104. *Constitution of Glycerophosphates. Part III.*

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α-GLYCEROPHOSPHATES have been synthesised by three methods : (1) by King and Pyman (J., 1914, **105**, 1238) by the action of trisodium phosphate upon α-monochlorohydrin, and isolation of the calcium salt (containing 0·9% H_2O, and solubility in water 1·9%); (2) by Bailly (*Compt. rend.*, 1915, **160**, 663) by oxidising sodium allylphosphate with potassium permanganate and isolation of the calcium salt (solubility in water 4·95% at 15°); (3) by Fischer and Pfähler (*Ber.*, 1920, **53**, 1606) by the hydrolysis of barium *iso*propylidene α-glycerophosphate and isolation as barium salt.

In view of Bailly's doubt (*Compt. rend.*, 1915, **161**, 677) as to the purity of King and Pyman's calcium α-glycerophosphate, Hill and Pyman (J., 1929, 2236) re-prepared α-glycerophosphates by the three methods indicated above and converted each preparation into the calcium and the quinine salt.

The King–Pyman and Fischer–Pfähler α-glycerophosphates readily gave pure quinine α-glycerophosphate, but the Bailly α-glycerophosphate gave an impure quinine salt. Further, the King–Pyman and Fischer–Pfähler α-glycerophosphates gave the same sparingly soluble, almost anhydrous calcium salt, whereas the Bailly glycerophosphate gave a calcium salt, containing $1H_2O$, which was soluble in water at 15° to the extent of 4·7%. Hill and Pyman concluded that pure α-glycerophosphates could be prepared by the methods of King and Pyman and Fischer and Pfähler, but threw doubt upon the purity of Bailly's calcium α-glycerophosphate, especially in view of the character of the quinine salt prepared from it. In the meantime, important contributions have been made to our knowledge of glycerophosphates from various sources, and the question of the purity of specimens prepared by the three methods has been reinvestigated in the light of this new information.

Three recently published new methods of analysis for estimating α-glycerophosphates in mixtures of α- and β-glycerophosphates have been tested. Kay and Lee's observation (*J. Biol. Chem.*, 1931, **91**, 135) that α- and β-glycerophosphates are hydrolysed at different rates by the enzyme takaphosphatase has been fully confirmed. By determining the rates of hydrolysis of pure α- and β-salts with that of a mixture of the two in unknown proportions, the composition of the latter can be calculated. The manipulations involved, however, are long and tedious, and the results are only trustworthy when the absence of enzyme-poisons is certain. Attempts to substitute hydrolysis with hydrochloric acid for enzyme hydrolysis were unsuccessful, since the rates of hydrolysis of the α- and β-glycerophosphates were identical.

Carrara (*Giorn. Chim. ind. appl.*, 1932, **14**, 236) has described a method of estimating α-glycerophosphates, which is based on the observation by Criegee (*Ber.*, 1931, **64**, 260) that lead tetra-acetate oxidises α-glycols quantitatively, but does not attack β-glycols. We found that the oxidation of sodium α-glycerophosphate by lead tetra-acetate in acetic acid solution proceeded very slowly, the reaction being incomplete after 24 hours at 18°. On allowing the reactants to stand for longer periods, the oxidation proceeded beyond the theoretical limit, the percentage of sodium α-glycerophosphate found in the pure salt being, for instance, 80·6% in 25 hours, 104·3% in 120 hours, and 145·9% in 288 hours. The apparent percentage of sodium α-glycerophosphate found by this method in pure sodium β-glycerophosphate was 6·0% in 25 hours and 15·5% in 120 hours. This method is therefore unsatisfactory as an absolute method for the quantitative estimation of α-glycerophosphates.

A third method, which has been recommended by Fleury and Paris (*Compt. rend.*, 1933, **196**, 1416) for the estimation of α-glycerophosphates in the presence of β-glycerophosphates, is based on their discovery that α-glycerophosphates are smoothly and quickly oxidised by periodic acid at the ordinary temperature in the sense of the equation $CH_2(OH)\cdot CH(OH)\cdot CH_2\cdot O\cdot PO(OH)_2 + HIO_4 = HCHO + CHO\cdot CH_2\cdot O\cdot PO(OH)_2 + HIO_3 + H_2O$ whereas β-glycerophosphates are not appreciably attacked even after 24 hours. This method proved to be entirely satisfactory and the details of the mode in which we employed it for sodium glycerophosphates with suitable modifications for the calcium and barium salts are given below. The results obtained demonstrate clearly that α-glycerophosphates prepared by the methods of King and Pyman and of Fischer and Pfähler are pure, but that α-glycerophosphates prepared by the method of Bailly contain varying amounts of some impurity. The purity of King and Pyman's calcium α-glycerophosphate has been confirmed further with the help afforded by the discovery of Charpentier and Bocquet (*Compt. rend.*, 1932, **194**, 104; *Bull. Soc. chim.*, 1932, **51**, 413) that sodium α-glycerophosphate can be obtained as a stable well-crystallised hexahydrate. When King and Pyman's calcium α-glycerophosphate was treated with sodium oxalate it gave a practically quantitative yield of sodium α-glycerophosphate hexahydrate, after inoculation with crystals of this salt kindly given to us by Messrs. Societé des Usines Chimiques Rhône-Poulenc, in whose laboratories it was discovered by the above authors. Charpentier and Bocquet comment on the difference in properties between sodium α-glycerophosphate as described by themselves and by King and Pyman respectively. It is shown below that the two forms are interconvertible.

Charpentier and Bocquet found that calcium α-glycerophosphate prepared from the sodium salt and calcium chloride showed an initial solubility of about 5% in cold water, but that the solution deposited a precipitate on keeping and did not then retain more than 2·8% of calcium α-glycerophosphate. We confirm this result, except that the solubilities were 4·7% and 2·0% in our hands, but find that the initially soluble form is a hydrate (Found: loss at 140°, 7·6. $C_3H_7O_6PCa,H_2O$ requires H_2O, 7·9%), and that in general the solubility of calcium α-glycerophosphate runs parallel with its state of hydration, specimens containing from 0·1% to 0·8% H_2O having solubilities in water ranging from 1·28% to 1·8% at 13°.

The results of the investigation fully confirm King and Pyman's claim to have been the first to prepare calcium α-glycerophosphate in a pure state.

EXPERIMENTAL.

Estimation of α-Salt in Sodium Glycerophosphates.—To about 0·15 g. of the salt, dissolved in 0·1*N*-hydrochloric acid (10 ml.), 0·05*M*-periodic acid (15 ml.) is added and the mixture is kept for 15 minutes and then neutralised with 0·1*N*-sodium hydroxide (phenolphthalein). After addition of a saturated aqueous solution of sodium bicarbonate (10 ml.) and potassium iodide (1 g.), an immediate titration is made with 0·1*N*-arsenite solution, a blank titration being performed with 0·05*M*-periodic acid (15 ml.) similarly treated. (In neutral solution, periodic acid liberates iodine from potassium iodide, but iodic acid does not.)

The results obtained are independent of the time of keeping; *e.g.*, pure sodium α-glycerophosphate hexahydrate showed 100·3% α-content after 15 minutes and 100·2% after 24 hours, and pure sodium β-glycerophosphate showed no α-content after 15 minutes and none after 24 hours.

Owing to the insolubility of calcium and barium periodates, the method had to be modified in order to be suitable for the analysis of calcium and barium glycerophosphates.

Estimation of α-Salt in Barium Glycerophosphates.—A weighed amount (*ca.* 0·2 g.) of the salt, dissolved in 0·1*N*-hydrochloric acid (15 ml.), is well shaken with sodium sulphate (0·2 g.). An aliquot (10 ml.) of the filtered solution is then treated as for the sodium salt.

Estimation of α-Salt in Calcium Glycerophosphates.—To about 0·1 g. of the salt, dissolved in water (5 ml.) and 5*N*-hydrochloric acid (0·5 ml.), 0·05*M*-periodic acid (15 ml.) is added, followed, after 15 minutes, by water (30—40 ml.), 5*N*-hydrochloric acid (2 ml.), and potassium iodide (2 g.). The liberated iodine is titrated with 0·1*N*-sodium thiosulphate, a blank experiment being performed as before. (In acid solution, both periodic and iodic acids liberate iodine from potassium iodide.)

By employing these methods for the estimation of α-glycerophosphate content, the following results were obtained: Calcium salts: *King and Pyman.* Found, in salt dried at 140°: Ca, 19·0; P, 14·8; α-salt 99·7 (Calc.: Ca, 19·1; P, 14·8%). Found, in two other batches, α-salt, 99·6; 100·1%. *Bailly.* Found, in salt dried at 140° (two batches): Ca, 19·2, 19·1; P, 14·9, 15·0; α-salt 88·5, 89·9%. In two further batches, in salt dried at 140° (1) Ca, 19·3; P, 14·7; (2) Ca, 19·1; P, 14·9; loss at 140°, (1) 15·7; (2) 6·3; in air-dried salt, α-salt, (1) 73·2, 73·2; (2) 86·5, 86·9; whence α-salt in anhydrous (1) 86·8; (2) 92·5%. Barium salts: *King* *and Pyman.* Found : loss at 140°, 0·6. In dried salt, Ba, 44·6; α-salt 100·1, 100·2% (Calc. : Ba, 44·7%). *Fischer and Pfähler.* Found : loss at 140°, 1·6. In dried salt, Ba, 44·6; α-salt 99·7, 99·9%.

Sodium α-Glycerophosphate Hexahydrate from Calcium Salt (King and Pyman).—Solutions of calcium α-glycerophosphate (King and Pyman) (36·3 g.) in water (3985 ml.) and sodium oxalate (23·25 g. of 99·6% $Na_2C_2O_4$) in water (1 l.) were stirred for 5 hours. After removal of calcium oxalate, the filtrate was evaporated (15 mm.) to 63 g. and kept for a week at 0°. The crystalline mass was triturated with alcohol, and the solid extracted and washed with alcohol, yielding a first crop (55·05 g.). The combined filtrate and washings were evaporated to about 1 g., allowed to dry in the air, and washed with alcohol, yielding a second crop (0·9 g.). Found, by titration with sulphuric acid (methyl-orange), $C_3H_7O_6PNa_2,6H_2O$, in first crop, 100·3; in second crop, 96·2%. Found, by periodic acid method, α-salt content of first crop, 100·1, of second crop, 93·2%. Whence, yield of pure sodium α-glycerophosphate hexahydrate=99·8%.

Interconversion of Hydrated and Anhydrous Sodium α-Glycerophosphate.—The hexahydrate (6 g.) was dried at 100°/15 mm., and the residue extracted with methyl alcohol (800 ml.) at −5°. On concentration of the extract to 100 ml. colourless deliquescent leaflets separated, which were washed with dry ether (yield, 3·34 g.; 83·5%) (Found : loss at 100°, 1·1%. In dried salt, 0·2064 g. required 9·77 ml. 0·1*N*-H_2SO_4. Calc. for anhydrous salt, 9·82 ml.).

The above almost anhydrous salt (2 g.) was dissolved in hot water (1·75 g.), and the solution kept at 0°. The crystalline mass was broken up, washed with alcohol, and air-dried (yield 2·7 g.; 90%) (Found : 0·3096 g. required 9·57 ml. 0·1*N*-H_2SO_4. Calc. for hexahydrate, 9·55 ml.).

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