8. An increase in the rate of progression of the bands down the column decreases the sharpness of the bands. On 'Zeo-Karb 215' (40–60 mesh/in.) a rate of progression of 10–15 cm./hr. gave satisfactory results.

9. Equations have been derived permitting the calculation of the proportion of the column occupied by a component, the width of the boundaries and the expected yield of pure components in separation experiments.

10. Details of a specimen separation are given, and the yields obtained compared with estimates derived from theory.

The authors wish to thank Dr E. C. Bate-Smith for his interest and encouragement. We are indebted to Mr J. R. Bendall and Dr N. E. Topp for much help and advice. Mr D. F. Elsdon carried out the partition chromatograms.

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REFERENCES


Purification of Alkaline Phosphatase


(Received 30 July 1948)

Thoai, Roche & Sartori (1944) claimed to have isolated and crystallized the alkaline phosphatase (phosphomonoesterase) of ox kidney. The isolation of similar crystals from dog intestinal mucosa by the same workers has been reported by Roche (1946). These authors pointed out that the crystals lost their activity after a few recrystallizations. This they attributed to a denaturation or loss of coenzyme. By dialysis and reactivation Thoai, Roche & Roger (1947) obtained amorphous preparations which were more active than the crystals. The procedure adopted for the preparation and purification of the enzyme was a modified Albers & Albers (1935) technique.

Following the same procedure, we have obtained very similar crystals which, in the first instance, showed high phosphomonoesterase activity. But after careful purification of these crystals it has been found that they are principally composed of inorganic matter which appears to be magnesium orthophosphate. After careful dialysis for a prolonged period the crystals can no longer be obtained from the dialyzed enzyme preparations by any procedure tried; but the enzyme activity is not affected and can be completely restored by adding magnesium to the buffer substrate mixture.

We have also tried other effective procedures for

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the further purification of phosphatase, e.g. trypic digestion followed by prolonged dialysis (cf. Schmidt & Thannhauser, 1943; Abul-Fadl & King, 1949). The products thus obtained show much higher activity than the mixtures containing the crystals; but several attempts to crystallize such protein material, of very high phosphatase activity, have been unsuccessful.

EXPERIMENTAL

Preparation of phosphatase extracts and purification by acetone

Fresh healthy ox kidneys are decapsulated, freed from fat, well washed and twice minced in a machine. They are then thoroughly mixed with an equal weight of 25% aqueous acetone containing 10% (v/v) of toluene and ethyl acetate (1:1). Autolysis is allowed to proceed at room temperature for 2–3 days with occasional thorough shaking. The mixture is strained through muslin, filtered clear, and then treated in the cold with acetone to a concentration of 55%. The precipitate obtained is separated by centrifuging, washed twice with pure acetone and dried in vacuo. A 2% aqueous filtered solution of the precipitate is then subjected to fractional precipitation with cold distilled acetone at 0°. The fraction obtained at an acetone concentration of 38–50% is mainly protein in nature, and possesses considerable phosphatase activity. When left for 24–36 hr. in the cold room (at 5°) it sets into a crystalline mass; these crystals were claimed by Thoai et al. (1947) to be the crystalline enzyme.
Dog intestines from normal animals have also been used for similar preparations. After thorough rinsing with saline solution, the gut is cut open longitudinally, and the mucosa is scrapped off with the aid of wooden spatulas. Autolyses of intestinal mucosa, prepared as described for kidneys, have yielded highly active enzyme concentrates from which similar crystals have also been obtained.

Purification by tryptic digestion and dialysis

The fractions obtained between 38–50% acetone concentration were treated with a 0·2% solution of a highly active purified trypsin obtained from Armour Ltd.

The pH was adjusted to 8·0 with Na₂CO₃ solution and the mixture incubated at 37° for 3–4 hr. The preparation was then shaken with 5% kaolin and centrifuged or filtered. This procedure was repeated twice to remove any remaining trypsin. The phosphatase activity was not affected. The enzyme was now precipitated in 0·9% intestinal mucosa, prepared as described previously, and dialyzed with 5% acetone. The precipitate was collected by centrifuging, and dialyzed in collidion bags at room temperature for 3–4 days, against a large volume of distilled water frequently changed. The activity and total N were determined each day, and dialysis was stopped when the activity/mg. total N remained constant for two successive estimations.

Activity determination

Activity has been determined in two ways: (a) in Roche P units, by estimation of orthophosphate liberated from 0·02% β-glycerophosphate in veronal buffer at pH 9 at 37°; (b) in King phenol units, by estimation of phenol liberated from 0·005% sodium phenylphosphate in Na₂CO₃-NaHCO₃ buffer at pH 10 at 37°. Determination of the activity by the two methods allows of comparison with former results published both by ourselves and by other authors.

Magnesium was added to give a concentration of 0·01 M.

Phosphorus was determined by Brigg's (1922) method or those of King (1932) and Allen (1940), phenol by the Folin-Ciocalteu method (for details see King, 1947), and total N by the micro-Kjeldahl procedure.

(a) Determination of activity in terms of Roche phosphate units. In a test tube marked at 10 ml. are placed 4 ml. veronal buffer (pH 9), together with 2 ml. 0·1 M-sodium glycerophosphate and 1 ml. 0·1 M-magnesium acetate. The volume is adjusted to 9·9 ml. with distilled water, and the tube allowed to stand in a 37° water bath for 5 min. The highly active enzyme solution (0·1 ml.) is added and hydrolysis is allowed to proceed for exactly 5 min. Trichloroacetic acid (25% 1 ml.) is added to stop enzyme action and to precipitate any proteins. (Generally the highly active preparations contain negligible amounts of precipitable proteins.) The solution is filtered if necessary, and the inorganic phosphate determination carried out.

Controls without any enzyme solution and blanks with enzyme added after acidification must be run parallel with the tests. If the amount of inorganic phosphate exceeds 0·3 mg. P in the whole tube (corresponding to about 5% hydrolysis) the determination is repeated with a conveniently diluted enzyme solution. The Roche unit of phosphatase is defined as the amount of enzyme which will liberate 1 μg. P/min.

(b) Activity by King phenol units. The Na₂CO₃-NaHCO₃ buffer of pH 10 (2 ml., Delory & King, 1945), 0·02 M-disodium phenylphosphate solution (1 ml.) and 0·04 M-magnesium acetate (1 ml.) are mixed in a test tube and warmed to 37°. After addition of 0·2 ml. of the enzyme solution suitably diluted, and incubation of the mixture for 15 min. at 37°, 1·8 ml. dilute (1 in 3) Folin-Ciocalteu reagent are added. The solution is mixed, centrifuged if necessary, and phenol determined in 4 ml. of the clear supernatant fluid (cf. King, 1947). Controls and blanks are also used. The unit of phosphatase is defined as the amount of enzyme which will liberate 1 mg. phenol/15 min.

RESULTS AND DISCUSSION

Dog intestinal phosphatase. Table 1 shows the activity of dog intestinal preparations at different stages of purification. The activity of the mucosa autolyses has been found to vary considerably from one preparation to another. It is important to work with intestines obtained from healthy, well-fed animals, showing no sign of abnormality. Preparations from ill or starving dogs yield solutions of low activity. The results shown in Table 1 are those normally obtained from satisfactory preparations.

Table 1. Phosphatase activity of dog intestinal preparations during different stages of purification

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Autolysis for 3 days in 25% acetone.</td>
<td>650–800</td>
</tr>
<tr>
<td>(Autolysate filtered clear, light yellow)</td>
<td>70–85</td>
</tr>
<tr>
<td>2. Crude Albers, precipitated by 55% acetone</td>
<td>2000–2400</td>
</tr>
<tr>
<td>from above autolysate</td>
<td>240–300</td>
</tr>
<tr>
<td>3. Fractional precipitation by acetone</td>
<td>5000–8000</td>
</tr>
<tr>
<td>38–50%, 0–4°</td>
<td>600–800</td>
</tr>
<tr>
<td>4. Tryptic precipitation followed by prolonged dialysis</td>
<td>9380–15700</td>
</tr>
</tbody>
</table>

The amount of mucosa obtained from the whole small intestine from a normal dog is generally about 100 g., which is left to autolyze as described above. The clear filtered autolysate is generally light straw-yellow in colour and has a total nitrogen content of about 0·7–1·6 mg./ml. The crude Albers preparation obtained by precipitating with 55% acetone in the cold has a higher activity (units/mg. total nitrogen) than the autolysate, but its comparatively low total nitrogen content (7–9%) indicates contamination with non-nitrogenous materials (possibly carbohydrates) as well as inorganic matter.

On fractional precipitation with cold pure redistilled acetone the fractions obtained below 38% acetone concentrations are relatively poor in enzyme activity. The fractions obtained with higher acetone concentrations (38–50%), on the other hand, are rich in enzyme activity. By carrying out this stage carefully in a cold room at approximately 0°, adding the chilled pure acetone gradually with constant stirring and separating every fraction by thorough
centrifuging in the cold room, it is possible to obtain highly active products, containing about 12–14 % nitrogen.

This fraction, however, is always contaminated with inorganic matter, consisting mainly of magnesium phosphate which crystallizes when the preparation is kept in the cold. The residual acetone evaporates slowly, and the inorganic crystals are gradually formed in the viscous protein solution, which adheres intimately and takes the shape of the crystals, thus giving the impression that the enzyme itself has crystallized.

The enzyme preparation at this stage could not be further purified by fractional precipitation with acetone. We have tried several ways of purification involving fractional salting out with ammonium sulphate, adsorption with alumina and kaolin, simple dialysis and electrodialysis, but none has given satisfactory results.

Tryptic digestion under the described conditions, however, followed by treatment with kaolin, salting out with ammonium sulphate and then prolonged dialysis, has been found very effective in achieving further purification. The relative stability of the intestinal phosphatase towards dialysis has been an additional favourable factor in effecting purification of this enzyme after tryptic digestion in this way. The preparation obtained after dialysis is an almost colourless, highly active, aqueous solution. If dialysis is very prolonged, the preparation begins to lose its activity with further decrease in total nitrogen. The activity, however, can be completely restored, as described by Thoai et al. (1947), by incubating the partially, but not completely, inhibited enzyme preparation with α-amino acids in a slightly alkaline medium (pH > 9). The best preparation contained 0-14 mg. nitrogen/ml., and had an activity of 15,700 Roche phosphate units or 1600 King phenol units/mg. total nitrogen.

The enzyme was obtained in the dry form without loss of activity by adding pure redistilled acetone to this solution in the cold to a concentration of 80–90 %. It separated as a light coagulated precipitate, which settled overnight in the cold, and was separated by decantation and centrifuging. Various attempts to crystallize this preparation, e.g. by allowing to stand in the cold in concentrated solution with and without spontaneous evaporation in a vacuum desiccator, by careful addition of ethanol and acetone, by allowing to stand at 0° in different concentrations of acetone, by additions of various cations or by addition of ammonium sulphate and other salting-out materials like sodium phosphate and magnesium sulphate, were all unsuccessful.

The total nitrogen content of the dry powders obtained from different preparations was 10–11 % nitrogen. No carbohydrate residue was detected in any of our final preparations. After prolonged hydrolysis with acid, no reduction of Benedict or Fehling solutions could be detected (cf. Schmidt & Thannhauser, 1943).

Ox-kidney phosphatase. Table 2 shows the activity of ox-kidney preparations during different stages of purification. Our experience with ox kidneys both in England and in France was entirely unsatisfactory. Preparations much weaker in activity than those from dog intestines have always been obtained. The autolysates, as well as the other preparations, are characterized by comparatively low activity and high total nitrogen (generally about 13–15 mg. nitrogen/ml.).

Table 2. Activity of ox-kidney preparations during stages of purification

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Phosphate (Roche units/mg. total N)</th>
<th>Phenol (King units/mg. total N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Autolysis at room temperature for 3 days in 25% acetone in presence of 0-1 vol. toluene-ethylacetate. Autolysate filtered clear, straw yellow</td>
<td>100–135</td>
<td>10–14</td>
</tr>
<tr>
<td>2. Crude Albers precipitated by 55% acetone from above autolysate</td>
<td>32–600</td>
<td>35–65</td>
</tr>
<tr>
<td>3. Fractional precipitation by acetone 38–50%, 0–4°</td>
<td>840–1200</td>
<td>90–130</td>
</tr>
<tr>
<td>4. Tryptic digestion and dialysis</td>
<td>2000–3500</td>
<td>250–400</td>
</tr>
</tbody>
</table>

The 38–50 % acetone fraction obtained from crude Albers's preparations contains about 14 % total nitrogen, and is relatively poor in activity. It has a considerable yield of crystals (much more than the corresponding intestinal preparation with higher activity), presumably because of the high phosphate content of this organ. Moreover, the relative instability of the kidney phosphatase during dialysis makes efficient purification of the enzyme by this procedure rather difficult. In this respect it differs from the intestinal enzyme which is more stable; also it is not reactivated to the same extent as the latter by amino-acids after partial inactivation through dialysis. This will be discussed in detail elsewhere. Tryptic digestion effected considerable purification of this enzyme.

In Table 3 are given some analytical data of two batches of crystals obtained from two kidney preparations. These were obtained from the first crystallization after thorough washing with cold water and rapid centrifuging. These crystals are soluble with difficulty in water, but are more soluble in dilute alkalioracid. They contained 12–15 % phosphorus, all of which was present as orthophosphate, 4–7 % carbon and very little nitrogen. The fact that the carbon and nitrogen contents are so small and so variable suggests that these are due to organic
impurities and are not chemical constituents of the crystals. The ash consisted entirely of magnesium pyrophosphate. Magnesium was determined microgravimetrically as oxinate by precipitation with 8-hydroxyquinoline (Berg, 1935), and phosphorus determined by the King (1932) method, after hydrolyzing the pyrophosphate into orthophosphate. The figures shown in the table are very close to those of $\text{Mg}_3\text{P}_2\text{O}_7$. Microtests for calcium and zinc were negative. No ammonia could be detected in the crystals. Comparison with two crystalline forms of magnesium orthophosphate, as shown in Table 3, shows the close similarity of these crystals with either one or the other, with only slight differences due to the presence of traces of organic impurities which are very difficult to remove, and also of water of crystallization which can easily vary when crystals are kept at room temperature. It is concluded that these crystals are magnesium orthophosphate. It appears, therefore, that the alkaline phosphatase has not been crystallized, but is easily adsorbed by magnesium orthophosphate, and, possibly, by other sparingly soluble mineral salts.

### Table 3. Analytical data for crystals obtained from kidney phosphatase

<table>
<thead>
<tr>
<th>Composition</th>
<th>Crystals from</th>
<th>Batch 1</th>
<th>Batch 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{MgH}_2\text{PO}_4$</td>
<td>$\text{MgH}_2\text{PO}_4$</td>
<td>$\text{MgH}_2\text{PO}_4$</td>
<td>$\text{MgH}_2\text{PO}_4$</td>
</tr>
<tr>
<td>Theory for</td>
<td>Theory for</td>
<td>Crystals from</td>
<td>Crystals from</td>
</tr>
<tr>
<td>$\text{MgH}_2\text{PO}_4\cdot \text{H}_2\text{O}$</td>
<td>$\text{MgH}_2\text{PO}_4\cdot \text{H}_2\text{O}$</td>
<td>$\text{MgH}_2\text{PO}_4\cdot \text{H}_2\text{O}$</td>
<td>$\text{MgH}_2\text{PO}_4\cdot \text{H}_2\text{O}$</td>
</tr>
<tr>
<td>C*</td>
<td>7.05</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>H*</td>
<td>2.36</td>
<td>2.04</td>
<td></td>
</tr>
<tr>
<td>N*</td>
<td>2.24</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>12.5</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>10.9</td>
<td>11.27</td>
<td></td>
</tr>
<tr>
<td>Ash (as $\text{Mg}_2\text{P}_2\text{O}_7$)</td>
<td>Ash of crystals</td>
<td>1.25</td>
<td>1.30</td>
</tr>
<tr>
<td>$\text{P}/\text{Mg}$</td>
<td>28.0</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td>$\text{Mg}$</td>
<td>21.6</td>
<td>22.5</td>
<td></td>
</tr>
</tbody>
</table>

* By Drs Weiler and Strauss, Oxford.

### SUMMARY

1. The purification of alkaline phosphatase from dog intestinal mucosa and ox kidney has been studied by different procedures, and highly active preparations were obtained from mucosa.

2. The crystals which were claimed to be pure alkaline phosphatase have been identified as magnesium orthophosphate, with adsorbed phosphatase.

3. Attempts to crystallize the highly active intestinal phosphatase were unsuccessful.

### REFERENCES


### Purification of Faecal Alkaline Phosphatase

BY M. A. M. ABUL-FADL AND E. J. KING, Postgraduate Medical School, London, W. 12

(Received 16 August 1948)

The presence of considerable amounts of alkaline phosphatase in dog faeces was first reported by Armstrong, King & Harris (1934). Since then, several attempts have been made to separate and purify this enzyme. Armstrong (1935) described a method by which a very potent phosphatase-containing powder could be obtained from dog faeces within 48 hr. King & Delory (1939) repeated this preparation, but did not succeed in raising its potency above that achieved by Armstrong. Chen, Freeman & Ivy (1940) concentrated the faecal phosphatase by adsorption on kaolin in an acid medium and elution by alkaline buffer. Their enzyme preparations, however, were less active than those described by Armstrong.

The present work was undertaken for the purpose of obtaining the faecal phosphatase in the purest condition possible, and to attempt its crystallization.