DISTRIBUTION OF ALKALINE PHOSPHATASE IN THE PROTEINS OF BOVINE LYMPH-NODES

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The extract of bovine lymph-nodes with physiological saline solution was found by electrophoresis to contain no less than four different proteins: albumin, nucleoprotein and two, if not more, globulins, in order of mobility (1).

The homogenate or extract of lymph-nodes is subject to autolysis due in part probably to the presence of kathepsin in it. The simultaneous remarkable increase in the amount of its content of inorganic phosphorus may be ascribed to the break-down of nucleoprotein. Presumably phosphatase, acting in the form of nucleotidase, takes part in the process. The physiological significance of the presence of alkaline phosphatase in various organs remains unexplained, but the enzyme may possibly be concerned in some way in the metabolism of nucleic acid. It appears at any rate probable that phosphatase has an interesting part to play in a lymph-node.

Setting aside for future investigation the dynamic relation each enzyme group bears to the metabolism in cells, a search for the particular protein component of tissue in which each kind of enzyme occurs may constitute a brach of the study of enzymes and tissue proteins. The author's study of the problem was started with an examination of the lymph-node for identification of its particular protein component or components in which phosphatase makes its appearance.

EXPERIMENTAL METHODS AND MATERIALS

Preparation of enzyme solutions: The bovine mesenterial lymph-nodes obtained immediately after slaughter were deprived of as much of their fatty and connective tissues as possible, washed three times with physiological saline solution, scissored into small pieces, and homogenized with a blade homogenizer. The homogenate obtained was further fractionated into several fractions, as will be described later.

The enzyme activity of alkaline phosphatase was determined as follows: 5 ml. of 1/15 M sodium glycerophosphate and 5 ml. of 0.1 M veronal buffer were added with a certain quantity of one of the enzyme solutions and water to make total volume of 15 ml. The mixture was kept at 39°C for 20 hours; the increase in the

amount of inorganic phosphate was measured by *Allen*'s (2) method and the enzyme activity was evaluated by subtruction from the value for the mixture that for a control containing no substrate.

Electrophoresis was carried out at 0° – 4° C by a *Hitachi* medium size *Tselius* apparatus using a veronal buffer of pH 8.7 and ionic strength 0.05. Samples to be examined were dialyzed against the same buffer solution for no less than 24 hours before being examined.

Fractionation by electrophoresis convection was performed in an apparatus devised by *Cann* et al. (3), with a top and a bottom reservoir of 50 ml. and 25 ml. in capacity, respectively. A citrate buffer of pH 6.0 and ionic strength 0.05 was used. The nitrogen content was measured by the usual semimicro *Kjeldahl* method.

EXPERIMENTAL PROCEDURE AND RESULTS OBTAINED

1. *Preparation of original solution of alkaline phosphatase, Fraction F_1*

Homogenized lymph-nodes were added with the equal volume of physiological saline solution, homogenized again, and centrifuged; the supernatant was filtered and a transparent brownish-red solution obtained was referred to as Fraction L_1.

Fraction L_1 was acidified to pH 4.5 by the addition of 10 per cent acetic acid, and the acidic protein (possibly nucleoprotein) was precipitated with some other proteins. The precipitate was centrifuged and filtered off. The transparent pale yellow solution obtained was referred to as Fraction F_1. It contained albumin and globulins, as determined from its electrophoretic pattern, but there still remained some amount of acidic protein, as will be described later.

The phosphatase activity per volume was nearly three times greater in Fraction L_1 than in Fraction F_1, but the phosphatase activity per unit of nitrogen was nearly twice as great in Fraction F_1 as in Fraction L_1, being the nitrogen content of the latter five times greater than that of the former. Besides, Fraction L_1 required a long time for the filtration from the original supernatant solution and was obtained in a limitted amount; whereas Fraction F_1 was prepared merely by acidifying the homogenate to pH 4.5, without using Fraction L_1 as starting material, so that the purification of phosphatase was more conveniently based on this fraction.

2. *Fractionation of Fraction F_1 with ammonium sulfate*

a. Inhibition of alkaline phosphatase by ammonium sulfate

If the inhibition of the alkaline phosphatase by ammonium sulfate were negligible, the measurement of the enzyme activity which must be carried out in each step of fractionation would be facilitated, since the rigorous dialysis to elimi-

nate ammonium sulfate can be dispensed with. As shown in Table I, ammonium sulfate has no marked inhibitory action in a concentration of 0.04 M and has no such action at all in 0.015 M or less (taken as saturated when occurring in a concentration of 5.7 M). On the other hand, quantitative determination of phosphoric acid by *Allen*'s method is not interfered with by the presence of ammonium sulfate in a concentration of 0.5 M or so. It was therefore neccessary that a solution to be fractionated with ammonium sulfate be dialyzed beforehand till ammonium sulfate in it was reduced to 0.05 M or less in concentration.

TABLE I

The inhibitory action of ammonium sulfate on the activity of alkaline phosphatase.

5 ml. 1/15 M sodium glycerophosphate, 5 ml. of 0.1 M veronal buffer solution of pH 9.0, 2.5 ml. of the enzyme solution, indicated amount of saturated solution of ammonium sulfate, and water were mixed to make the total amount 15 ml. After being kept at 39°C for 24 hours, the increase in the liberated phosphate was measured.

Amount of ammonium sulfate added.	Increase in P, mg./ml.
2.5 ml. saturated ammonium sulfate	0.024
2.0 〃 〃 〃 〃	0.039
1.0 〃 〃 〃 〃	0.060
0.5 〃 〃 〃 〃	0.100
0.1 〃 〃 〃 〃	0.232
2.5 〃 1/25 saturated ammonium sulfate	0.229
2.0 〃 〃 〃 〃 〃	0.230
1.0 〃 〃 〃 〃 〃	0.266
0.5 〃 〃 〃 〃 〃	0.269
0.0	0.269

TABLE II

The change of activity by the salting-out and dialysis of the enzyme solution.

Experimental conditions: same as those in Table I, except that the saturated ammonium sulfate was not added.

No. of exper.	Enzyme solution	Dialysis	Increase in volume*	Increase in P, mg./ml.
1	F_1	−	−	0.32
	F_1	+	2×	0.113
	Filtrate (1/2 saturation with $(NH_4)_2SO_4$).....	+	4.6(5)	0.001
	Precipitate	+	2(5)	0.03
2	F_1	+	2.2(7)	0.022
	Filtrate (1/2 saturation with $(NH_4)_2SO_4$).....	+	6.7(7)	0.37
	Precipitate	+	2.4(7)	0.00
3	F_1	+	2.5(7)	0.047
	Filtrate (1/2 saturation with $(NH_4)_2SO_4$).....	+	6.0(7)	0.019
	Precipitate	+	2.2(7)	0.128

*) The numbers in brackets in the column for the increase in volume show the corrected volumes by the addition of water.

b. Dialysis of Fraction F_1.

Fraction F_1 was added with its volume of a saturated solution of ammonium sulfate; the precipitate and the filtrate was each dialyzed for 24 hours against one litre of water (to be renewed at the end of the first 12 hours) per 10 ml. of the solution. The dialysis was sufficient to reduce the content of ammonium sulfate to 0.05 M in concentration. The original solution F_1 was also dialyzed in the same way. The dialyzed solutions were made equal in volume by the addition of water, and 5 ml. of each solution were examined for enzyme activity.

As shown in Table II the results obtained were barely reproducible. Since this could probably be ascribed to the loss of co-enzyme during the procedure of dialysis, histidine was added (4). As can be seen in Table III, an apparent increase in the enzyme activity of the solution was brought about, but without any satisfactory reproducibility.

TABLE III

The effects exerted by 1 mg. of histidine added.
Experimental conditions: same as those in Table II.

No. of exper.	Enzyme solution	Addition of histidine	Increase in P, mg./ml.
1	F_1	−	0.160
	Globulin fraction*	−	0.013
	Globulin fraction	+	0.055
2	F_1	−	0.251
	F_1	+	0.211
	Globulin fraction	−	0.013
	Globulin fraction	+	0.229

*) Precipitate obtained from F_1 bay hlf-saturation with ammonium sulfate.

3. *Fractionation of Fraction F_1 with ethanol*

All the procedures were carried out by keeping the solution at 0°– 5 °C; the precipitates obtained by fractionation were usually washed three times with two to three times its volume of alcohol and three times more with ether, kneaded immediately to be dried and pulverized, and stored in a refrigerator. The fractionation procedures followed were as follows.

Al_1: Solution F_1 was added with one third of its volume of 95 per cent alcohol; the precipitate obtained was referred to as Fraction Al_1.

Al_2: After the fraction Al_1 was centrifuged off, the supernatant solution was added with a sufficient volume of alcohol to make the ratio in volume of alcohol to the original solution F_1, 1 : 2; the precipitate obtained was referred to as Fraction Al_2.

Al_3: The supernatant solution obtained by centrifuging off the fraction Al_2 was added with a sufficient volume of alcohol to make the ratio in volume of alcohol to the original solution F_1, 1 : 1; the precipitate obtained was referred to as Fraction Al_3.

The fractions obtained from one litre of Fraction F_1 ranged in weight from 3.7 to 5.2 g. for Fraction Al_1, from 0.7 to 0.8 g. for Fraction Al_2, and from 2 to 2.5 g. for Fraction Al_3, all white when pulverized. They were not easily soluble in water, but could be almost completely dissolved in water when added with a drop of 10 per cent NaOH or in a veronal buffer of pH 9. The fact that the fraction F_1 yielded no measurable precipitate when dialyzed against water showed that it must in itself be water-soluble. Thus it follows that considerable amount of proteins contained in each alcoholic fraction was denatured and became water-insoluble during the procedure of alcoholic fractionation.

In Table IV, the phosphatase activity of each fraction in 0.5 per cent solution in veronal buffer was shown. The phosphatase content of each fraction varied slightly in different batches of preparations, but as a whole it was least for Fraction Al_2 and equally larger for Fractions Al_1 and Al_3. Addition of histidine to Fraction Al_3 caused no marked increase in the enzyme activity.

TABLE IV

The enzyme activities of the alcoholic fractions.
Experimental conditions: same as those in Table II. Enzyme solution was obtained by dissolving each fraction in a veronal buffer in the proportion by weight of 0.5 to 100.0.

No. of exper.	Enzyme solution.	Increase in P, mg./ml.
1	Al_1	0.170
	Al_2	0.070
	Al_3	0.210
2	Al_3	0.170
	Al_3	0.123 (+ histidine)

4. *Fractionation of the alcoholic fraction Al_3, by the electrophoresis-convection*

Of Fractions Al_1, Al_2 and Al_3, the last named was further fractionated by electrophoresis-convection. As has been stated, Fraction Al_3 was almost completely soluble in a veronal buffer of pH 9, fairely well soluble in physiological saline solution, but not easily soluble in water. After insoluble parts were removed, phosphatase activities of dissolved fractions were compared. As can be seen from Table V, enzyme activities were nearly the same in buffer solution, in physiological saline, and in water. The insoluble part of Fraction Al_3 left after water extraction had practically no phosphatase activity. Fraction Al_3 dissolved in a veronal buffer and the same fraction extracted with water contained nitrogen in the ratio of 20:3, indicating that the enzyme activity per unit of nitrogen was 7 times higher for the latter than for the former.

The water extract of Fraction Al_3 was subjected to electrophoresis-convection for further purification. It was first dialyzed against a citrate buffer of pH 6.0 and ionic strength 0.05, and was introduced into the electrophoresis-convection apparatus. After operation for 20 hours, three fractions were withdrawn from

the apparatus: one from the upper reservoir (Al_3-AcT), one from the bottom reservoir (Al_3-AcB) with some precipitate, and another from the channel, and their phosphatase activities were measured. As can be seen from Table VI, the

TABLE V

The enzyme activity of each solution prepared from the alcoholic fraction Al_3.

Experimental conditions: same as those in TABLE IV. The enzyme solution was prepared by dissolving Al_3 in the solvent in proportion by weight of 0.5 to 100.0.

Enzyme solution	Relative nitrogen content	Increase in inorganic P, mg./ml.
Water extract (Al_3-A)	0.15	0.098
Residue of water extract	—	0.004
Extract with buffer	1.0	0.119
Extract with physiological saline	—	0.101

activity for the fraction from the bottom reservoir was found to be about three times greater than that from the upper, whereas the nitrogen content was about 6 times greater for the former fraction than for the latter. Thus the activity per unit of nitrogen was nearly two times higher for the former than for the latter fraction.

TABLE VI

The fraction obtaind by electrophoresis convection from water extract of Fraction Al_3.

Experimental conditions: same as those in TABLE V.

Enzyme solution	Nitrogen content, mg./ml.	Increase in inorganic P, mg./ml.
Top cut (Al_3-AcT)	0.35	0.03
Fraction in channel	0.31	0.01
Bottom cut (Al_3-AcB)	2.2	0.09

5. *Electrophoretic investigation of fractions by the Tiselius method*

Fraction L_1: The electrophoretic pattern of the physiological saline extract of lymph-node, fraction L_1, with its peaks provisionally given their respective names was shown in Flg.1. The proteins occurring as peaks were protein A, apparently albumin, protein N, an acidic protein, and protein G's of two or three different forms, in order of mobility. That protein N was an acidic protein could be inferred from the fact that Fraction L_1, when acidified, lost the peak of protein N, as can be seen from the pattern of its filtrate, Fraction F_1 (Fig. 3). Further, when the acid precipitate from Fraction L_1 was purified by repeated dissolution and reprecipitation with dilute alkali and, and added to the original solution L_1, the peak of N became larger as shown in Fig. 2. The acidic protein N purified by reprecipitation contained no more than 0.5 per cent phosphorus, and gave a faint orcinol reaction, making it difficult to be decided whether the protein was a nucleoprotein or not.

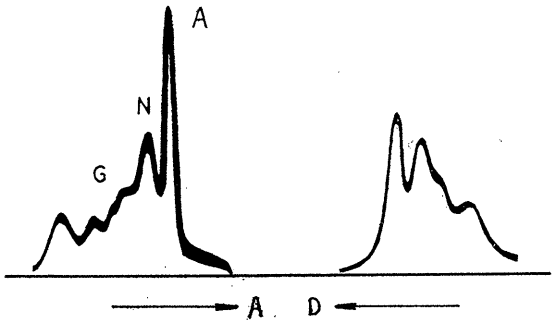

Figure 1. Electrophoretic pattern of physiological saline extract of bovine lymph-nodes. Explanation, see text. Phosphate buffer, pH 7.8, ionic strength 0.05. 20mA., 170V. 120min.

Fraction F_1: When Fraction L_1 was acidified to pH 4.5 by the addition of dilute acetic acid, proteins contained in it were precipitated, leaving only one fifth of total protein in solution. As mentioned above, protein N was most thoroughly precipitated. Hence in the electrophoretic pattern of Fraction F_1 in Fig. 3, Protein A alone formed a well-defined peak and no other protein present manifested itself in any clear-cut peak.

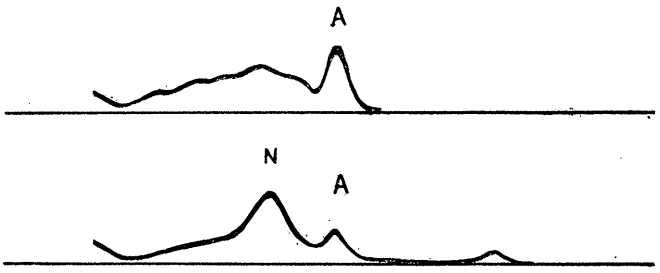

Figure 2. Electrophoretic pattern of fraction L_1 added with acidic protein.
Upper Figure: Fraction L_1, part of acidic protein precipitated off.
Lower Figure: The same fraction as above, added with acidic protein.
Explanation, see text. Veronal buffer, pH 9.0, ionic strengh 0.05. 17 mA., 120V. Upper, 50 min., lower, 60 min.

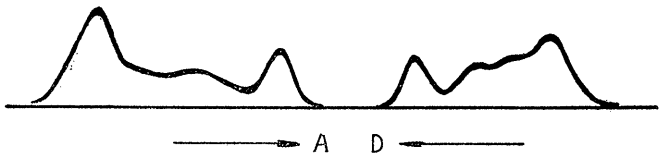

Figure 3. Electrophoretic pattern of fraction F_1. (Slightly modified).
Explanation, see text. Veronal buffer. 13 mA., 174 V. 45 min.

Fraction $Al_{1,2}$: This was a fraction obtained from Fraction F_1 by the addition of half its volume of ethanol, instead of preparing Fraction Al_1 and Al_2 separately by the successive addition of ethanol to one third and then to one half of

the volume of mother fraction F_1. In the electrophoretic pattern of Fraction $Al_{1,2}$ shown in Fig. 4, there can be seen three peaks showing the presence of Proteins A, N and G. It is evident that this fraction contained Protein N as its main protein component, in contrast to Fraction F_1, the starting material, from which Fraction $Al_{1,2}$ was fractionated and of which the main protein component was Prptein A.

Fraction Al_3: This was obtained by adding to the filtrate of Fraction Al_2 a sufficient quantity of ethanol to make the ratio in volume of Fraction F_1 to ethanol 1 : 1; it was dried to powder and dissolved in a veronal buffer of pH 9.0 for electrophoretic observation. The proteins contained were Protein A and two to three different forms of Protein G's as shown in Fig. 5.

Fraction Al_3–A: This fraction, the water extract of Fraction Al_3, contained Protein N and G's, as shown in Fig. 6.

Figure 4. Electrophoretic pattern of fraction $A_{1,2}$. (Slightly modified) Explanation, see text. Veronal buffer. 16 mA., 180 V. 70 min.

Figure 5. Electrophoretic pattern of fraction Al_3. (Slightly modified) Explanation, see text. Veronal buffer. 14 mA., 180 V. 60 min.

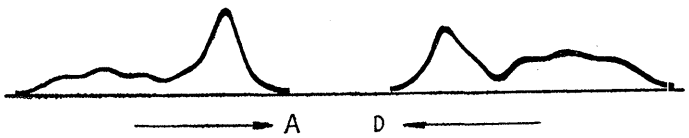

Figure 6. Electrophoretic pattern of fraction Al_3–A. (Slightly modified) Explanation, see text. Veronal buffer. 14 mA., 180 V. 60 min.

Fraction Al_3–AcT: This was a fraction obtained from the top reservoir of the electrophoresis-convection apparatus, after Fraction Al_3–A had been fractionated. Fig. 7 shows that this fraction was almost identical with the starting fraction Al_3–A in its electrophoretic pattern.

Fraction Al_3–AcB: This fraction was obtained from the bottom reservoir of the electrophorsis convection apparatus after Fraction Al_3–A had been fractionated. In its electrophoretic pattern in Fig. 8, three peaks can be seen. As has been stated, the proteins contained were six times as concentrated in this fraction as in Fraction Al_3–AcT. The fraction gave off nearly all its protein content as precipitate when acidified to pH 4.5, so that the filtrate obtained could not be

analyzed electrophoretically without lyophilyzing. Thus the main component of Fraction Al_3–AcB must be acidic N Protein. However, this circumstance is rather difficult to explain; the thickning of any protein which could bave occurred in the bottom reservoir can not exceed three fold as compared with the used original

Figure 7. Electrophoretic pattern of fraction Al_3-AcT. (Slightly modified). Explanation, see text. Veronal buffer. 14 mA., 180 V. 45 min.

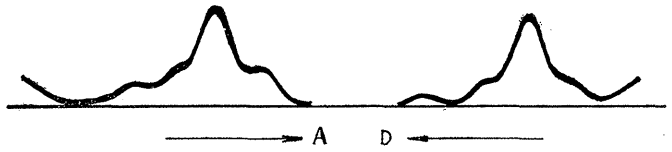

Figure 8. Electrophoretic pattern of fraction Al_3-AcB. (Slightly modified). Explanation, Ver see text. Veronal buffer. 14 mA., 190 V. 60 min.

protein solution, since the volume of the bottom reservoir is half as large as that of the top. It is almost improbable, that the acidic protein in this fraction Al_3–AcB was only three times as concentrated as that in its mother fraction Al_3–A, to become the main component, because the latter fraction contained it only in an insignificant proportion.

Discussion

There is no established procedure available in the extraction of proteins and enzymes. In the purification of an enzyme, it is essential, first of all, to obtain a fraction containing as small quantity of other proteins than the enzymes as possible. Hence for the purification of enzyme alone, it is not neccessary to analyze electrophoretically all the fractions obtained successively. However, the mobility can hardly be taken as the only criterion by which one particular peak observed in the pattern can be identified with one of the proteins fractionated. For the determination of the position where the enzyme in question makes its appearance in the electrophoretic pattern of the extract used, it is necessary to investigate the electrophoretic pattern of each fraction obtained successively by the fractionation.

In the physiological saline extract of lymph-nodes several kinds of proteins provisionally named Proteins A, N and G in this paper show their distinct presence. Protein N is precipitated when the extract is acidified to pH 4.5, so that the main protein components of the filtrate (Fraction F_1) were Proteins A and G's, but not any acidic protein. But a fraction obtained from Fraction F_1 by electrophoresis-convection from the bottom reservoir of the apparatus contained

as its main protein component acidic protein. As mentioned above, it is doubtful, whether it were due to the thickning of the acidic protein left uneliminated in Fraction F_1, since the concentration must be within three fold. On the other hand the larger the mobility of a protein, the more thickning of it must occur. Thus the acidic protein cannot be more highly concentrated than any other proteins such as Protein A which migrates faster than it. It is now probable that proteins were denatured during the process of fractionation, especially during the alcohol-fractionation, and had become altered in their solubility, though in this paper any protein precipitated when acidified is referred to as the Protein N.

In the fraction obtained by electrophoresis-convection from the bottom reservoir, acidic protein and other proteins of higher mobility become concentrated, and in that from the top reservoir those of lower mobility. The activity of alkaline phosphatase per unit of protein nitrogen is twice as higher for the fraction from the top resevoir as for the one from the bottom, showing probably that the enzyme is contained in Protein G's of low mobility.

The activity of alkaline phosphatase is here determined according to the amount of inorganic phosphorus liberated from a definite quantity of glycerophosphate. The value determined by this method has its limitation and is more qualitative than quantitative, but the results obtained here can serve as the bases for further works.

SUMMARY

Homogenate of bovine lymph-nodes was extracted with the same volume of physiological saline solution and acidified to pH 4.5. The extract obtained was fractionated with ethanol. The ethanol fraction obtained by the addition of one third to one half of its volume of alcohol was extracted with water and fractionated by electrophorsis convection, using citrate buffer of pH 6.0 and ionic strength 0.05. The phosphatase-activity per unit of nitrogen was higher for the fraction from the top reservoir than for the one from the bottom reservoir.

All the fractions obtained at different stages in the procedure of purification were analyzed by electrophoresis. It was found that the alkaline phosphatase of bovine lymph-node was contained in the protein fraction of lower mobility.

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