V. THE HYDROLYSIS OF ORGANIC PHOSPHORUS COMPOUNDS BY DILUTE ACID AND BY DILUTE ALKALI.

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Plimmer and Scott [1908] have shown that phosphoproteins may be distinguished from other organic phosphorus compounds occurring in animal tissues by their behaviour with dilute caustic soda. The phosphorus of the phosphoproteins is completely separated as inorganic phosphate by one per cent. caustic soda at 37° C. in twenty-four hours. Dilute caustic soda at 37° C. does not decompose the other natural organic phosphorus compounds but their hydrolysis at higher temperatures has never been investigated. Neumann's method of preparation of nucleic acid, in which the tissues are heated with caustic alkali, shows that nucleic acid in comparison with phosphoprotein is much more stable; it is not definitely known if the nucleic acid is hydrolysed by alkali. Hexosephosphoric acid is readily decomposed by alkali. The synthetical esters of phosphoric acid with methyl, ethyl and other alcohols are stable to alkali, as was shown by Lossen and Köhler in 1891.

In order to determine the composition of the natural organic phosphorus compounds they are hydrolysed by heating with acid at a high temperature and very frequently under pressure. The hydrolysis of glycerophosphoric acid by acids has recently been investigated by Malengreau and Prigent [1911] who find that the decomposition proceeds according to the laws of a monomolecular reaction and that it corresponds to the hydrolysis of ethyl phosphoric acid and other esters of phosphoric acid studied by Cavalier [1899].

There appear to be no further data concerning the hydrolysis of the natural organic phosphorus compounds by acids and alkalies. The statement is usually made that they are decomposed very easily and that loss, due to hydrolysis, occurs in their isolation from tissues when the solutions are being concentrated. More definite information as to their hydrolysis in solution,

both alone and in the presence of acid and alkali, is therefore required. Such data are not only of use in their preparation, but may also serve for the elucidation of their constitution. More practical knowledge concerning the hydrolysis of phytic acid by acids was required for the estimation of inorganic phosphoric acid in the presence of phytic acid and for the comparison of the hydrolysis of the various compounds with the hydrolysis by enzymes (see preceding paper). The experiments have been carried out for these purposes and no attempt has been made to ascertain the kinetics of the reaction.

Experimental.

Dilute acid and dilute alkali of concentrations varying from normal to twice normal have been used. The hydrolysis by acids was carried out in glass vessels, that by alkali in copper vessels. Porcelain vessels were as unsuitable as glass owing to the solvent action of the alkali upon the glaze. The copper vessels were also attacked by the alkali: the solution generally contained dissolved copper as shown by the blue-green colour; the copper compound was precipitated on neutralising with acid and a colourless filtrate was obtained. The solution of the organic phosphorus compound was either mixed with the acid or alkali and samples of equal volume were placed in separate vessels which were maintained at the desired temperature, or the entire mixture was placed in a thermostat. The vessel was then removed, cooled to room temperature and samples taken for analysis.

The same preparations of glycerophosphoric acid, phytic acid, ethyl phosphoric acid and the same trypsin digest were used as described in the previous paper: hexosephosphoric acid was again kindly placed at my disposal by Dr Young: the nucleic acid was again prepared for me by Miss Skelton by Kossel's method and Mr Page gave me a further quantity of hydroxymethyl-phosphinic acid.

The analyses of phosphoric acid were made by precipitation with ammonium magnesium citrate and conversion into magnesium pyrophosphate. Total phosphoric acid in the solution was estimated in a separate sample by Neumann's method. All results were then calculated out in terms of P_2O_5 for the same volume.

As mentioned in the previous paper, inorganic P_2O_5 is not precipitated in the presence of phytic acid. Inorganic P_2O_5 was therefore estimated by precipitation with ammonium molybdate. The hydrolysis by acids was carried out with nitric acid. In most experiments the phytin was dissolved in the nitric acid; there was no necessity to remove the calcium as its presence in the solution does not interfere with the precipitation of the

ammonium phosphomolybdate. When alkali was used the cooled sample was neutralised with nitric acid, an equal volume of 2N nitric acid, and then 20–30 c.c. of 10 per cent. ammonium molybdate solution added; the precipitation began almost immediately and in 24 hours the solution was quite colourless. The precipitate was then filtered off and estimated by the Neumann method by solution in seminormal caustic soda and titration with seminormal sulphuric acid after boiling off the ammonia. The analytical figures obtained are evidence that inorganic P_2O_5 can be estimated by this method in the presence of phytic acid. Details concerning the inhibition of the precipitation of inorganic P_2O_5 by ammonium magnesium citrate in presence of phytic acid and the estimation by ammonium molybdate at room temperature will be given in a later paper dealing with the analysis of phytin.

The details of these experiments are:

I. *Hydrolysis of Glycerophosphoric Acid.*

(*a*) *By Acid.*

i. By $\frac{N}{4}$ H_2SO_4 at 86° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. with water. 50 c.c. samples + 50 c.c. $\frac{N}{2}$ H_2SO_4 kept at 86° C.

	P_2O_5 in gm.
At commencement	0·0069
After 3 hours ...	0·0092
,, 6 ,, ...	0·0112
,, 15 ,, ...	0·0391
,, 24 ,, ...	0·0494
,, 2 days ...	0·0756
,, 3 ,, ...	0·0855
,, 6 ,, ...	0·0906
Total	0·2675

ii. By $\frac{N}{4}$ H_2SO_4 at 92° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. with water. 50 c.c. samples + 50 c.c. $\frac{N}{2}$ H_2SO_4 kept at 92° C.

	P_2O_5 in gm.
At commencement	0·0102
After 1 day ...	0·0547
,, 2 days ...	0·0683
,, 3 ,, ...	0·0928
,, 4 ,, ...	0·0998
,, 6 ,, ...	0·1578
,, 10 ,, ...	0·2629
Total	0·2587

(*b*) *Autohydrolysis.*

i. At 95° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. 50 c.c. samples kept at 95° C.

	P_2O_5 in gm.
At commencement	0·0074
After 2 hours ...	0·0114
,, 4 ,, ...	0·0154
,, 6 ,, ...	0·0179
,, 8 ,, ...	0·0194
,, 16 ,, ...	0·0342
Total	0·2612

ii. At 75° C.

25 c.c. glycerophosphoric acid diluted to 500 c.c. and kept at 75° C.

	P_2O_5 in gm.
At commencement	0·0157
After 2 days ...	0·0216
,, 3 ,, ...	0·0281
,, 4 ,, ...	0·0341
,, 5 ,, ...	0·0404
,, 6 ,, ...	0·0458
,, 7 ,, ...	0·0512
,, 9 ,, ...	0·0623
Total	0·1374

(*c*) *By Alkali.*

i. By $\frac{N}{1}$ NaOH at 95° C.

25 c.c. glycerophosphoric acid diluted with 700 c.c. $\frac{N}{1}$ NaOH.

	P_2O_5 in gm.
At commencement	0·0137
After 2 days ...	0·0144
,, 3 ,, ...	0·0118
,, 5 ,, ...	0·0150
,, 7 ,, ...	0·0195
,, 10 ,, ...	0·0179
Total	0·2194

ii. By $\frac{2N}{1}$ NaOH at 75° C.

6 gm. sodium glycerophosphate dissolved in 550 c.c. $\frac{2N}{1}$ NaOH (5 c.c. = 20·1 c.c. $\frac{N}{2}$ H_2SO_4) and kept at 75° C.

	P_2O_5 in gm.
At commencement	——
After 2 days ...	0·0011
,, 4 ,, ...	0·0023
,, 8 ,, ...	0·0010
,, 16 ,, ...	0·0018
,, 32 ,, ...	0·0033
,, 81 ,, ...	0·0078
Total	0·1750

II. *Hydrolysis of Ethyl Phosphoric Acid.*

(*a*) By acid $\left(\frac{N}{1}\ HCl\right)$ at 75° C.

300 c.c. sodium ethyl phosphate solution + 300 c.c. $\frac{2N}{1}$ HCl.

	P_2O_5 in gm.
At commencement	0·0001
After 8 hours ...	0·0003
,, 1 day ...	0·0013
,, 2 days ...	0·0031
,, 3 ,, ...	0·0045
,, 4 ,, ...	0·0065
,, 5 ,, ...	0·0077
,, 6 ,, ...	0·0091
,, 8 ,, ...	0·0115
,, 11 ,, ...	0·0146
,, 16 ,, ...	0·0204
,, 20 ,, ...	0·0240
Total	0·0469

(*b*) By alkali $\left(\frac{N}{1}\ NaOH\right)$ at 75° C.

300 c.c. sodium ethyl phosphate solution + 300 c.c. $\frac{2N}{1}$ NaOH.

	P_2O_5 in gm.
At commencement	0
After 1 day ...	0
,, 5 days ...	0
,, 13 ,, ...	0
,, 33 ,, ...	0
Total	0·0926

(*c*) Autohydrolysis at 75° C.

200 c.c. ethyl phosphoric acid solution.

	P_2O_5 in gm.
At commencement	0·0002
After 1 day ...	0·0018
,, 2 days ...	0·0033
,, 4 ,, ...	0·0064
,, 6 ,, ...	0·0094
,, 50 ,, ...	0·0526
Total	0·0746

III. *Hydrolysis of Phytic Acid.*

(*a*) *By Acid.*

i. By $\frac{N}{1}$ HNO_3 at 37° C.

125 c.c. phytic acid solution + 125 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0127
After 1 day ...	0·0144
,, 2 days ...	0·0142
,, 5 ,, ...	0·0134
Total	0·2156

ii. By $\frac{2N}{1}$ HNO_3 at 37° C.

1 gm. phytin dissolved in 250 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0055
After 2 days ...	0·0062
,, 5 ,, ...	0·0062
,, 8 ,, ...	0·0066
Total	0·0976

iii. By $\frac{N}{1}$ HNO_3 at 65° C.

1 gm. phytin dissolved in 130 c.c. H_2O + 130 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0051
After 1 hour ...	0·0056
,, 2 hours ...	0·0057
,, 4 ,, ...	0·0054
,, 6 ,, ...	0·0058
Total	0·0938

iv. By $\frac{2N}{1}$ HNO_3 at 64° C.

1 gm. phytin dissolved in 260 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0046
After 15 mins. ...	0·0051
,, 30 ,, ...	0·0053
,, 1 hour ...	0·0053
,, 2 hours ...	0·0057
Total	0·0913

v. By $\frac{2N}{1}$ HNO_3 at 64° C.

1 gm. phytin dissolved in 260 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0049
After 3 hours ...	0·0056
,, 6 ,, ..	0·0060
,, 9 ,, ...	0·0061
,, 24 ,, ...	0·0065
Total	0·1040

vi. By $\frac{N}{1}$ HNO_3 at 75° C.

1 gm. phytin dissolved in 260 c.c. $\frac{N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0063
After 1 day ...	0·0134
,, 2 days ...	0·0232
,, 4 ,, ...	0·0280
,, 8 ,, ...	0·0439
Total	0·0926

vii. By $\frac{N}{1}$ HNO_3 at 75° C.

1 gm. phytin dissolved in 260 c.c. $\frac{N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0056
After 1 day ...	0·0116
,, 2 days ...	0·0165
,, 4 ,, ...	0·0259
,, 8 ,, ...	0·0436
Total	0·1014

viii. By $\frac{2N}{1}$ HNO_3 at 75° C.

2·5 gm. phytin dissolved in 500 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0077
After 1 day ...	0·0216
,, 2 days ...	0·0349
,, 4 ,, ...	0·0571
,, 6 ,, ...	0·0777
,, 8 ,, ...	0·0976
,, 9 ,, ...	0·1040
,, 11 ,, ...	0·1147
,, 12 ,, ...	0·1192
,, 14 ,, ...	0·1243
,, 15 ,, ...	0·1255
,, 16 ,, ...	0·1274
,, 17 ,, ...	0·1312
Total	0·1471

(*b*) *By Alkali.*

By $\frac{N}{1}$ NaOH at 75° C.

300 c.c. sodium phytate solution + 300 c.c. $\frac{2N}{1}$ NaOH. (5 c.c. = 9·7 c.c. $\frac{N}{2}$ H_2SO_4.)

	P_2O_5 in gm.
At commencement	0·0093
After 1 day ...	0·0089
,, 2 days ...	0·0091
,, 4 ,, ...	0·0079
,, 8 ,, ...	0·0094
,, 16 ,, ...	0·0106
,, 32 ,, ...	0·0119
Total	0·1407

(*c*) *Autohydrolysis at* 75° C.

600 c.c. phytic acid solution.

	P_2O_5 in gm.
At commencement	0·0034
After 1 day ...	0·0071
,, 2 days ...	0·0112
,, 3 ,, ...	0·0151
,, 4 ,, ...	0·0191
,, 5 ,, ...	0·0235
,, 7 ,, ...	0·0314
Total	0·0527

IV. *Hydrolysis of Hexosephosphoric Acid.*

(*a*) By acid $\left(\frac{N}{1}\text{ HCl}\right)$ at 75° C.

100 c.c. hexosephosphoric acid solution neutralised with NaOH + 100 c.c. $\frac{2N}{1}$ HCl. (5 c.c. = 11·2 c.c. $\frac{N}{2}$ NaOH.)

	P_2O_5 in gm.
At commencement	0·0034
After 7 hours ...	0·0348
,, 1 day ...	0·0443
,, 2 days ...	0·0480
,, 3 ,, ...	0·0486
,, 5 ,, ...	0·0508
,, 8 ,, ...	0·0508
Total	0·0527

(*b*) By alkali $\left(\frac{1·5N}{1}\text{ NaOH}\right)$ at 75° C.

40 c.c. hexosephosphoric acid solution + 500 c.c. $\frac{2N}{1}$ NaOH. (5 c.c. = 16·5 c.c. $\frac{N}{2}$ H_2SO_4.)

	P_2O_5 in gm.
At commencement	0·0136
After 1 day ...	0·1610
,, 2 days ...	0·1598
,, 4 ,, ...	0·1641
Total	0·1978

(*c*) Autohydrolysis at 75° C.

50 c.c. hexosephosphoric acid solution + 220 c.c. water. (5 c.c. = 0·2 c.c. $\frac{N}{2}$ NaOH.)

	P_2O_5 in gm.
At commencement	0·0037
After 1 day ...	0·0494
,, 2 days ...	0·0698
,, 3 ,, ...	0·0791
,, 18 ,, ...	0·1152
Total	0·1243

V. *Hydrolysis of Nucleic Acid.*

(*a*) By acid $\left(\frac{N}{1}\text{ HCl}\right)$ at 75° C.

3·5 gm. thymus nucleic acid dissolved in 200 c.c. H_2O + 10 c.c. $\frac{2N}{1}$ NaOH; 210 c.c. $\frac{2N}{1}$ HCl then added; the precipitate went into solution in 15 minutes at 75° C.

	P_2O_5 in gm.
At commencement	——
After 4 hours ...	0·0250
,, 1 day ...	0·0429
,, 2 days ...	0·0545
,, 3 ,, ...	0·0612
,, 4 ,, ...	0·0968
,, 8 ,, ...	0·1106
Total	0·1103

(*b*) By alkali $\left(\frac{N}{1}\text{ NaOH}\right)$ at 75° C.

6 gm. nucleic acid dissolved in 600 c.c. $\frac{N}{1}$ NaOH. (5 c.c. = 10 c.c. $\frac{N}{2}$ H_2SO_4.)

	P_2O_5 in gm.
At commencement	0
After 1 day ...	0·0040
,, 2 days ...	0·0106
,, 4 ,, ...	0·0179
,, 16 ,, ...	0·0369
,, 32 ,, ...	0·0510
,, 76 ,, ...	0·0990
Total	0·1128

(*c*) By alkali $\left(\frac{N}{1}\ NaOH\right)$ at 70° C.

4 gm. nucleic acid dissolved in 200 c.c. H_2O + 200 c.c. 2N NaOH.

	P_2O_5 in gm.
At commencement	0·0020
After 1 day	0·0108
,, 2 days	0·0145
,, 4 ,, ...	0·0236
,, 9 ,, ...	0·0305
,, 22 ,, ...	0·0447
Total	0·0951

VI. *Hydrolysis of Phosphoprotein by Alkali.*

i. 200 c.c. trypsin digest of caseinogen + 50 c.c. $\frac{2N}{1}$ NaOH. (= 1·6 per cent. NaOH.)

	P_2O_5 in gm.
At commencement	0·0194
After 1 day ...	0·0320
,, 2 days ...	0·0321
,, 3 ,, ...	0·0328
Total	0·0342

ii. 175 c.c. trypsin digest of caseinogen + 25 c.c. $\frac{2N}{1}$ NaOH. (= 1 per cent. NaOH.)

	P_2O_5 in gm.
At commencement	0·0199
After 1 day ...	0·0327
,, 2 days ...	0·0342
Total	0·0380

iii. 180 c.c. trypsin digest of caseinogen + 20 c.c. $\frac{2N}{1}$ NaOH. (= 0·8 per cent. NaOH.)

	P_2O_5 in gm.
At commencement	0·0209
After 1 day ...	0·0272
,, 2 days ...	0·0300
,, 4 ,, ...	0·0334
Total	0·0406

VII. *Hydrolysis of Hydroxymethyl-phosphinic Acid by Acid.*

130 c.c. sodium hydroxymethyl-phosphinate solution + 130 c.c. $\frac{2N}{1}$ HNO_3. (5 c.c. = 10·3 c.c. $\frac{N}{2}$ NaOH.)

	P_2O_5 in gm.
At commencement	0·0006
After 1 day	0·0004
,, 2 days	0·0006
,, 4 ,, ...	0·0006
,, 8 ,, ...	0·0018
Total	0·1179

Glycerophosphoric acid is slowly hydrolysed by dilute acid, complete separation of the phosphoric acid requiring 10 days at 92° C. The glycerol, which is formed, was isolated in another experiment by removing the sulphuric and phosphoric acids by baryta, concentrating and distilling *in vacuo*: the glycerol distilled at 162° C. at 10–15 mm. pressure and a yield of 44 per cent. was obtained. Autohydrolysis is considerably slower; one-eighth of the glycerophosphoric acid was decomposed in 16 hours at 95° C., and only one-half in 9 days at 75° C. It is not hydrolysed by alkali; the slight increase in the amount of inorganic phosphate observed after 81 days by the action of twice normal caustic soda was due to slight evaporation through the cork; the total P_2O_5 at the end of the period being 0·1750 gm., which is a little greater than that at the beginning—0·1509 gm.

Glycerophosphoric acid is stable to alkali like ethyl phosphoric acid; Lossen and Köhler's result has been confirmed and autohydrolysis and acid hydrolysis of ethyl phosphoric acid have been carried out at 75° C. for comparison with the other compounds. Autohydrolysis of ethyl phosphoric acid was not complete in 50 days and the hydrolysis by acid seems to be slower than in the case of glycerophosphoric acid.

Phytic acid is the most stable of the organic phosphorus compounds. At 37° C. dilute acid effected no hydrolysis; at 64° C. a slight hydrolysis could be detected in 24 hours; at 75° C. normal nitric acid separated about half of the phosphorus as inorganic phosphate in 8 days. Complete hydrolysis was not effected by twice normal nitric acid in 17 days at the same temperature. Autohydrolysis at 75° C. resulted in the splitting off of about half the phosphoric acid in 7 days.

Phytic acid is also quite stable to alkali; the slight increase in the amount of inorganic phosphate noted after 32 days by normal alkali at 75° C. is due to concentration of the solution by evaporation through the cork.

Hexosephosphoric acid is easily hydrolysed by both acid and alkali. Complete hydrolysis was effected by normal hydrochloric acid in 3–5 days, the greater part of the phosphoric acid being separated in 1 day. Autohydrolysis at 75° C. was much slower than acid hydrolysis; half the phosphoric acid was separated in 2 days; complete hydrolysis occurred in about 18 days. Dilute alkali produced complete hydrolysis in 1 day. In all the experiments a small quantity of organic phosphorus remained undecomposed: it is very probable that another organic phosphorus compound was present as impurity in the solution.

Nucleic acid is hydrolysed by both acid and alkali. Normal hydrochloric acid effected complete hydrolysis in 8 days at 75° C. With normal caustic soda a slow hydrolysis was observed; about one-third of the phosphoric acid was split off in 8 days, about one-half in 32 days; even after 76 days a small amount of organic phosphorus remained in solution.

The organic phosphorus compound which remains in a prolonged tryptic digest of caseinogen is also completely hydrolysed by 1 per cent. caustic soda; there is no difference in its behaviour from that of caseinogen; the former observation of Plimmer and Bayliss [1906] was incorrect.

Hydroxymethyl-phosphinic acid is not hydrolysed by dilute acid at 75° C.

Summary and Conclusion.

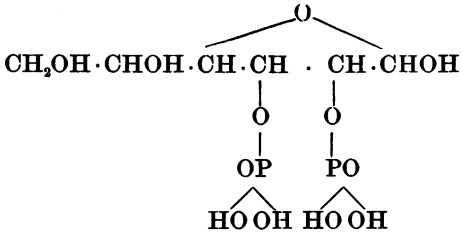
Ethyl phosphoric acid, glycerophosphoric acid and phytic acid are hydrolysed by acid, but are stable to alkali. Stability to alkali is therefore a property of the esters of phosphoric acid.

Hexosephosphoric acid and phosphoprotein are so different in their behaviour to alkali from the above three compounds that some difference in their constitution from that of the esters must exist.

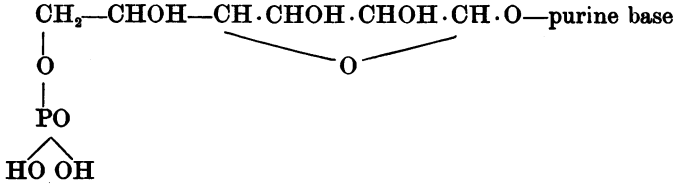
It is not known how the phosphoric acid is combined in phosphoprotein, but it is probably united with one of the amino-acids.

Hexosephosphoric acid reduces Fehling's solution which points to the presence of the functional aldehyde or ketone group in the molecule.

The phosphoric acid radicles are most probably combined with two of the hydroxyl groups leaving the reducing group free. The action of the alkali will destroy this grouping and the whole carbohydrate molecule will be decomposed leaving the phosphoric acid:



Nucleic acid, since it is hydrolysed like hexosephosphoric acid by both acid and alkali, seems to occupy an intermediate position between the stable esters and the very unstable hexosephosphoric acid. If the purine, or pyrimidine, base be attached to the functional aldehyde group in the same way as the alcohols in the glucosides, the action of alkali may be to destroy the purine base leaving the aldehyde group for decomposition of the molecule, and phosphoric acid will remain. A formula such as



would explain the slow decomposition by alkali.

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