# Zone Electrophoresis of Intestinal Alkaline Phosphatase.* (22085)

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It has been shown(1) that soluble phosphatase prepared by treating the microsomal fraction of bovine intestinal mucosa with butanol traveled at a discrete peak during zone electrophoresis on filter paper, and that its solubility behavior makes it a promising starting material for further purification. Recently, Roche and Bouchilloux(2) reported purification of dog intestinal phosphatase by electrophoresis on filter paper. Morton(3) reported in detail upon application of acetone fractionation to material obtained by butanol treatment of microsomes from calf intestine, which yielded a highly purified preparation. The present paper deals with the use of electrophoresis in the starch block for purification of bovine intestinal alkaline phosphatase, and with changes in electrophoretic mobility during the purification process. Purity as great as achieved by Morton does not appear to have been reached in our study, but additional information regarding change in behavior of phosphatase during purification has been obtained.

*Materials and methods. Crude intestinal phosphatase.* Even with a homogenizer designed for treatment of relatively large volumes of material(4), isolation of the microsomal fraction from quantities of starting material required, was cumbersome. Consequently, butanol treatment was applied directly to the intestinal tissue. Thirty-five pounds of calf duodenum, obtained from 40 to 60 animals, were rinsed with tap water, slit, and placed in washing machine with aluminum agitator and porcelain-lined tank. Other exposed metal parts had been replaced with stainless steel and rubber with polyethylene. Five gallons distilled water and 3 gallons n-butanol were added and agitator started. After extracting 30 minutes at approximately 25°C, the intestinal tissue was removed and butanol-water emulsion transferred to glass carboys for storage at −5°C. After 12 to 18 hours, the emulsion had separated into an upper butanol layer, a middle gel layer containing much denatured protein, and aqueous layer containing the enzyme. The aqueous layer was siphoned, and pH adjusted to 4.9-5.0 with acetic acid. Precipitation and equilibration took place for 24 hours at 0°C. The supernatant solution was then siphoned and clarified by passage through a Sharples Supercentrifuge. The inactive precipitate was discarded, and an equal volume of acetone, pre-cooled to −5°C, was slowly added, with stirring, to the clarified solution. The enzyme-containing precipitate, formed upon addition of acetone, was equilibrated at −5°C for 24 hours and then removed by decantation and centrifugation at −5°C. The precipitate of crude enzyme was washed in the cold, twice with 50% acetone and twice with 100% acetone. It was then dried in a vacuum desiccator over sulfuric acid. Average yield of crude, dry enzyme was 23 g. *Starch block electrophoresis.* 1.5 g of crude enzyme were suspended in 7 ml of veronal buffer at pH of 8.6, ionic strength 0.02, and extracted with stirring for 90 minutes at 5°C. The suspension was then centrifuged in Spinco Model L preparative ultracentrifuge 45 minutes at 140000 x g, and supernatant solution recovered. This solution was subjected to electrophoresis in starch according to the method of Kunkel and Slater(5). The buffer for electrophoresis was the same as used in extraction. The starch block was 30 cm long, 5 cm wide, and one cm deep. The voltage gradient was 6.7 v/cm, and the current 7-9 ma. Migration occurred for 12 hours at 0°C. At termination of migration, the starch block was cut into 1 cm segments, each eluted by

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suspending it in 5 ml of pH 8.6 veronal buffer. Supernatant solutions obtained after centrifuging starch suspensions were used for enzyme and protein analyses. *Determination of phosphatase.* The substrate was disodium p-nitrophenyl phosphate, made as a 1% solution in 0.05 M bicarbonate buffer containing 5 x $10^{-4}$ M magnesium acetate, pH 10.1. Enzymatic activity was measured by incubating 2 ml of substrate solution with 0.05 ml of enzyme solution for 20 minutes at 37°C. The reaction was stopped by addition of 10 ml of 0.002 N NaOH to the reaction mixture. Absorbance of the solution was then measured at 420 mμ, setting absorbance to zero with appropriate reagent blank. Concentration of enzyme was adjusted to produce an increase in absorbance of 0.1 to 0.3. The disodium p-nitrophenyl phosphate was prepared by the method of Bessey and Love(6), and recrystallized by the method of Aschaffenburg(7). A unit of phosphatase activity is that amount of enzyme which will release one millimole of p-nitrophenol in one hour, the specific activity as the number of units per mg nitrogen. Nitrogen was determined by either micro-Kjeldahl digestion and titration, or by the ultra-micro method of Tompkins and Kirk (8). Protein in the eluates from starch blocks was measured by the method of Lowry *et al.* (9). Since color development was carried out directly on eluates, without precipitating the protein, other chromogenic material may have influenced the apparent protein distributions.

*Results.* The crude material used had a specific activity of 1.5, approximately 30 times greater than the average mucosal homogenate. The dried, crude material was extracted with pH 8.6 buffer, and the extract subjected to electrophoresis in starch with results shown in Fig. 1A. Positions of the peaks are not corrected for endosmotic flow. However, position of the major enzyme peak is essentially that of an immobile component. The minor peak accounts for approximately 10% of the entire enzyme found on the block. After removal from the block, the major enzyme component had a specific activity of 1.9, or 35 times that of the mucosal homogenate, and a 30-minute pre-incubation at 37°C with 0.01 M $Mg^{++}$ and 0.01 M alanine resulted in 1.4

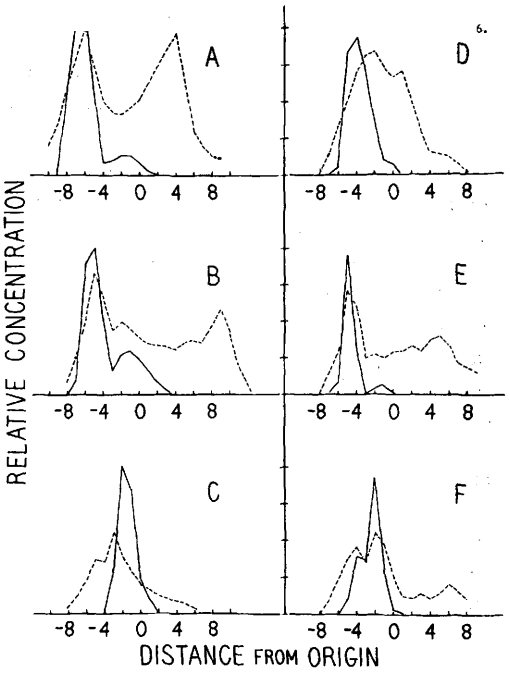


FIG. 1. Distribution of enzyme (solid line) and protein (dotted line) from starch blocks after electrophoresis for 12 hr at a voltage gradient of 6.7 v/cm and 0°C. Veronal buffer, pH 8.6, $\Gamma/2 = 0.02$. Distances from the origin (0) are indicated in cm toward the anode or −cm toward the cathode. The concentration scales are arbitrary, and not comparable for the various blocks. See text for details.

fold increase in activity over that found without pre-incubation. All specific activities are obtained after pre-incubation with $Mg^{++}$ and alanine. The solution obtained from mid-portion of the major enzyme peak, which contained two-thirds of the total enzyme of that peak, was subjected to a second starch block electrophoresis under conditions identical with those for the first block. These results are presented in Fig. 1B. Although only the middle of the major peak found on Block 1 was used, 2 enzyme peaks were again obtained and the minor enzyme peak of Block 2 represented approximately 25% of the total. Specific activity of the major enzyme component was 4.4, indicating an 83-fold purification when compared with average mucosal homogenate. At this stage, pre-incubation with magnesium and alanine produced a 1.65-fold increase in activity. Distribution of protein on Block 2 was also unexpected, since material was found which migrated more rapidly toward the

anode than did the material on Block 1 from which it was taken. The eluate from the section of Block 2, containing the middle portion of the larger enzyme peak, was subjected to electrophoresis a third time, again using same conditions as in the first 2 blocks. These results are shown in Fig. 1C. A single enzyme peak was now found, having the mobility of what had previously been the minor component. The specific activity was 13.7, representing a 260-fold purification. Pre-incubation with magnesium and alanine produced a 6.4-fold increased activity. Protein distribution of the third starch block also differed from that observed on the 2 previous blocks. Although 2 components are discernible, the enzymatic activity does not seem to be definitely associated with either of them. It appears that enzymatically active protein on this block could not have represented more than 30% of the total. During each period of electrophoresis, 40 to 50% of the enzyme placed on the block was lost, as judged by summing enzymatic activities found in individual segments. This was a considerably greater loss than that observed in recovering enzyme added to starch. Combination of eluates from individual sections, in an attempt to reconstitute the original enzyme solution, did not produce any increase in activity over that calculated from the sum of activities in individual eluates. Since storage in solution at 0°C for the same time as required for electrophoresis produced a much smaller loss in activity, it did not appear that losses on storage and in recovery from starch, could amount to more than 15 or 20% of enzymatic activity. The larger loss during electrophoresis remains unexplained. Since the shift in mobility, accompanied by increased response to magnesium activation, suggested that tightly bound magnesium might be lost and contribute to the change in mobility, a second series of starch blocks was studied with 0.001 M magnesium acetate in the buffer. This was again 0.02 M veronal buffer at pH 8.6. The results are shown in Fig. 1D, 1E, and 1F. As before, Fig. 1D is the crude enzyme, now subjected to electrophoresis in the presence of $Mg^{++}$. The distribution shown in Fig. 1E is that obtained on taking the middle portion of the major enzyme component from the first block and subjecting it to electrophoresis in the presence of $Mg^{++}$, and the process repeated for Fig. 1F. Although presence of $Mg^{++}$ appears to have suppressed appearance of the more mobile enzyme peak, the same general trend of results is observed as was found for electrophoresis in the absence of $Mg^{++}$.

*Discussion.* The results obtained confirm the previous conclusion 1) that alkaline phosphatase in the material obtained from n-butanol treatment of microsomes represents only a small part of the total protein. A similar conclusion can, of course, be drawn from the work of Morton(3) and the many papers of Roche and coworkers. It also serves to emphasize the problems associated with losses in activity during any purification procedures which have been investigated. Alteration in mobility during the series of electrophoretic separations indicates that purification procedures may not simply remove inactive protein, but may result in changes in the enzyme. We do not know whether a similar change would be observed during repeated fractionation with acetone. There seem to be two possible explanations for change in mobility of the enzyme. One possibility would be that the enzyme tends to form complexes with other proteins or ions which are sufficiently stable to yield discrete zones in starch block electrophoresis, but which may become less stable, as components of the original mixture are removed in electrophoretic separation. Magnesium alone is not responsible for this behavior, but magnesium does appear to have some influence on the process as judged by reduced tendency of enzyme to shift into the more mobile component in the presence of magnesium. A serious objection to this is found, however, in the behavior of the non-enzymatic protein. In general, if a complex were being disrupted and giving rise to a more highly negatively charged fraction, a somewhat comparable amount of a more highly positively charged material should be formed. This does not appear to be the case, unless such material has migrated off the block. A second possibility would be that the enzyme is altered by a process of pro-

teolysis, and that some such process is in general responsible for the fact that a portion of one block behaves so differently from what would be predicted when subjected to electrophoresis a second time. Changes in the electrophoretic behavior of enzyme preparations on standing and the degree of reproducibility in the same and different preparations do not, however, support this explanation. The problem requires further study, but we are inclined more to the view that some explanation involving dissociation of a complex provides the answer.

*Summary.* A crude preparation of alkaline phosphatase from bovine intestinal mucosa, obtained by treatment with butanol to release enzyme from the microsomes, was subjected to sequential electrophoresis on starch blocks. The results indicate that the phosphatase may have been present initially in a complexed form and that the complex was disrupted during repeated electrophoretic separation. Such behavior, together with the rather small amounts of enzyme protein which appear to be present in the starting material, may account for some of the difficulties encountered in attempts to purify intestinal alkaline phosphatase. An approximately 260-fold purification was achieved, but the amounts of final product obtained were too small for further study.

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## Effect of Season on Appetite and Food Consumption of the Lizard, *Anolis carolinensis.* (22086)

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The problem of the regulation of food intake by vertebrate animals has received much attention in recent years(1). Among the factors which seem to be implicated in appetite control are the fat stores of the animal and differences between arterial and venous blood glucose levels. Since the lizard, *Anolis carolinensis,* undergoes marked seasonal changes in total body fat as well as changes in both the concentration and distribution of other metabolites(2,3), a study of the food consumption of lizards at different times of the year was undertaken to determine if such changes in composition are correlated with variations in appetite.

*Methods.* Adult male specimens of *Anolis carolinensis* which had reached the plateau stage of growth were used. Colonies of 8 to 15 specimens, used to determine seasonal changes in food consumption, were formed in different months of the year from lizards recently captured in the New Orleans area. Colonies were kept for duration of each experiment (1.5 to 2.5 months) in small cages in a constant temperature (28° ± 2°C), exposed to natural hours of light (30° latitude) through a north window. An excess of meal worms, the larval form of the insect, *Tenebrio mollitor,* was kept in cages except during the 4-day fasting period preceding body weight measurements. Food intake was determined from the weight of meal worms eaten. Fortunately, large specimens of meal worms are relatively constant in gross composition if