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THE ELECTROPHORETIC FRONTAL ANALYSIS OF THE ALKALINE PHOSPHATASE OF LYMPHNODES

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The method of the electrophoretic frontal analysis of enzymes has been proposed by *Nakamura* and *Tanaka* (1). The method was applied to the bovine serum (2) and the extract of the intestinal mucosa by *Tanaka* (3). But he did not analyze the extract *per se*, but mixed it to the serum. The electrophoretic pattern of the serum was thereby not disturbed by the addition of the extract, as this was little enough added. In other words, the tissue extract was added merely to increase the activity of the alkaline phosphatase of the serum, avoiding the change of the electrophoreretic patterns. Accordingly the method is not yet tested with any tissue extract *per se*. As has already been suggested by *Nakamura*, it is very interesting to make clear whether the method can be applied to the tissue extract or not.

The extract of bovine mesenterial lymph nodes is considerablv rich in alkaline prosphatase and the composition in proteins or in enzymes is probably not so intricated as in liver. In these points it is an advantageous tissue extract to investigate the applicability of the frontal analysis to the enzymes of tissue.

EXPERIMENTAL

Experimental methods of the frontal analysis of alkaline phosphatase are the same as described by *Nakamura* and *Tanaka* (1) and *Tanaka*(2).

Material:- The materials were prepared according to the method of *Nakamura* et al. (4) and *Takahashi* and *Hayashi*(5). Bovine mesenterial lymph-nodes washed with physiological saline solution and removed of fatty and connective tissues were first chopped with scissors and then homogenized for five minutes with a cooled blade homogenizer. To the tissue brei the same quantity of 0.95 per cent saline solution was added and again homogenized for 5 minutes. The homogenate was centrifuged for 30 minutes at 3000 r.p.m. The supernatant obtained was filtered in a refrigerator overnight with No. 5c Toyo filter paper and referred to as L_1. L_1 was dialyzed in a refrigerator against a veronal buffer of pH 7.8 and ionic strength 0.05. The volume increased to about 3/2 of the original after 20 hours.

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The same homogenate was, on the other hand, acidified to pH 4.5 with 10 per cent acetic acid, the precipitate was centrifuged off and the supernatant was neutralized with 5 per cent NaOH, acidified again, centrifuged, and so on. By reprecipitation of three times, the supernatant became clear and referred to as F_1. After neutralization, F_1 was lyophilized to 1/3 of the original volume and dialyzed in a refrigerator against the veronal buffer. The volume increased to about 2/3 of the original filtrate after 20 hours.

Analytical methods:– Nitrogen was determined by the usual micro-Kjeldahl method and the activity of alkaline phosphatase by the *p*-nitrophenyl phosphate method (6), as applied to the frontal analysis by *Nakamura* and *Tanaka* (1): To one drop of enzyme solution forced from the electrophoresis cell, 1 ml. of substrate solution was added, which was made up of 0.5 ml. 0.4 per cent sodium *p*-nitrophenyl phosphate and 0.5 ml. 0.1 M carbonate buffer of pH 10.0 containing 1 mM $MgCl_2$ as activator. After the incubation of 30 minutes at 38°C, 5 ml. of 0.1 N NaOH was added and the extinction at 410 mμ. was estimated with a Beckman DU type spectrophotometer.

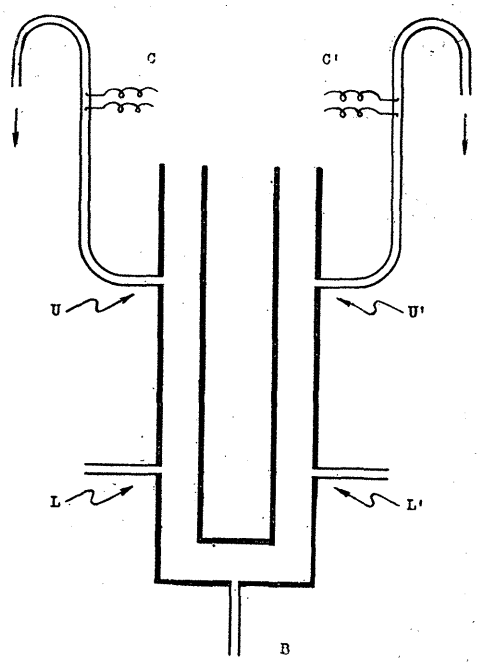

Fig. 1. Diagram of Cell Assembly.
U, U′: Upper holes and capillaries.
L, L′: Lower holes and cabillaries.
B : Bottom hole and capillary.
C, C′: Conductivity cells.

Procedures of electrophoretic frontal analysis: The electrophoresis cell was the same as describeb by *Nakamura* and *Tanaka* (1). As shown diagrammatically in Fig. 1, the cell is provided with five holes to displace protein or buffer solution. This type of cell was first described by *Svensson* and *Brewer* et (7). The filling of the cell was carried out as described by the latter. The electrophoresis was carried out at 4°C with a veronal buffer of pH 7.8 and ionic strength 0.05. After the electrophoresis of 3 to 4 hours, the protein solution in each limb was forced from the upper holes by the introduction of the original solution from the bottom hole. The conductivity of the solution was determined by the micro conductivity cells set up in polyvinyl capillary tubes inserted in the upper holes. Conductivity measurements enable us to depict the electrophoretic patterns, as reported by *Svensson* (8). Thus it becomes possible to correlate exactly a displaced drop and its place on the electrophoretic pattern, as the volume of capillary tube from the conductivity cell to the opening can easily be measured.

Results

L_1: The alkaline phosphatase activities of the forced drops of the extract,

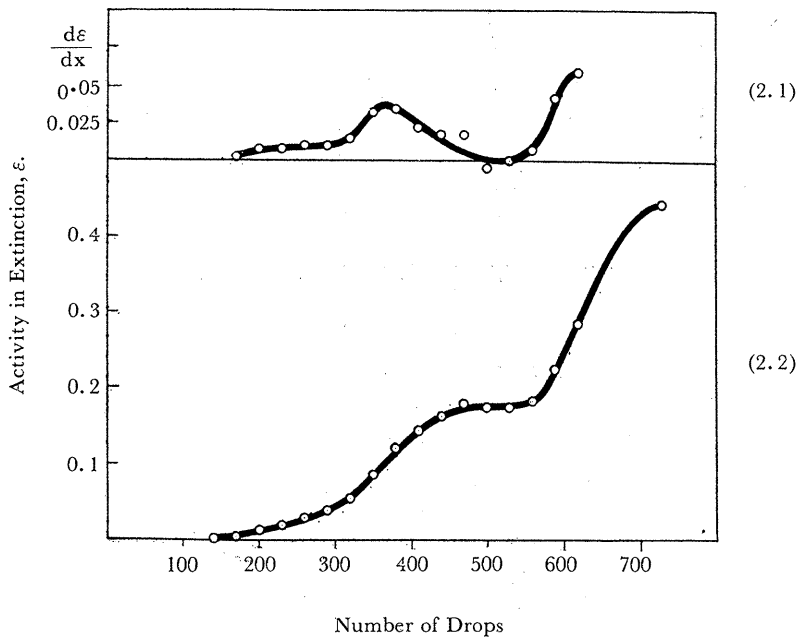

Fig 2. Activity Curves of Alkaline Phosphatase in the Ascending Channel.

(1) The differential acivity curve.
(2) The activity curve of the alkaline phosphatase. Explanation, see text.

L_1, from the ascending and descending channels are shown in Figures 2 and 3, respectively. The plotted lines represent the change of activities along the line of electrophoresis.

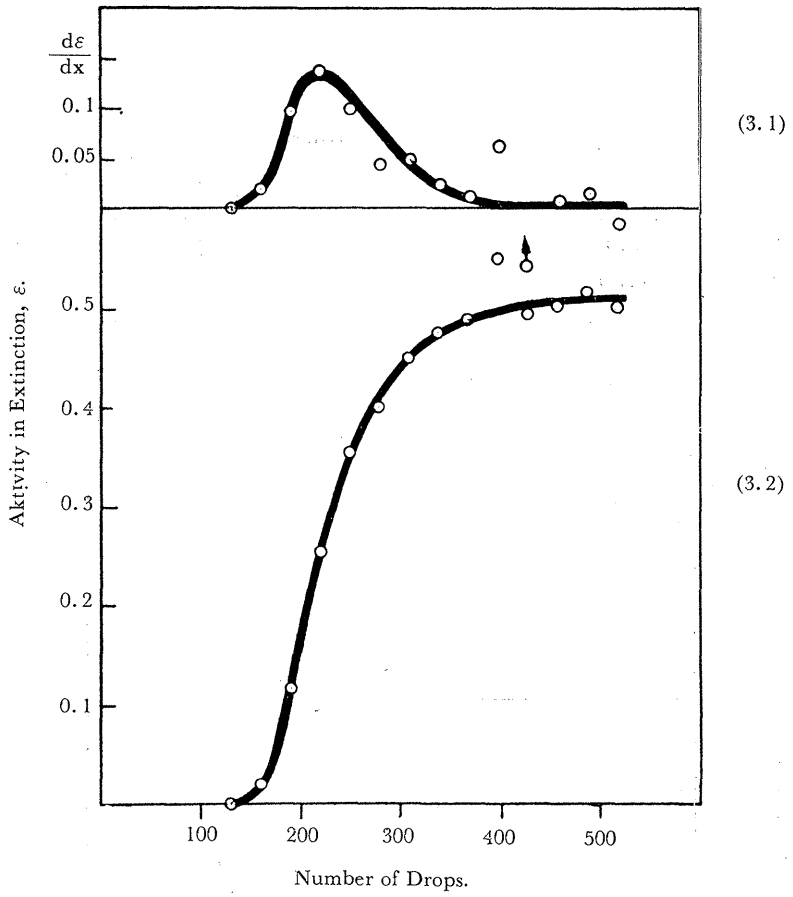

Fig. 3. Activity Curves of Alkaline Phospatase in the Descendig Channel.
(1) The differential activity curve.
(2) The activity curve of the alkaline phosphatase. Explanation, see text.

The patterns of the alkaline phosphatase can be obtained by differentiating the activity curves, i.e. by plotting the differences of two adjacent activity values. Thus Curves 2.2 and 3.2 represent the activity curves, and 2.1 and 3.1 the differential curves of them, namely the patterns of the alkaline phosphatase in the ascending and descending channels, respectively.

The conductivity curves of the displaced solutions from the ascending and descending channels are shown in Figures 4 and 5, respectively. The differential curves of them are also shown therein. The peaks of anomalies are especially

prominent. The peak of δ-anomaly of ascending side situated on 600th drop and that of ε-anomaly on 100th.

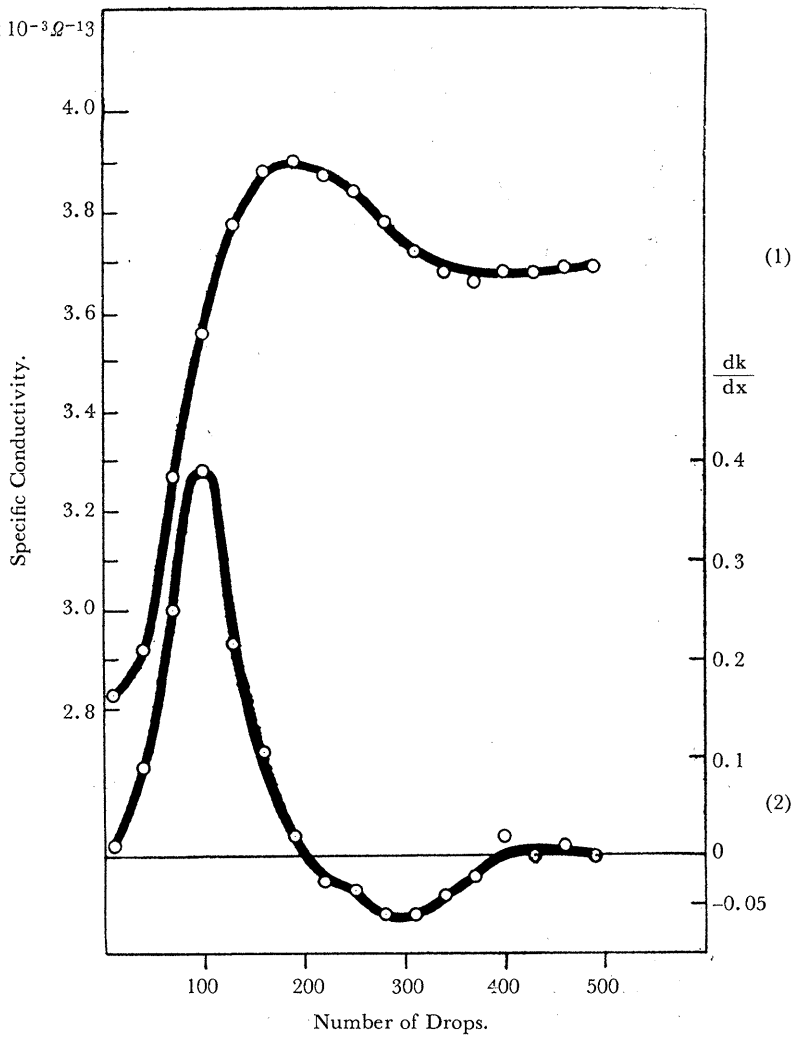

Fig. 4. Conductometric Curves of the Solution in the Ascending Channel.
(1) Specific conductivitycurve.
(2) Curve of conductivity gradient. Explanation,see text.

The electrophoretic patterns obtained optically are shown in Figures 6 and 7 together with the patterns obtained conductometrically. The latter patterns were not sharp enough to be correctly coordinated with the former, even though the coordination of the two patterns must be equal. Therefore the peaks of the anomalies were taken as starting points and the number of displaced drops (or the length along the line of electrophoresis) as reference to correlate the two

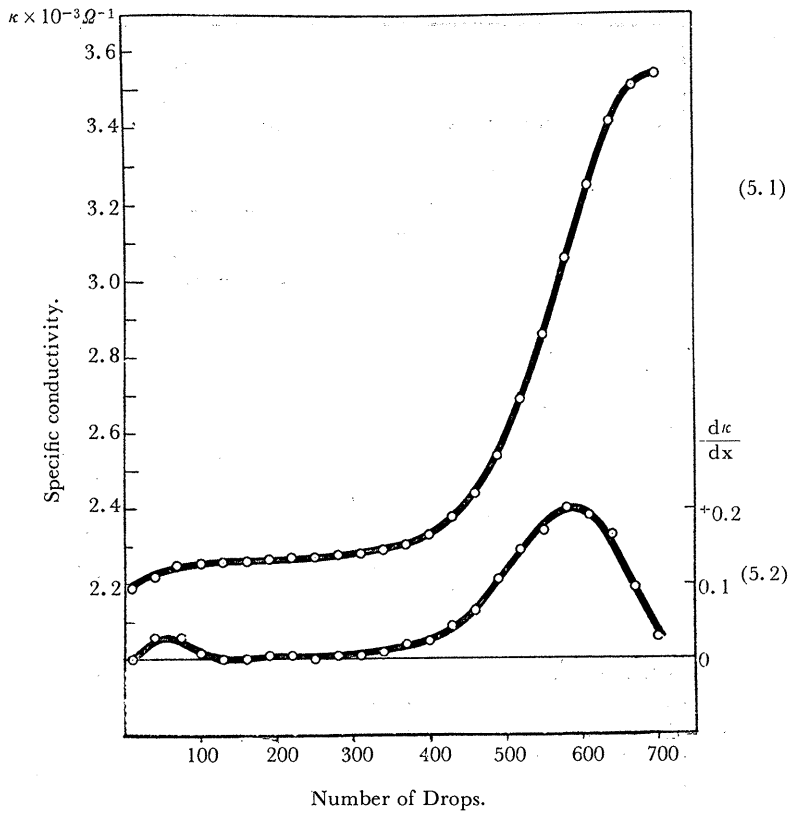

Fig. 5. Conduc tometric Curves of the Solution in the Descending Channl.
(1) Specific conductivity curve.
(2) Curve of conductivity gradient. Explanation, see text.

curves. The third patterns represent the curves of the alkaline phosphatase, which are correlated exactly with the conductometric curves.

As already pointed out by *Nakamura* and *Tanaka*(1) the alkaline phosphatase moves electrophoretically slowly. Its mobility, U, on the ascending side can be calculated as follows: The peak of the alkaline phosphatase lay on about 370th drop, the peak of δ-anomaly on about 600th. The volume of the capillary from the conductivity cell to the outlet opening measured 15 drops. Thus the number of drops displaced between the peaks of alkaline phosphatatase and δ-anomaly was about 215. Hence the displaced vlume, V, was 3.55 ml., as one drop measured 0.0165 ml. At 355 th drop the specific conductivity, κ, was 2.295×10^{-3}mho. The total time, t, of electrophoresis was 11,400 seconds and

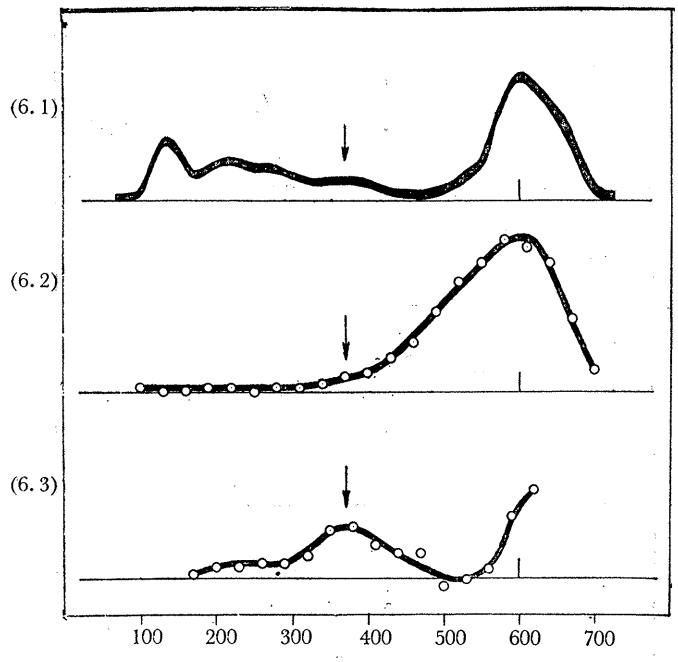

Fig. 6. Comparison of Electrophoretic, Conductometric and Enzymatic Analyses of the Extract, L_1, of Bovine Lymp node in the Ascending Channel.
(1) Pattern of refractive index gradient.
(2) Pattern of conductivity gradient.
(3) Pattern of alkaline phosphatase. Explanation, see text.

the current, i, 20 mA. According to the formula,

$$U=\frac{V}{t}/\frac{i}{\kappa},$$

$$U=3.58\times10^{-5}\ \text{cm}^2./\text{sec. volt.}$$

The mobility on the descending side can be calculated in quite the same way. The peak of the enzyme lay on 320th drop and the ε anomaly on 200th. The displaced volume between them amounted to 1.95 ml., since one drop measured 0.0186 ml. $\kappa=3.73\times10^{-3}$ mho. Thus,

$$U=3.20\times10^{-5}\text{cm}^2./\text{sec. volt.}$$

The values fall in the region between α and β-globulin of serum.

F_1 : Unfortunately the protein concentration of the fraction, F_1, was so little that no clear pattern was obtained. In Fig. 8 only the patterns obtained from the descending channel at the beginning are shown. The mobility of the alka-

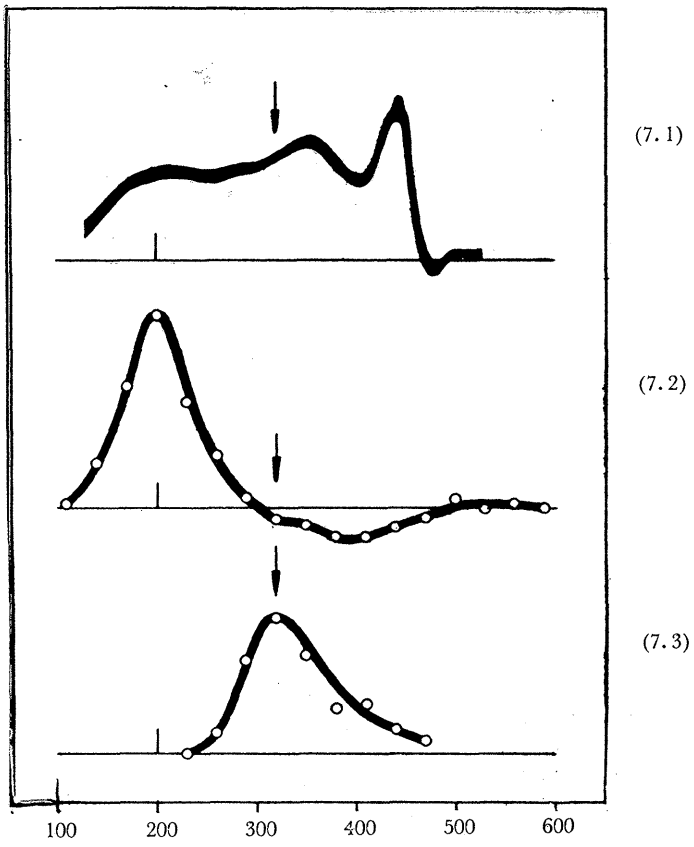

Fig. 7. Comparison of Electrophoretic, Conductometric and Enzymatic Analyses of the Extract, L_1, of Bovine Lymphnode in the Descending Channel.
(1) Pattern of refractive index gradient.
(2) Pattern of conductivity gradient.
(3) Pattern of alkaline phosphatase. Explantion, see text.

line prosphatase can be calculated from the results obtained, as follws: The peak of the enzyme lay on 200th and that of ε-anomaly on 120th drop. The displaced volume, V, was 1.44 ml., since one drop measured 0.0222ml. $t=$ 11,820 sec. $\kappa=5.91\times10^{-3}$ mho. $i=20$ mA. Thus,

$$U=3.60\times10^{-5}\ \text{cm}^2./\text{sec. volt.}$$

DISCUSSION

The mobility of the alkaline phosphatase could be determined by the present experiment but the patterns obtained optically or conductometrically showed no

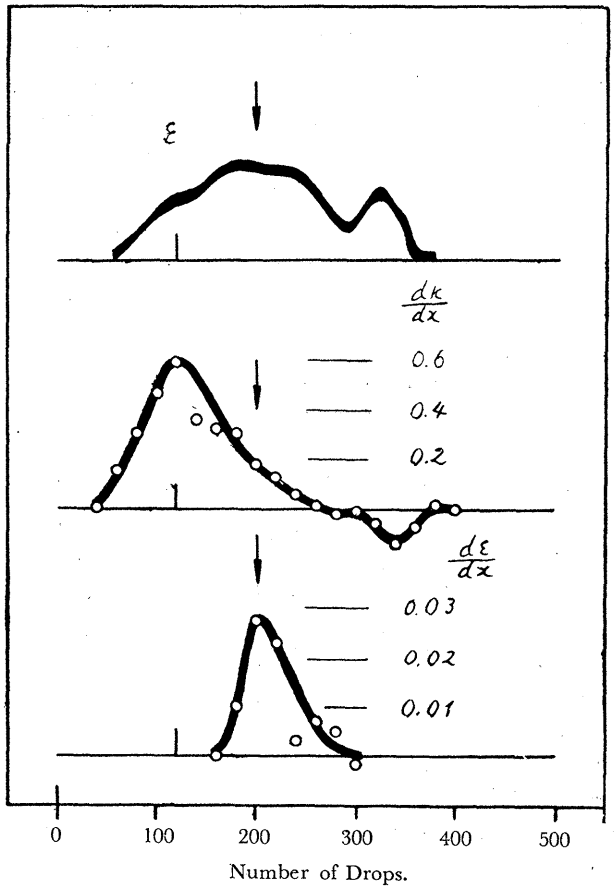

Fig. 8. Comparison of Electrophoretic, Conductometric and Enzymatic Analysis of the Fraction, F_1, of Bovine Lymphnode in the Descending Channel.
(1) Pattern of refractive index gradient.
(2) Pattern of conductivity gradient
(3) Pattern of alkaline phosphatase. Explanation, see text.

distinct peak which might correspond to it. The reason for this would be the low concentration of the extract in proteins, but it is also unprobable that the phophatase protein would be contained therein enough, to be distinguished optically, if the total protein concentration were raised as a whole.

The pattern of alkaline phosphatase on the ascending side shows two peaks, whereas that on the descending side only one. As already discussed by *Nakamura* (1), the second peak of the descending pattern would result probably from the concentration difference between the protein solution and the buffer solution, i.e. the δ-anomaly peak. But the absenece of a second peak on the descending pattern remains, in regard to this aspect, to be further investigated,

since an anomaly peak should here also appear.

The present author and *Takahashi* (5) have previously attempted a purification of the alkaline phophatase of the bovine lymph nodes. The fraction, F_1, prepared at that time retained ca. 1/3 of the total phosphatase activity of the original homogenate. But the fraction now prepared showed only 1/10 of the original activity, owing partly to the repeated reprecipitation of the acid proteins in the extract.

The difference in salt concentration between the tissue extracts prepared and the buffer soltuion is considerably large, namely,

$$0.16\ \mathrm{M}\ (0.95\,\%\ \mathrm{NaCl}) : 0.05\ \mathrm{M} \fallingdotseq 3 : 1.$$

Thus an increase in volum of about two fold occurred during the dialysis (20 hours) of the extract against the buffer solution. This circumstance is not favorable, if smaller protein components should be distinguished and the fractionation of the extract should be carried out. Lyophilization profitted a little hereto.

The alkaline phosphatase is one of the enzymes of which the estimation is microchemical, simple and fairly accurate. Thus it is a very favourable ezyme to test the applicability of the electrophoretic frontal analysis to the tissue extract. The range of accuracy, however, of the measuremement is 3 to 4 per cent of the obtained values. Therefore it is altogether impossible to distinguish an enzyme from others of the same kind and similar mobility, if the activity of the former is smaller than about 5 per cent of the latter. Moreover for the differentiation of the activity curve, the differences of values of two adjacent points must fulfil this condition. Therefore, as a matter of fact the activity of the former should be 10 per cent or more of the latter.

These cicumstances would make the proposed method unprofittable for the application to other enzymes except phosphatase. Even for the phosphatase the procedure of differentiation seems to be unfavourable.

SUMMARY

1. The extract of lymph nodes was first analyzed electrophorecally, then forced dropwise and the activities of the alkaline phosphatase in the drops were measured (electrophoretic frontal analysis).

2. By differentiating the activity curves, patterns of the alkaline phosphatase of lymph node were obtained, which were correlated with the optical patterns, by means of differential conductivity curves of the drops.

3. The peak of the alkaline phosphatase had a mobility of 3.8×10^{-5} cm^2./sec. volt. at pH 7.8 and ionic strength 0.05, which corresponds to that between the α and β globulin of serum.

4. Applicability of the method to other enzymes was also discussed.

LITERATURE

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