ELECTROPHORETIC STUDIES OF PHOSPHATASES

II. FRONTAL ANALYSIS OF ALKALINE AND ACID PHOSPHATASES OF BOVINE TISSUES

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The distribution of enzymes in the proteins of cells or tissues may be studied in regard to the organization of materials. Of some enzymes, especially of the respiratory enzymes (1), their localization in the cell have already been clarified. Functions of a cell seem to be so differentiated that they are related to some difinite sites in the cell, as in the case of whole body and its functions. Now the main part of the proteins which make up the cell, is contained in the homogeneous protoplasm, in so far as the latter materially occupies largest part of cell. But the questions of what kinds of proteins are in the protoplasm and what their roles are, is scarcely known, except some conjectures. The protein synthesis could be closely related to the protoplasm, as the main part of cell proteins. But even the site of protein synthesis might be ascribed to the particulate part, not to the homogeneous part of cell.

Thus the studies of tissue proteins have been undertaken in this institute, first to make clear the protein composition of tissues and then the metabolism of, or the physiological relations among, the tissue proteins (2). The studies on the distribution of enzymes in the tissue proteins can perhaps be related to both parts of the studies undertaken. Hither-to the determination of the mobility of an enzyme has been realizable only when the enzyme was sufficiently enriched in a solution to be analyzed by the Tiselius method of electrophoresis. Otherwise, the enzyme can not be separated and observed as a boundary, since the concentration of enzyme protein in the crude extract of tissue is usually small. Thus the mobility has been determined only with the crystallized enzymes as one of their physico-chemical properties.

By means of the mobility, however, the distribution of a protein, consequently of an enzyme, in the protein system of a tissue may be determined. The present work makes it possible to measure the mobility of an enzyme without profound purification.

The phosphatase is one of the enzymes, which are long known and yet their

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nature is chemically as well as physiologically obscure. Numerous works have been done without succeeding as yet in crystallizing it (3). The mobility and the distribution of phosphatase in the tissue proteins could be determined by the present work. But much work will be neccessary for the elucidation of its physiological nature.

EXPERIMENTAL

Experimental methods and procedures are the same as described previously (4).

The activities of the alkaline and acid phosphatases were measured by the *p*-nitrophenyl phosphate method according to *Bessey et al* (5). The substrate solution for the alkaline phosphatase: consisted of 0.5 ml. of 0.4 per cent sodium *p*-nitrophenyl phosphate and 0.5 ml. of 0.1 M carbonate buffer of pH 9.8; for the acid phosphatase: 0.5 ml. of 0.1 M acetate buffer of pH 5.0 instead of carbonate. To the collected drops of protein solutions, 1 ml. of substrate solution was added at 38°C in the thermostat. After the predetermined time of incubation, 5 ml. of 0.1 N NaOH was added and the extinction at 410 mμ was determined with a Beckman DU type spectrophotometer.

Nitrogen was measured by the usual micro-Kjeldahl method followed by the Nesslerization and colorimetry. The protein concentration of the serum or the extracts were sometimes measured also by the refractometric method.

Bovine sera used were obtained by centrifuging the clotted blood and dialyzing them against a veronal buffer of pH 7.8 and ionic strength 0.1, in a refrigerator for 48 hours, changing once the buffer.

The tissue extracts were prepared as described in the corresponding section, asfollows: Bovine kidney or small intestine was freed from fatty and connective tissues after being throughly washed. The intestinal lumen cut open and the mucosa was scratched off. The washed kidney or intestinal mucosa was homogenized with a blade homogenizer with the same quantity of solvent solution. As described in the next section, the solution used for the extraction was0.1 M acetate buffer of pH 4.5. The supernatant obtained by centrifugation was neutralized with 1N NaOH, if neccessary. After dialysis against the veronal buffer, itwas mixed appropriately with the serum to make up the test solutions.

The proced ures of electrophoresis, conducto metry and collection of the displaced drops were carried out as described previously (4), using the same cell as before.

Acid Extracts of Kidney and Intestinal Mucosa

The extract of tissue can be prepared with the same volume of physiological saline solution, as in the previous works done in this institute (2), for the sake of

uniformity of method. The extracts thus obtained, however, were turbid. As the extract, however, must be clear to be analyzed electrophoretically, a complication might be brought about by the prolonged filtration in the refrigerator. Moreover, they contained tissue proteins in considerably high concentration, and the separation of proteins into components by the electrophoresis was insufficient. Therefore, it was desirable to prepare a extract which could be easily clarified. And a simple acid extraction of the tissue homogenate was attempted to remove the turbidity from the extract, precipitating the inert proteins at the same time.

To determine the most favourable pH to precipitate the inert tissue proteins together with the turbidity, the homogenate of kidney was extracted with the

TABLE I

Relative activity of alkaline and acid phosphatase in the acid extracts of bovine kidney.

pH of buffer used for extraction.	Activity in extinction coefficient per mg. N per ml.		mg. Nitrogen per ml.
	Alkaline phosphatase	Acid phosphatase	
3.6	0.430	4.30	0.21
4.0	0.615	3.57	0.28
4.5	10.25	8.9	0.15
5.0	12.6	8.15	0.33
5.5	6.6	11.7	0.36
Physiological saline solution	7.2	14.5	0.34
Distilled water	3.52	15.8	0.33

same quantity of 0.1 M acetate buffer of various pH values. As can be seen from Table I, the highest relative activity of the alkaline phosphatase per nitrogen was obtained with the buffer of pH 5.0. The results obtained were not always strictly reproducible, probably because the pH of the homogenate, when mixed with solvent-solution, was not adjusted. As to the acid phosphatase, relative activity in the acid extract was rather decreased, compared to the extract with physiological saline solution. But on the other hand, the extract with the buffer of pH 4.5 could be most readily clarified by centrifugation at 3,000 r.p.m. Consequently the extract obtained was utilized as the phosphatase solution of kidney, in spite of the decrease in acid phosphatase activity. Unfortunately the protein concentration of the extract was so small that the proteins contained could not be separated to be observed individually by the boundary electrophoresis. Hence the extract was added to the serum to utilize the serum proteins as the reference substances. Thereby enough enzyme activity could be added, without changing practically the relative concentrations of the serum proteins,

since the quantity of tissue proteins added was very small compared with the former, as can be seen from Table II.

The enzyme solution submitted to the following experiments, was composed of 45ml. of the dialyzed serum and 5ml. of the dialyzed extract of kidney. The activities of alkaline and acid phosphatases were measured with 0.1 ml. of each solution and are presented also in Table II.

TABLE II

Alkaline and acid phosphatase activitiy of kidney extract and serum.

	Activity in extinction coefficient per 0. 1 ml. solution used.		Protein concentration
	Alkaline phosphatase	Acid phosphstase	
Serum	0. 660	0. 085	6. 3 per cent.
Kidney extract	0. 42 × 6	0. 553 × 6	0. 37 〃 〃
Mixture used for electrophoresis	0. 940	0. 470	5. 8 〃 〃
Calculated for the mixture	0. 846	0. 408	5. 7 〃 〃

As to the acid extracts of the intestinal mucosa, almost the same can be said. A series of the results obtained was shown in Table III. The remaining activity in each extract was not always the same, partly because the pH of the homogenate was not adjusted, when it was mixed with the extracting buffer. In the run of experiments shown, the activity of alkaline phosphatase per nitrogen in the extract with the buffer of pH 4.5 was about 3 times as much as that with physiological NaCl solution.

TABLE III

Relative activity of alkaline and acid phosphatase in acid extracts of bovine intestinal mucosa.

pH of buffer used for extraction.	Activity in extinction coefficient per mg. N per ml.		mg. Nitrogen per ml.
	Alkaline Phosphatase	Acid phosphatase	
3. 6	13. 15	3. 44	1. 15
4. 0	35. 7	7. 78	1. 35
4. 5	46. 6	12. 4	1. 68
5. 0	51. 1	14. 25	2. 25
5. 5	36. 2	22. 6	3. 75
Physiological saline solution	15. 3	11. 2	7. 50

Morton (3) reported that the alkaline phosphatase of tissue was contained in the particulates, i.e. the microsomes, of the cell, and he used butanol to liberate the

enzyme. In the experiments reported here, special attention was not payed to it. The acid extracts with buffers of pH 3.6, 4.0 and 4.5 were always completely clear, whereas that of pH 5.0 was sometimes turbid. Therefore the former three extracts must have been free from particulates. As shown in Table IV, the activity of alkaline phosphatase in the extract at pH 4.5 was considerably reduced

TABLE IV

Alkaline phosphatase activity in acid extract and physiological saline solution extract.

pH of buffer used for extraction.	Activity in extinction coefficient per ml.			
	Liver	Lymph-node	Kidney	Intestinal Mucosa
3.6	3.4	4.9	6.0	0.2
4.0	6.6	1.7	8.0	44.5
4.5	5.5	4.3	4.1	81.6
5.0	10.4	19.0	9.0	108
5.5	30.0	31.9	29.3	113
Physiological saline solution	44.3	32.3	42.0	182

in the case of the liver and the lymph-node. But the decrease in activity barely exceeded 50 per cent in the case of the kidney and the intestinal mucosa. It is of course not excluded that not all the alkaline phosphatase were dissolved and that the decrease were due to the coprecipitation of the enzyme. But in any case, enough enzyme was dissolved in the solution, at least to meet the need under the present experimental conditions.

Frontal Analysis of Alkaline and Acid Phosphatase of Kidney and Intestinal Mucosa

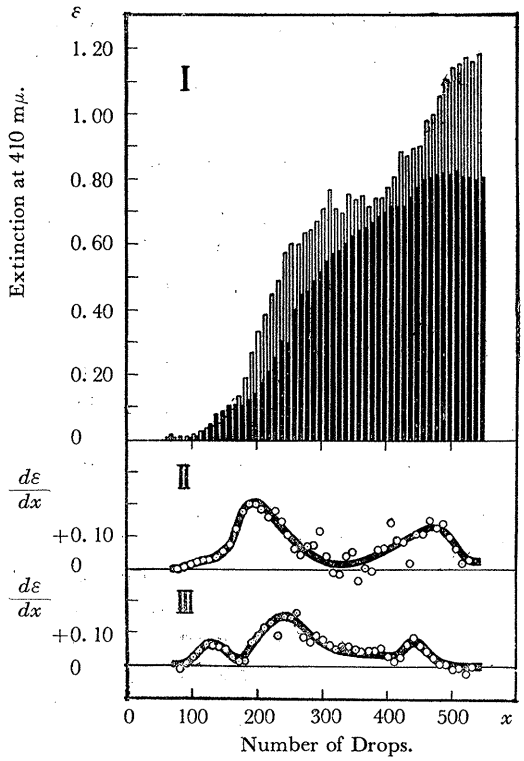
Kidney.

After the electrophoretic boundaries were sufficiently separated, the protein solutions from ascending and descending channels were forced dropwise. Five drops at a time were collected alternately for the measurements of alkaline and acid phosphatase. The time of incubation was 45 minutes for the former and 60 minutes for the latter.

In Figs. 1 and 2 are presented the results obtained on the ascending and descending sides, respectively.

A sufficiently large number of drops must be forced from each side from the unseparated original solution, which occupied the channel between the peak of anomaly on the ascending side and that of albumin on the descending side. Therefore the activities of these drops must be equal. But as the volume of a drop from

the ascending side differs from that of the descending side, the enzyme activities measured on either sides differ from each other. In the present case, the volume of 5 drops on the ascending side was 0.127 ml. and that on the descending side, 0.133 ml., and the activities (in extinction coefficient) were on an average 1.15 and 1.35, respectively.



I: Solid lines represent the activity of acid phosphatase and blank ones, the alkaline phosphatase.
II: Activity gradient curve of alkaline phosphatase.
III: Actility gradient curve of acid phosphatase.

Fig. 1. Activity of phosphatases in the mixture of bovine serum and kidney extract in the ascending channel.

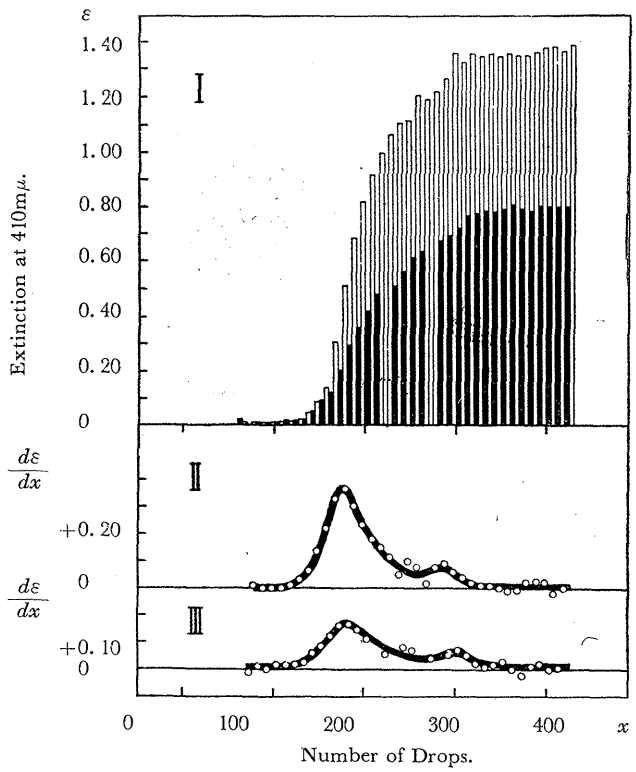
The differential activity curves were obtained by plotting the differences of activity values at intervals of two on the ascending side and at intervals of one on the descending side.

The activity curve of the alkaline phosphatase on the ascending side shows obviously two steps, while on the descending side only one. But on the differential curve of the latter, either two peaks or one main peak with a strong tailing can be seen, which should correspond to the steps on activity curve.

On the activity curves of acid phosphatase, only one step is obtained, even on the ascending side. If differentiated, one peak with considerable taling on the descending side, and three peaks on the ascending side become obvious. It is noteworthy, that three peaks of activity appear here, instead of two as in the case of alkaline phosphatase.

As fully discussed previously (4), the activity curve is correlated to the number of drops. The latter, however, is not immediately correlated to the pattern of electrophoresis. Accordingly, the conductivity of the drop is measured and its gradient curve, which corresponds to the optical pattern, is plotted against the number of drops. Thus the differential curve of the enzyme activity, i.e. the pattern of the enzyme, can be correlated with the pattern of electrophoresis by

means of the conductometric pattern. In Figs. 3 and 4, the differential curves of alkaline and acid phosphatase activities, the conductometric and the optical patterns of the solution on the ascending and the descending sides are

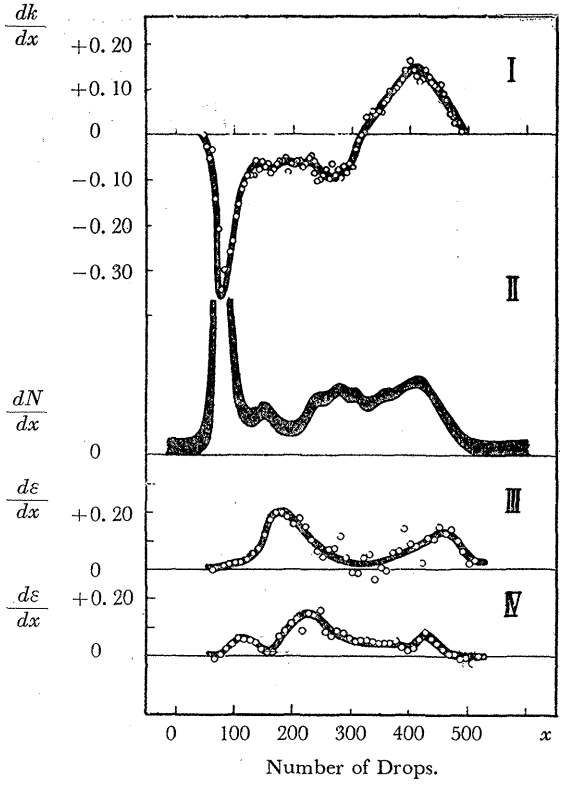


I: Solid lines represent the activities of acid phosphatase and blank ones, the alkaline phosphatase.
II: Activity gradient curve of alkaline phosphatase.
III: Activity gradient curve of acid phosphatase.

Fig. 2. Activity of phosphatases in the mixture of bovine serum and kidney extract in the descending channel.

brought together. As can be seen from the presented figures, the alkaline phosphatase shows two peaks on the ascending as well as on the descending side. In both cases the first peak is larger than the second one, and situated on almost identical position, namely between α- and β-peaks of serum globulins. Hence it must represent the same activity on either side and can be referred to as the main peak of alkaline phosphatase. On the other hand, the second peaks of the two sides differ totaly from each other: The second peak on the descending side must occur electrophoretically before the main peak, whereas that on the ascending side, behind it, since the solution was forced from the upper holes of both chan-

nels. Accordingly the former lies near the site of albumin and the latter near that of δ-anomaly. Thus only the main peak can be confirmed as the peak of alkaline phosphatase, and the two second peaks remain indefinite. As discussed previously, one of the latter peaks, i.e. the one which occurs near δ-anomaly,



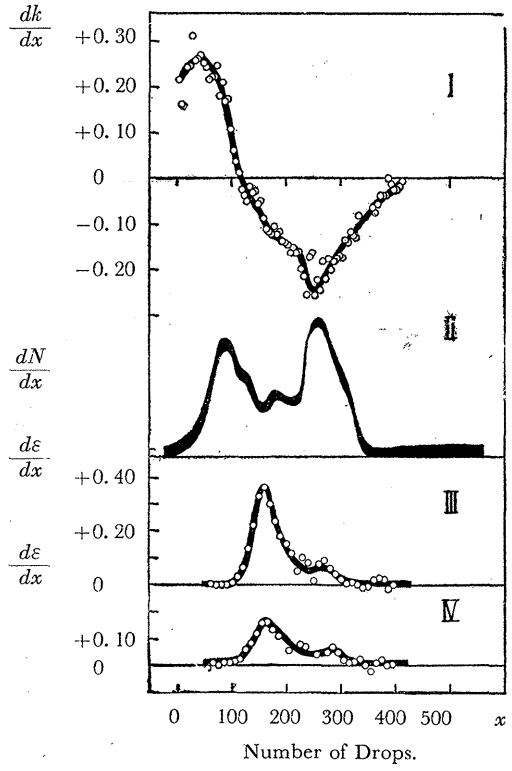
I: Conductometric pattern.
II: Optical pattern.
III: Pattern of alkaline phosphatase.
IV: Pattern of acid phosphatase.

Fig. 3. Patterns of phosphatases in the mixture of bovine serum and kidney extract, compared with optical and conductometric patterns in the ascending channel.

would have arisen from the initial concentration difference as the "anomaly" itself and would not represent the real entity of the enzyme.

Now, the solution tested is a mixture of the extract of kidney and the serum. As presented in Table II, the ratio of mixture, serum: kidney extract, was in protein concentration 100: 0.7 and in the activity of alkaline phosphatase 100: 45. Therefore, if the alkaline phosphatase of serum and that of kidney had different mobilities, it would have been able to differentiate them by the present

method. But in so far as the second peak on either side can be set aside as not representing real entity of the enzyme, there remains only one peak to represent the enzyme in the mixture. Thus the alkaline phosphatase of kidney must be electrophoretically identical with that of serum.



I: Concductometric pattern.
II: Optical patten.
III: Pattern of alkaline phosphatase.
IV: Pattern of acid phosphatase.

Fig. 4. Patterns of phosphatases in the mixture of bovine serum and kidney extract, compared with optical and conductometric patterns in the descending channel.

As to the acid phosphatase, three peaks can be observed on the ascending side and two on the descending side. Of the former three, the second one is largest and most distinct. It corresponds to the first one on the other side, in regard to their electrophoretic position, between α- and β-globulins. Hence they must represent the same main mass of acid phosphatase and can be referred to as the main peak of it. Other peaks than the main ones are small and not distinct. One of them, the third one on the ascending side, stands near δ-anomaly and might be ascribed to the initial concentration difference, as observed above in the case of alkaline phosphatase. As to the first one on the descending side and the third one on the ascending side, it is difficult to decide whether they are really existing ones or artifacts, though they stand at the similar site near albumin, for the experimental error of about 5 per cent and the considerable "tailing" must be taken into account.

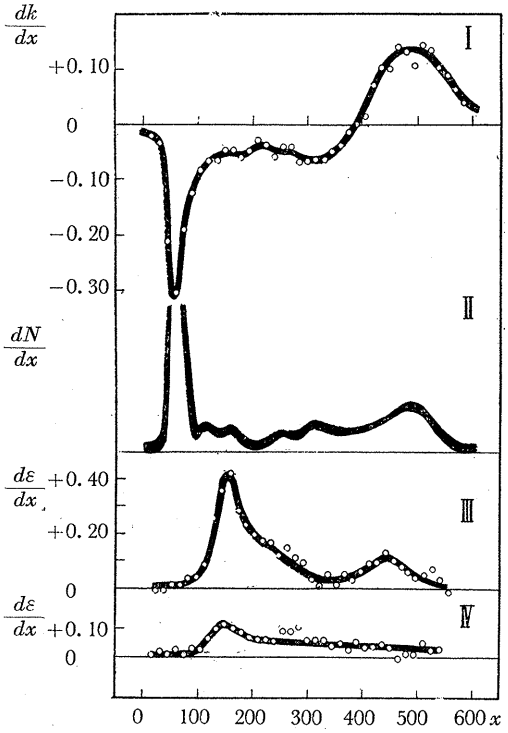
As there is practically no acid phosphatase in the serum, the activity observed in the mixture can be ascribed to the kidney only.

Intestinal Mucosa.

In Figs. 5 and 6 are shown the results obtained with intestinal mucosa. Exper-

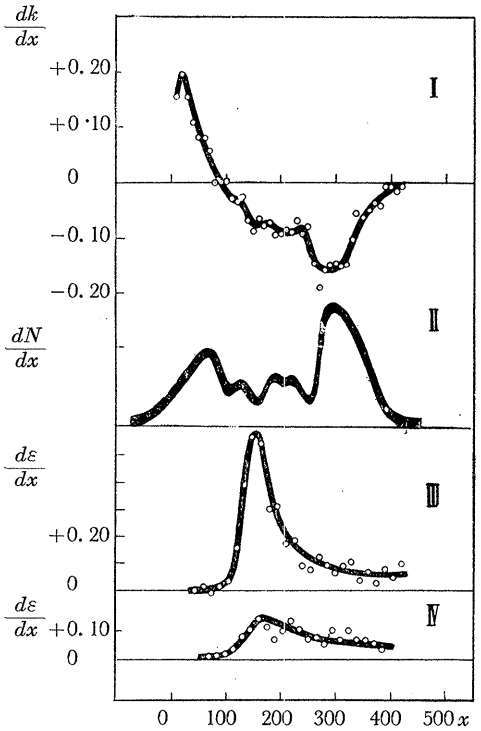
imental conditions were the same as for kidney, except that 3 drops were used for the measurement of alkaline phosphatase and 12 drops for acid phosphatase, with 60 minutes of incubation.

As can be seen from the figures, the alkaline phosphatase of the mixed solution



I: Conductometric pattern.
II: Optical pattern.
III: Pattern of alkaline phosphatase.
IV: Pattern of acid phosphatase.

Fig. 5. Patterns of phosphatases in the mixture of bovine serum and the extract of intestinal mucosa, compared with optical and conductometric patterns in the ascending channel.



I: Conductometric pattern.
II: Optical pattern.
III: Pattern of alkaline phosphatase.
IV: Pattern of acid phosphatase.

Fig. 6. Patterns of phosphatases in the mixture of bovine serum and the extract of intestinal mucosa, compared with optical and conductometric patterns in the descending channel.

of the extract of intestinal mucosa and the serum showed two peaks on the ascending side and one, on the descending side. The first peak on the former is larger and can be referred to as the main peak. As discussed above in the case of kidney the smaller second peak of the alkaline phosphatase on the ascending side seems to correspond to the site of initial concentration difference, namely δ-anomaly. But it is not without question as to whether the second peak resulted from this ground alone. Why it appears on the ascending side only is as yet

obscure. In so far as the second peak can be set aside as a mere concentration peak, there remains only one peak of alkaline phosphatase in the mixture of the extract of the intestinal mucosa and the serum. Therefore the alkaline phosphatase of intestinal mucosa must also be electrophoretically identical with that of serum. Moreover, since the alkaline phosphatase of kidney was electrophoretically identical with that of serum, it follows that there exists electrophoretically only one kind of alkaline phosphatase in serum, kidney and intestinal mucosa.

The acid phosphatase of intestinal mucosa showed only one peak on both sides. They stand on the identical position between α- and β-globulin. They must represent the enzyme. Its position appears to be slightly nearer to β-globulin than that of alkaline phosphatase. But the difference is insignificant, if the range of experimental error is taken into account. For the close analysis further works are needed. In any way, from the results presented above it can be said, that the alkaline phosphatase of serum, kidney and intestinal mucosa are electrophoretically identical and the same holds with the acid phosphatase of the latter two tissues as well. Moreover, the alkaline and acid phosphatase of the tissues tested can not be distinguished electrophoretically from each other.

Summary

With acetate buffer of pH 4.5 clear extracts of tissues could be obtained. They contained the alkaline and acid phosphatases.

The mixture of the serum and the acid extract obtained was analyzed electrophoretically and the solution was forced dropwise from both channels. Enzymatic activity and conductivity of the collected drops were measured.

By means of the gradient curves of conductivity, the activity curves of the enzymes could be correlated with the optical pattern of the solution analyzed. The alkaline phosphatases of serum, kidney and intestinal mucosa were electrophoretically identical. The same held with the acid phosphatases of the latter two tissues.

The alkaline and the acid phosphatases of the tissues tested showed almost the same mobility between α- and β-globulin.

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