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

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

	Composition			
	Magnesium phosphate		Crystals from phosphatase	
	Theory for $\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$	Theory for $\text{MgHPO}_4 \cdot 7\text{H}_2\text{O}$	Batch 1	Batch 2
C*	—	—	7.05	4.3
H*	4.02	3.02	2.36	2.04
N*	—	—	2.24	Trace
P	17.7	12.6	12.5	14.7
Mg	13.9	9.86	10.0	11.27
Ash (as $\text{Mg}_2\text{P}_2\text{O}_7$)	62.1	45.2	45.8	60.2
P/Mg	1.27	1.27	1.25	1.30
	Theory for $\text{Mg}_2\text{P}_2\text{O}_7$		Ash of crystals	
P	28.0		26.5	27.8
Mg	21.6		21.7	22.5

* By Drs Weiler and Strauss, Oxford.

figures shown in the table are very close to those of $\text{Mg}_2\text{P}_2\text{O}_7$. 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.

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.

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Purification of Faecal Alkaline Phosphatase

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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.

By the use of several methods of purification, it has been possible to obtain preparations with higher activities than those obtained by Armstrong (1935). Several attempts at crystallization were unsuccessful. With the procedure outlined by Armstrong, we encountered many difficulties, and the preparations obtained were, in the majority of cases, much less active than his. Methods of purification have been studied, and an improved procedure is described.

EXPERIMENTAL

The main features of the method adopted were those originally described by Armstrong (1935). The following precautions, modifications and additions, some of them taken from the procedures used for kidney and intestinal phosphatase by Abul-Fadl, King, Roche & Thoai (1949) have been found useful. (1) Avoidance of any change of the pH towards the acid side, as the faecal phosphatase is very sensitive to acid, even at 0°. (2) Fractionation of the aqueous extract with $(\text{NH}_4)_2\text{SO}_4$. (3) Decolorization with minimum loss of activity. (4) Removal of inactive protein material from the final enzyme concentrates by short tryptic digestion followed by careful electrodialysis. The whole procedure is outlined below.

Estimation of activity was in terms of King phenol units as described by Abul-Fadl *et al.* (1949).

A. Preparation of a clear extract from the faeces

About 1 kg. of dog faeces, collected within 24 hr. of being passed, is placed in a beaker together with cracked ice, and tap water added to make the total volume about 2 l. The contents are stirred thoroughly till uniform and then strained through a wire sieve. The sludge is tested by litmus paper and made alkaline with ammonia. It is then poured into a fluted filter paper and left in the ice box to filter overnight. The filtrate, if not absolutely clear, should be treated with 5% kieselguhr or kaolin and refiltered with suction.

The dark brown filtrate, which should be free from any turbidity, shows an activity of 100–150 King phenol units/ml. and contains about 1.2–0.6 mg. protein N/ml. With a few drops of toluene added, the solution retains its activity for several days at 0°. To assure a high degree of activity, faeces should be collected from healthy, well-fed dogs, discarding any samples which have become dry.

It will be noted that preliminary autolysis, as in the case of kidney and intestine, is here unnecessary since the faecal phosphatase is readily obtained in a soluble form.

B. Precipitation with ammonium sulphate

Preliminary experiments showed that precipitation with acetone at this stage, as was used in the case of kidney and intestinal preparations, gives preparations of poor activity whilst MgSO_4 , even in full saturation, was of little use in salting out the enzyme. Half saturation with $(\text{NH}_4)_2\text{SO}_4$, however, precipitates about 90% of the phosphatase, while with 0.8 saturation precipitation is almost complete. To each 100 ml. clear filtrate, cooled to 0°, 40 g. $(\text{NH}_4)_2\text{SO}_4$ (A.R.) are added with stirring. On standing for 10–15 min. in the cold a precipitate is formed which rises to the surface. Most of the clear underlying liquid can be removed by siphoning,

and the precipitate readily collected and separated by rapid centrifuging.

The use of acetone at this stage to help salting out, as described by Armstrong (1935), leads to much loss of activity in the aqueous acetone solution. The loss in some experiments amounted to about 40%.

The precipitate thus obtained gives a dark brown turbid solution. The activity is about 600–750 units/ml., with a high protein N amounting to as much as 6.5 mg. N/ml. in some experiments.

C. Decolorization by activated charcoal

This is the most critical step in the purification of the faecal phosphatase. Much enzyme activity may be lost if the steps are not carefully controlled. The difficulties encountered are due to the following: (1) The faecal enzyme is unstable in slightly acid medium even in the cold, but for effective decolorization the medium must be slightly acid. (2) Adsorption of the enzyme on the activated charcoal cannot be reversed.

Glycerophosphate buffer adjusted with acetic acid to pH 5.2–5.4 and containing 0.5 M- MgSO_4 is the best medium for effective decolorization with removal of inactive protein material and minimum loss of enzyme activity. β -Glycerophosphate appears to protect the enzyme against inactivation due to changes in pH, while Mg increases the enzyme affinity for substrate, thus minimizing the risk of adsorption on the charcoal.

The precipitate obtained from B is dissolved in 150 ml. 0.02 M-sodium β -glycerophosphate solution previously adjusted with dilute acetic acid to pH 5.2–5.4, and 15 g. crystalline $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in the solution. The procedure is conducted in the cold at about 0°. Activated charcoal (1.5 g.) is now added and the mixture is well stirred for 10 min. It is then filtered through a folded filter paper previously prepared by passing through it 1% charcoal suspension in water. The filtrate is almost colourless, or only slightly coloured, and shows an activity of 570–700 units/ml.

The solution is transferred into collodion or cellophane bags of 50 ml. capacity and left to dialyze against distilled water overnight to eliminate most of the Mg, in the presence of which inactivation is likely to occur, and has been observed in many cases. The dialyzed solution, increased in volume by osmosis, is now subjected to further purification as it still contains a lot of inactive protein.

D. Digestion with trypsin

The effect of tryptic digestion on purification of phosphatase has been discussed by Abul-Fadl & King (1949). A potent commercial trypsin (Armour Ltd.) is thoroughly mixed with the enzyme solution in a concentration of 0.2%. The pH is adjusted to 8.0 with Na_2CO_3 and the mixture left to stand in a thermostat at 37° for 3–4 hr. At the end of this time 5% kaolin is added, stirred for 10 min. and then separated by centrifuging. This procedure is twice repeated in order to eliminate any residual trypsin. If any colour is still present, it can be removed at this stage by a second charcoal treatment (1%), which will also help in removing traces of trypsin, with practically no loss in enzyme activity. The medium need not be acidified in this operation since the colour is only slight and complete decolorization can be easily effected.

The activity now ranges between 400 and 500 units/ml. and the protein N from 0.2 to 0.3 mg. N/ml. Thus a high degree of purification has been achieved at this stage; but the preparation is still rich in non-protein N, which can be eliminated by prolonged dialysis in collodion or cellophan bags against running distilled water.

E. Further purification by electro dialysis

Kutscher & Wolbergs (1935) claimed to have obtained the prostatic phosphatase in a high state of activity by long electro dialysis. On the other hand, Albers & Albers (1935) reported a continuous loss of activity in kidney-phosphatase preparations subjected to electro dialysis.

We have studied the behaviour of kidney, intestine and faecal phosphatase towards electro dialysis and the detailed results will be published later. With the faecal phosphatase preparations we have been able to attain a further degree of purification by virtue of the denaturation of the remaining proteins which occurs during the electro dialysis.

The apparatus described by King & Dolan (1934) has been used. The clear faecal phosphatase solution is placed in a 150 ml. capacity cell, and distilled water is run continuously in the two parchment thimbles containing the electrodes. The current is kept low (10–20 ma.) throughout the experiment which lasts usually for 24–30 hr. Care must be taken to keep water running in the thimbles and to stir the dialyzed solution continuously. If a current of N_2 is used for stirring, it must be slow, so as not to form excessive froth which causes surface inactivation of the enzyme. Drops of chloroform are added as a preservative and to minimize frothing.

No physical change is observed in the enzyme solution during dialysis. The activity and protein N, which are tested regularly, show no significant variation throughout the experiment. The enzyme solution remains clear, and only starts to show turbidity near the end of the experiment when diminution in activity is also noticed. On the other hand, turbidity is always found inside the cathode chamber, owing to elimination of nitrogenous and other impurities. If the dialysis is unduly prolonged or the water circulation is stopped, the enzyme solution starts to show turbidity, and precipitation may occur with complete loss of activity.

F. Precipitation with acetone

At the end of electro dialysis there is no change of activity in relation to the protein N, and the solution is perfectly clear. The enzyme solution is now cooled to 0° and pure cold acetone is added to a concentration of about 60%. A flocculent precipitate collects at the bottom on leaving to stand in the cold. This can be separated by pouring off the supernatant fluid, centrifuging and washing with pure acetone.

The precipitate thus obtained, however, is not now completely soluble in water. It seems that part of the proteins has been denatured. Distilled water (20 ml.) is now thoroughly stirred with the acetone precipitate, allowed to stand in the cold for about 30 min., and any insoluble part spun down. The clear supernatant fluid is reprecipitated with 60–80% acetone in the cold, separated and washed with acetone by centrifugation. It is finally dried over $CaCl_2$ or soda lime in a vacuum desiccator.

The precipitate dries in thin, almost transparent scales. It can be pulverized into a white or buff-coloured powder showing an activity of 1166–1300 units/mg. N. The total N is 10–11% and protein N is 5.6–6.2%. The yield is 0.2–0.4 g./kg. faeces.

DISCUSSION

The activity/mg. dry powder is of the order of 95–120 units which corresponds to 190–240 King-Armstrong units. This activity is higher than that recorded by Armstrong (1935), whose best preparation was of about 185 King-Armstrong units.

These purified faecal phosphatase preparations are similar to the intestinal preparations described by Abul-Fadl *et al.* (1949) in their nitrogen content and in the absence of any detectable carbohydrate residue. On the other hand, there is a tendency to get more active enzyme preparations from the mucosa since our best faecal preparation shows an activity of 1300 units/mg. nitrogen as compared with that from mucosa which was about 1600 units/mg. nitrogen. In general, it has been noticed that the most active purified preparations were always obtained from starting materials which were originally of a very high order of activity. This suggests that the enzyme may be originally present in the tissues in quite different amounts or degrees of activity.

As in the case of intestinal phosphatase, repeated attempts at crystallization of the purified faecal enzyme have been unsuccessful.

SUMMARY

1. A method for the purification of faecal alkaline phosphatase is described.
2. Amorphous phosphatase preparations of very high activity have been prepared, but all attempts to crystallize them have failed.

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