The Isolation and Identification of Phosphoethanolamine from the Urine of a case of Hypophosphatasia

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Rathbun (1948) described an unusual and previously unreported type of faulty bone development in a male child 3 weeks old. It was characterized by gross changes in long bones and skull, superficially similar to those in rickets, associated with a very low level of serum alkaline phosphatase. Post-mortem examination of bones, kidney and other organs revealed very low levels of alkaline phosphatase in these tissues also. Rathbun considered the low alkaline phosphatase to be the primary defect responsible for the clinical condition and called the new disease hypophosphatasia. Several other cases have subsequently been described (reviewed by Fraser, 1957), which suggests that the disease is not excessively rare but rather had not been previously recognized as a separate entity. McCance, Morrison & Dent (1955) and Fraser, Yendt & Christie (1955) independently and simultaneously reported finding phosphoethanolamine to be one of the main ninhydrin-reacting spots on the amino acid chromatograms of urine from two further cases in children. Phosphoethanolamine is not detected in normal urine by their chromatographic techniques. Cusworth & Dent (1956) confirmed these findings in six further child cases and one adult case of the disease and pointed out that although traces of phosphoethanolamine are occasionally found in the urine in other diseases, such as liver disease, it is then only a temporary phenomenon, whereas in hypophosphatasia the excretion of the compound is both large and constant. A small constant excretion of phosphoethanolamine is found in certain relatives of known cases of hypophosphatasia (see Fraser, 1957), suggesting an inborn condition. Cusworth & Dent (unpublished observations) have subsequently found the same large excretion of phosphoethanolamine in a further four child cases and two adult cases, bringing the total number of cases examined in this department to 13 and suggesting that excretion of the compound is to be considered as an essential feature of the disease.

The identification of phosphoethanolamine was in each case based on two-way chromatograms for urine amino acids and as such could be only tentative. Although identical chromatographic

behaviour with synthetic phosphoethanolamine in several solvents and also by paper ionophoresis at various pH values was strongly suggestive, the ultimate proof of identity must, if possible, depend on isolation of the pure compound from the urine of a case of hypophosphatasia followed by its chemical analysis and study of physical properties. This has now been achieved.

EXPERIMENTAL

The patient who provided the material for this isolation was a woman of 41 years with a history of 'rickets' in childhood. She had been well until the last 4 years, when she began to get severe pains in her thighs and was found to have pathological fractures in both femora in the mid-shaft region. She was shown at the Royal Society of Medicine in 1953 by Dr G. H. Jennings as a case for diagnosis. Dr C. E. Dent, who saw her there, thought she had some unusual form of osteomalacia, but the chromatogram for urine amino acids then carried out in this department showed a large excretion of a substance considered to be phosphoethanolamine. The significance of this was not realized at the time. Subsequently, increased excretion of phosphoethanolamine was found in several child cases of hypophosphatasia and it was realized that this was a diagnostic feature of the disease. The original chromatogram was noted during a review of previous work in the department and the possibility that the adult patient might be a case of this disease was therefore considered. This was fully confirmed after detailed study in the Metabolic Ward, University College Hospital; no clinical details of these studies are yet published.

Urine collection. During the patient's stay in the Metabolic Ward, continuous 24 hr. urine collections were made. After portions had been removed for other investigations, the remainder was bulked and stored under toluene at 4°. A total of 32 l., corresponding to 21 days' output, was collected.

Preliminary isolation. In the early stages, the general procedure of Westall (1952) was followed. Two-way paper chromatograms (Dent, 1948) and high-voltage paper ionophoresis at pH 2 and 110 v/cm. (Gross, 1955) were used extensively throughout the isolation to check the amino acid content of the various fractions and the purity of the product. The 32 l. of urine was concentrated under reduced pressure in a circulating evaporator (Van Heyningen, 1949) to 2 l., 3 vol. of ethanol were added and the mixture was allowed to stand at 4° overnight and filtered. The filtrate contained phosphoethanolamine and amino acids and has

not yet been worked up. The precipitate also contained some amino acids and phosphoethanolamine, but the ratio of phosphoethanolamine to other amino acids was higher than in the filtrate, so the precipitate was dissolved in water and the residue removed, by centrifuging, and washed. The volume of extract and washings was 900 ml. and contained most of the phosphoethanolamine from the ethanol precipitate. The extract was treated with 5 g. of charcoal, boiled to remove CO_2 , and the filtrate passed through a train of three columns of bed size 6 cm. (diam.) $\times$ 11 cm. and 3.5 cm. (diam.) $\times$ 13 cm. packed with Dowex 2 ($\times$ 8) anion-exchange resin (50–100 mesh; OH^- ion form). After washing with CO_2 -free water, displacement was commenced with N-HCl and 250 ml. fractions were collected. Owing to the limited amount of resin available this step was carried out in three batches.

The amino acid-containing fractions from the Dowex 2 were diluted to 10 l. and passed through an 8 cm. $\times$ 33 cm. column of Zeo-Karb 215 cation-exchange resin (H^+ ion form). Most of the amino acids are retained by this resin but taurine and phosphoethanolamine pass through into the effluent. This was boiled for a short time to remove CO_2 and passed through the original set of Dowex 2 columns previously regenerated to the OH^- ion form. Displacement from the Dowex 2 was carried out with 0.2N-HCl and 25 ml. fractions were collected on a time-operated fraction collector. Phosphoethanolamine was very slightly retarded in coming off the column compared with taurine, but most fractions contained the two substances in equal amounts and no fraction contained one component free from the other.

Separation of phosphoethanolamine and taurine. These two substances are very similar in their behaviour on ion-exchange resins and consequently are difficult to separate on columns on the preparative scale, especially when they are present in equal amounts. Results of attempts to carry out the separation by eluting bands from one-way paper chromatograms and precipitating the barium salt of phosphoethanolamine in ethanol (cf. Plimmer & Burch, 1937; Kurtz & Luck, 1935) were not very encouraging, but electrodialysis seemed more promising. A three-compartment electrodialysis cell was set up as described by Campbell & Work (1952), the volume of each compartment being about 750 ml. A Permutit anion-exchange membrane (A10) was used between anode and centre compartments and a cellophan membrane between cathode and centre compartments. A carbon rod was used for the cathode and a platinum wire for the anode. The fractions containing phosphoethanolamine and taurine were diluted to 500 ml., brought to pH 6.5 with 2N-NaOH and placed in the centre compartment. The cathode and anode compartments were filled with water and brought to pH 9 with aq. NH_3 soln. and to pH 3 with dil. H_2SO_4 respectively. A d.c. potential of 190 v was applied, when a current of 40 ma passed which rose to 900 ma over the course of 2 hr. At this point two-thirds of the contents of the anode compartment were removed and replaced with water, when the current dropped to 400 ma and gradually fell to 100 ma during the next 4 hr., when the run was stopped. Analysis showed that the anode fractions contained phosphoethanolamine and a small amount of taurine. The fractions were put on a train of two columns of bed size 3.5 cm. $\times$ 12.5 cm. and 2 cm. $\times$ 6 cm. packed with the Dowex 2 resin used previously. Displacement was carried out with 0.1N-HCl

and fractions were collected as before. The first three amino acid-containing fractions contained decreasing amounts of taurine and increasing amounts of phosphoethanolamine, the next three fractions contained large amounts of phosphoethanolamine, apparently free from other ninhydrin-reacting material, and the following fractions contained decreasing amounts of phosphoethanolamine and increasing amounts of chloride. The fractions containing only phosphoethanolamine were evaporated on a rotary evaporator, when crystals separated out. These were filtered off, twice recrystallized from 80% (v/v) methanol and once from water and dried at 100°. Yield 40 mg., which represents a very small portion of that present in the original urine.

Confirmation of identity. The isolated material showed identical behaviour with synthetic phosphoethanolamine (Clarke, Datta & Rabin, 1955) on paper chromatography in phenol-water (NH_3), lutidine-water (diethylamine) and butanol-pyridine-water, and on paper ionophoresis at pH 2 and pH 5.5, and in all cases gave only one spot on the paper. Its behaviour on ion-exchange resins and in the electrodialysis cell during the isolation was that expected of phosphoethanolamine. The m.p. of the isolated material was 242–243° (corr.), of synthetic phosphoethanolamine 243–244° (corr.) and mixed m.p. 241–242° (corr.) [Analysis (Organic Microanalytical Laboratory, Imperial College): C, 17.3; H, 5.8; N, 10.1; P, 22.1. Calc. for $\text{C}_6\text{H}_{10}\text{O}_4\text{NP}$: C, 17.0; H, 5.7; N, 9.9; P, 22.0%]. Infrared spectroscopy was carried out by Dr G. Wilkinson (King's College, London) who found that the isolated material and synthetic phosphoethanolamine gave identical absorption spectra. The isolated material was considered to be pure phosphoethanolamine.

DISCUSSION

The positive identification of phosphoethanolamine confirms the original suggestion of McCance *et al.* (1955) and Fraser *et al.* (1955) that it is excreted in excess in hypophosphatasia. Recently, Fraser's group in Toronto have succeeded in isolating a small amount of crystalline material from the urine of the physically normal father of a child case of hypophosphatasia. Examination of the melting point, X-ray-diffraction pattern, infrared spectrum and phosphorus:nitrogen ratio has led them to conclude that the material is phosphoethanolamine (Dr D. Fraser, personal communication). The finding of increased excretion of phosphoethanolamine in all cases so far examined suggests that it is part of the biochemical disturbance which occurs in hypophosphatasia. The original suggestion of Rathbun (1948) that the primary defect in this disease is a deficiency of alkaline phosphatase is still accepted and it is now considered to be genetically determined, so that this may be an inborn error of metabolism (Garrod, 1908) in which phosphoethanolamine is an intermediary metabolite excreted as a consequence of the metabolic block. The metabolism of other phosphate esters may also be abnormal but this has not so far been studied in detail; phosphoethanolamine has been

detected because it reacts with ninhydrin on amino acid chromatograms whereas most other phosphate esters do not. McCance, Fairweather, Barrett & Morrison (1956) have tentatively reported the excretion of adenosine monophosphate in one of their cases but this has not yet been confirmed (Dr T. A. J. Pranker, personal communication).

Robison (1923) described the occurrence of phosphatase in ossifying cartilage and suggested that the hydrolysis of 'some phosphoric ester' might be a factor in bone formation. Since that time there has been much speculation as to the nature of the physiological substrate for phosphatase, and the role of this enzyme in bone formation is still uncertain (Bourne, 1956). It is tempting to link together the findings in hypophosphatasia of reduced phosphatase activity, phosphoethanolamine excretion and deranged bone growth and to suggest that phosphoethanolamine might be a substrate for phosphatase in bone formation. Phosphoethanolamine occurs abundantly in tissues (Colowick & Cori, 1939; Tallan, Moore & Stein, 1954), and may be concerned in phospholipid metabolism (Ansell & Dawson, 1952), but apart from this its function in the body is not known. It is not normally detected in plasma or urine and thus is probably usually an exclusively intracellular substance. Phosphoethanolamine is hydrolysed by alkaline phosphatase *in vitro* (McCance *et al.* 1956), but, owing to the low specificity of this enzyme, the fact that phosphoethanolamine can act as a substrate does not prove that it is a physiological substrate for phosphatase, so this can be no more than a tentative suggestion.

The exact significance of excretion of phosphoethanolamine in hypophosphatasia is not known, but from a practical point of view it can be of use in the diagnosis of this disease. Increased excretion of phosphoethanolamine is sometimes seen in liver disease but is then part of a generalized amino aciduria (Walshe, 1951); hypophosphatasia is the only condition so far encountered in which there is a gross excretion of phosphoethanolamine with no other change in the pattern of excretion of amino acids.

SUMMARY

Phosphoethanolamine has been isolated in pure form from the urine of an adult case of hypophosphatasia and identified by chemical and physical examination.

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