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Artefacts Appearing at the Crossing Points on the Lines of Reactants in the Crossing Electrophoresis*)

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Nakamura and his associates (1-3) have developed the method of crossing electrophoresis, which has been applied to the various regions of biochemical research by them(4-10) and by others.(11-13) The principle of the method consists in making two lines of different substances encounter on the filter paper or on other supporting media.(5) As shown in Fig. 1, if two substances of different electrophoretic mobility are applied, one on line AB and the other on line XY, the two lines will encounter in the course of electrophoresis. If the two substances react with each other, a groove will be formed at the crossing point in the line of one substance, because the electrophoretic migration of the latter will be hindered by the reaction. Thus the method of the crossing electrophoresis has offered an appropriate means to investigate whether two substances react each other or not. But as the deformation of the lines of reactants at the crossing may arise from other causes than the interaction of the two reactants, the most essential problem in the crossing electrophoresis is the question of the formation of artefacts at the crossing. Such artefacts can arise when the mobility of a substance is changed locally at the point where an other solution was applied, owing not to the interaction with the specific solute, but to the general properties of the solution; for instance, as the solutions to be applied contain usually electrolytes, local change of pH and ionic strength can arise, when their compositions differ considerably from those of the buffer solutions used. These changes can naturally affect the mobility of a substance which

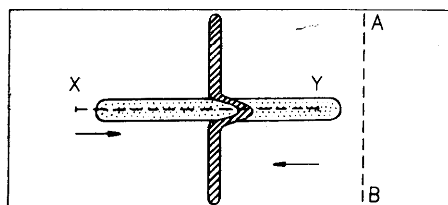

Fig. 1. Diagram of the crossing electrophoresis. Explanation, see text.

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migrates over this region, and form a groove in its line. Thus it is essential for the technique of crossing electrophoresis either to avoid the formation of such artefacts or at least to be able to distinguish them from the change due to real interaction of the two reactants.

THEORETICAL

As suggested above, some artefacts can be predicted from the theories of mobility.(14)

1) Factors affecting the electrophoretic mobility of a substance.

A charged particle in an electrical field of voltage gradient, E, will be driven or migrate by a force, F,

$$F = qE \qquad (2.1)$$

where q is the charge of the particle. On the other hand, its movement through the medium will be counteracted by a resistance, R, which is proportional to its velocity, v. Thus,

$$R = vC \qquad (2.2)$$

where C is the coefficient of resistance. The particle will move or migrate with a constant velocity, when the driving force, F, is equal to the resistance, R. Thus in regard to its velocity of migration,

$$qE = vC \qquad (2.3)$$

Hence,

$$v = \frac{q}{C} E \qquad (2.4)$$

This shows that the electrophoretic velocity of a substance is proportional to the voltage gradient. If the velocity in a unit electrical field, i. e. in the voltage gradient 1 volt/cm is defined as its mobility, u, it will be given by

$$u = \frac{v}{E} = \frac{q}{C} \qquad (2.5)$$

and is independent of the voltage gradient.

2) The effect of pH.

As is apparent from equation (2. 5), the mobility of a charged particle is proportional to its electrical charge, q, which depends on the pH of the medium. Hence the mobility must be determined by the latter. When a solution is applied on a line parallel to the electrical field causing a considerable local change

of pH, but not affecting other conditions, the line of an appropriate substance applied perpendicularly to the field will be deformed at the crossing, owing to the local change of the mobility of the substance, with the progress of electrophoresis.

3) Effect of viscosity.

According to equation (2. 5), the mobility depends not only on pH of the medium, but also on the resistance. On the other hand, the resistance coefficient will be determined by the shape and size of the particle as well as by a) the viscosity and b) the ionic composition of the medium, because the movement of the particle will be influenced by the friction with the medium and by the interaction with the ionic atmosphere. Of these factors, the shape and size of the particle can be set aside from consideration, since here the question is the effects on mobility of one and the same substance.

The frictional coefficient, f, can be given by

$$f = 6\pi\eta r \qquad \cdots\cdots(2.\ 6)$$

when the particle is spherical, where r is its radius and η the viscosity of the medium. Hence the mobility can be presentend by the equation,

$$u = \frac{q}{6\pi\eta r} \cdot \frac{1}{\alpha} \qquad \cdots\cdots(2.\ 7)$$

where α is the resistance by ionic atmosphere. This equation indicates that the mobility of a substance decreases in proportion to the increase of the viscosity of the medium. Hence the mobility of the test substance will be decreased on the filter paper in the region where a solution of high viscosity was applied, provoking an artefact.

4) Effect of ionic strength.

The ionic atmosphere around a charged particle may resist the movement of the latter by, as it were, an attraction. Such resistance[15] depends on the thickness of the ionic atmosphere, $1/\kappa$, which will be given by

$$\frac{1}{\kappa} = \left(\frac{DkT}{8\pi e^2 \mu}\right)^{\frac{1}{2}} \qquad \cdots\cdots(2.\ 8)$$

where D is dielectric constant, k the Bolzmann constant, T the absolute temperature, μ the ionic strength, and e the unit charge.

The mobility, u, of a charged particle may be given by the following equation, provided that the charge of the particle will not be influenced by the ionic strength of the medium:

$$u = \frac{Kf(\kappa r)}{\mu} \qquad \cdots\cdots(2.\ 9)$$

where $f(\kappa r)$ a function of κr, and K a constant. The value of $f(\kappa r)$ approximates to 2/3 and to 1, when ionic strength is large or small, respectively. Thus the mobility becomes small when the ionic strength increases, as will be understood from the above equation. Hence it can be inferred that the local increase of ionic strength on the filter paper may cause an artefact in the line of test substance.

EXPERIMENTAL

A paper electrophoresis apparatus of horizontal type was used. Buffer solutions of phosphate, pH 7.0 and ionic strength 0.05 or 0.1, were used for electrophoresis. For the electrophoresis of serum, barbiturate buffer of pH 8.6 and ionic strength 0.05 was used at times. Buffer solutions of ionic strength 0.1 of citrate of pH 2.0 to 5.0 and of barbiturate of pH 9.0 were used to change the pH of the lines applied with them.

Solutions of dyes (bromophenol blue, bromocresol green, and orange GG), amino acids (arginine, lysine, histidine, glutamic acid, glycine, and threonine), starch, and other substances (ethanolamine, glucosamine sulfonamides, dextrin, gum arabic, and gelatin) were prepared with the same buffer solution as used for the electrophoresis. The concentration of dye was about 2 mM and that of amino acid was about 0.02 M. Starch solutions were prepared with soluble starch to 1, 2.5, 5, and 10%. Salt solutions of ionic strength 1, 0.5, 0.2, 0.1, 0.05, and 0.005 were prepared with sodium chloride, sodium sulfate, potassium chloride, or ammonium sulfate.

Normal human serum was prepared as usual. Ovalbumin was crystallized from chicken egg white and dialyzed against water and the buffer solution used.

Filter paper, Toyo No. 50, 51, or 52.

At the tip of micropipett a short thread was inserted to avoid scratching the filter paper (cf. discussion).

Amino acids and amines were developed with ninhydrin.[16] Sulfonamides were stained with dimethylaminobenzaldehyde.[17] Proteins were stained with amidoblack 10 B or with bromophenol blue.[16]

RESULTS

1) Artefact caused by the local increase of viscosity.

In Fig. 2 is shown one of the electropherograms obtained with dyes varying the local viscosity. On line AB, which is perpendicular to the direction of the electrical field, a solution of the dye, bromophenol blue (BPB), was applied, and on the lines, X_1Y_1, X_2Y_2, $\cdots$, drawn in parallel to the electrical field starch solutions of various concentrations were applied. As the starch does not migrate

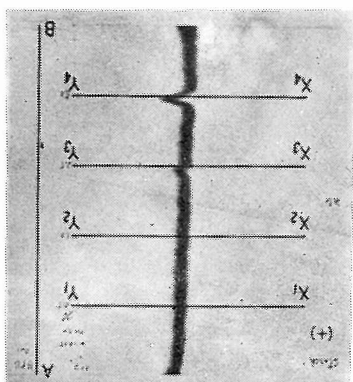

Fig. 2. Electropherogram of the bromophenol blue line crossing over the lines of starch solutions of various concentrations.

AB: 0.01 ml/15 cm of a 2mM BPB solution. X_1Y_1, X_2Y_2, X_3Y_3, and X_4Y_4: 0.02 ml/10 cm of 0.5, 1, 2.5, and 5% starch (soluble) solution, respectively. Phosphate buffer, pH 7.0, ionic strength, 0.05. Filter paper, Toyo No. 51. Electrophoresis at 150 V and 5 mA, for 2 hours.

under the experimental conditions, the line of dye migrated with the electrophoresis over the lines of starch solutions, and formed grooves at the crossing points with the lines of 2.5 and 5% starch solutions, whereas no groove was formed at the crossing with the lines of 1 and 0.5% starch solutions.

Fig. 3 represents the result obtained with glutamic acid, varying the local viscosity with starch solutions. In this case also, the line of the amino acid formed grooves only at the crossing points with starch solutions of more than 2.5%.

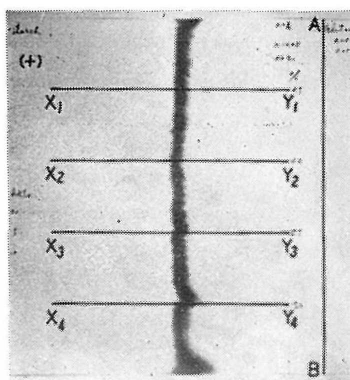

Fig. 3. Electropherogram of the glutamic acid line crossing over the lines of starch solutions of various concentrations.

AB: 0.01 ml/15 cm of a 50 mM L-glutamic acid solution. X_1Y_1, X_2Y_2, X_3Y_3, and X_4Y_4: 0.02 ml/10 cm of 0.5, 1, 2.5, and 5% starch solution, respectively. Other conditions were the same as in Fig. 2.

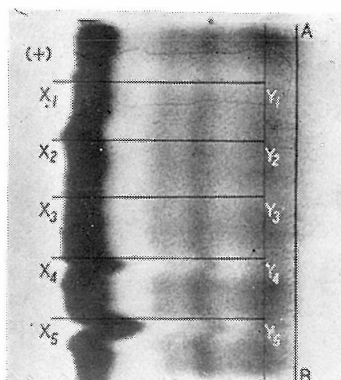

Fig. 4. Electropherogram of the serum protein lines crossing over the lines of starch solutions of various concentrations.

AB: 0.05 ml/19 cm of human serum. X_1Y_1: 0.02 ml/5 cm of a 10% solution of glucose. X_2Y_2, X_3Y_3, X_4Y_4, and X_5Y_5: 0.02 ml/5 cm of 1, 2, 5, and 10% starch solutions, respectively. Phosphate buffer, pH 7.0, ionic strength 0.05. Electrophoresis at 60 V and 3 mA, for 15 hours. Filter paper, Toyo No. 51. Stained with amidoblack 10 B.

In Fig. 4 is shown the result obtained with serum. As in the other cases, the lines of serum proteins, especially that of serum albumin, were grooved at the crossing points with the lines of starch solutions of more than 2.5%.

The same results could also be obtained with other dyes, amino acids, and proteins, and with other buffer solutions. Thus the formation of the groove in the lines of test substances does not depend on the nature of them nor on the medium, but solely on the concentration of the starch solutions applied locally. Of other substances tested, which give high viscous solutions, gum arabic and gelatin reacted specifically with some test substances, whereas the dextrin solutions showed an uniform effect in that those more than 5% formed grooves at the crossing with the line of any test substances. But no comparison of viscosities of them with starch solutions was made.

2) Artefact caused by the local change of ionic strength.

The electropherogram shown in Fig. 5 was obtained by local changes of ionic strength. The dye, BPB, was at first applied on line AB drawn perpendicularly to the direction of the field. Buffer solutions diluted or added with sodium chloride to increase ionic strength were applied on the lines, X_1Y_1, X_2Y_2, $\cdots$, parallel to the field. With the progress of the electrophoresis, the line of BPB migrated over the lines, X_1Y_1, X_2Y_2, $\cdots$, where ionic strength was changed locally. As can be seen from the figure, the line of the dye formed depressions at the crossing points with the lines where solutions of ionic strength higher than 0.2 were applied, whereas no depression was formed at the crossing points with solutions of lower ionic strength.

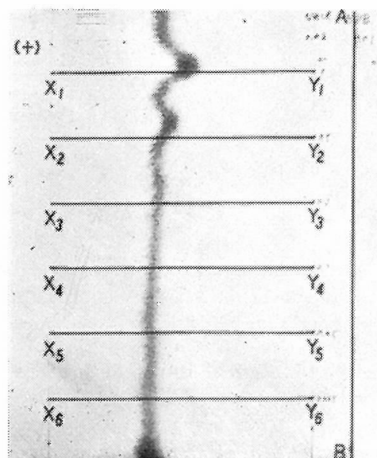

Fig. 5. Electropherogram of the bromophenol blue line crossing over the zones of various ionic strengths.

AB: 0.01 ml/17 cm of a 2 mM BPB solution. X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, X_5Y_5, and X_6Y_6: 0.02 ml/10 cm of buffer solutions of ionic strength, 1.0, 0.5, 0.2, 0.1, 0.05, and 0.005, respectively. Ionic strengths larger than 0.1 were established by adding NaCl, and ionic strength, 0.05 without and 0.005 by the dilution of the buffer solution used for electrophoresis. Phosphate buffer, pH 7.0 and ionic strength 0.05. Electrophoresis at 150 V and 6 mA, for 3 hours 30 min.

Fig. 6 shows the result obtained with arginine. The result is the same as with dyes.

As shown in Fig. 7, the same result was obtained also with human serum.

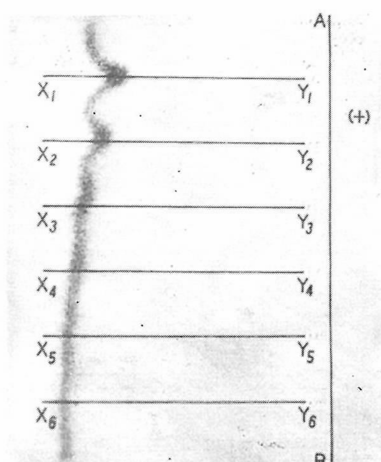

Fig. 6. Electropherogram of the arginine line crossing over the zones of various ionic strengths.

AB: 0.02 ml/17 cm of a 50 mM L-arginine solution. X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, X_5Y_5, and X_6Y_6: 0.02 ml/10 cm of buffer solutions of ionic strength, 1.0, 0.5, 0.2, 0.1, 0.05, and 0.005, respectively. Electrophoresis at 150 V and 6 mA, for 3 hours. Other conditions were the same as in Fig. 5.

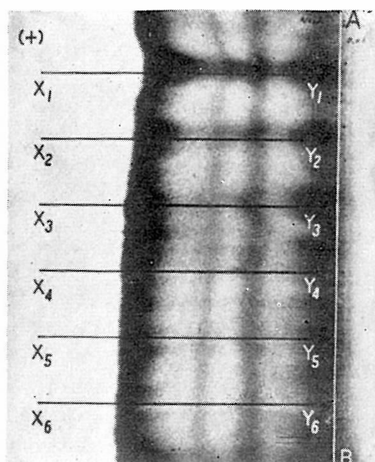

Fig. 7. Electropherogram of the serum protein lines crossing over the zones of various ionic strengths.

AB: 0.06 ml/17 cm of a human serum. X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, X_5Y_5, and X_6Y_6: 0.02 ml/10 cm of buffer solutions of ionic strength, 1.0, 0.5, 0.2, 0.1, 0.05, and 0.005, respectively. Electrophoresis at 150 V and 6 mA, for 8 hours. Stained with BPB. Other conditions were the same as in Fig. 6.

The same results could be obtained with other dyes, amino acids, and proteins, and also when salt solutions other than sodium chloride were used to change local ionic strength or buffer solutions of other pH values were used. Thus it is evident that the depressions formed in the line of test substance were caused by the local increase of ionic strength.

3) Artefact caused by the local change of pH.

In the experiment shown in Fig. 8, buffer solutions of various pH values and of the same ionic strength as the electrophoresis medium were applied on the lines, X_1Y_1, X_2Y_2, $\cdots$, drawn in parallel to the field, and solutions of BPB and orange GG were applied on line AB, and CD, respectively, drawn perpendicularly. With the electrophoresis, the lines of BPB and orange GG migrated over the zones of changed pH, but formed no depression at any crossings. The same results were obtained with serum proteins and with various amino acid except histidine.

Fig. 9 presents the electropherogram of the histidine line after it had migrated over the lines where buffer solutions of various pH values were applied. It formed hollows at the crossings with the lines where pH's were decreased, and formed projections at those with the lines where pH's were increased. The

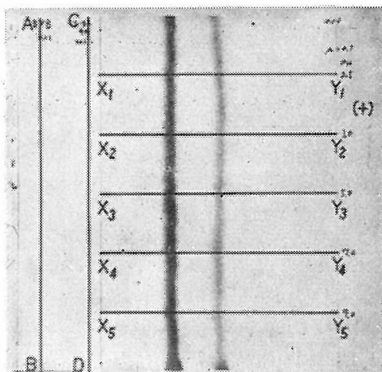

Fig. 8. Electropherograms of the lines of dyes crossing over the zones of various pH values.

AB: 0.01 ml/15 cm of a 2 mM solution of BPB, CD: 0.02 ml/15 cm of a 2 mM solution of orange GG. X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, and X_5Y_5: 0.02 ml/10 cm (on X_5Y_5 0.01 ml) of buffer solutions of ionic strength 0.1 and pH values of 2.5, 3.0, 5.0, 7.0, and 9.0, respectively. Buffer solutions of pH 2.5, 3.0, and 5.0 were prepared with citrate, that of pH 7.0 with phosphate, and that of pH 9.0 with barbiturate. Electrophoresis with phosphate buffer of pH 7.0 and ionic strength 0.05, at 150 V and 6 mA, for 2 hours. Filter paper, Toyo No. 51.

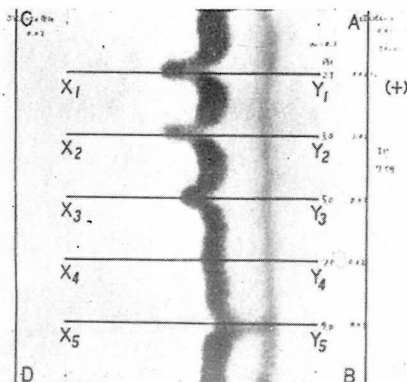

Fig. 9. Electropherogram of the histidine line crossing over the zones of various pH values.

AB: 0.01 ml/15 cm of a 50 mM solution of L-histidine. CD: 0.02 ml/15 cm of a 2 mM solution of orange GG. X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, and X_5Y_5: 0.02 ml/10 cm (on X_5Y_5 0.01 ml) of buffer solutions of pH 2.5, 3.0, 5.0, 7.0, and 9.0, respectively. Electrophoresis at 150 V and 5 mA, for 4 hours. Stained with ninhydrin. Other conditions were the same as in Fig. 8.

reason why only histidine showed changes in its line may be interpreted as follows: In these experiments, buffer solutions were not sufficiently applied on the lines parallel to the field, to cause locally large change of pH values. On the other hand, the substances tested, dyes, serum proteins, and amino acids except histidine, show scarcely any change in their mobilities, when the pH of the medium is changed to some extent in the region of pH 7.0, at which the buffer solution of the electrophoresis was fixed. On the contrary, the mobility of histidine changes considerably in the region of pH 7, even when the pH of the medium will be changed slightly, since its isoelectric point lies at pH 7.6. Thus the reason why only the histidine line was susceptible to the local change of pH may depend on the fact that the pH value of the medium was near that of isoelectric point of histidine, where its mobility changes steeply.

This could be demonstrated experimentally with other substances. A result obtained with glutamic acid is shown in Fig. 10. Here the buffer solution used for electrophoresis was of pH 3.5, which is near the isoelectric point of glutamic acid. As can be seen, the line of glutamic acid formed hollows or projections,

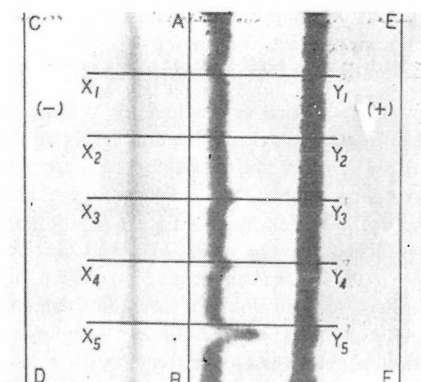

Fig. 10. Electropherogram of the glutamic acid line crossing over the zones of various pH values.

AB: 0.005 ml/15 cm of a 50 mM solution of L-glutamic acid. CD: 0.02 ml/15 cm of a 2 mM solutions of orange GG. EF: 0.01 ml/15 cm of a 50 mM solution of L-arginine. X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, and X_5Y_5: 0.02 ml/10 cm (on X_5Y_5 0.01 ml) of buffer solutions of ionic strength 0.1 and pH 2.5, 3.0, 5.0, 7.0, and 9.0, respectively. Electrophoresis with citrate buffer of pH 3.5 and ionic strength 0.05, at 150 V and 5 mA, for 70 min. Stained with ninhydrin. Other conditions were the same as in Fig. 8.

according to whether the local pH was decreased or increased. The same could be demonstrated also with ovalbumin, when a buffer solution of pH value near its isoelectric point was used for electrophoresis.

On the other hand, the degree of the local change of pH depends obviously on the quantity and the strength of the buffer action of the solution applied, so long as the effect of ionic strength does not appear.

DISCUSSION

1) Artefacts induced by other causes.

From the experiments presented above, it can be shown that the local changes of viscosity, ionic strength, and pH can cause artefacts in the line of test substance applied perpendicularly to the field. Besides these theoretically predictable effects, however, two other causes were found to induce artefacts occasionally.

a) One of them has been described already by Nakamura and his colaborators,[(4)] and was ascribed to the mechanical demages of the filter paper: The scratch made by the tip of a glass capillary in applying the test solution affects the uniformity of the paper thickness, and consequently the electrical field will not be uniform. This effect became especially prominent, when it was made in the direction of the field. The test substance applied on a line perpendicular to the field may form a projection when it crosses over the scratch groove, since the migration of the substance would be accelerated at the groove, owing to the increased electrical resistance.

b) The other artefact was observed[(18)] in a case of the crossing electrophoresis of serum albumin. Fig. 11 presents the two-dimensional crossing electrophoresis of chicken ovalbumin with the homologous rabbit antiserum: By the first electrophoresis antiserum was separated into its fractions, then chicken ovalbumin was applied on the line perpendicular to the line of the

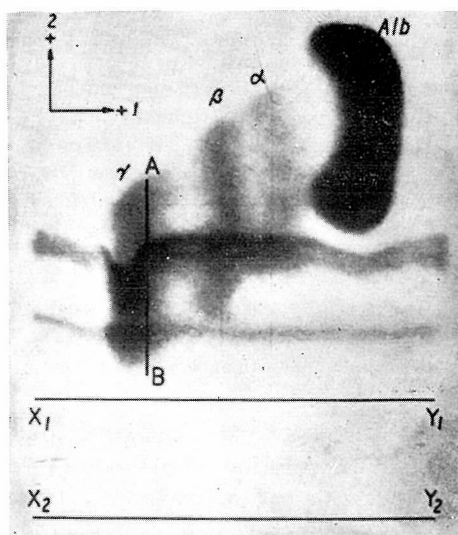

Fig. 11. Artefact appeared in the line of chicken ovalbumin, in the crossing electrophoresis against the homologous rabbit anti-serum.

First electrophoresis: On line AB, 0.12 ml/8 cm of the anti-chicken ovalbumin rabbit anti-serum; at 100 V and 6 mA for 17 hours, in direction 1. Then second electrophoresis: On line X_1Y_1, 0.15 ml/16 cm of a 5% solution of crystallized chicken ovalbumin, and on line X_2Y_2, 0.01 ml/16 cm of a 5% solution of purified duck ovalbumin. At 120 V and 8 mA for 8 hours, in direction 2. Barbiturate buffer of pH 8.6 and ionic strength 0.05. Filter paper, Toyo No. 52. Stained with BPB.—The line of chicken ovalbumin formed a groove at the crossing with γ-globulin of the antiserum, whereas that of duck ovalbumin formed none, indicating that the antibody reacted totally with chicken ovalbumin.

antiserum and the second electrophoresis was carried out in a direction perpendicular to the first one. As can be seen the line of ovalbumin is depressed blow the zone of serum albumin, without crossing it. The depression can not be caused by any substance which might be contained in serum albumin fraction and would have migrated slower than it, remaining below it to react with ovalbumin, because the serum albumin fraction had already been separated by the first electrophoresis and could not contain any substance of low mobility. Hence the depression must have been caused by the adsorbed serum albumin on the filter paper.

2) Measures to prevent artefacts.

As the artefacts mentioned above might occur in the "crossing electrophoresis", measures to prevent them are necessary.

a) Cautions in preparing the test solutions.

i) Viscosity of test solutions: As the local increases in viscosity can cause an artefact in the line of one reactant which is applied perpendicularly to the field, application of a test solution of high viscosity must be avoided. But as shown experimentally, a starch solution of 1–2 per cent did not affect the mobility of test substances. Hence the application of a solution of so high viscosity as to cause artefacts might not usually occur, except special cases.

ii) pH of test solution: When the pH of the test solution is different from that of electrophoresis medium, especially when the latter is near the isoelectric point of test substance, an artefact might arise. Hence it is better to use a buffer solution of a pH value apart from the isoelectric point of the test substance, which will be applied on the line perpendicular to the field. On the

other side, the degree of the local pH change caused by the application of a solution depends not only on the difference of pH but on the quantity and the concentration of the latter. Thus when a more concentrated solution is used, the pH adjustment must not be ignored. But in such a case the difference of ionic strength must be considered sooner than that of pH, since the former can affect the mobility more severely.

iii) Ionic strength of test solutions: The values of the ionic strength of buffer solutions used usually for electrophoresis range from 0.05 to 0.1. Hence the local change of ionic strength caused by the application of a solution of low ionic strength can not exceed this range to cause any artefact. When, however, a test solution of higher ionic strength than that of electrophoresis medium is to be applied, an artefact might arise, especially when the difference is larger than 0.2. This must be taken into consideration not only in regard to the salts contained in the test solutions, but also the reactants themselves. Particularly when their molecular weights are small, their concentrations should not be unnecessarily large. On the contrary it is advisable to apply as dilute a solution as possible, so they can be detected.

The above cautions apply not only to the solutions applied to the lines parallel to the field, but also to those applied to the perpendicular lines. When a too concentrated solution is applied, the lines of the other reactants applied in parallel to the field may be stopped there owing to the decrease in mobility due to the local increase of ionic strength. Fig. 12 shows an example of the erroneous application of a too concentrated test solution[19]: Here the serum alone was separated into fractions by the first electrophoresis in direction 1, then a solution of a sulfonamide, sulfisomidine, was applied on the line perpendicular to the line of serum and the second electrophoresis was carried out in the direction perpendicular to the first. Through this procedure the lines of serum pro-

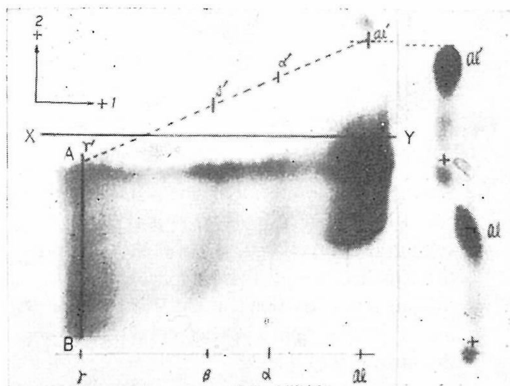

Fig. 12. Two-dimensional crossing electrophoresis of serum proteins against sulfisomidine.

First electrophoresis: On line AB, 0.04 ml/7 cm of human serum; at 100 V and 6 mA for 10 hours, in direction 1. Second electrophoresis: On line XY, 0.07 ml/15 cm of a 8% sulfisomidine solution; at 100 V and 4 mA for 8 hours in direction 2. At the marks (+) human serum was applied to indicate the position of albumin after proper electrophoresis in the second run. Barbiturate buffer of pH 8.6 and ionic strength 0.05. Filter paper, Toyo No. 52. Stained with BPB.

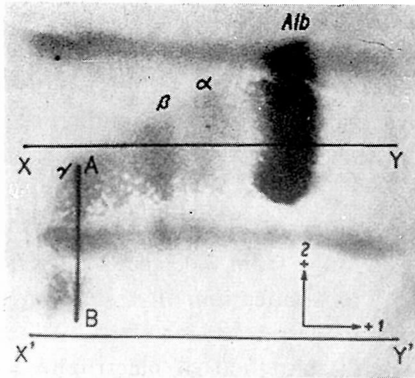

Fig. 13. Two-dimensional crossing electrophoresis of serum proteins against sulfisomidine.

First electrophoresis: On line AB, 0.06 ml/6 cm of human serum, at 40 V and 3 mA for 16 hours, in direction 1. Second electrophoresis: On line XY, 0.01 ml/14 cm of a 0.05% sulfisomidine solution; at 50 V and 6 mA for 8 hours, in direction 2. Barbiturate buffer of pH 8.6 and ionic strength 0.05. Stained at first with ninhydrin, then with dimethylaminobenzaldehyde. Other conditions were the same as in Fig. 12.

teins crossed the line of sulfonamide. But as can be seen, the former lines were stopped at the region of the latter. That this comes from the excessive concentration of the latter can be confirmed in comparison with the example of the crossing electrophoresis of serum proteins with sulfisomidine in low concentration as shown in Fig. 13. Here, sulfisomidine migrated toward anode from line XY, where it had been originally applied, with a mobility same as or somewhat smaller than that of serum albumin. The mobilities of the serum proteins were not affected at the crossing with the start line, XY, of sulfisomidine.

On the other hand, the reaction of sulfisomidine with serum albumin seems to be weak, since its line formed hardly a groove, whereas the line of sulfisoxazole formed a groove at the crossing with serum albumin, as shown in Fig. 14; it is evident that they formed a complex. In this connection it way be worthy of note that the application of a dilute solution of low molecular substance is also favorable in such case, when it is applied on the line parallel to the field and the other reactant, a protein, on the line perpendicular to it, since the molecular ratio of them can be reduced to make their reaction more obvious.

However, this does not always apply to the solution of a protein of high

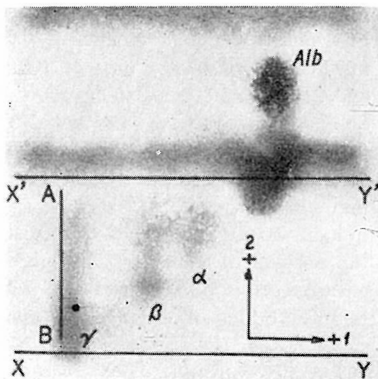

Fig. 14. Two-dimensional crossing electrophoresis of serum proteins against sulfisoxazole.

First electrophoresis: On line AB, 0.04 ml/6 cm of human serum, at 40 V and 3 mA for 16 hours, in direction 1. Second electrophoresis: On line XY, 0.02 ml/14 cm of a 0.05% sulfisoxazole solution; at 50 V 5 mA for 8 hours, in direction 2. Other conditions were the same as in Fig. 13.

molecular weight, which may be used up to 10 per cent without disturbing the mobility of other substances. The only precaution is to dialyze it against the buffer solution used for electrophoresis, to exclude the effect of difference in ionic strength.

b) Use of control.

The formation of artefacts due to local changes of viscosity, pH and/or ionic strength can be avoided by several precautions in preparing test solutions. But even with these precautions, artefacts can not always be completely ruled out. As other sorts of artefacts may arise, a control must be used for this purpose; If any appropriate substance can be found which forms no groove in its line at the crossing points with test substances when it is applied on the line parallel to the field, it is evident that this substance does not react with them and that no local disturbance exists on the filter paper. Such controls can serve any substance without restriction, so long as they do not react with the test substances, although a substance with the same mobility as the reactant to be tested may be most favorable. We have used dye stuffs most frequently, as they can be visually followed without staining throughout the electrophoresis.

By the use of the appropriate controls, practically all the local disturbances which may cause artefacts can be detected. But the artefact due to adsorption of protein on filter paper can not always easily be avoided. In such a case reexaminations with other sorts of filter paper or with other supporting media to eliminate the effect of adsorption might be necessary.

c) Cautions for the application of test solution.

As already shown,[4] mechanical damage of the filter paper may cause an artefact in the line of test substance applied perpendicularly to the field. This artefact can usually be detected easily, since it appears as a projection against the direction of the migration of the reactant, whereas the real interaction causes depression. But difficulties were experienced in some cases, when a groove (or peak) formed by the interaction of two reactants was further deformed by the artefact to show two apices, as though three reactants had reacted. Thus it is necessary to avoid the mechanical damage of the paper in applying the test solutions. For this purpose micropipetts inserted with a few threads have been used successfully.

In regard to the application of test solution, another caution may also be useful: Uniformity of the application is as important in crossing electrophoresis as in the usual one. Thus the application is so regulated by the inserted threads and by the inclination of the micropipett, as to apply the necessary quantity in 4 to 5 courses on the planned line. It might also deserve attention that the design should be drawn lightly with pencil, since the lead might contain interfering substances.

SUMMARY

When one substance is applied on a line parallel to the electrical field and the other substance on a line perpendicular to it, the line of the latter may be grooved at the crossing point by the electrophoresis, if they reacted each other. Thus the formation of the groove can be used as a criterion to detect whether two reactants react or not. (Principle of crossing electrophoresis.)

Artificial groovings in the line of a substance were theoretically predicted to be due to high viscosity, high ionic strength, and/or large difference in pH of the solution of the other reactant from those of the buffer solution used for electrophoresis. Artefacts due to mechanical damage of the filter paper and those due to adsorption of protein were also shown to occur.

Discussions were presented on the prerequisites for avoiding the formation of artificial grooves in the line of reactant, due to the above causes, and the use of appropriate controls was recommended.

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