LVI. ESTERS OF PHOSPHORIC ACID

III. PHOSPHORYLAMINOETHANOL AND PHOSPHORYLCHOLINE

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AMONGST the esters of phosphoric acid those of colamine (aminoethanol) and choline are of interest on account of their connexion with kephalin and lecithin. Like other esters of these bases they are preferably designated phosphorylaminoethanol, or phosphorylcolamine, and phosphorylcholine to distinguish them from the salts, colamine phosphate and choline phosphate. These salts have been described by Grün & Limpacher [1926] and by Renshaw [1910].

Neither by the interaction of phosphoric acid and phosphorus pentoxide, nor of phosphoryl chloride, with choline did Schmidt [1904] obtain a specimen of the ester. Langheld [1911] and Abderhalden *et al.* [1925] tried to obtain the ester by the action of trimethylamine on the reaction products of chloroethyl alcohol and bromoethyl alcohol with ethyl metaphosphate. This reaction, as we have shown [1929] leads to a mixture of products very difficult to separate. Renshaw & Ware [1925] failed to obtain the ester by the action of phosphoryl chloride on choline, but Renshaw & Hopkins [1929] prepared the choline ester of dimethylphosphoric acid. Jackson [1935] isolated an amorphous calcium salt by the action of phosphoric acid and phosphorus pentoxide upon choline chloride and also obtained tricholyl phosphate from trichloroethyl phosphate.

There was no record of the preparation of phosphorylaminoethanol at the time of the commencement of our experiments, but since our work with this compound was completed the isolation of the ester from tumours and its synthesis have been described by Outhouse [1936]. Our work has been the preparation of both esters by the action of phosphoryl chloride, phosphoric acid and pentoxide, upon choline chloride and aminoethanol and by the action of ammonia and trimethylamine upon chloroethylphosphoryl chloride. The esters have been isolated as their calcium or barium salts in pure form and converted into the free esters. Their hydrolysis by acid, alkali and phosphatase has been investigated. Some further investigations have been made upon chloroethyl and hydroxyethyl hydrogen phosphates.

EXPERIMENTAL

Interaction of phosphoryl chloride and aminoethanol

5 g. of aminoethanol were added, 4-5 drops at a time, to 12.5 g. of ice-cold POCl_3 , and the mixture was kept in a flask connected with NaOH traps to absorb HCl for 20 days and subsequently over NaOH and H_2SO_4 for 14 days. The resulting yellowish white mass was dissolved in ice-water and the solution treated with excess of baryta water till the reaction was alkaline to phenolphthalein and filtered from barium phosphate. Chlorides were removed with

a slight excess of silver sulphate, and the solution, freed from silver with H_2S , was again treated with baryta water and filtered from barium phosphate. The solution after concentration *in vacuo* contained 1.45 g. of combined phosphorus. On adding an equal volume of absolute alcohol a crystalline precipitate was produced, which was filtered off, washed with 50 % alcohol and air-dried. Analysis indicated that the crystals were barium phosphorylaminoethanol contaminated with barium carbonate. A further crop of crystals, which became sticky in the air, was obtained by adding more alcohol to the filtrate. These crystals appeared to be a mixture of the neutral and acid barium salts of phosphorylaminoethanol. Recrystallization of these products did not give purer preparations. It was not observed till our experiments had been made with phosphoric acid and pentoxide that the solution must remain alkaline if a pure specimen of the barium salt is to be obtained.

Interaction of $\text{H}_3\text{PO}_4 + \text{P}_2\text{O}_5$ and aminoethanol

Several experiments were made to ascertain the best conditions for the formation of the ester and the most suitable way of isolating the barium or calcium salt, which, owing to the basic character of the aminoethanol, was easily contaminated with the acid salt or with carbonate. In each experiment, 5 g. of aminoethanol were added slowly with cooling between each addition to a mixture of 4 g. of P_2O_5 and 40 g. of H_3PO_4 and allowed to stand for 120 hours or better heated under a reflux on a water-bath for 6 hours. Powdered ice was added to dissolve the resulting thick syrup and the solution poured into about 1500 ml. of ice-water. Powdered barium hydroxide, or a paste of calcium hydroxide, was well shaken with the solution until the reaction was alkaline to phenolphthalein. The precipitated barium or calcium phosphate was filtered off and washed with dilute baryta or lime water. In one experiment with baryta the precipitate was extracted with water and a further quantity of ester was obtained. Estimation of the combined phosphorus in the solution showed amounts of 0.6 g. in the cold reaction and 2.6 g. when heated, and up to 4.1 g. when the precipitate was extracted. With calcium hydroxide the amount was 1.75 g. The yield of ester was thus almost quantitative in one experiment. The solutions were evaporated *in vacuo* to a volume of 100–200 ml.

Barium salt. This salt was precipitated on adding slowly an equal volume of alcohol. If the reaction was only faintly alkaline a crystalline precipitate was formed, which after washing with 60 % alcohol was sticky and on analysis was found to consist of the neutral salt mixed with some acid salt. If the reaction was not alkaline an oil was precipitated. It was found best to redissolve the precipitate with the addition of a few ml. of conc. HCl and to add to the alcoholic solution clear baryta water and more alcohol keeping the reaction just alkaline to phenolphthalein. Shining platelets then separated out. A further small crop was obtainable on concentrating the filtrate *in vacuo* and treating it in the same way. In one experiment a yield of 24 g. was obtained = 94 %.

Analysis. Found: N, 4.24; P, 9.49; Ba, 41.71; H_2O at 100° , 10.8 %. $\text{C}_2\text{H}_6\text{O}_4\text{NPBa} \cdot 3\text{H}_2\text{O}$ requires N, 4.24; P, 9.39; Ba, 41.51; H_2O , 16.36 %.

Except for water of crystallization the figures are in agreement. As in the case of other esters of phosphoric acid all water is probably not evolved at 100° .

Purification by solution in water and addition of alcohol gave specimens containing barium carbonate or some acid salt. The purest specimens of the salt were obtained by dissolving in water, passing in CO_2 , evaporating to a smaller

volume *in vacuo*, filtering from BaCO_3 and adding alcohol until an oily precipitate began to form. On now adding clear baryta water and also more alcohol, keeping the solution alkaline, shining platelets were precipitated. These were rapidly filtered off and washed with 50 %, 60 % and absolute alcohol. Found: N, 4.18; P, 9.29; Ba, 41.69; H_2O , 10.57 %. The water of crystallization is again too low.

Acid barium salt. 20 g. of a mixture of neutral and acid barium salts from another preparation were dissolved in water, CO_2 passed into the solution, and the whole evaporated *in vacuo*. To the filtered solution absolute alcohol was added till oil separated out. The alcoholic layer was decanted and the oil covered with fresh alcohol. On allowing to stand for 3–4 weeks the oil gradually hardened and finally crystallized. The crystals were separated and washed with absolute alcohol; yield, 8.6 g. These crystals contained N, 5.85; P, 12.93; Ba, 26.59; H_2O , 13.14 %, and at 100° melted in their water of crystallization. The salt on exposure to the air gradually took up water to 17.78 %. The analysis then corresponded with $\text{C}_4\text{H}_{14}\text{O}_8\text{N}_2\text{P}_2\text{Ba}$, $5\text{H}_2\text{O}$ which requires N, 5.52; P, 12.23; Ba, 27.02; H_2O , 17.75 %.

The original low value for water was probably due to dehydration by the treatment with absolute alcohol.

Calcium salt. This salt began to crystallize out during evaporation of the solution to a smaller volume in very fine crystals which were most difficult to filter off. All the portions analysed contained carbonate. A pure specimen of the calcium salt was not obtained.

Ammonium salt. Owing to the difficulty experienced with the calcium salt, the various portions were combined and converted into the ammonium salt by dissolving in water with the aid of a little hydrochloric acid and treating with ammonium oxalate and ammonia. The filtrate from calcium oxalate was evaporated *in vacuo* until ammonium chloride began to crystallize. This was just redissolved in water and on adding alcohol an oil started to separate. On saturating the solution with ammonia gas, the ammonium salt crystallized out. Further crops were obtained from the filtrate by adding alcohol saturated with ammonia. The crystals on analysis had the composition: N as NH_3 , 7.61, 8.24, 7.32; total N, 13.12, 13.93, 15.27; P, 14.26, 15.37, 15.57; H_2O , 27.97, 19.86, 23.39 %, and correspond best with $\text{C}_2\text{H}_{11}\text{O}_4\text{N}_2\text{P}$, $2\frac{1}{2}\text{H}_2\text{O}$, which requires N as NH_3 , 6.90; total N, 13.79; P, 15.27; H_2O , 22.16 %.

A further preparation of ammonium salt was made from the filtrates from the barium salt. The alcohol was evaporated off *in vacuo*, a slight excess of ammonium sulphate added to the aqueous solution and then barium chloride exactly to remove sulphate. The solution after filtration from barium sulphate was evaporated *in vacuo* to a small volume and treated with alcohol saturated with ammonia. 2.2 g. of crystals resulted. Air-dried they had the composition: N as NH_3 , 7.17; total N, 13.96; P, 16.32; H_2O , 20.9 %, which corresponds best with $\text{C}_2\text{H}_{11}\text{O}_4\text{N}_2\text{P}$, $2\text{H}_2\text{O}$.

Other salts. A solution of the barium salt treated with appropriate solutions of other metallic salts gave no insoluble salt by double decomposition. A solution of phosphorylaminoethyl alcohol of about 1 % concentration was not precipitated with basic lead acetate. All salts of the ester are thus soluble in water.

Phosphorylaminoethanol. The free ester was prepared from 16 g. of barium salt containing some barium chloride by dissolving in water, and treating with a suspension of silver oxide, filtering from AgCl and excess of Ag_2O , treating with H_2S , filtering from Ag_2S and evaporating *in vacuo*. The solution was then freed from Ba and SO_4 ions and evaporated to about 50 ml. Absolute alcohol

added in portions of 10 ml. to the extent of 200 ml. gave crystals on standing consisting of long prisms. They were filtered off, washed with 80 % alcohol and absolute alcohol and air-dried: yield, 5.7 g. On analysis they corresponded with phosphorylaminoethanol. Found: N, 9.67, 9.73; P, 22.26, 21.75 %. $\text{C}_2\text{H}_5\text{O}_4\text{NP}$ requires N, 9.93; P, 21.99 %. They melted with decomposition at $232\text{--}233^\circ$ on slow heating.

*Interaction of phosphorylchloroethanol and phosphorylchloroethyl
chloride with ammonia*

The products of this reaction were complicated and it was necessary to carry out four experiments to isolate phosphorylaminoethanol and study the other products.

Exp. 1. 21 g. of phosphorylchloroethanol (chloroethyl dihydrogen phosphate) were slowly added to 300 ml. of NH_4OH (sp. gr. 0.880) and the solution was heated in an autoclave at 120° for 4 hours. On cooling 1.5 g. of $(\text{NH}_4)_2\text{HPO}_4$ separated out. The solution was evaporated *in vacuo* and at the same time treated with barium hydroxide solution to remove NH_3 . After filtering from $\text{Ba}_3(\text{PO}_4)_2$ the concentrated solution on gradual addition of absolute alcohol gave 3 crops of crystals amounting to 13.1 g. which analysis indicated to be impure barium phosphorylaminoethanol. They were dissolved in water, treated with Ag_2SO_4 to remove Cl and Ba, H_2S to remove Ag, boiled with $\text{Ba}(\text{OH})_2$ to remove NH_3 and finally treated with H_2SO_4 to remove Ba. The concentrated solution on treatment with absolute alcohol gave 2.1 g. of phosphorylaminoethanol, melting at 228° and containing N, 9.36; P, 22.56; H_2O , 0.9 % (calc. N, 9.93; P, 21.99 %). The filtrate on addition of acetone gave an oil, which on solution in water and neutralization with $\text{Ba}(\text{OH})_2$ gave 2.5 g. of barium phosphorylhydroxyethanol: Ba, 43.30, 43.63; P, 9.44, 10.10; H_2O , 9.03, 10.49 % at 100° *in vacuo*. $\text{C}_2\text{H}_5\text{O}_5\text{PBa}, 2\text{H}_2\text{O}$ requires Ba, 43.84; P, 9.89; H_2O , 11.49 %. The solution on neutralization with $\text{Ba}(\text{OH})_2$ gave 7.3 g. of barium phosphorylhydroxyethanol: Ba, 45.66; P, 10.59; H_2O , 6.65, 8.34 % at 100° *in vacuo*. $\text{C}_2\text{H}_5\text{O}_5\text{PBa}, \text{H}_2\text{O}$ requires Ba, 46.51; P, 10.49; H_2O , 6.09 %. The dried substance contained 49.54 % Ba; 11.31 % P corresponding with the calc. 49.52 % Ba, 11.17 % P. It took up 6.18 % water on exposure to air. The salt thus appears to crystallize with one or two molecules of water.

The original solution on concentration and addition of alcohol gave mixtures of barium phosphorylhydroxyethanol and chloroethanol, which could not be separated.

The chief product of the reaction was phosphorylhydroxyethanol.

Exp. 2. 20 g. of chloroethylphosphoryl chloride were slowly added to 200 ml. of ice-cold NH_4OH (sp. gr. 0.880); on reaching room temperature the solution was heated in an autoclave at 130° for 1 hour. The solution was diluted with water and evaporated *in vacuo* and at the same time treated with calcium hydroxide solution to remove NH_3 . The solution filtered from $\text{Ca}_3(\text{PO}_4)_2$ contained 1.3 g. N and 3.0 g. P. On further evaporation very fine crystals separated which clogged the filter on filtration. They were redissolved with a small quantity of dilute hydrochloric acid and the solution at once made faintly alkaline with calcium hydroxide. The concentrated solution on treatment with increasing quantities of absolute alcohol gave several crops of crystals containing from 4 to 9 % N and from 14 to 18 % P. The nitrogen was present partly as amide-N and partly as amino-N in each crop. The crystals appeared to be amides of phosphorylhydroxyethanol and aminoethanol. Attempts to separate them into pure compounds were not successful.

Exp. 3. 35 g. of chloroethylphosphoryl chloride were gradually added to 450 ml. of ice-cold NH_4OH (sp. gr. 0.880) and the solution was heated in an autoclave at 130° for 6 hours. On cooling 5 g. of $(\text{NH}_4)_2\text{HPO}_4$ separated out. The solution on evaporation gave 17.3 g. of NH_4Cl . The concentrated solution was then treated with absolute alcohol saturated with ammonia and gave 11.5 g. of ammonium phosphorylaminoethanol (yield, 11.3%) from which 7.6 g. of the pure compound melting at 230° were obtained on recrystallization from alcohol and ammonia. Found: N, 14.48; P, 15.68; H_2O , 18.93%. $\text{C}_2\text{H}_{11}\text{O}_4\text{N}_2\text{P} \cdot 2\text{H}_2\text{O}$ requires N, 14.43; P, 16.00; H_2O , 18.56%. Ammonia was removed from the original filtrate by evaporation *in vacuo* and addition of baryta solution, and alcohol was added gradually to the concentrated solution. Several crops of crystals were produced of which the N and P contents indicated that they were mixtures of barium phosphorylaminoethanol and barium phosphorylhydroxyethanol. Separation by fractional crystallization was not successful. From 9 g. of such a mixture Cl and Ba were removed with Ag_2SO_4 , Ag with H_2S and the concentrated solution gave with alcohol 1.3 g. of phosphorylaminoethanol, melting at 230° and containing N, 9.96; P, 22.0%; yield, 0.9%. The total yield of phosphorylaminoethanol in this experiment was thus about 12%.

Exp. 4. 20 g. of chloroethylphosphoryl chloride were slowly added to 200 ml. of ice-cold NH_4OH (sp. gr. 0.880) and the solution was heated in an autoclave at 130° for 8 hours. The solution was diluted with about 1 litre of water, treated with excess of $\text{Ca}(\text{OH})_2$ suspension, filtered from $\text{Ca}_3(\text{PO}_4)_2$ and evaporated *in vacuo*, keeping the solution alkaline with lime solution. The concentrated solution contained 1.2 g. N and 1.6 g. P. Carbon dioxide was then passed through the solution and it was evaporated to 300 ml. and treated with 500 ml. of absolute alcohol. 12.8 g. of crystals corresponding to impure calcium phosphorylaminoethanol separated out (yield, 42.5%). The salt was dissolved in water, Ca and Cl removed with silver sulphate and alcohol, and the solution concentrated. On treating with 2 volumes of absolute alcohol 5.4 g. of phosphorylaminoethanol melting at 231° were obtained containing N, 9.85; P, 22.43; H_2O , 0.47%; yield, 30.8%.

Interaction of phosphoryl chloride and choline chloride

As preliminary trials indicated that some phosphorylcholine was formed in this reaction, numerous other experiments were made to ascertain how the yield could be increased. The best yield of 0.6 g. of combined phosphorus was obtained by slowly adding 8 g. of ice-cold POCl_3 to 5 g. of ice-cold choline chloride and allowing to stand in connexion with NaOH traps for 11 days. The choline chloride dissolved and in a few days crystals formed in the liquid. The yields were almost nothing if pyridine were used in the reaction. The product was dissolved in ice-cold water, the solution made faintly alkaline with barium hydroxide solution and filtered from barium phosphate. Separation of barium phosphorylcholine from barium chloride by crystallization was found impossible, all specimens consisting of mixtures of the two salts. Difficulty was also experienced after quantitatively removing barium chloride with silver sulphate. The solution after making faintly alkaline with barium hydroxide and concentrating *in vacuo* and treating with alcohol gave beautiful glistening platelets. On analysis these crystals were always found to be contaminated with barium carbonate. It seemed that the trimethylamine group displaced barium hydroxide from the salt, which during filtration and drying absorbed carbon dioxide from the air. The experiments were not continued as it was found that better yields of the ester were formed by the action of $\text{H}_3\text{PO}_4 + \text{P}_2\text{O}_5$, and that the ester was preferably isolated as the calcium salt of the ester chloride.

Interaction of $H_3PO_4 + P_2O_5$ and choline chloride

5 g. of choline chloride were added in small portions to 40 g. of $H_3PO_4 + 4$ g. of P_2O_5 . In the first experiment the reaction was allowed to proceed at room temperature for 120 hours. In the second and subsequent ones the mixture was heated under a reflux with a $CaCl_2$ tube for 6 hours and allowed to cool. Crushed ice was added to dissolve the resulting syrup and the solution poured into about 1.5 litres of ice-water. The solution was thoroughly shaken with powdered barium hydroxide or a paste of calcium hydroxide until it was alkaline to phenolphthalein and filtered from barium or calcium phosphate and excess of reagent. The yield of combined phosphorus in the cold was 0.3 g., whilst on heating it averaged 1 g., almost the theoretical amount.

Barium salt. Barium carbonate always separated on evaporation of the clear solution and again during filtration. The solution was therefore just acidified with HCl, filtered and made just alkaline. On now adding about an equal volume of alcohol glistening platelets separated out. These when filtered off, washed with 50 % alcohol and absolute alcohol weighed 8.5 g. (air-dried). Their analysis corresponded with barium phosphorylcholine chloride. Found: N, 3.1; P, 7.4; Ba, 32.4; Cl, 8.4; H_2O at 100° , 8.4 %. $C_5H_{13}O_4NPClBa \cdot 4H_2O$ requires N, 3.3; P, 7.3; Ba, 32.2; Cl, 8.3; H_2O , 16.88 %. It was found later that the water of crystallization was not completely given off at 100° *in vacuo* over P_2O_5 .

Further quantities mixed with barium chloride were precipitated from the filtrate after concentration and addition of alcohol. Separation by fractional crystallization was not entirely successful.

It was found that the salt was best crystallized by dissolving in water, making the solution exactly neutral to litmus and adding an equal volume of alcohol. A specimen thus obtained from a mixed sample with barium chloride had the composition: N, 3.04; P, 7.34; Ba, 32.16; H_2O , 8.79, 12.33 % at 100° *in vacuo* over P_2O_5 .

Calcium salt. On evaporation of the solution calcium carbonate precipitated in the same way as in the case of the barium salt. After evaporation and filtration from carbonate to a volume of 140 ml. glistening crystals consisting of fine needles were precipitated by adding an equal volume of absolute alcohol; they were filtered off, washed with 60 %, 80 % and absolute alcohol and dried in the air. Their composition corresponded with that of calcium phosphorylcholine chloride. Found: N, 4.25; P, 9.55; Ca, 12.5; Cl, 10.65; H_2O , 15.0 %. $C_5H_{13}O_4NPClCa \cdot 4H_2O$ requires N, 4.25; P, 9.46; Ca, 12.16; Cl, 10.75; H_2O , 21.86 %. A further quantity was obtained on concentrating the filtrate and adding an equal volume of alcohol. The total yield of calcium salt was 9.2 g. air-dried or 78 %.

After re-dissolving in water, filtering clear from calcium carbonate and adding an equal volume of alcohol 7.8 g. of pure salt were obtained. Found: N, 4.34; P, 9.33; Ca, 12.21; Cl, 10.25; H_2O , 10.47 %, at 100° *in vacuo* over P_2O_5 , 19.49 %. The calcium salt is preferable to the barium salt for isolation of phosphorylcholine.

Other salts. On testing a 1 % solution of the calcium salt with appropriate solutions of other metallic salts there was not any formation of an insoluble salt. No precipitate was given on adding a solution of basic lead acetate to a 1 % solution of phosphorylcholine. All salts of phosphorylcholine are thus soluble. A double salt with platinum chloride separated in sheaves from an alcoholic solution.

Phosphorylcholine. The free ester was prepared from 12.5 g. of the calcium salt, which were dissolved in 500 ml. of water and treated with a suspension of Ag_2O . The reaction of the solution became alkaline and it was made slightly acid with H_2SO_4 and filtered. The filtrate was treated with Ag_2SO_4 solution to remove the last trace of Cl and again filtered. It was then treated with H_2S , filtered from Ag_2S , boiled, neutralized with lime water and evaporated *in vacuo* to a small volume. The addition of an equal volume of absolute alcohol removed CaSO_4 and the filtrate was evaporated to dryness. The residue except for a small amount of CaSO_4 dissolved in 60 % alcohol. The solution was treated with absolute alcohol until a turbidity was no longer given, and on standing an oil separated out. The flask and its contents were placed in a vacuum desiccator over CaO and the alcohol slowly evaporated during 4–5 weeks. Crystals appeared on the top of the solution, which was stirred with more absolute alcohol and left in the desiccator. Gradually the oil became a hard pale brownish crystalline solid, which was recrystallized by dissolving in hot absolute alcohol, filtering and allowing to stand over CaO . As crystals did not separate, 2 ml. of ether were added from time to time and a hard crystalline solid slowly separated. This was filtered off rapidly, washed with absolute alcohol and ether and placed in a desiccator over CaCl_2 . The yield was 5 g. of slightly brown, extremely hygroscopic crystals. Analysis indicated that they were phosphorylcholine anhydride. Found: N, 7.43; P, 16.64; H_2O , 1.76 %, at 100° *in vacuo*, 2.05 %. Calc. for

N, 7.65; P, 16.94 %. The slightly lower values are accounted for by the water due to the hygroscopic character of the compound.

Interaction of chloroethylphosphoryl chloride with trimethylamine

In comparison with the interaction of chloroethylphosphoryl chloride with ammonia the reaction with trimethylamine was less complex and phosphorylcholine was easily isolated as the chloride of the calcium salt.

35 g. of chloroethylphosphoryl chloride were gradually added to 250 ml. of ice-cold 33 % aqueous trimethylamine and the solution was heated in an autoclave for 4 hours at 130° . The pale brown solution was diluted with 1 litre of water, treated with $\text{Ca}(\text{OH})_2$ solution till distinctly alkaline to phenolphthalein and filtered from 2.9 g. of calcium phosphate and chloroethyl phosphate. The filtrate was evaporated *in vacuo* keeping the solution alkaline by addition of lime water so as to remove trimethylamine. Several crops of crystals amounting to 13.9 g. consisting of impure calcium phosphorylhydroxyethanol separated out. This substance was very slightly soluble in water and was purified by dissolving in very dilute hydrochloric acid, making the solution just alkaline with $\text{Ca}(\text{OH})_2$ solution and evaporating *in vacuo*. The compound which separated corresponded on analysis with basic calcium phosphorylhydroxyethanol. Found: C, 11.40; H, 4.26; P, 14.03; Ca, 18.52; H_2O , 8.81 % at 100° *in vacuo* over P_2O_5 . $\text{C}_2\text{H}_5\text{OPO}(\text{OH})\text{OCaOH}$, H_2O requires C, 11.15; H, 4.20; P, 14.80; Ca, 18.55; H_2O , 8.33 %. The filtrate from these crops contained 1.4 g. N and 3.0 g. P and on treatment with successive portions of 50 ml. of absolute alcohol gave crops of crystals amounting to 35 g. (yield, 60 %), each of which corresponded on analysis with calcium phosphorylcholine chloride. On recrystallization by solution in water, exact neutralization and addition of successive portions of alcohol the pure compound separated out in masses of glistening needles in a yield of 26.8 g.

(47 %). The several fractions analysed as follows: N, 4.02, 4.14, 4.21, 4.14; P, 9.41, 9.27, 9.38, 9.26; Ca, 12.17, 11.93, 12.06, 12.06; Cl, 10.38, 10.21, 10.08, 10.37 %. H_2O at 100° *in vacuo* over P_2O_5 , 21.26, 21.27, 21.45, 21.99 %. $\text{CH}_2\text{N}(\text{CH}_3)_3\text{Cl} \cdot \text{CH}_2\text{OPO}(\text{OH})\text{OCa} \cdot 4\text{H}_2\text{O}$ requires N, 4.25; P, 9.46; Ca, 12.16; Cl, 10.75; H_2O , 21.86 %. A fair yield of the compound is thus obtained by this method.

Chloroethylphosphoryl chloride

The isolation of this compound was not attempted in our previous work [1928]. It is readily obtained by mixing slowly 1 mol. of POCl_3 with 1 mol. of ethylene chlorohydrin and cooling in ice. The mixture is then boiled under a reflux at 140° for 1 hour and distilled *in vacuo*. After unchanged ethylene chlorohydrin and POCl_3 has distilled over, chloroethylphosphoryl chloride distills as a colourless liquid at 103° and 12 mm. in a yield of 62 %. Found: P, 15.06; Cl, 52.74 %. $\text{C}_2\text{H}_4\text{O}_2\text{PCl}_3$ requires P, 15.60; Cl, 53.6 %.

Barium phosphoryldi-(chloroethanol). The undistilled residue consisted principally of di-(chloroethyl)phosphoryl chloride and was converted into the barium salt of phosphoryldi-(chloroethanol) by treatment with water, neutralization of the solution to phenolphthalein with barium hydroxide, evaporation to a small volume *in vacuo*, and addition of alcohol. The barium salt was then precipitated as a very fine white crystalline powder. Found: at 110° *in vacuo* H_2O , 11.3 %. Calc. for 4 H_2O , 11.03 %; anhydrous salt: Ba, 24.12; P, 10.4; Cl, 23.76 %. $\text{Ba}[(\text{C}_2\text{H}_4\text{Cl})_2\text{PO}]_2$ requires Ba, 23.58; P, 10.67; Cl, 24.44.

Lead phosphoryldi-(chloroethanol). The lead salt was precipitated on adding a solution of lead acetate to a solution of the barium salt, as a white amorphous powder, insoluble in hot water.

Phosphorylchloroethanol. This compound is formed on decomposing chloroethylphosphoryl chloride with water, but its isolation as barium or calcium salt in a pure form proved difficult owing to its ready conversion in faintly alkaline solution into phosphorylhydroxyethanol.

Barium salt. 5 g. of chloroethylphosphoryl chloride were dropped slowly into 1 litre of water and well shaken. When the oil had completely decomposed, the solution was made faintly alkaline with baryta and filtered from a small precipitate of barium phosphate. The filtrate on evaporation *in vacuo* gave a slight precipitate and did not filter clear. It was neutralized to litmus with HCl and again evaporated. The crystals so obtained were filtered off, washed with 10 %, 30 %, 60 % alcohol and air-dried; wt. 1.7 g. The filtrate on treatment with alcohol in 5 ml. portions until an equal volume had been added gave a further crop which weighed 1.4 g. air-dried. Neither crop on analysis corresponded with barium phosphorylchloroethanol. Found: 1st crop: P, 8.69; Ba, 39.06; H_2O , 8.74 %. 2nd crop: P, 8.59; Ba, 37.87; Cl, 7.55; H_2O , 8.85 %, 10.39 % at 100° *in vacuo*. $\text{C}_2\text{H}_4\text{O}_4\text{ClPBa} \cdot 3\text{H}_2\text{O}$ requires P, 8.85; Ba, 39.26; Cl, 10.13; H_2O , 15.44 %. A purer specimen was not obtained on recrystallization.

10 g. of chloroethylphosphoryl chloride were covered and shaken with 500 ml. of water which was kept alkaline with baryta until the oil was completely decomposed. After filtration the filtrate was evaporated till crystallization began. It was then boiled and the crystals so produced filtered off, washed with boiling water, 60 % alcohol and dried. The yield was 6.4 g. (43 %). Found: P, 11.80; Ba, 45.61; Cl, 11.70; H_2O , 0.32 %. $\text{C}_2\text{H}_4\text{O}_4\text{ClPBa}$ requires P, 10.47; Ba, 46.43; Cl, 11.98 %.

The crystals were dissolved in a minimum of water and an equal volume of alcohol added. After filtering and washing with 60 % alcohol, the air-dried crystals weighed 7.3 g. and corresponded most closely with basic barium phos-

phorylchloroethanol. Found: H_2O at 100° *in vacuo*, 10.92; Ba, 39.27; P, 8.86; Cl, 9.93; C, 7.79; H, 3.49 % (by Dr Weiler). $\text{C}_2\text{H}_5\text{O}_5\text{ClPBa} \cdot 2\text{H}_2\text{O}$ requires H_2O , 10.30; Ba, 39.26; P, 8.85; Cl, 10.13; C, 6.86; H, 2.87 %. The anhydrous salt contained P, 9.90; Ba, 44.04; Cl, 10.84 %. Calc. P, 9.87; Ba, 43.80; Cl, 11.30 %.

Calcium salt. 5 g. of chloroethylphosphoryl chloride were dropped into 1 litre of water and shaken till completely decomposed. The solution was made slightly alkaline with calcium hydroxide solution and filtered from a small precipitate of calcium phosphate. On filtering calcium phosphorylchloroethanol started to crystallize out and a yield of 1.3 g. was obtained. Found: Ca, 18.47; P, 12.69; Cl, 13.47; H_2O , 7.84 %, at 100° *in vacuo*, 9.15 %. The filtrate on standing deposited 1.7 g. air-dried salt: Ca, 16.88; P, 13.07; Cl, 14.74; H_2O , 7.21 %. After neutralizing to litmus with HCl and further evaporating, 2.5 g. of air-dried salt resulted: Ca, 18.81; P, 13.37; Cl, 12.55; H_2O , 7.67 %; and a further crop of 0.4 g. air-dried: Ca, 17.05; P, 11.89; Cl, 11.75; H_2O , 7.93 %. None of these crops corresponded with calcium phosphorylchloroethanol, $\text{C}_2\text{H}_4\text{O}_4\text{ClPCa} \cdot 2\text{H}_2\text{O}$: Ca, 17.08; P, 13.20; Cl, 15.11; H_2O , 15.35 %.

Except for the first crop, the percentage of Cl became less and it seemed that the chlorine was exchanged for hydroxyl during evaporation. The several crops were covered with 50 ml. of water and boiled for half an hour with 5 ml. of 2N NaOH. The solution was then neutralized with HCl and made just alkaline with lime water. 4.4 g. of impure calcium phosphorylhydroxyethanol separated out. The salt was recrystallized by dissolving in dilute HCl and neutralizing with NaOH and lime water. 4.1 g. air-dried substance were obtained which consisted of basic calcium phosphorylhydroxyethanol. Found: H_2O *in vacuo* at 100° , 10.94; Ca, 17.19; P, 13.03 %. $\text{CH}_3\text{OH} \cdot \text{CH}_2\text{OPO}(\text{OH})\text{OCaOH} \cdot 2\text{H}_2\text{O}$ requires H_2O , 15.39; Ca, 17.12; P, 13.23 %. The anhydrous salt contained Ca, 19.83; P, 15.21 %. Calc. Ca, 20.23; P, 15.64 %.

The rapid conversion of phosphorylchloroethanol into phosphorylhydroxyethanol was also tested by heating a solution of 0.5 g. of the barium salt in 50 ml. water with 50 ml. 0.14N $\text{Ba}(\text{OH})_2$. All the chlorine was removed in 6 hours (Cl, 0.0043 in 10 ml.; total Cl in 10 ml., 0.0042 g.). Slow conversion occurred on allowing the solution to stand at room temperature. Inorganic Cl increased from 0 to 0.0007 in $3\frac{1}{2}$ days, to 0.0014 g. in 16 days.

Note on the estimation of Cl in ethylene chlorohydrin, chloroethylphosphoryl chloride and salts of phosphorylchloroethanol

The estimation of chlorine in these compounds by the Neumann method [Plimmer, 1904] was not possible owing to volatility of some halogen compound during the distillation with the nitric acid mixture. Better results were obtained by treating the solution of the compounds with sodium or with sodium amalgam. The result became quantitative on subsequently heating on a water-bath under a reflux for 2 hours. It was finally found that quantitative results were obtained by simply evaporating the solution with 5 ml. of aqueous KOH on the water-bath and dissolving the residue in dilute HNO_3 . In each case the Cl in the solution was estimated by the Volhard method.

Ethylene chlorohydrin: 3.3708 g. in 100 ml. 1 ml. = 0.01487 g. Cl, 44.1 %.

1 ml. portions were used in each estimation:

	Cl g.	Cl %
(a) By Neumann's method	0.0007	2.1
(b) By NaHg	0.0137	40.5
(c) By Na	0.0146	43.2
(d) By KOH	0.0149	44.2

Chloroethylphosphoryl chloride: 2.358 g. in 100 ml. water. 1 ml. = 0.01271 g. total Cl, 53.92 %. 1 ml. = 0.00847 g. inorg. Cl, 35.95 %.

1 ml. portions were used in each estimation:

	Cl g.	Cl %
(a) Inorganic	0.00852	36.13
(b) By Neumann	0.00923	39.14
(c) By NaHg alcohol	0.01083	45.92
(d) By NaHg water	0.01101	46.6
(e) By Na alcohol	0.0091	38.39
(f) By Na alcohol heat 2 hours	0.0123	52.27
(g) By KOH	0.01232	52.27

The figures for chlorine in the salts of phosphorylchloroethanol are given above under these salts.

Hydrolysis of the esters

The simple alkyl esters of phosphoric acid were found by us [1928] to be hydrolysed by acid, but not by alkali. They are also hydrolysed by phosphatase. The hydrolysis of the new esters was therefore also investigated.

Hydrolysis by acid. 1 g. portions of the calcium salts of phosphorylaminoethanol, phosphorylcholine and phosphorylhydroxyethanol were dissolved in 50 ml. of water and mixed with 52 ml. of 2*N* HCl. In the case of phosphorylchloroethanol the sodium salt was prepared from the barium salt by adding an equivalent of sodium sulphate and filtering from BaSO₄. The solution was then mixed with an equal volume of 2*N* HCl. The solutions of phosphorylaminoethanol and phosphorylcholine were first kept at 37°. At intervals, 10 ml. were withdrawn and treated with NH₄Mg citrate mixture and NH₄OH. Neither after 17 or 72 hours or even 9 days with phosphorylcholine was there any separation of phosphate. All the solutions were heated under a reflux on the water-bath, and at intervals after cooling 10 ml. samples were taken and precipitated with NH₄Mg citrate mixture. The precipitate was filtered off, washed with ammonia, dissolved in 2*N* HNO₃ and the phosphorus estimated by Plimmer's micro-method [1933]. The hydrolysis was slow and is indicated by the figures in the following table:

Hydrolysis by N HCl at 100°

Time hours	mg. P	Hydrolysis %	Time hours	mg. P	Hydrolysis %
Phosphorylaminoethanol			Phosphorylcholine		
5	0.92	5.3	2	0.89	13.1
21	3.28	19.0	5	0.99	15.0
36	4.87	28.2	9	1.33	19.7
68	7.81	45.1			
Total	17.27		Total	6.75	
Phosphorylchloroethanol			Phosphorylhydroxyethanol		
6	0.13	3.1	4	1.66	12.0
36	0.58	13.7	24	5.54	40.0
124	1.54	36.5	33	8.03	58.0

Hydrolysis by alkali. 1 g. portions of the calcium salts of phosphorylaminoethanol, phosphorylcholine and the sodium salt of phosphorylhydroxyethanol were dissolved in water and mixed with an equal volume of 2*N* NaOH, filtered and heated under a reflux on the water-bath. Samples were withdrawn after varying intervals, acidified with HCl and treated with NH₄Mg citrate mixture and ammonia. The precipitate was dissolved in 2*N* HNO₃ and phosphorus estimated in the solution. The precipitate consisted mainly of silicates from the glass and

the solutions up to 24 hours in each case contained no phosphate. The precipitate increased after 24 hours and contained phosphorus. The amount so found up to 102 hours was about 2.5% of the total phosphorus and was really organic phosphorus not washed out of the bulky precipitate whilst washing with ammonia. The esters must be considered stable to hydrolysis by alkali.

Hydrolysis by phosphatase. Phosphatase solutions were prepared from bones, kidneys and intestines of rabbits. Bones were broken up and extracted with chloroform water for 6–11 days. Kidneys were minced and extracted with toluene water. The mucous membranes of the small intestines were scraped off, ground with sand and extracted with toluene water for several days. The extracts were strained through calico. Solutions of the sodium salts of the several esters were prepared from the calcium or barium salts by treatment with the equivalent quantity of sodium oxalate or sulphate and filtration from the insoluble calcium or barium salt. Equal volumes of enzyme solution or boiled enzyme solution were added to the same volumes of the solutions of the esters, 1% of toluene was added, and the mixtures were placed in an incubator at 37°. Samples of 5 or 10 ml. were withdrawn at once and at intervals, acidified with 5 ml. of 2N HCl, treated with 5 ml. of NH_4Mg citrate mixture and 5 ml. of NH_4OH (sp. gr. 0.880). After standing for 12 or 24 hours, the precipitate of protein matter and NH_4MgPO_4 was filtered off, washed with ammonia solution,

Table I

Time hours	Bone phosphatase			Kidney phosphatase			Intestinal phosphatase		
	Enzyme mg. P	Boiled enzyme mg. P	Hydro- lysis %	Enzyme mg. P	Boiled enzyme mg. P	Hydro- lysis %	Enzyme mg. P	Boiled enzyme mg. P	Hydro- lysis %
Phosphorylaminoethanol									
0	0.11	0.12	—	0.82	0.86	—	0.80	0.82	—
24	—	—	—	3.54	0.83	77.7	—	—	—
48	2.27	0.07	45.4	4.04	0.82	92.0	4.15	0.74	76.1
96	2.32	0.14	45.4	4.60	0.61	100	4.21	0.85	77.3
136	2.32	—	45.4	—	—	—	4.32	0.86	79.8
Total	4.98	—	—	4.32	5.20	—	5.20	4.98	—
Phosphorylcholine									
0	0.14	0.13	—	0.74	0.84	—	0.68	—	—
24	—	—	—	2.21	0.90	27.7	—	—	—
48	3.32	0.06	53.5	3.21	0.87	46.2	4.76	0.73	78.7
88	3.71	0.12	60.0	4.15	0.82	63.7	—	—	—
140	3.82	—	62.0	—	—	—	4.98	0.71	83
Total	6.09	4.98	—	6.09	6.09	—	5.87	5.54	—
Phosphorylhydroxyethanol									
0	0.17	0.12	—	0.89	0.52	—	1.22	1.05	—
24	4.43	0.21	74.7	3.16	0.66	42.7	4.71	1.01	80.6
48	4.43	0.23	74.7	4.37	1.07	65.5	4.65	1.05	79.2
96	4.32	0.19	72.8	5.31	1.22	83.2	4.87	1.11	84.3
Total	5.87	5.31	—	6.20	6.20	—	5.55	5.55	—
Phosphorylchloroethanol									
0	0.18	0.25	—	1.01	0.99	—	0.98	0.86	—
24	1.16	0.20	48.3	1.83	0.94	35.5	—	—	—
48	1.16	0.22	48.3	2.16	0.94	49.8	2.33	0.89	57.5
96	1.33	0.17	56.6	2.32	1.11	56.7	2.49	0.99	64.4
Total	2.21	2.77	—	3.32	3.32	—	3.32	3.32	—

dissolved in 2*N* HNO₃ and the phosphorus content estimated by Plimmer's micro-method [1933]. Total P in the solutions was estimated in 1 ml. by the same method. The results were positive in all cases and are given in Table I. All these esters are hydrolysed by phosphatase.

SUMMARY

Phosphorylaminoethanol and phosphorylcholine have been prepared (1) by the action of phosphoryl chloride on the bases, (2) by the action of H₃PO₄ + P₂O₅ on the bases, (3) by the action of ammonia or trimethylamine upon chloroethyl-phosphoryl chloride. The isolation was usually effected as the barium or calcium salts. Phosphorylaminoethanol can also be isolated as ammonium salt. The other salts of these esters are soluble in water.

The free esters have been obtained from the salts.

Phosphorylchloroethanol rapidly changes into phosphorylhydroxyethanol on heating its solutions with dilute alkali.

The halogen in the chloro-compounds, ethylene chlorohydrin, chloroethyl-phosphoryl chloride, and phosphorylchloroethanol is best estimated by evaporating the solution with aqueous KOH and determining the chloride by Volhard's method.

The esters are hydrolysed by heating with *N* HCl, but are stable to *N* NaOH at 100°. They are hydrolysed by phosphatase of bone, kidney and intestine.

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