Preparation and Properties of Highly Purified Alkaline Phosphatase from Swine Kidneys*

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This report concludes an investigation directed toward the isolation of alkaline phosphatase (1, 2). Highly purified preparations were desired for the unequivocal characterization of this enzyme. The degree of purification obtained is indicated by the fact that the specific activity of the enzyme in the source tissue is approximately 1 unit per mg. of total nitrogen, whereas that of our most active preparations was 10,500 units per mg. of protein nitrogen. Furthermore, our better material appeared to contain 80 to 90 per cent of one component as judged by analysis in the ultracentrifuge and by free electrophoresis. Evidence is also presented for a close association between the activity of the enzyme and its content of zinc. Fully active preparations of maximal purity contained 0.15 to 0.18 per cent of zinc.

EXPERIMENTAL

Enzyme Assay—Activity was estimated by the procedure of King et al. (3) with only minor modification (1, 4). All assays were performed in the presence of magnesium for activation unless otherwise indicated. The routine assay also included preliminary activation by incubation of the final enzyme dilution for 3 hours at 25° in 0.01 M dl-alanine buffered at pH 9.7 with 0.05 M carbonate. The enzyme unit is defined as that amount of enzyme which will liberate 1 mg. of phenol from disodium phenyl phosphate in 15 minutes at 25°. The pH optimum and kinetics of substrate hydrolysis were redetermined, and it was found that the assay, as developed with crude enzyme preparations, was a valid measure of activity for highly purified, amino acid-activated enzyme.

Protein—Protein nitrogen was determined as indicated previously (1) for the data in Procedure I (see below) and by the procedure of Lowry et al. (5) for the remaining data. The reproducibility of the latter determination was satisfactory when the various reaction steps were carried out with careful timing at 25°. Twice crystallized ovalbumin was used as a standard protein, and occasional Kjeldahl determinations (6) were carried out on enzyme solutions to secure the validity of the use of ovalbumin as a reference protein. The stock ovalbumin solution was diluted to approximately 0.2 units per ml. in the solution to be tested. After the solution was incubated at 25° for the desired length of time, its activity was determined. Except where otherwise stated, the incubation time was 3 hours, the activator was 0.01 M, and the buffer was 0.05 M carbonate adjusted to pH 9.7. For the control incubations the activator was omitted. The chromogenicity of each of the compounds in the assay was determined as were also the possible effects of these compounds on the chromogenicity of phenol. The amino acids and other compounds tested were in the purest state obtainable and, when necessary, were recrystallized.

Zinc—For quantitative zinc determinations, enzyme samples were digested with a small amount of glass-redistilled concentrated sulfuric acid in quartz tubes. When the digest became clear, the remaining acid was neutralized by exposing it to ammonia gas, and the solution was evaporated to dryness, dissolved in 0.01 M HCl, and analyzed by the dithizone procedure (7).

RESULTS

Isolation of Enzyme

Preparation of a crude concentrate of the enzyme is outlined in Procedure I. The basic steps employed are solubilization of the enzyme with pancreatin, thermal denaturation of contaminating proteins at room temperature in the presence of 24 per cent ethanol, fractional precipitation with ethanol at 5°, fractional precipitation with ammonium sulfate at 5°, and dialysis.

The dialyzed enzyme solution is then carefully titrated to pH 4.75 at 0-5° with acetic acid, and the precipitate, containing the enzyme, is separated by high speed centrifugation (Procedure II). Alkaline phosphatase is relatively unstable at acid pH values, therefore this step is carried out at low temperature and the precipitate is promptly dissolved in 0.01 M NaHCO₃. Occasionally, preparations were obtained that gave little precipitation at this pH. Stock solutions used were prepared to require dilutions of 1:500 or 1:1000 in order to yield activities suitable for assay. The stock solution solvent, which greatly stabilized the enzyme during storage at 5°, was 0.05 ionic strength phosphate buffer of pH 7.2, prepared in 80 per cent aqueous glycerol.

The manner of cleaning the glassware and the type of glass used are factors to be considered. Pyrex tubes, cleaned with commercial detergent (Alconox), gave greater control activation than Pyrex tubes cleaned with chromic acid cleaning solution or nitric-sulfuric acid cleaning solution, even when the latter was prepared with glass-redistilled acids. Deliberate extensive etching of acid-cleaned Pyrex tubes greatly reduced control activations.

* A summary of this investigation was reported at the meeting of the American Society of Biological Chemists at Atlantic City in April, 1954 (Federation Proc., 13, 290 (1954)).
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stage. This was overcome by the use of a different lot of pancreatin, preferably one which had been aged for a few weeks. Attempts to carry out this step with the undialyzed extract resulted in no precipitation, indicating that the presence of ammonium sulfate interfered, presumably by means of a non-specific salt effect. The precipitation is probably dependent upon the formation of an insoluble complex with some other component in the mixture, possibly nucleic acid, since Albers' preparations (8) of horse kidney alkaline phosphatase gave very little precipitation when treated in this manner. Repeated acid precipitation did not increase the purity of the enzyme.

The third stage in the purification of the enzyme is outlined in Procedure III. Inactive protein along with most of the brown pigmentation was removed by adsorption on Ca₃(PO₄)₂ gel (9). This step must be carried out with care since adsorption of the enzyme on the gel can result in the loss of the entire preparation. This step must be carried out with care since adsorption of the enzyme on the gel can result in the loss of the entire preparation.

Procedure III. Inactive protein along with most of the brown pigmentation was removed by adsorption on Ca₃(PO₄)₂ gel (9). The resulting solution is treated with kaolin to remove sufficient protamine is then removed by autolysis followed by adsorption on kaolin. The final solution is concentrated by ammonium sulfate precipitation.

Additional (NH₄)₂SO₄ fractionation of the concentrate described in Procedure I was not very effective. However, the treatments outlined in Procedures II and III gave a preparation that could be fractionated with saturated (NH₄)₂SO₄, an example of which is described in Procedure IV. Occasionally two consecutive fractionations were required to obtain material with the specific activity obtained in Step 5. Less active preparations were stored and added to subsequent purifications at an appropriate stage of specific activity or were simply refractionated.

Continued application of this scheme of (NH₄)₂SO₄ fractionation has yielded preparations with measurements of specific activity up to 5000.

Procedure V outlines the preparation of material with a specific activity of 8000 starting from 1800. This purification was obtained with a combination of Sevag's CHCl₃ treatment (10) with electrophoresis-convection (11). The enzyme is relatively resistant to surface denaturation and thus allows removal of a significant proportion of the more sensitive contaminating protein by emulsification with CHCl₃. An example of the final steps in the preparation of enzyme of maximal activity is presented in Procedure VI. Generally, the specific activity was increased from 1000 to between 2000 and 3000 by ammonium sulfate fractionation, from between 2000 and 3000 to between 4000 and 7000 by treatment with CHCl₃ and final purification was obtained with electrophoresis-convection.

Procedures—In the descriptions which follow PU is phosphatase units, TPKU is the total phosphatase units X 10⁻³, and PU/mg. PN is units per mg. of protein nitrogen.

Procedure I—Preparation of Crude Concentrate of Alkaline Phosphatase

1. Mix 8 kg. of ground swine kidney cortex, 4 l. of H₂O, 400 ml. of toluene, 160 gm. of Viokase, and 200 ml. of 20 per cent Na₂CO₃.

2. Digest overnight at room temperature, and then add another 200 ml. of 20 per cent Na₂CO₃. Digest for 2 more days.

3. Add 4 l. of 95 per cent ethanol, shake at room temperature for 4 hours, and then let stand overnight.

4. Add 1 1/2 kg. of Hyflo Super-Cel, and filter with suction (2 l. of suspension per filtration with 32-cm.-diameter Buchner funnels). The filter cakes are rinsed with a total of 2 l. of 24 per cent ethanol. Filtrate.

5. Add 25 ml. of cold 95 per cent ethanol per 100 ml. of filtrate at 5° slowly with stirring. Add 200 gm. of Hyflo Super-Cel and filter with suction. Wash the cake with 800 ml. of 38 per cent ethanol. Filtrate.

6. Slowly add 60 ml. of cold 95 per cent ethanol per 125 ml. of filtrate as above. Add 160 gm. of Hyflo Super-Cel and filter with suction. Wash the filter cake with 800 ml. of 38 per cent ethanol. Filtrate.

7. Add 25 gm. of (NH₄)₂SO₄ per 100 ml. of filtrate, and dissolve with slow stirring at 5°. Add 80 gm. of Hyflo Super-Cel and filter with suction. Wash the filter cake with 800 ml. of H₂O in which 192 gm. of (NH₄)₂SO₄ has been dissolved. Filtrate.

8. Add 30 gm. of (NH₄)₂SO₄ per 112.5 ml. of filtrate and dissolve as above. Add 30 gm. of Hyflo Super-Cel and filter with suction. Wash the filtrate with 400 ml. of H₂O in which 224 gm. of (NH₄)₂SO₄ has been dissolved. Discard the filtrate. Stir the filter cake with 400 ml. of 0.05 M NaHCO₃ for 30 minutes at 5°. Filter with suction, and wash the filter cake with H₂O to give 500 ml. of filtrate plus washings. Dialyze the filtrate against 0.1 per cent and finally 0.05 per cent NaHCO₃ at 5° until free of sulfate.

Procedure II—Acid Precipitation of Alkaline Phosphatase—Crude Concentrate

1. Dialyze alkaline phosphatase crude concentrate.

2. Enzyme solution cooled to 0° and titrated with efficient stirring to pH 4.75 with M acetic acid. The suspension was then centrifuged at 60,000 X g for 20 minutes at 5°. The supernatant was discarded, and the precipitate was taken up in 25 ml. of 0.1 M NaHCO₃. The solution was rinsed from the centrifuge bowl with 0.001 M NaHCO₃ to give a final total volume of 50 ml.

The values noted in this procedure were obtained without alanine activation.

Viobin Laboratories, 4 X U.S.P. pancreatin.

1.19 units per mg. of total nitrogen.
Procedure III—Preparation of Purified Concentrate of Alkaline Phosphatase

1. The acid-precipitated crude concentrate from 16 kg. of cortex is cooled to 5°.

2. Stir for 30 minutes with 30 ml. of 8 to 10 per cent Ca₃(PO₄)₂ gel. The gel is then removed and washed twice with 0.001 M NaHCO₃ by centrifugation at 5°. Supernatant.

3. The solution is titrated to pH 8 with 20 per cent Na₂Os₄, cooled to 5°, and treated with 10 ml. of gel as above. Supernatant.

4. The supernatant is stirred for 30 min. at 5° with 20 gm. of acid-washed kaolin. The kaolin is removed and washed by centrifugation. A 2 per cent dialyzed protamine sulfate solution is added to the enzyme solution at room temperature to the point at which there is no additional precipitation. The suspension is immediately centrifuged for 5 minutes, and the clear supernatant is allowed to stand at room temperature for 1 hour. After repeating the above kaolin treatment, 60 gm. of (NH₄)₂SO₄ are added per 100 ml. of enzyme solution and dissolved slowly at 5°. The precipitated enzyme is removed by suction filtration with the aid of 1 gm. of Hyflo Super Cel. The filter cake is extracted with 20 ml. of 0.05 M NaHCO₃ for 30 minutes, and the resulting extract is dialyzed overnight against 0.1 per cent NaHCO₃. Extract.

Procedure IV—Fractionation of Alkaline Phosphatase with Saturated Ammonium Sulfate at 5°

1. The dialyzed enzyme solutions from several preparations were combined.

2. Brought to 0.55 saturation with solid (NH₄)₂SO₄. 1 gm. of Hyflo Super-Cel was added and the preparation was filtered with suction. Precipitate extract.

3. The 0.55 saturated filtrate was taken to 0.60 saturation with saturated (NH₄)₂SO₄. 0.5 gm. of Hyflo Super-Cel was added and the preparation was filtered with suction. Precipitate extract.

4. The 0.60 saturated filtrate was taken to 0.65 saturation as in Step 3. Precipitate extract.

5. The 0.65 saturated filtrate was taken to 0.70 saturation as in Step 3. Precipitate extract.

6. The 0.70 saturated filtrate was taken to 0.80 saturation with solid (NH₄)₂SO₄ as in Step 2. Precipitate extract.

Procedure V—Purification by Electrophoresis-Convection and Agitation with Chloroform

1. A number of preparations were pooled and dialyzed against 0.01 M alanine in 0.1 ionic strength phosphate buffer of pH 7.2. Enzyme solution.

2. Agitated vigorously with an equal volume of CHCl₃ for 30 minutes at room temperature. Concentrated the enzyme solution and rinsings by ultrafiltration.


4. Fractionated three times by electrophoresis-convection, stripping out three top-cuts. Final bottom-cut.

5. Treated bottom-cut as in Step 2.

6. The CHCl₃ treated bottom-cut was then fractionated by electrophoresis-convection to obtain two more top-cuts. Final bottom-cut.

7. The five top-cuts from Steps 4 and 6 were combined, concentrated by ultrafiltration, and refractionated by electrophoresis-convection. The bottom-cut was treated with CHCl₃ as in Step 2 and then refractionated by electrophoresis-convection. Final bottom-cut.

8. The two top-cuts from Step 7 were combined and concentrated by ultrafiltration.

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Test for trypsin by adding 0.5 ml. of the protamine solution to 5 ml. of the supernatant. If the precipitate redissolves significantly within 10 minutes, the kaolin treatment must be repeated.

Expressed in terms of saturation at 0°, based on a solubility of 70.6 gm. per 100 ml. of water.

The filter cakes and glassware in this and subsequent precipitation steps were rinsed with a small volume of saturated (NH₄)₂SO₄ solution of the appropriate concentration.

Precipitate extracts were prepared by stirring the filter cake with a small volume of 0.1 ionic strength phosphate buffer of pH 7.2 for 30 minutes, filtering with suction, and rinsing with the same buffer.

The saturated (NH₄)₂SO₄ solution was added very slowly with constant stirring over a period of several hours. This was accomplished by the use of a syringe equipped with a mechanical drive. In this manner volumes as small as 2 ml. could be added over a period of 4 to 5 hours.

The electrophoretic fractionations were carried out with the use of 0.1 ionic strength phosphate buffer of pH 7.2 and a field strength of 1.5 volts per cm., for 16 to 20 hours at 5°. Repeated fractionation involved removal of the top-cut at the end of a run, dilution of the remaining solution to fill the cell, and repetition of the electrophoresis.
Procedure VI—Preparation of Enzyme with Maximal Specific Activity

1. An enzyme preparation was dialyzed against 0.01 M alanine in 0.1 ionic strength phosphate buffer of pH 7.2.

2. Shook vigorously for 15 minutes with an equal volume of CHCl₃ at room temperature. Aqueous layer plus rinsings.

3. Concentrated by ultrafiltration and fractionated by electrophoresis-convection at 5°C and 1.6 volts per cm. for 20 hours. Bottom-cut.

Top-cut.

Physicochemical Characterization

4 ml. of the preparation from Step 8 of Procedure V were dialyzed for 24 hours against glass-redistilled water adjusted to pH 7.25 with 0.2 M Na₂CO₃, lyophilized in a quartz tube, and analyzed for elementary composition. The percentages obtained were 45.03, 7.21, 12.61, 1.21, and 1.69 for carbon, hydrogen, nitrogen, sulfur, and ash, respectively. Conditions of ultracentrifugation involved 1.2 and 1.5 mg. of protein nitrogen per ml., respectively; solvent, 0.01 M m-alanine in 0.1 ionic strength phosphate buffer of pH 7.2; 70 minutes at 30°C; 32,000 r.p.m. rotor speed.

A systematic study of the amino acid activation of our highly purified preparations was carried out. Over 50 compounds, including a wide variety of amino acids, peptides, their derivatives, and analogous compounds were employed. Results obtained with some of the compounds tested are given in Table I. Considering first the effect of increasing separation of the amino and carboxyl groups, one sees that the α-configuration was by far the most effective. The β-configuration gave intermediate activation whereas further separation (γ-aminobutyric to ε-aminocaproic acids) resulted in complete loss of capacity for activation. The requirement for α-amino group hydrogen atoms was considered next, and it was observed that substitution by one or two methyl groups was possible without reduced activation (serosine and dimethylglycine). Further methylation (betaine) resulted in a completely inactive structure.

Activation by α-aminobutyric acid indicates that replacement of the hydrogen at the α-carbon does not impair the power to activate, whereas activation by α-amino-n-valeric acid, α-amino-n-butyric acid, α-alanine, and norleucine demonstrates the lack of dependence upon the length of the hydrocarbon chain. Acyl substitution on the amino group has a marked effect, as can be seen by the lack of activation by hippuric acid, acetylglycine, benzoylglycylglycine, and acetylglycylglycine. Glycine anhydride seemed to be a moderately effective activator; however, its effect decreased with decreasing pH which indicated that hydrolysis to glycglycine, an efficient activator, may have taken place in the more alkaline test solutions.

Glycine ethyl ester, taurine, 3-aminopropanol, glucosamine, imidazole, and histamine are of particular interest for testing the effects of carboxyl group modification or substitution. The activation effect of these compounds indicates that sulfonic acid, carbonyl, hydroxyl, and unsubstituted heterocyclic nitrogen groups may substitute for the carboxyl in the activating molecule.

Results with 3-aminopropanol, taurine, and α-alanine indicated that β-ethanolamine and aminomethanesulfonic acid might activate. These two compounds were then found to be moderately effective. 2-Amino-2-methyl-1,3-propanediol and 2-amino-2-(hydroxymethyl)1,3-propanediol, analogues of β-ethanolamine...
with two and three correctly positioned hydroxyl groups, respectively, were then tested and found to be efficient activators.\textsuperscript{15} Since chelation ability appeared to be of primary importance in an activating molecule, the effects of KCN and α,α′-dipyridyl were also determined. Both of these compounds gave nearly optimal activation at a concentration of 10^{-4} \text{ M}.

### Analysis for Metals

Activation characteristics of our enzyme preparations were such as to indicate that metal ion interactions were involved. It then became of interest to ascertain which metals were present in our best preparations. Qualitative spectrographic observations are presented in Table II.\textsuperscript{14} The preparation analyzed was that used to obtain the electrophoretic pattern of Fig. 1. Elements of major importance seem to be zinc, magnesium, and copper, in that order. The presence of magnesium was not unexpected. With respect to the presence of copper, it is of interest to note that the enzyme solution was pale green before and after dialysis against bicarbonate. This color changed to very pale yellow after dialysis against 0.1 M alanine which indicated that an appreciable amount of the copper had been removed. The effect of adding trace amounts of cupric ion to dilute enzyme solutions was then investigated (Fig. 3). The inactivating effect of the added cupric ion was nearly completely reversed by the subsequent addition of alanine.

Quantitative analysis of the specimen analyzed spectrographically revealed the presence of substantial amounts of zinc (Table III). It was also observed that 40 per cent of this zinc could be removed by dialysis against alanine, a process that greatly diminished the alanine activation of the preparation. The resulting material, containing 0.15 per cent of zinc, had a specific activity of over 10,000 units per mg. of protein nitrogen. Dialysis of this preparation against Versene (disodium salt of ethylenediamine tetraacetic acid, Dow Chemical Company) caused an inactivation that was accompanied by a proportional loss in zinc. Moreover, this inactivation by Versene occurred in the presence of excess magnesium, which indicated that the effect was not a result of loss of magnesium by the enzyme molecule.

To test further the possibility that swine kidney alkaline phosphatase was a zinc metalloenzyme, the increase in zinc content accompanying the increase in enzyme specific activity was determined (Fig. 4). A clear linear relation was obtained between enzyme purity and zinc concentration.
Figure 3. Effects of cupric ion and alanine on the activation of alkaline phosphatase. The enzyme preparation had a specific activity of 6000 protein units per mg. of protein nitrogen. Conditions were those of the routine preliminary activation except for the addition of CuSO₄, the altered timing of the addition of the alanine, and the use of specially purified reagents and quartz tubes.

**TABLE III**

Alteration of zinc content and activity of alkaline phosphatase by dialysis

<table>
<thead>
<tr>
<th>Dialysis against</th>
<th>0.01 M NaHCO₃</th>
<th>0.1 M Alanine</th>
<th>0.01 M Versene</th>
<th>0.02 M MgSO₄</th>
<th>0.01 M in Versene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc (%)</td>
<td>0.26</td>
<td>0.15</td>
<td>0.01</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>PU/mg. PN†</td>
<td>8550</td>
<td>10440</td>
<td>1020</td>
<td>2700</td>
<td></td>
</tr>
</tbody>
</table>

* The pH of the dialyzing solutions was adjusted to approximately 8 with 0.2 M Na₂CO₃ (recrystallized). Versene was removed before analysis by dialysis against dilute NaHCO₃.
† Phosphatase units per mg. of protein nitrogen.

Figure 4. Relation of specific activity of alkaline phosphatase to zinc content. The three preparations with specific activities over 2000 were dialyzed against 0.01 M alanine in 0.1 ionic strength phosphate buffer of pH 7.2. Specific activity is expressed in phosphatase units per mg. of protein nitrogen.

**DISCUSSION**

Purification—The specific activity of our best preparations seems to be higher than that of the intestinal preparations described by Morton (12), Roche and Bouchilloux (13), and Schramm and Armbruster (14), and the renal preparation discussed by Binkley et al. (15). Calculations based on the relative size of the major peaks in each of the two ultracentrifuge patterns and the specific activities of these preparations give values in reasonable agreement with the assumption that a pure enzyme would have a specific activity of 10,000 to 11,000 units per mg. of protein nitrogen. Additional support for this assumption is provided by the nature of the electrophoretic pattern and the fact that at no time has it been possible to prepare material with activities in excess of this figure. Thus, treatment of the enzyme with an activity of 10,000 units per mg. of protein nitrogen with CHCl₃ destroyed enzymic activity at the same rate that protein was denatured. Furthermore, exhaustive fractionation of material with a specific activity of 8000 units by electrophoresis-convexion gave activities that reached a plateau in the 10,500 range.

Much the same problem was encountered in our work on alkaline phosphatase as has been reported by other investigators of this enzyme. The amount of the final, highly active preparation obtained was quite limited despite the fact that 5 kg. of cortex were processed every week for several years. It can be calculated that not more than 0.003 per cent of the protein of swine kidneys is alkaline phosphatase. The illusion that this tissue is an abundant source of the enzyme stems from the turnover number of approximately 100,000 moles per minute per 100,000 gm. of protein and from the sensitivity of the assay procedure.

**Amino Acid Activation**—Amino acid activation has been investigated a number of times since the first observations of Bodansky (16). Abuhl-Fadl and King (17) obtained results which indicated that amino acids did not activate kidney alkaline phosphatase. Akama et al. (18, 19) observed that the enzyme was efficiently activated by all amino acids. Schales and Mann (20), on the other hand, obtained activation after simple alkaline incubation. Roche et al. (21) and Fischer and Groep (22), among others, have observed activation by amino acids.

We have observed that our preparations are activated not only by amino acids but by a number of other compounds. Our findings with amino alcohols parallel those of Granger and Fraux (23), and our observations on the effects of cyanide are in accord with those of Aso (24). When the structural requirements of an activating molecule are considered closely, it seems that ability to enter into complex formation with metal ions is of primary importance. Proper chelation strength seems necessary, since the powerful metal binding agent, Versene, inactivated the enzyme at all concentrations tested, and excessive amounts of KCN, histidine, and histamine were also inhibitory.

**Metals and Enzyme Activity**—The literature contains numerous references to metals and alkaline phosphatase (17, 25–33). For the most part, the findings described are based on the effects of various inhibitors and activators. In discussing the significance of such evidence Vallee (34) concludes that such studies are of restricted utility in the absence of direct analytical data. The present investigation provides evidence based on direct analysis to amplify and support inferences from data obtained with activators and inhibitors.

Our preparations of alkaline phosphatase contain approximately 0.15 per cent of firmly bound zinc, which seems to be essential for the activity of the enzyme. On the assumption that pure enzyme contains 0.17 to 0.18 per cent of zinc, a minimal molecular weight of approximately 37,000 can be calculated. Diffusion (35) and ultracentrifuge measurements (sₑ = 6 to 6.5) indicate an intermediate molecular weight; therefore it is probable that one molecule of the enzyme contains several atoms of firmly bound zinc.

With respect to the relation of the metal content to the amino acid activation characteristics of the enzyme, the evidence im-
plicates both zinc and copper. It is probable that variable amounts of an inhibitory excess of zinc are present in the form of a loosely bound zinc-enzyme complex. The portion of the metal so involved may be removed by treatment with amino acids, and thus it can be differentiated from the more tightly bound, intrinsic zinc. The copper would appear to be exclusively inhibitory in nature.

It is generally accepted that magnesium is essential for alkaline phosphatase activity, and consequently it is probable that two metals are involved in the action of this enzyme. It is therefore of interest to note that under standard conditions of assay, the optimal concentration of magnesium is $5 \times 10^{-3}$ M whereas the actual enzyme concentration is of the order of $10^{-8}$ M or less. In general, the magnesium concentration required for optimal activation approximates that of the substrate and leads one to speculate on a relation between this fact and the observations of Bannam and Nowotny (36) that certain metal ions, when combined with phosphoric acid esters, have a phosphatase-like effect. It may well be that the actual substrate for the enzyme is the magnesium salt of the phosphate ester under attack.

**SUMMARY**

Details are presented for a method of isolation of the alkaline phosphatase of swine kidneys with which a purification of approximately 10,000-fold has been obtained. The specific activity of the enzyme was increased from 1 to 2 units per mg of total nitrogen in the source tissue to 10,500 units per mg of protein nitrogen. The material obtained consisted of 50 to 90 per cent of one component by analysis in the ultracentrifuge and by free electrophoresis.

The activation characteristics of the enzyme were studied with a wide variety of amino acids, peptides, amino acid derivatives, and analogous compounds, and it was observed that the structural requirements for an activating molecule were very similar to the structure required of organic compounds for the formation of chelates with metal ions. It was concluded that amino acid activation of alkaline phosphatase results from complex formation with inactivating or inhibiting ions, or with both.

Qualitative spectroscopic analysis of a preparation of the enzyme revealed the presence of significant amounts of zinc, magnesium, and copper. The zinc content of this preparation was found to be 0.26 per cent. This value could be reduced to 0.15 per cent by dialysis against alanine, and the resulting enzyme was fully active without alanine activation. Removal of any part of the remaining zinc by dialysis against Versene or magnesium Versenate resulted in directly proportional losses in enzymic activity. A direct proportionality was also demonstrated between increases in specific activity and zinc content of the enzyme during the course of purification.

**REFERENCES**

Preparation and Properties of Highly Purified Alkaline Phosphatase from Swine Kidneys
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