of liquid in the paper from anode to cathode, the rate of flow diminishing from the anode end to the cathode end. Even with non-uniform liquid flow of this type it is possible to choose a path such that migration of an ion is linear with respect to both time and potential gradient.

Proteins having high isoelectric points are strongly absorbed on the paper at all pH values, and other proteins are absorbed at pH values below their isoelectric points.

After applying corrections for liquid flow, the mobility of bovine plasma albumin on paper has been shown to approximate that observed in free electrophoresis. The isoelectric point in buffers of ionic strength 0.025 was found to be at pH 4.65. The isoelectric points of the proteases and esterases produced by the mould *Aspergillus oryzae,* as measured by paper electrophoresis are: esterases 3.9, 5.1; proteases 4.2, 5.1. The pH-mobility curve of one of the mould proteases is identical with that of one of the esterases.

I. Introduction

The technique of electrophoresis on filter paper has found great success recently in the examination of mixtures of inorganic salts (McDonald, Urbin, and Williamson 1950), amino acids (Wieland and Fischer 1948; McDonald, Urbin, and Williamson 1951*a*, 1951*b*), of normal and pathological sera (Durrum 1950; Turba and Enenkal 1950; Grassman and Hannig 1950), and of mixtures of enzymes (Mills and Smith 1951; Gillespie, Jermyn, and Woods 1952).

Most workers have developed their own design of equipment for filter paper electrophoresis and amongst the most satisfactory are those due to Cremer and Tiselius (1950) and Kunkel and Tiselius (1951). These workers have also been responsible for developments concerning the theoretical basis of the subject.

In this laboratory the technique has been successfully used in studies of enzymes in culture filtrate from a strain of *Aspergillus oryzae* (Gillespie, Jermyn, and Woods 1952). Most of this work was concerned with the elucidation of the composition of the mixture of enzymes with a view to achieving complete separation of each component so that its concentration and specific activity could be measured. In the present study a critical examination has been made

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of the factors affecting the migration of proteins on paper, in order that measurements could be made of the mobilities and isoelectric points of the various components, which is normally accomplished with larger samples of protein solution by free electrophoresis in the Tiselius apparatus. As reported by earlier workers, one of the major difficulties in paper electrophoresis arises from the fact that a protein spot moving under the influences of an electrical potential gradient on paper is also affected by a complex flow of liquid; the buffer solution moves from anode to cathode, the rate of flow being greatest at the anode end and decreasing towards the cathode. At least two movements of liquid can be recognized, firstly that due to electro-endosmosis producing a flow towards the cathode, and secondly a movement of liquid from each end towards the centre of the paper to replace that lost by evaporation. In order, therefore, to make quantitative measurements of protein migration in an electric field, it is necessary to determine the extent of these liquid movements and apply corrections for them. The first part of this paper is concerned with the measurement of these interfering factors and the second part with the estimation of mobility and isoelectric points of some proteins.

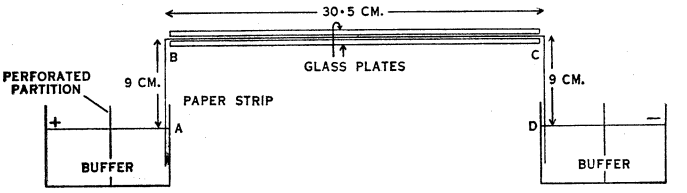

Fig. 1.—Diagram of electrophoresis apparatus.

II. Methods

The apparatus used (Fig. 1) is a modification of that described by Cremer and Tiselius (1950). The filter paper strip* 3.5 cm. wide was placed between two plate glass strips 4 cm. wide and tightly clamped by means of four strong spring clamps. The ends of the strips were immersed in buffer in 1 l. capacity "Perspex" electrode vessels having a perforated partition in the centre of each to keep the ends of the paper strips away from the carbon electrodes. The recommendation of Cremer and Tiselius (1950) to use a non-conducting cooling fluid, such as chlorobenzene, to prevent evaporation was not followed because of the unknown effect of organic liquids on the particular enzymes investigated. It was found that if the exposed length of the paper strip (9 cm.) between the ends of the glass plates and the surface of the liquid in the electrode vessels were shortened the entire strip became very wet during an experiment and inferior separations were obtained.

A well-regulated voltage was applied between the electrodes and the magnitude of this and the total current were constantly monitored. The voltage between *B* and *C* (Fig. 1) was also measured at intervals during each experiment with a voltmeter of 20,000 ohms per volt resistance,† the shunting effect of

* Whatman 3MM filter paper.

† Avo model 8 on the 1000 V. range.

which could be neglected. The voltage between *B* and *C* rose to a maximum and then fell slowly, as shown in Figure 2. This fall in voltage persisted even if the power was switched off overnight and reapplied next day. Similar results were obtained with any voltage within the range 100-1050 V. The total current increased by about 50 per cent. during an experiment and both this and the corresponding voltage drop can be ascribed to an increase in conductivity of the buffer in the filter paper due to evaporation of water. This change of conductivity could be decreased by working at low temperatures or by immersing the filter paper assembly in a non-conducting fluid as recommended by Cremer and Tiselius (1950).

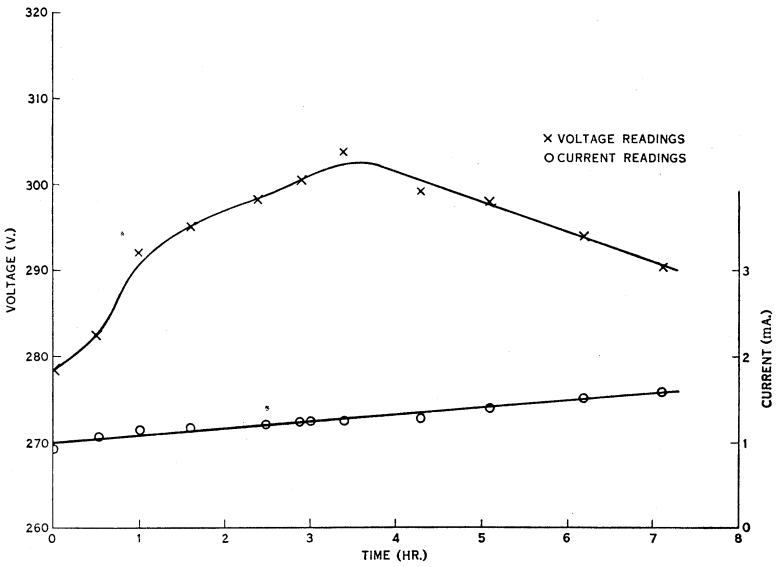

Fig. 2.—Variations in voltage between *B* and *C* (Fig. 1) and total current for a voltage of 400 V. between the electrodes. Veronal buffer Γ/2 0.025 pH 8.6.

The mean value of the voltage measured between *B* and *C* was used in subsequent calculations of potential gradient.

To set up the apparatus, the paper strips were wetted with the buffer solution and blotted to remove excess liquid, and 0.015 ml. of a solution containing between 2 and 10 per cent. protein was applied to a marked position on each strip. They were clamped between the glass plates, the ends were immersed in buffer in the reservoirs, and the desired potential was then applied. After the required period the papers were removed from the apparatus, stained with bromphenol blue (Durrum 1950), and the distance of migration from the point of application of the protein to the most intensely stained portion of the spot was measured. If the protein had enzymic properties, the filter paper strips were cut transversely at 1-cm. intervals along the length and extracts of these portions were tested for specific enzyme activities.

As a convenient standard protein, crystalline bovine plasma albumin (Armour) was used to determine the influence of time and potential gradient on distance moved, whilst an electrodialysed culture filtrate from *A. oryzae* (Gillespie and Woods, unpublished data) was used for experiments with enzymes. Protease activities were determined using sulphanilamide-azocasein as substrate at pH 8 (Charney and Tomarelli 1947). The method used for the determination of esterase was based on that of Huggins and Lapides (1947) using *p*-nitrophenyl acetate as substrate at pH 7 and 37°C.

The ionic strength of the buffers employed (0.025) was sufficiently low to minimize heating by the current yet high enough to ensure adequate buffering capacity. Except where otherwise indicated, measurements were made at 20-25°C.

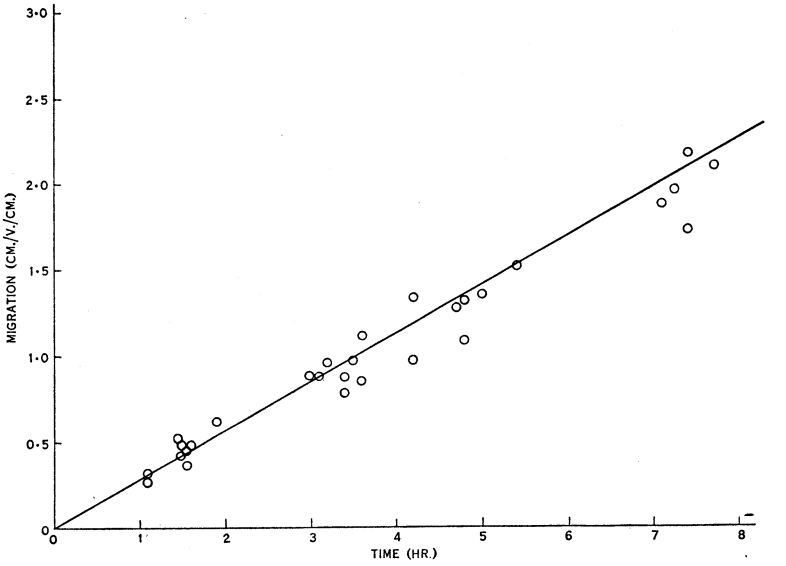

Fig. 3.—Movement of bovine plasma albumin as a function of time. Veronal buffer Γ/2 0.025 pH 8.6.

III. Examination of Factors Affecting Movement on Paper

(*a*) *Effect of Time and Electrical Potential Gradient on Migration*

Figure 3 shows that only an approximate straight-line relation exists between migration and time with bovine serum albumin in veronal buffer at pH 8.6. A similar deviation was found for the relation between rate of movement and potential gradient. These deviations were found to vary with the point of application of the protein on the paper, and the path over which it moved. The protein moved faster on the anode side of the centre of the paper strip than on the cathode side. Durrum (1950, 1951*a*, 1951*b*) has discussed these deviations from linearity and has reported the phenomenon of "mobility equilibrium," claiming that an ion moves to an equilibrium position on the paper irrespective of its point of application owing to variation in the liquid flow along

the paper. Experimentally it was found possible to overcome these deviations. If a protein spot was placed at 6 cm. from the cathode end of the glass strip and allowed to migrate 18 cm. until it was 6 cm. from the anode end, then a straight-line relation was observed between distance moved and potential gradient. This can be seen in Figures 4 and 5. It seems that when a protein moves the same distance on either side of the centre of the strip, variations in liquid flow due to evaporation can be neglected. However, the mobilities must be corrected for liquid movement due to electro-endosmosis.

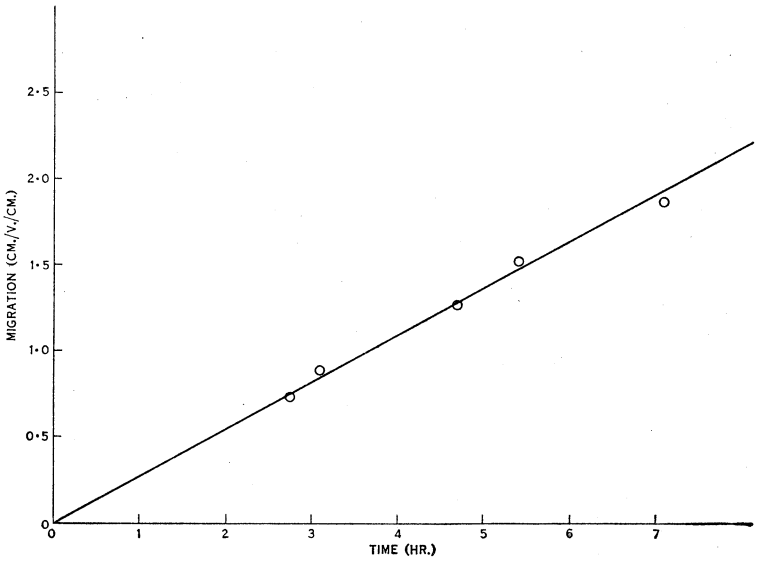

Fig. 4.—Movement of bovine plasma albumin over a fixed 18 cm. path as a function of time. Veronal buffer Γ/2 0.025 pH 8.6.

(*b*) *Investigation of Liquid Flow along the Paper*

Liquid flow was investigated by placing spots of glucose at intervals along the paper, as recommended by Kraus and Smith (1950) and Schwarz (1951), and determining their positions at the end of an experiment by heating the paper at 110°C. to char the glucose and produce brown spots. The distance moved towards the cathode from the point of application was plotted against the initial position of the spot on the paper. Displacement of glucose provides an accurate measure of the movement of liquid in the paper.

Figure 6 shows the variation in rate of liquid flow along the paper for veronal buffer of 0.025 ionic strength in some typical experiments. It can be seen that the rate of liquid flow progressively increased from the cathode end of the paper to the anode end approximately in proportion to the distance from the cathode. The results obtained for the migration of bovine plasma albumin, reported in this paper, support the view that the liquid flow is greater on the anode side of the centre than on the cathode side. These results can be explained on the basis of two combined liquid movements, that due to electro-endosmosis, which causes a flow of liquid from anode to cathode, and that

caused by evaporation resulting in a movement of liquid from each end to the centre. It has been observed that considerable shifts of liquid into the paper occur without the passage of the electric current. These movements, which continue for many hours, are symmetrical; they decrease from each end towards the centre and depend to some extent on the method of wetting the paper. It has been found that the liquid flow gradient is a function of time, potential gradient, position of the spot on the paper, method of wetting the paper, the extent of evaporation (which depends on the particular experimental technique adopted), and the pH and ionic strength of the buffers used.

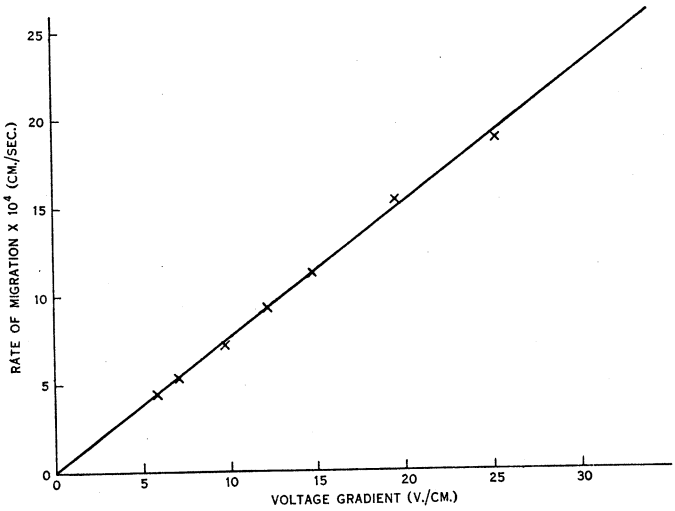

Fig. 5.—Movement of bovine plasma albumin over a fixed 18 cm. path as a function of potential gradient. Veronal buffer Γ/2 0.025 pH 8.6.

The effect of potential gradient on liquid flow is shown in Figure 6. It will be seen that there is a marked increase in liquid flow on increasing the voltage from 9.5 to 19.5 V./cm. At 19.5 V./cm. a spot of glucose placed in the centre of the paper moves 8.2 cm. towards the cathode in 5 hr. This corresponds to a mobility of 2.3×10^{-5} cm.^{2}/V./sec., and so a negatively charged substance with this mobility would remain stationary on the paper. The migration velocity of an ion at any point on the paper will be the resultant of the liquid movement at that point and the electrophoretic mobility. Experiments have shown that the liquid flow at a particular position changes slightly with time, and consequently only under certain operating conditions would it be possible for an ion to reach a stationary position on the paper, irrespective of the starting point. These conditions apparently apply in Durrum's experiments (1951*b*). In the experiments demonstrating the attainment of "mobility equilibrium" Durrum used a potential gradient of 35 V./cm. and he supported his strips over a glass rod in a closed vapour space. Moreover the total distance between the electrodes was 30 cm. as compared to more than 50 cm. in the present investigation. We have found that if the distance *BC* (Fig. 1) is too

short liquid flow into the paper is excessive. Durrum (1951*b*) has also reported that increasing the apical angle decreased the flow gradient and gave better resolution in this method, and hence the technique used in the present study should greatly reduce liquid flow. The contradiction between the finding of McDonald, Urbin, and Williamson (1951*a*, 1951*b*) and of McDonald *et al.* (1951) and that of Durrum (1950, 1951*a*, 1951*b*) is also probably explained by differences in the techniques used. The potential gradient, distances between

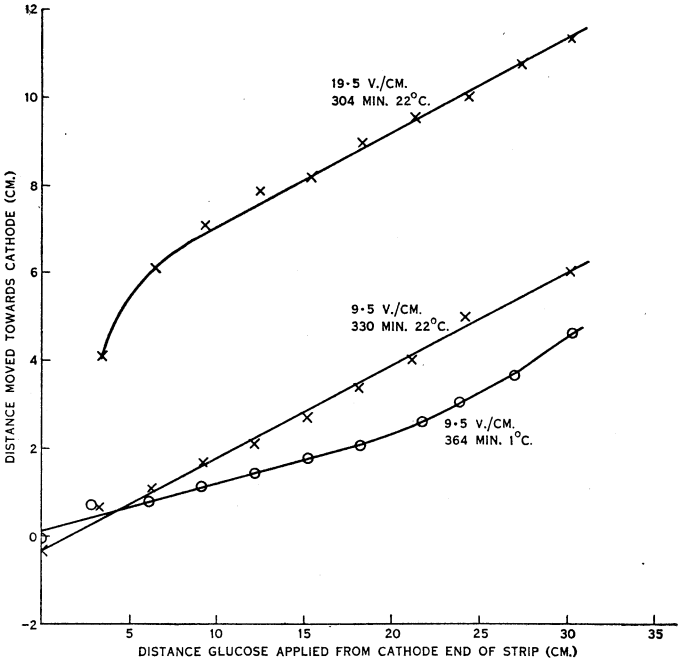

Fig. 6.—Liquid flow in paper electrophoresis as indicated by movement of glucose at various points along the paper. Veronal buffer Γ/2 0.025 pH 8.6.

electrodes, volume of enclosed vapour space, and the method of supporting the strip will all influence the volume of liquid flowing into the paper from the electrode vessels. In the present investigations it has been possible to obtain a linear migration of protein with time and potential gradient, as shown also by McDonald *et al.* (1951), simply by choosing a suitable migration path even though the liquid flow was not uniform along the paper. In determining the isoelectric points of amino acids, McDonald *et al.* (1951) made no correction for liquid flow although this must have occurred in their experiments. Electro-endosmosis always occurs in such a system and corrections must be applied to compensate for this even if there are no complications due to evaporation. In electro-endosmosis only, the liquid flow should be uniform along the paper and, in the absence of adsorption, movement of ions will be directly proportional to time and potential gradient.

In conformity with the experience of other workers, it has been observed in this laboratory that evaporation is diminished by working at a lower temperature. This is evident from Figure 6 where the liquid flow is shown for an experiment carried out at 1°C., but even at this temperature evaporation was not completely eliminated and non-uniformity of liquid movement was still observed.

(*c*) *Adsorption of Proteins on Paper*

For satisfactory separations of protein mixtures on paper and for the determination of ionic mobilities, adsorption on the paper should not occur. Brattsten and Nilsson (1951) found that the red-coloured algal protein, phycoerythrin, was strongly adsorbed on filter paper. Schwarz (1951) found that the pH-mobility curve of serum proteins was flatter below pH 5.0 than the corresponding curve obtained in free electrophoresis, and he attributed this to adsorption of the positively charged protein on the negatively charged filter paper. In this laboratory adsorption on paper has been found to occur very strongly during the paper electrophoresis of proteins on the acid side of their isoelectric points. Proteins and polypeptides with high isoelectric points were also strongly adsorbed at pH values between 2 and 11. Figure 7 shows the type of pattern obtained with papain in buffer at pH 4.8. This enzyme has an isoelectric point of about pH 9.0 (Balls and Lineweaver 1939). The basic polypeptide secretin,* and the basic protein, salmine, behaved similarly to papain on paper electrophoresis. Even at pH values above the isoelectric points, adsorption still occurred. These proteins contain a high proportion of arginine residues which would be ionized to give a net positive charge up to pH 12.5, which is the pK value for the guanidine residue (Cohn and Edsall 1943). Paper electrophoresis of basic proteins at pH values sufficiently high to remove the charge on the side chains of arginine presented technical difficulties for the filter paper became weak and difficult to handle and the high conductivity of the buffers resulted in excessive heating.

In the paper chromatography of amino acids, similar streaking has been found to be due to the presence of heavy metals (Consden, Gordon, and Martin 1944), and this was eliminated by washing the paper with dilute acid or more effectively by treating it with a metal-chelating agent, such as 8-hydroxyquinoline or ethylene diamine tetra-acetate. Attempts to eliminate adsorption during paper electrophoresis of papain by either of these means were unsuccessful. Bovine plasma albumin showed strong adsorption on filter paper below pH 4.5 and in the separation of the enzymes of *A. oryzae* by paper electrophoresis at several pH values, adsorption by the paper of some of the components of the mixture became pronounced at pH values below 5.0. Figure 8 shows the patterns obtained by staining with bromphenol blue after electrophoresis of the electrodialysed enzyme preparation at several pH values. Hall and Wewalka (1951) consider that the protein spots which Franklin and Quastel (1949, 1950) obtained by chromatography of plasma were not different chemical entities but artifacts, the nature of which depended on the properties of the proteins in

* Sample kindly prepared by Mr. G. E. Rogers by a modification of the method of Greengard and Ivy (1938).

contact with the paper. The same considerations may also apply to the movement of proteins on paper under the influence of an electric field. Hence in any separation obtained by paper electrophoresis it is desirable to eliminate the possibility of adsorption and test the separate identity of the components by some other means. It has already been shown (Gillespie, Jermyn, and Woods 1952) that many of the enzymes in the culture filtrate from *A. oryzae* are multiple in nature. Later work has shown that the various components separated by paper electrophoresis are not artifacts, for they retain their separate identities on fractionation with ethanol under conditions of low ionic strength and low temperature, and furthermore two of the protease components have differing specificities (Gillespie and Woods, unpublished data) as do many of the β-glucosidases (Jermyn 1952).

IV. Application of the Method to the Determination of the Mobilities and Isoelectric Points of Bovine Plasma Albumin and some Mould Enzymes

From the results given in Figures 4 and 5 a mean apparent mobility of 7.6×10^{-5} cm.^{2}/V./sec. is derived for bovine plasma albumin in 0.025 ionic strength veronal buffer at pH 8.6. This value was corrected for liquid flow due to electro-endosmosis and evaporation by running at the same time as the protein, parallel strips with spots of glucose at 3 cm. intervals along the paper, and taking the mean value of the liquid flow at the beginning and end of the path of migration. This only gives an approximation for the liquid flow; it is not practicable to apply an accurate correction as the protein moves along a path with a varying rate of flow of liquid. In systems where evaporation occurs, the ionic composition will also vary along the paper (Durrum 1951*b*) and this will cause variations in the calculated mobilities for which it is not possible to apply any correction. Four replicate determinations of the corrected mobility of bovine plasma albumin gave a mean value of 9.1×10^{-5} cm.^{2}/V./sec. Thus liquid movement accounts for nearly 20 per cent. of the migration velocity in this case. By electrophoresis in the moving boundary apparatus at 0°C. in veronal buffer of 0.1 ionic strength a value of 6.5×10^{-5} cm.^{2}/V./sec. was obtained by Alberty (1948) for the mobility of the same protein. Since the migration velocity increased by about 2 per cent. per 1°C., the value for bovine plasma albumin at 23°C. is approximately 9.5×10^{-5} cm.^{2}/V./sec. The value obtained in paper is thus in close agreement with that obtained in free electrophoresis.

Both the work of Kunkel and Tiselius (1951) and that described here, although using slightly different assumptions as to the factors operative in the process, have given approximately correct values for the mobilities of plasma albumins. On the one hand Kunkel and Tiselius introduce the factor l/l' which is claimed to take into account the tortuous path a protein may follow in its passage through the paper. On the other hand we have made a correction for the velocity gradient along the paper which arises from non-uniform liquid flow. This does not appear to have been taken into account by Kunkel and Tiselius for the only liquid flow movement considered by them was that de-

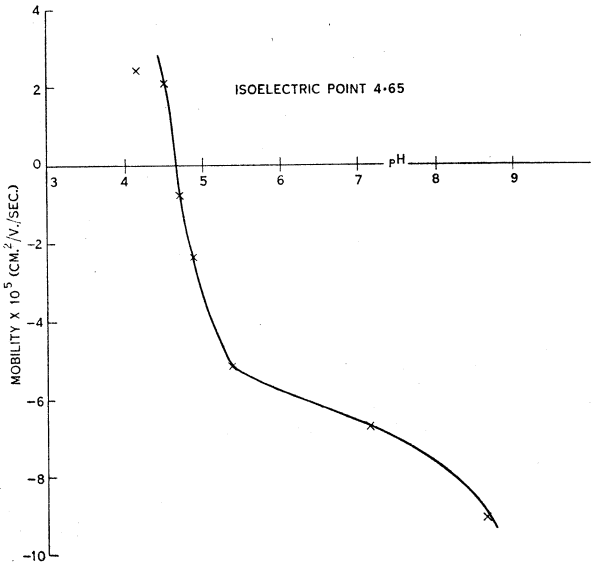

Fig. 7.—pH-mobility curve for bovine plasma albumin.

fined by the shift of a single dextran spot from the origin. The fact that the two methods give correct mobilities suggests that all the factors operative in filter paper electrophoresis are not as yet fully defined and understood.

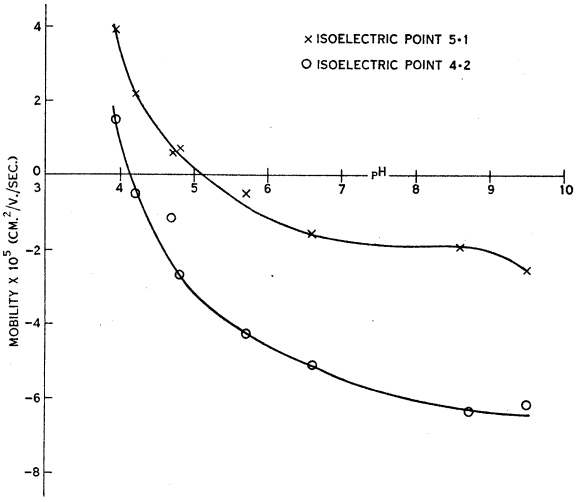

Fig. 8.—pH-mobility curves for two proteases from *A. oryzae*.

Having achieved a method for measuring mobilities it is possible to extend the work to the calculation of isoelectric points of proteins both in the pure state and in admixture with other proteins. The mobility of bovine plasma albumin was measured at a number of pH values and the results are plotted in Figure 7. It can be seen that the isoelectric point obtained, namely pH

4.65, is in good agreement with the most recently determined value (4.7). This procedure was then used for determining the isoelectric points of some of the proteases and esterases of the mould enzyme mixture. Paper electrophoresis experiments were run on electro-dialysed mould enzyme at several pH values and the enzyme activities of aqueous extracts of strips of the paper were determined. The pH-mobility curves are shown in Figures 8 and 9. The esterases have iscelectric points of 3.9 and 5.1 and the proteases 4.2 and 5.1. One of the proteases and one of the esterases move together at all pH values and have identical pH-mobility curves within the limits of the accuracy of the method. It was not possible to employ lower pH values because of the complications due to adsorption, as evidenced by the patterns in Plate 1, Figure 2, and also because at pH values below 4 the protease activity is lost rapidly (Crewther and Lennox 1953). The values obtained for the isoelectric points are in agreement with the observation that crystals, containing protease (Crewther and Lennox 1950, 1953) from *A. oryzae* culture filtrate, form in ammonium sulphate solutions over the pH range 4.0-5.2 but most readily at pH 4.6. Paper electrophoresis showed that these crystals contained the protease and esterase of isoelectric point 5.1 but only traces of the protease of isoelectric point 4.2.

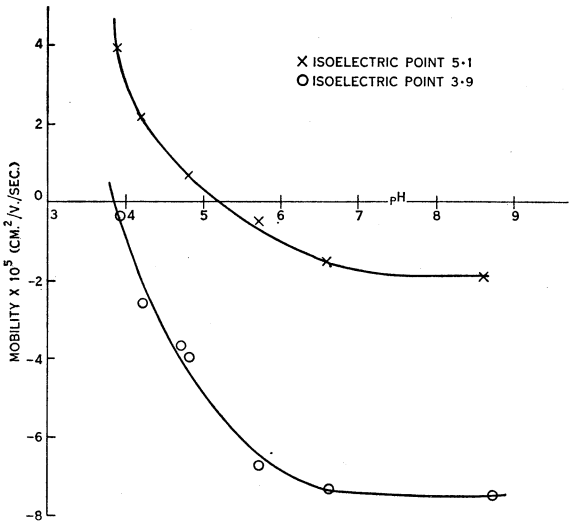

Fig. 9.—pH-mobility curves for two esterases from *A. oryzae*.

V. Acknowledgments

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PAPER ELECTROPHORESIS OF PROTEINS

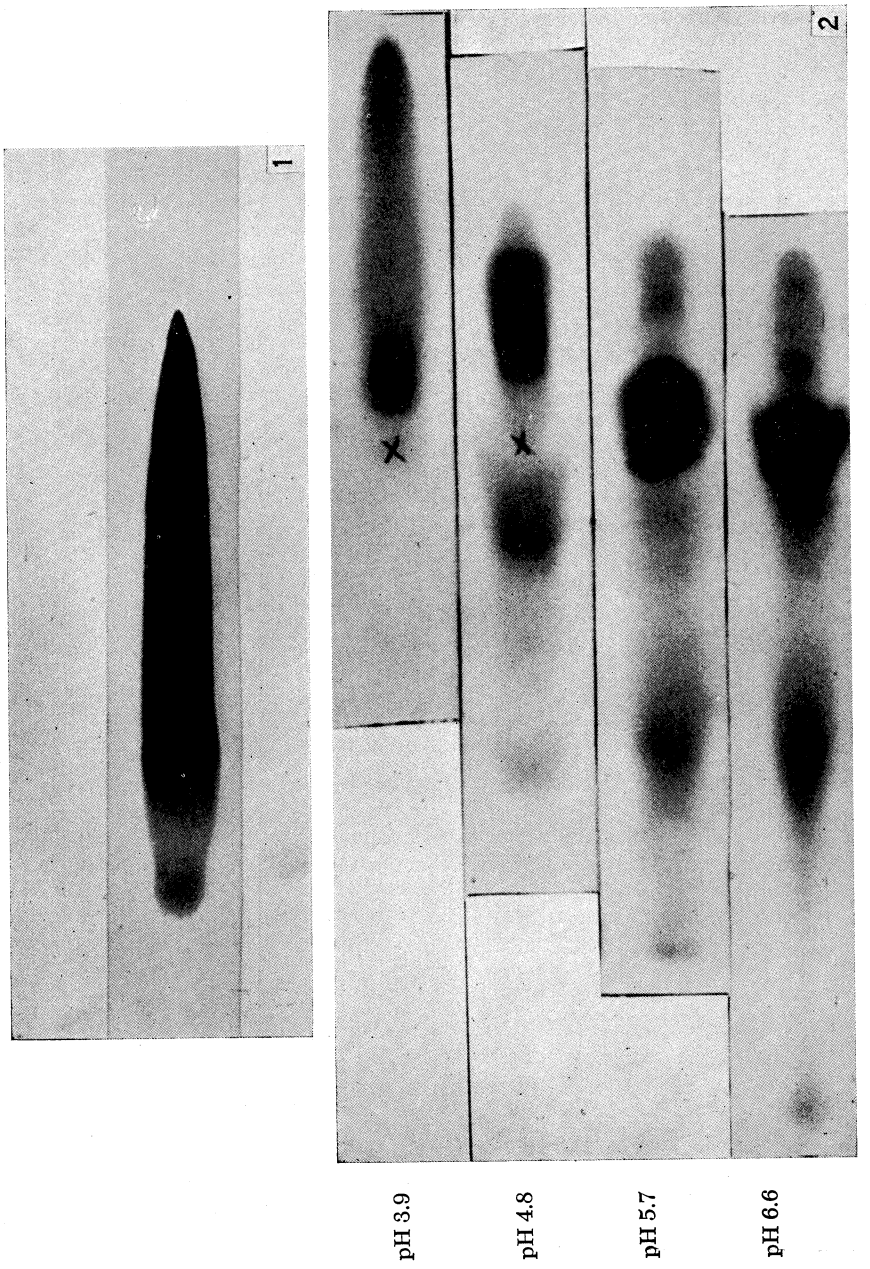

Fig. 1.—Paper electrophoresis of water-soluble fraction of papain in acetate buffer Γ/2 0.025 and pH 4.8.
Fig. 2.—Paper electrophoresis of enzymes of *A. oryzae* at various pH values and buffer Γ/2 0.025, 9.5 V./cm. for 6 hr. Cathode at right end of both figures.

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