CCXXXV. PURIFICATION OF THE ACTIVE PHOSPHATASE FOUND IN DOG FAECES.

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It was observed [Armstrong *et al.*, 1934] that a phosphatase, active at p_H 9·6, was present in dog faeces. The activity of the faeces is variable but usually exceeds that found in the case of intestinal mucosa which is the tissue having, weight for weight, the greatest phosphatase activity in the body.

It seemed worth while to attempt to isolate, if possible, a crystalline enzyme from this cheap and unlimited source. Whilst a crystalline material was not obtained, the degree of purification reached was sufficiently great to warrant publication in view of the fact that the work must now be temporarily abandoned.

The underlying principles involved in the procedures employed were as follows: preparation of a water-clear aqueous extract of the faeces, salting out the protein from this extract, solution of the protein and removal of foreign material by acidification and adsorption, reprecipitation of the protein with minimum contamination by inorganic salts. These procedures presented considerable difficulty for two reasons: (1) the colloidal nature of concentrated mixtures of faeces and water and (2) the fact that the phosphatase active at p_H 9·6 is unstable in acid media. No difficulty was ever encountered owing to faecal extracts containing large amounts of unhydrolysed protein. The protein content was always less than 1 % and a large part of this was insoluble in acid.

The procedure outlined below avoids the difficulties mentioned and makes possible the preparation of a dry powder of unusual potency within 48 hours.

Experimental.

A. *To obtain a water-clear filtrate from the faeces.*

About 1 kg. of dog faeces, collected within 24 hours of being passed, is placed in a 3 l. beaker. Cracked ice is placed in the beaker and tap water is added to make the total volume about 2 l. The contents are now stirred thoroughly until uniform. When completely mixed the coarse particles are removed by straining through three grades of wire sieve, the final one being 40-mesh.

The sludge is now cooled to 0° and 20 ml. glacial acetic acid are added, causing some of the phosphates to dissolve. The fluid is now brought to p_H (about) 8 by adding approximately 30 ml. concentrated ammonia. This causes the dissolved phosphates to precipitate and the precipitate entrains fine particles which otherwise will pass through a filter paper. The sludge is now poured into a pleated coarse filter-paper and left in the ice-box filtering overnight. The dark brown filtrate should be free from any turbidity. If turbidity is present the suspension must be filtered through a thin layer of diatomaceous earth under suction.

If kept near 0° this solution retains its phosphatase activity for some days.

B. *To obtain a washed protein-like precipitate from "A".*

To the ice-cold filtrate "A", ammonium sulphate is added to complete saturation. A precipitate forms which tends to rise.

A quarter volume of 65–70 % acetone is now stirred in. The precipitate rises rapidly and collects at the interface between the acetone and ammonium sulphate layers. This precipitate is skimmed off in a spoon and placed in a separating funnel. The saturated ammonium sulphate solution which accompanies it is drained through the stopcock, and the supernatant acetone decanted.

Successive amounts of 65 % acetone are added to the precipitate in the funnel, shaken and decanted; the supernatant liquid finally becomes nearly colourless. The precipitate is then run through the stopcock into a filter-paper and allowed to drain thoroughly. This material is greyish brown in colour and turns dark brown after exposure to air for a few minutes.

C. *Decoloration and purification of "B".*

The washed precipitate, "B", is dissolved in 100 ml. water kept at p_H 8–9 with 1 % ammonia. It is brought to 0° and then adjusted to p_H 4–4·5 with about 0·5 ml. glacial acetic acid. A variable amount of relatively inactive material precipitates.

1 g. of activated charcoal is shaken in. After 10 min. the mixture is filtered in the ice-box through a filter-paper prepared by filtering through it 100 ml. 1 % charcoal suspension. This latter technique prevents any fine charcoal particles, rendered semi-colloidal by the protein, from passing through the filter. Filtration takes from 0·5 to 12 hours, depending upon the physical state of the protein thrown down by the acid. The ice-cold filtrate is now brought to p_H 7·0 by adding about 1 ml. strong ammonia solution.

D. *To obtain a dry powder nearly free from ammonium sulphate and other inorganic salts.*

To the neutralised filtrate, "C", acetone is added to make 60 % concentration. Most of the active material is precipitated and on standing much of the turbid supernatant fluid can be decanted. The residue is poured into a filter and allowed to drain thoroughly. It forms a gummy mass which is readily removed from the filter-paper. This is placed on a watch glass, dried in a vacuum desiccator overnight and ground to a fine powder. It is grey to faintly brown in colour.

Yield and activity.

There is a considerable variation in the yield of powder obtained. From an amount of faeces sufficient to occupy a volume of about 1 l. yields of 0·1–0·5 g. have been obtained. This is to be expected, for the amount of active material per g. of faeces will vary according to the amount of undigested material in the stool.

The activity of the powders has been estimated by the phenylphosphate method [King and Armstrong, 1934] with the modification that excess Mg^{++} ion was added to the buffered substrate. This was considered advisable since in the process of purification this co-enzyme is reduced to a trace. The activities of successive batches lay between 130,000 and 185,000 units per g. (For comparison it may be recalled that 1 g. wet weight of adult dog long bone has an activity of about 2 units by the same method.)

A clearer conception of the tremendous activity is obtained by the following computation based on the definition of a "unit", *viz.* that 1 g. of the best powder (185,000 units per g.) acting on an excess of disodium phenyl phosphate in the presence of Mg ion and in sufficient dilution in a buffer solution of suitable p_H at 37·5° will liberate 185 g. of phenol every 30 min., *i.e.* more than 6 g. per min.

The activity obtained is considerably diminished if the sludge and filtrates are not kept cool during the preparation, especially when the p_H is lower than 7·0. Allowing the solutions to stand for longer times than mentioned also causes some loss. Apart from these causes, variations in activity may be expected, if for any reason any protein-like material behaving chemically in a manner similar to the powder should be passed in the faeces. Apparently however such a material is not encountered in any considerable quantity.

Optimum p_H. Between p_H 3·0 and 10·5 only one zone of optimum activity was observed when disodium phenyl phosphate was used as substrate. The optimum p_H lay approximately at 9·6 measured at 37°; practically no activity was observed below p_H 6·0. This suggests the identity of the enzyme with bone phosphatase.

Enhancement of activity by Mg^{++} *ions.* An increase in the activity of the enzyme using the buffered substrate of King and Armstrong [1934] occurred when Mg^{++} ions were added; approximately 0·0003 *M* Mg^{++} produced a 25 % increase. Further increases to 0·003 *M* produced no definite additional enhancement.

Qualitative analysis. On analysis the material was found to contain 2·7 % ash of which 0·24 % was silica. The powder gave qualitative tests for nitrogen and phosphorus; a trace of halogen was present and faint traces of sulphur and iron.

Adsorption by kaolin. The material may be adsorbed on kaolin and eluted with a recovery as high as 90 %. No attempt has been made to reprecipitate a powder from such a recovered solution. The conditions for adsorption and elution are as follows: 1 ml. of 0·5 % solution of phosphatase powder and 1 ml. 5 % kaolin suspension are brought to 0° and acidified with 1 % acetic acid to p_H 4·5. The kaolin is quickly centrifuged, washed with ice-cold water at p_H 5 and again quickly centrifuged. To the washed kaolin is now added 1 ml. of 0·5 % diammonium hydrogen phosphate and after stirring for 15 min. the kaolin is finally removed by centrifuging. The active material is now in the phosphate solution.

Summary.

A method is described by which a very potent phosphatase-containing powder can be obtained from dog faeces within 48 hours. Its optimum p_H is in the region of 9·6 measured at 37·5° when acting on disodium phenyl phosphate. No other zone of optimum p_H has been found. Using the same substrate the optimum Mg^{++} ion concentration is below 0·0003 *M*.

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References.

Armstrong, King and Harris (1934). *Can. Med. Assoc. J.* **31**, 14.
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