ELECTROPHORETIC STUDIES OF PHOSPHATASE

I. FRONTAL ANALYSIS OF ALKALINE PHOSPHATASE OF BOVINE SERUM

KAZUSHIGE TANAKA

Institute of Medical Chemistry, Yamaguchi Medical School, Ube

Director: Prof. S. Nakamura

(Received March 8, 1957)

The Tiselius method of electrophoresis has been amply utilized in the enzyme chemistry. It has been proved to be useful especially in separating the enzymes and in testing their uniformity. As to the study of the distribution of enzymes in the proteins of biological fluids and organ extracts, the usual moving boundary method has some limitations (1). Because the separation by boundaries is only a partial one, even if the compensation is carried out. On the other hand paper electrophoresis has an advantage to achieve complete separation of components. Thus it has become widely used in studies of enzymes. The paper electrophoresis, however, has also certain limitations, as discussed by *Kunkel et al* (2). In the study of enzyme distribution the method of the electrophoretic frontal analysis proposed by *Nakamura* (6) seems to be promising. The present author has applied it to the alkaline phosphatase of bovine serum.

A. PRELIMINARY EXPERIMENTS

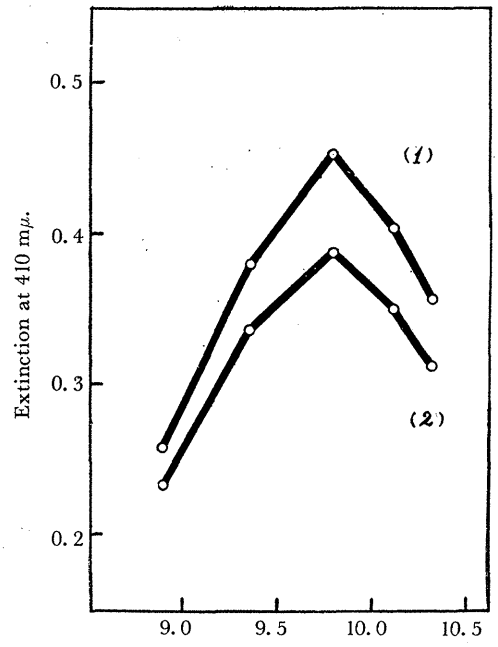
The alkaline phosphatase can be studied usually by measuring either the inorganic phosphate liberated by the enzyme from the substrate, or by measuring the colour developed by the alcoholic groups of the ester, e. g. *p*-nitrophenyl phosphate. For the electrophoretic frontal analysis, a rapid and simple micro-method is neccessary. Thus the method using *p*-nitrophenyl phosphate as substrate is preferred.

The method was used first by *Ohmori* (3). In the present work the method of *Bessey et al* (4) was utilized substituting the 0.1 M carbonate for glycine buffer. To 0.5 ml. of carbonate buffer of pH 8.95, 9.44, 9.80, 10.10 and 10.40 containing 1 mM $MgCl_2$, 0.5 ml. of 4 per cent sodium *p*-nitrophenyl phosphate and 0.1 ml. of bovine serum were added. The change in pH after incubating the solutions for 30 minutes at 38°C was within 0.1 pH.

The bovine serum was obtained by centrifuging the blood clotted in centrifuge tube for 20 minutes at 2,000 r.p.m.

田 中 一 成

Optimal pH: Using the same buffer and substrate with or without $MgCl_2$, the optimal pH of the alkaline phosphatase in the bovine serum was investigated. After the incubation of 30 minutes at 38°C 0.02N. NaOH was added to make the total volume of the solution 5 ml. The extinction coefficient of each solution at 410 mμ. was measured by a Beckman DU type spectrophotometer (*Shimazu*). Fig. 1 represents the activity pH curves. The optimal pH was 9.8, which lies between that of human (10.0 -10.1) and rabbit serum (9.1-9.7) (5).



(I) Activity with Mg^{++}-ion.
(II) Without Mg^{++}-ion.

Fig. 1. Activity-pH curves of alkaline phosphatase of bovine serum.

The activation of the alkaline phosphatase by Mg^{++}-ion was also observed, but not striking. From the results obtained by the preliminary experiments, the conditions of enzymatic analyses are chosen as follows: To the enzyme solution to be analyzed, 1 ml. of buffered substrate solution is added, after both solutions have been warmed to 38°C, in a thermostat. The buffered substrate solution consists of 0.5 ml. of substrate solution and 0.5 ml. of carbonate buffer as described above.

B. Electrophoretic Frontal Analysis

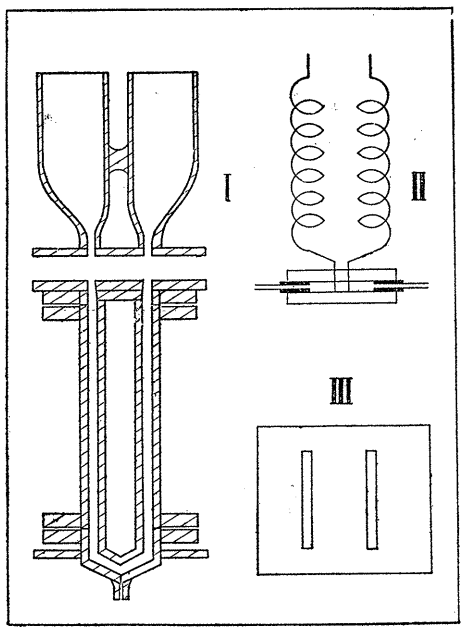
The principle of the method applied here was described by *Nakamura* and *Tanaka* (6). It is the same as devised by *Tiselius* and his collaborators (7) for the chromatography: After a boundary pattern by the usual Tiselius method is obtained, the solution in each limb of electrophoresis cell is forced through a microconductivity cell. The forced solution are collected dropwise and analyzed enzymatically. The conductivities of the drops are measured to correlate the optical and activity patterns.

Apparatus and Procedure:

Two sorts of apparatuses were used. At first the cell described by *Svensson* (8) for the measurement of conductivity was used. It consists of usual top,

center, bottom pieces and "cut-off" and "take-off" plates between top and center pieces. In the "take-off" plate are inserted two capillary tubes with conductivity cells. After the boundaries are obtained in the usual manner, the original solution was introduced from the capillary tube sealed to the bottom piece by a syringe. Thus the solutions in the ascending and descending channels were forced dropwise through the capillary tubes inserted in the take-off plate.

In filling and making the initial boundaries in the *Svensson* cell, center section or other must be displaced several times. As the cell assembly is fairely complicated with "cut-off" and "take-off" plates and three delicate glass capillary tubes, the manipulation is somewhat annoying. Afterwards special cells according to the ideas of *Svensson* (9) and *Kekwick et al* (10) was built from "Acrylite" (polyacrylate) by *Nakamura*. Fig. 2 represents diagrammatically the cell assembly and a microconductivity cell. The main part of the cell, i.e. the center and the bottom section is non-separable one piece. It has five knobs, bored by capillary hole to be inserted with a polyvinyl capillary tube.



(I) Cell assembly.
(II) Micro-conductivity cell.
(III) Cross-section at the bottom plate.

Fig. 2. Electrophoresis cell assembly for frontal analysis of enzyme.

Each of the upper two capillaries is provided with a conductivity cell made of an "Acrylite" block ($3 \times 7 \times 22$mm.). The platinum wire electrodes are sealed through the wall of capillary hole (diameter 1.5mm.) bored in the block. The distance between the two electrodes is 2.5mm.

Filling of the cell is carried out according to *Kekwick et al* (10). In the present work, a three way cock of "Acrylite" was attached to the end of each capillary tube to facilitate the procedure. In filling the cell, i. e. in forming the initial boundaries, the total cell assembly remaines unmoved. First, all the apparatus is filled with a veronal buffer of pH 7.8 and ionic strength 0.1, the electrode vessels are arranged for use and the vessels and the cappillary tubes are all closed. Then by means of a syringe the protein solution is introduced from the bottom and the buffer is forced out from the lower capillary on the

ascending side, then from the upper on the descending side. Sharp boundaries are made at the level of holes. Finally a caution is taken to fill upper capillaries with buffer and lower ones with protein solution.

After the boundaries are sufficiently separated, the protein solution is forced dropwise through the upper capillaries, by introducing the original solution from the bottom. Two drops at a time are collected serially in a test tube and analyzed of the alkaline phosphatase activity. The conductivities of the solution are measured in the course.

The material used is the bovine serum obtained as described in the section of preliminary experiments and dialysed against veronal buffer for 24 hours. The measurement of alkaline phosphatase is carried out as in preliminary experiments.

RESULTS AND DISCUSSION

Figures 3 and 4 show the activity of alkaline phosphatase versus the number of drops forced from the ascending and the descending channel, respectively. On the pattern of ascending limb, two steps, i. e. two boundaries, can be observed, wheras on the descending only one. These boundaries become more distinct, if the activity changes, i. e. the differences of two adjacent values, are plotted against the number of drops. In Figs. 3.2 and 4.2 the changes in activity are shown. Two peaks of activity on the ascending and one on the descending side are readily seen. It is the same procedure as in the case of refractive index gradient, provided that the latter is observed immediately instead of calculating the differences.

Under the experimental conditions adopted here, the obtained activity values are subjected to a considerable error. Thus the curve of differences between two just adjacent activity values fluctuates remarkably, as can be seen from the Curves 3.3 and 4.3. On the other hand the Curve 3.2 was obtained by plotting the differences between the activity values at intervals of two and the Curve 4.2 at intervals of one. Here the fluctuation of values obtained is small enough compared with the differences of them, to give smooth curves. The peak is made up of about 7 points which are sufficient in number to make the peaks well defined. This is also true with conductivity curves.

Since the peak of alkaline phosphatase could be depicted in correlation to the number of drops, it must now be coordinated with the optical pattern of serum. The number of drops and the distance of electrophoretic migration stand in inverse relation on the ascending side and in direct relation on the descending side. But the exact position of any drop on the optical pattern can not be determined from the volume of drops only. Because the volume from a certain

point on the pattern to the outlet opening of the capillary can not be measured exactly, and moreover the volume of drops forced is not always the same as expected from the distance in the pattern. Therefore the corresponding position of a certain drop on the optical pattern must be determined by other means.

As investigated by *Svensson* (9), the pattern of conductivity gradient cor-

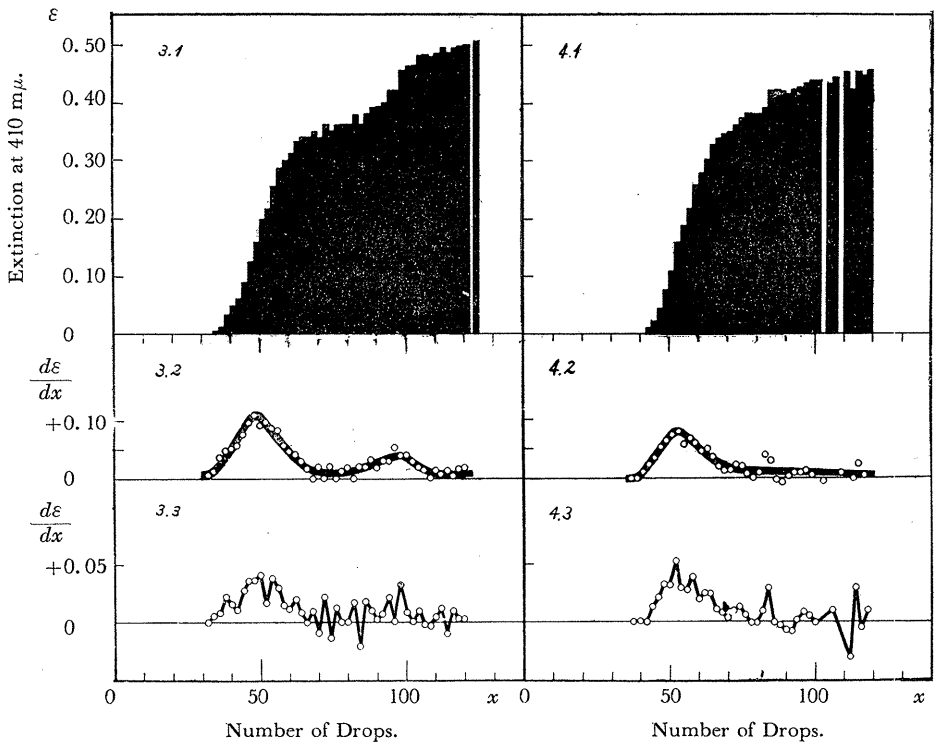


Fig. 3. Activity of alkaline phosphatase of bovine serum in the ascending channel.

Fig. 4. Activity of alkaline phosphatase of bovine srum in the descending channel.

responds to the optical pattern of refractive index gradient. The conductivity of a certain drop can be measured by inserting a micro-conductivity cell in the outlet capillary. The volume of the capillary from the cell to the opening can be measured easily. Thus the pattern of enzyme activity can be coordinated with the optical pattern, by means of a conductometric pattern. Of the curves shown in Figs 5 and 6, 5.1 and 6.1 represent the conductivity and Figs. 5.2 and 6.2 their gradient in the ascending and descending channels, respectively. In 5.2 and 6.2 severel peaks can be observed, which must correspond to those of the optical pattern.

In Figs.7 and 8 are brought together (I) the pattern of alkaline phosphatase, (II) that of conductivity change, and (III) the optical pattern.

In the curve of alkaline phosphatase on the ascending side, there can be observed two peaks, one at about 49th and the other at 98th drop. As can be seen from the figures, the second peak on the pattern (I) of ascening side correspons to the δ-anomaly peak of the conductometric pattern (II), since the two patterns are coordinated by means of the number of drops. From this fact it would be very probable that the second peak is due to the initial concentration

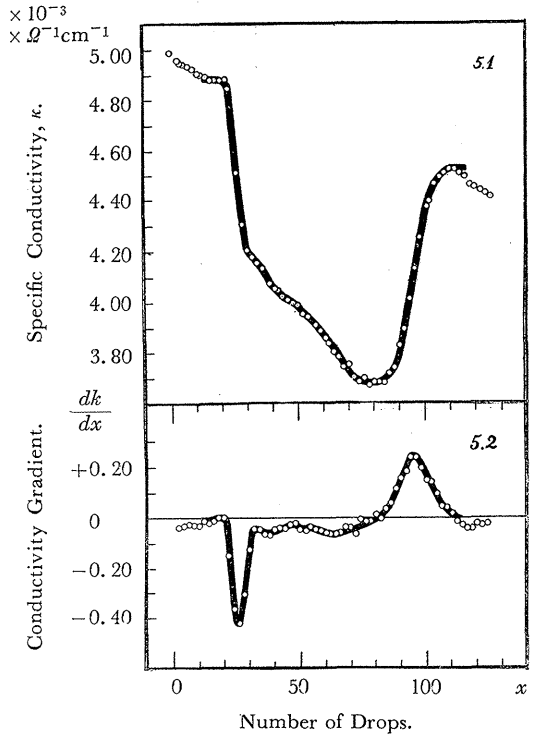


Fig. 5. Conductivity curve and conductometric pattern of bovine serum in the ascending channel.

difference between serum and buffer solution. It can be supported also from the following fact: The results obtained by *Hayashi* (11) show a second, more distince, larger peak. As the concentration differnce was larger in his experiments than in ours, it can be assumed that the larger the concentration difference, the larger the second peak. The absence, however, of the corresponding second peak on the descending side, is to be investigated further.

In any way, only the first peak will be regarded as that of alkaline phosphatase.

As to the coordination of patterns (II) and (III), it may be noted that the position of each pair of corresponding peaks on them must be identical. Unfortunately the conductometric peaks are not clear enough, except the two remarkable peaks of albumin and anomaly (δ or ε). In order to coordinate the patterns (II) and (III) these two peaks are sufficient, since the two patterns can be set in position so as to locate the two peaks on the same positions.

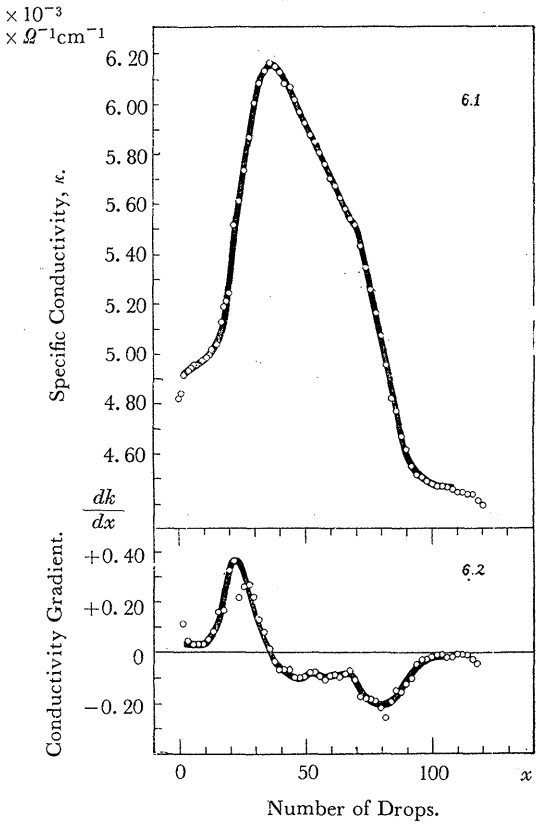


Fig. 6. Conductivity curve and conductometric pattern of bovine serum in the descending channel.

Thus the pattern of alkaline phospatase, (I), could be coordinated with that of refractive index gradient, (III). As can be seen from figures 7 and 8, the peak of the alkaline phosphatase lies between those of α- and β-globulins. The relative mobility of it is 69 on the ascending and 55 on the descending side, refering to that of albumin as 100. *Mathies* (12) separated the fraction containing alkaline phosphatase from swine kidney by the electrophresis-convection. By usual method of electrophoresis it was confirmed that the fraction had a main component of mobility $1.7 \times 10^{-5} cm^2/v.$ sec. Any conclusion, however, was withheld as to whether the alkaline phosphatase coincided with it or not. According to the present experiments the mobility of alkaline phosphatase seems to be somewhat larger than that of the component mentioned. *Roche et at* (13) separated a strongly active fraction by paper electrophoresis, the homogeneity of which was discussed by *Morton* (14). But they have also reserved to discuss any values of the mobility of the fraction obtained.

The distribution of an enzyme may be studies by the electrophoretic frontal analysis proposed here, as can be seen from the results. In the method proposed, however, the volume of enzyme solution for one measurement is limited and the number of measurements is fairely large, so that not all enzymes can be in-

vestigated by it. The alkaline phosphatase is an enzyme very favourable in testing the applicability of the method, since its measurement is microchemical and simple. But considering the accuracy of the enzymatic measurement, it would be very difficult to distinguish two enzymes of the same kind with similar mobility, if the activity of the one of them were under about 10 per cent of the other.

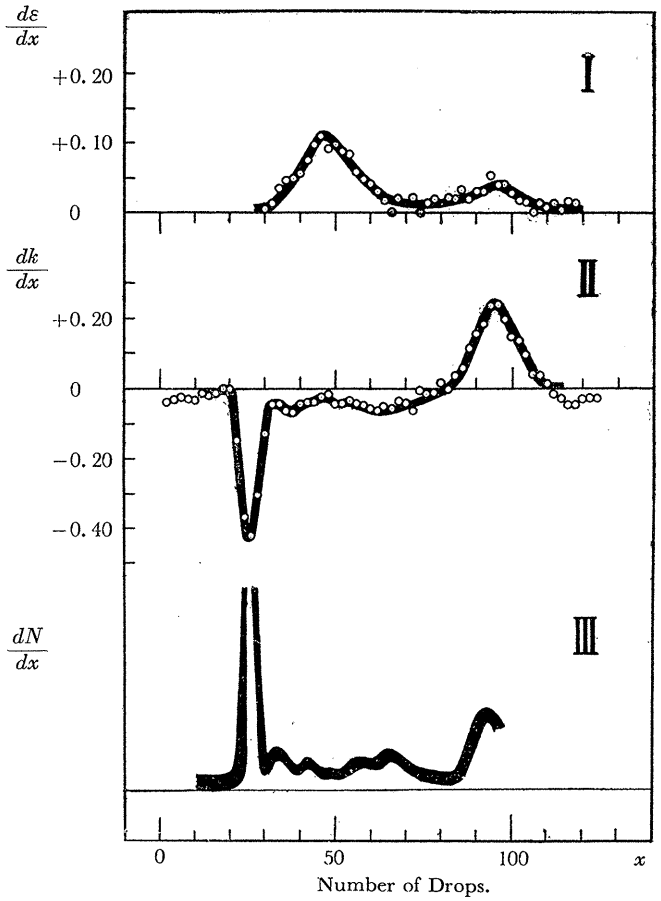


Fig. 7. The pattern of alkaline phosphatase of bovine serum, compared with the optical and conductometric patterns in the ascending channel.

SUMMARY

The alkaline phosphatase of bovine serum showed the pH optimum at 9.8 with a carbonate buffer.

After the boundaries were obtained by the usual method of electrophoresis,

the protein solution was forced dropwise. Conductivities and enzyme activities of the drops were measured.

By differentiating the activity curves, a pattern of alkaline phosphatase was obtained, which could be correlated with the optical pattern of serum by means of a differential conductivity curve.

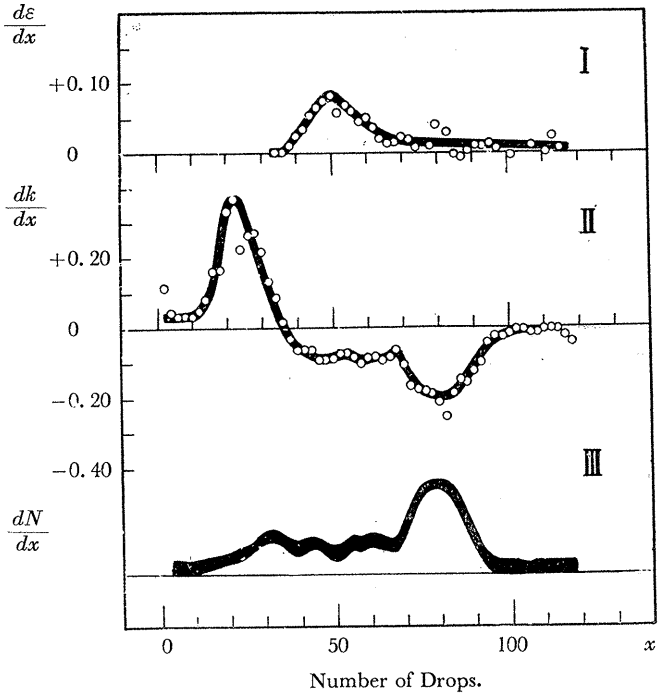


Fig. 8. The pattern of alkaline phosphatase of bovine serum, compared with the optical and conductometric patterns in the descending channel.

The alkaline phosphatase of bovine serum migrates between α- and β-globulin. Its relative mobility referring to albumin as 100, was 69 and 55 in the ascending and descending channels, respectively, of the electrophoresis cell, at pH 7.8 and ionic strength 0.1.

LITERATURE

1) TISELIUS, A. AND FLODIN, P.: *Advances in Protein Chem.*, **8**, 461, 1953.
2) KUNKEL, H. G. AND SLATER, R. J.: *Proc. Soc. Exptl. Biol. Med.*, **80**, 42, 1952.
3) OHMORI, Y.: *Enzymologia*, **4**, 217, 1937.
4) BESSEY, O. A., LOWRY, O. H. AND BLOCK, M. J.: *J. Biol. Chem.*, **164**, 321, 1946.
5) MORTON, K.: *Biochem.J.*, **60**, 573, 1955.
6) NAKAMURA, S. AND TANAKA, K.: *J. Biochem.* (Japan) **44**, 309, 1957.
7) TISELIUS, A.: *Advances in Protein Chem.*, **3**, 67, 1947.
8) SVENSSON, H.: *J. Am. Chem. Soc.*, **72**, 1974, 1950.
9) SVENSON, H.: *Arkiv Kemi. Mtneral. Geol.*, **22A**, No. 10., 1946. Cited from (8).
10) KEKWICK, R. A., LITTELETON, J. W., BREWER, E. AND DREBLOW, E. S.: *Bioch. J.*, **49**, 253, 1951.
11) HAYASHI, Y.: *Bull. Yamaguchi Med. School*, **5**, 25, 1957.
12) MATHIES, J. C.: *Science*, **115**, 144, 1952.
13) ROCHE, J. AND BOUCHILLOUX, S.: *Bull. soc. chim. biol.*, **35**, 567, 1953.
14) MORTON, R. K.: *Biochem. J.*, **57**, 595, 1954.