IV. THE METABOLISM OF ORGANIC PHOSPHORUS COMPOUNDS. THEIR HYDROLYSIS BY THE ACTION OF ENZYMES.

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INTRODUCTION.

Our knowledge concerning the composition of the organic phosphorus compounds occurring in both plants and animals is still very rudimentary. Five groups of organic phosphorus compounds occur in nature:—

1. *Phospholipines*, of which lecithin is the principal example, and in which the phosphorus is regarded as being in ester combination with glycerol as glycerophosphoric acid;

2. *Phytin*, in which phosphoric acid is in combination with inositol as inositol-phosphoric acid, the compound itself being regarded as the calcium magnesium salt;

3. *Hexosephosphate*, in which a hexose is combined with phosphoric acid;

4. *Nucleic acid*, in which again a carbohydrate (hexose or pentose) is combined with phosphoric acid, and attached to the carbohydrate is a purine base;

5. *Phosphoprotein*, in which phosphoric acid is in an unknown form of combination, presumably with one of the amino-acids composing the protein molecule.

The phospholipines occur chiefly in animals and only to a small extent in plants; phytin or phytic acid has only been found in plants; hexosephosphate is a product formed in alcoholic fermentation by yeast; nucleic acid is said to be the chief constituent of the cell nuclei in both plants and animals; phosphoprotein is present only in the animal kingdom and forms the chief protein foodstuff of embryo birds and suckling mammals.

Considering how little is known as to the chemical composition of these

compounds it is not to be wondered at that the knowledge of their metabolism is vague. Most investigators will agree that plants can synthesise organic phosphorus compounds from inorganic phosphates and that they are also able to carry out the reverse process, but how these changes are effected, or by what enzymes they are caused, is practically unknown.

Most of the experiments on phosphorus metabolism in animals have been feeding experiments in which animals have been fed with organic and inorganic phosphorus compounds and the total intake and output of phosphorus estimated, together with that of nitrogen in most cases. The conclusions which were drawn from the general condition of the animal at the end of the time, and from the effect of the phosphorus compounds upon the nitrogen metabolism, were summarised by F. C. Cook in 1909. They led to the belief that organic phosphorus compounds are directly assimilated and are preferable to inorganic phosphates as the source of phosphorus for animals. Against this belief the recent work of Fingerling [1912; 1, 2] shows that the animal organism can synthesise organic phosphorus compounds from inorganic phosphates. He fed ducks for long periods on food containing only organic phosphorus, or only inorganic phosphorus, as the source of phosphorus. Both sets of ducks laid approximately the same number of eggs and the lecithin and nuclein content of the eggs was about the same in either case. He also analysed the milk of goats which were fed with organic and inorganic phosphorus in the same way as he fed the ducks. In both cases the composition of the milk was the same. Gregersen [1911] has also found that the organic phosphorus compounds can be built up in the animal body from inorganic phosphate, thus confirming the work of Fingerling.

Both organic and inorganic phosphorus compounds can therefore be utilised for the formation of the organic phosphorus compounds in animals; the feeding experiments with organic phosphorus compounds do not show in what form the phosphorus is assimilated, i.e. whether it is absorbed in an organic form or whether it is first hydrolysed and then resynthesised. Phosphorus is excreted in the urine of animals almost entirely as inorganic P_2O_5 , whether the intake be organic or inorganic; only a small quantity is excreted in an organic form [Mathison, 1909; Plimmer, Dick and Lieb, 1909; and Gregersen, 1907], and the composition of this organic phosphorus compound is unknown although it is stated to be glycerophosphoric acid. The phosphorus in the faeces is also mainly inorganic P_2O_5 . These facts point to the hydrolysis or decomposition of organic phosphorus compounds in the animal organism.

It seemed to me that the only possible means by which the metabolism and assimilation of the natural organic phosphorus compounds could be studied was to ascertain if these compounds were hydrolysed by the enzymes of the digestive tract. The action of the enzymes of the pancreas, intestine and liver upon the various organic phosphorus compounds has therefore been investigated and in addition the action of some plant enzymes has been included so as to obtain a complete and comparative survey of the whole series of organic phosphorus compounds.

I. GLYCEROPHOSPHORIC ACID.

The literature on phosphorus metabolism in plants contains no statement as to the hydrolysis or synthesis of glycerophosphoric acid or of lecithin, and until quite recently the knowledge of the metabolism of these substances in animals was equally indefinite. The experiments made by Marfori [1905], Bülow [1894], Mathison [1909], Plimmer, Dick and Lieb [1909] and others had shown that glycerophosphoric acid was completely absorbed when introduced into the body either by the mouth or subcutaneously, and that the phosphorus appeared in the urine as inorganic phosphate and not as glycerophosphoric acid. At the time when these investigations were made¹ no one had tried if the organs of the animal body were able to hydrolyse glycerophosphoric acid and convert it into glycerol and phosphoric acid. It was generally assumed that glycerophosphoric acid was either assimilated as such, or that, since it is an ester, it was hydrolysed like the fats by the lipase in the liver, pancreas and other organs. In 1912 Grosser and Husler [1912] first published experiments which proved that an enzyme (glycerophosphatase) able to hydrolyse glycerophosphoric acid was present in certain animal tissues. They found that the kidney and intestine could completely hydrolyse glycerophosphoric acid, that the lung hydrolysed 62 per cent. and the liver 16 per cent. The spleen, blood and muscle did not hydrolyse glycerophosphoric acid.

The fact that glycerophosphatase is present in the intestine, kidney and lung but not in the pancreas and liver suggests that the hydrolysis is not effected by lipase since the pancreas and liver are the chief animal tissues which contain lipase, whereas the other organs mentioned only contain small amounts of this enzyme.

An enzyme which can hydrolyse glycerophosphoric acid is also present in plants, especially in castor oil seeds. Like the lipase in these seeds it is only

¹ The results of my investigations upon the metabolism of glycerophosphoric acid were communicated to the Biochemical Club at their meeting in July 1911.

liberated from a precursor by the action of dilute acids, but unlike the lipase it is soluble in water [Connstein, Hoyer and Wartenberg, 1902; Armstrong and Ormerod, 1906; Tanaka, 1910]. The action of the two enzymes is thus distinct although it has been impossible to prove this absolutely, as after repeated extraction with water the final extract always contained some glycerophosphatase.

Extracts of yeast and of bran, which contain the enzymes hexosephosphatase [Harden and Young, 1908] and phytase [Suzuki and Takaishi, 1907] respectively, are also able to hydrolyse glycerophosphoric acid.

EXPERIMENTAL.

The general method for determining the action of extracts of various tissues upon glycerophosphoric acid has been to mix a known volume of the extract with either a known volume of a solution of glycerophosphoric acid previously neutralised with soda, or with a solution of sodium glycerophosphate; to withdraw a sample of known volume immediately after mixing and after subsequent intervals of time; and to estimate, after filtering if necessary, the inorganic phosphate in these samples by precipitation with ammonium magnesium citrate in the presence of ammonia, and conversion into magnesium pyrophosphate. By the use of magnesium citrate instead of magnesia mixture the precipitation of organic matter with the ammonium magnesium phosphate is avoided, as was shown by Plimmer and Bayliss in 1906. The total phosphorus content of the mixture was estimated in another portion by Neumann's method as modified by Plimmer and Bayliss [1906]. All results were then calculated for the same volume. In some cases a control experiment consisting of the same volumes of water and of extract was carried out simultaneously. Toluene was added as an antiseptic and the mixtures were kept at 37° C. after the removal of the first sample.

The commercial preparations of glycerophosphoric acid and of sodium glycerophosphate, which are synthetically prepared, were employed. The synthetical compound except for its optical activity is apparently identical with the natural compound obtained from lecithin. Both products seem to be mixtures [Willstätter and Lüdecke, 1904; Power and Tutin, 1905; Tutin and Hann, 1906].

It was originally supposed that the glycerophosphoric acid, since it is an ester, would be hydrolysed by the lipase of the pancreas and other tissues: for this reason the preparations and extracts of tissues used have been purposely made by the usual methods for obtaining an active lipase.

The action of the pancreas was investigated with :

1. The residue from an aqueous extract of trypsin (Fairchild); such residues have been shown by Dietz [1907] to be very strongly lipoclastic in their action. The residue was suspended in a solution of sodium glycerophosphate and the inorganic P_2O_5 was estimated after filtering off and washing the precipitate.

2. A glycerol extract of pig's pancreas prepared by extracting the organ for 24–36 hours with ten times its bulk of a mixture consisting of 90 parts of glycerol and 10 parts of 1 per cent. sodium carbonate, and then straining through muslin. In one experiment this extract was used in aqueous solution, in another in about 50 per cent. glycerol solution. The mixture containing the extract of sodium glycerophosphate was then made just alkaline to phenolphthalein and maintained at 37° C. in the presence of toluene. The samples from the glycerol solution were diluted with water and filtered, and inorganic P_2O_5 was estimated in the filtrate and washings.

3. Fresh pancreatic juice which is known to contain an active lipase. The pancreatic juice in one experiment was kindly collected for me by Prof. Starling, in another experiment by Prof. Bayliss; in the first experiment the juice was activated by enterokinase by adding a small amount of intestinal extract; in the second experiment the juice was not activated.

The action of the liver was investigated with :

1. An extract of dog's liver made by the method of Loevenhart and Peirce [1906] for obtaining an active lipase.

2. An extract of calf's liver prepared according to the method of McCollum and Hart [1908] in their study of phytase in animal tissues. The samples removed at intervals were acidified with a drop of glacial acetic acid, and were boiled and filtered. The coagulum was thoroughly washed and inorganic P_2O_5 was estimated in the filtrate and washings.

The action of the small intestine was investigated by extracts made from the mucous membrane, which was ground up with sand and extracted for 24–48 hours with water containing toluene as antiseptic; and then strained through lint or muslin. In the earlier experiments this extract which was milky in appearance was used directly; the samples, which were removed at intervals, were acidified with acetic acid; the precipitate so formed was filtered off and thoroughly washed and the inorganic P_2O_5 estimated in the clear filtrate and washings. In the later experiments the aqueous extract of the mucous membrane was acidified with acetic acid, and filtered from the precipitate: the clear extract so obtained was found to be active, either when still acid, or after neutralising with sodium carbonate. These clear extracts

were used in all the later experiments with the other organic phosphorus compounds.

The action of the kidney and liver was investigated with extracts made by grinding the organ with sand, and extracting the mass with water, to which toluene was added, for 1-2 days. In the first experiment with the kidney the extract was acidified with acetic acid, the filtrate neutralised with sodium carbonate and used after again filtering. In the other experiments the milky extract was used; each sample was acidified and diluted with the same volumes of acetic acid and water. Inorganic phosphate was estimated in a measured volume of the clear filtrate.

A suspension of shelled castor oil seeds in dilute acetic acid was used for the detection of a glycerophosphatase, samples being removed and the inorganic P_2O_5 estimated in the filtrate and washings. A suspension was also employed when the seed was treated with acid, washed and the extracted residue tested for glycerophosphatase.

Extracts of castor oil seeds were prepared by treating the shelled seeds ground as finely as possible, sometimes after the fat had been previously removed by extraction with ether, with water or with decinormal acetic acid for 1-2 days in the proportion of 5-20 grams of seed and 100-300 c.c. of the acetic acid. A clear filtrate was obtained on filtration. These filtrates were used directly and in some experiments after neutralisation of the acid extract with sodium carbonate.

Preliminary experiments to ascertain if a glycerophosphatase were present in yeast were made with an aqueous extract of pressed yeast prepared in the presence of toluene and by a suspension of zymine; in the latter case the estimations of inorganic P_2O_5 were made in the filtrate and washings from the insoluble matter.

The later and all subsequent experiments with yeast enzymes were made with extracts of the commercial "zymine" of Schroder and Co. of Munich. These extracts were prepared by treating the powder with ten times its weight of water for 24-48 hours in the presence of toluene and then filtering. A pale brown solution resulted.

By simply covering wheat bran with five times its weight of water in the presence of toluene a clear and active extract was always obtained on filtering. The bran extract if kept without contact with air was of a pale brown colour, but when it was exposed to the air and filtered it became rapidly darker in colour.

The following are the analytical data:

Pancreas.

(1) Residue from 1.5 gm. trypsin (Fairchild) suspended in 500 c.c. water containing 5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0121
After 31 days	0.0115
Total	0.1712

(2) 100 c.c. glycerol extract of pig's pancreas + 5 gm. sodium glycerophosphate in water. Mixture made alkaline to phenolphthalein and volume diluted with water to 500 c.c.

P_2O_5 in gm.	
At commencement	0.0128
After 9 days	0.0135
Total	0.1775

(3) 100 c.c. glycerol extract of pig's pancreas + 5 gm. sodium glycerophosphate dissolved in 200 c.c. glycerol + 200 c.c. water and mixture made alkaline to phenolphthalein.

P_2O_5 in gm.	
At commencement	0.0120
After 8 days	0.0126
Total	0.1712

(4) 20 c.c. glycerophosphoric acid diluted to 500 c.c. with water and neutralised with caustic soda + 20 c.c. pancreatic juice of dog + 4-5 drops intestinal juice. Total volume made up to 1000 c.c.

P_2O_5 in gm.			
At commencement	...	0.0126	After 8 days 0.0759
After 1 day	...	0.0517	" 10 " 0.0799
" 2 days	...	0.0569	" 13 " 0.0824
" 3 "	...	0.0633	" 20 " 0.0891
" 5½ "	...	0.0723	" 36 " 0.1110
Total	0.2606		

(5) 2.5 gm. sodium glycerophosphate in water + 50 c.c. pancreatic juice of dog. Total volume made up to 500 c.c.

P_2O_5 in gm.	
At commencement	0.0135
After 14 days	0.0124
Total	0.1661

Liver.

(1) A. 100 c.c. extract of dog's liver by Loevenhart and Peirce's method + 400 c.c. H_2O .
B. 100 c.c. same extract + 400 c.c. water containing 5 gm. sodium glycerophosphate.

P_2O_5 in gm.		
	A	B
At commencement	0.0006	0.0147
After 1 day	0.0013	0.0165
" 3 days	—	0.0157
" 5 "	—	0.0122
Total	0.0177	0.1889

(2) A. 125 c.c. extract of calf's liver by McCollum and Hart's method + 125 c.c. water.
B. 125 c.c. same extract + 125 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.			
	A	B	Difference
At commencement	...	0.0232	0.0529 0.0297
After 5 days	...	0.0339	0.0679 0.0340
Total	0.0659	0.2460	0.1801

Small Intestine.

(1) A. 100 c.c. milky extract of dog's intestine + 400 c.c. water. B. 100 c.c. same extract + 400 c.c. water containing 5 gm. sodium glycerophosphate.

P ₂ O ₅ in gm.		
A	B	Difference
At commencement:		
0.0042	0.0444	0.0402
After 1 day:		
0.0062	0.1506	0.1444
After 2 days:		
0.0061	0.1539	0.1478
After 6 days:		
0.0061	0.1539	0.1478
Total:		
0.0080	0.1864	0.1784

(2) 80 c.c. clear acid extract of cat's intestine + 5 gm. sodium glycerophosphate in water made up to 500 c.c. with water.

P ₂ O ₅ in gm.	
At commencement	0.0256
After 17 hours ...	0.0313
„ 24 „ ...	0.0346
„ 5 days ...	0.0680
„ 12 „ ...	0.0963
Total	0.1458

(3) 80 c.c. clear acid extract of intestines of 2 cats neutralised with soda + 2.5 gm. sodium glycerophosphate in water made up to 250 c.c. with water.

P ₂ O ₅ in gm.	
At commencement	0.0236
After 1 day ...	0.0877
„ 4 days ...	0.1176
„ 9 „ ...	0.1270
Total	0.1496

Kidney.

(1) A. 100 c.c. clear acid extract of dog's kidneys neutralised with soda + 150 c.c. water. B. 100 c.c. same neutralised extract + 150 c.c. water containing 2.5 gm. sodium glycerophosphate.

P ₂ O ₅ in gm.		
A	B	Difference
At commencement:		
0.0080	0.0090	0.0010
After 3 days:		
0.0075	0.0763	0.0688
After 10 days:		
0.0073	0.0838	0.0765
Total:		
0.0076	0.1661	0.1585

(2) A. 200 c.c. extract of sheep's kidneys + 50 c.c. water. B. 200 c.c. same extract + 50 c.c. water containing 2.5 gm. sodium glycerophosphate.

P ₂ O ₅ in gm.	
A	B
At commencement:	
0.0256	0.1300
After 1 day:	
0.0321	0.2085
After 4 days:	
0.0332	0.2092
Total:	
0.0406	0.2130

(3) A. 200 c.c. extract of dog's kidneys + 50 c.c. water. B. 200 c.c. same extract + 50 c.c. water containing 2.5 gm. sodium glycerophosphate.

P ₂ O ₅ in gm.	
A	B
At commencement:	
0.0071	0.0176
After 1 day:	
0.0084	0.1204
After 5 days:	
0.0092	0.1723
Total:	
0.0241	0.1750

Lung.

A. 200 c.c. extract of dog's lungs + 50 c.c. water. B. 200 c.c. extract of dog's lungs + 50 c.c. water containing 2.5 gm. sodium glycerophosphate.

P ₂ O ₅ in gm.			
	A	B	Difference
At commencement	0.0069	0.0148	0.0079
After 1 day ...	0.0110	0.0344	0.0234
„ 2 days ...	0.0128	0.0472	0.0344
„ 4 „ ...	0.0161	0.0730	0.0569
Total	0.0241	0.2232	0.2041

Castor oil seeds.

(a) 5 gm. ground seeds suspended in 250 c.c. $\frac{N}{10}$ acetic acid containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0089
After 1 day ...	0.1199
„ 2 days ...	0.1395
„ 3 „ ...	0.1538
„ 8 „ ...	0.1852
Total	0.2308

(b) 60 c.c. aqueous extract of ground seeds + 200 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0121
After 12 days ...	0.0105
Total —	

(c) 130 c.c. $\frac{N}{10}$ acetic acid extract + 120 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0217
After 1 day ...	0.0888
„ 2 days ...	0.1027
„ 3 „ ...	0.1109
„ 6 „ ...	0.1204
Total	0.2143

(d) 100 c.c. $\frac{N}{10}$ acetic acid extract of ground seeds + 150 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0356
After 1 day ...	0.0920
„ 2 days ...	0.1008
„ 7 „ ...	0.1179
„ 12 „ ...	0.1263
Total	0.2003

50 c.c. extract contained—

At commencement	0.0477 gm. inorg. P_2O_5
After 12 days ...	0.0504 „ „
Total P_2O_5 ...	0.0520 gm.

Increases are thus due entirely to hydrolysis of the glycerophosphate.

(e) 200 c.c. $\frac{N}{10}$ acetic acid extract of ground seeds freed from fat by extraction with ether and kept for several months so as to convert all organic phosphorus into inorganic phosphate + 50 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0410
After 1 day ...	0.0579
„ 2 days ...	0.0711
„ 3 „ ...	0.0806
„ 6 „ ...	0.1030
Total	0.2003

(f) Residue of seeds in expt. (c) thoroughly washed by decantation and filtration suspended in 250 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0165
After 3 days ...	0.0651
„ 10 „ ...	0.0889
„ 20 „ ...	0.1011
Total	0.1826

(g) Residue of seeds in expt. (e) after thorough washing was suspended in 250 c.c. of 1 per cent. solution of sodium glycerophosphate in water.

P_2O_5 in gm.	
At commencement	0.0005
After 2 days ...	0.0259
„ 4 „ ...	0.0338
„ 7 „ ...	0.0522
Total	0.1500

Yeast.

(a) 80 c.c. extract of yeast + 170 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.0396
After 1 day ...	0.0776
„ 3 days ...	0.0869
„ 7 „ ...	0.0952
Total 0.2244	

50 c.c. yeast extract contained—
 0.0804 gm. inorg. P_2O_5
 0.0930 gm. total P_2O_5

Increases are therefore due to hydrolysis of glycerophosphate.

(b) A. 10 gm. zymin suspended in 250 c.c. water. B. 10 gm. zymin suspended in 250 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.			
	A	B	Difference
At commencement ...	0.0215	0.0847	0.0632
After 1 day ...	0.0527	0.1323	0.0796
„ 2 days	0.0582	0.1424	0.0842
„ 6 „	0.0632	0.1539	0.0907
Total	0.0850	0.2637	0.1787

(c) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.			
	A	B	Difference
At commencement	0.0304	0.0774	0.0470
After 1 day ...	0.0341	0.0907	0.0566
„ 8 days ...	0.0379	0.1068	0.0689
Total	0.0621	0.2409	0.1788

Bran.

(1) 200 c.c. extract of bran + 50 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
At commencement	0.1430
After 1 day ...	0.1696
„ 2 days ...	0.1839
„ 4 „ ...	0.1877
Total 0.2904	

(2) A. 125 c.c. extract of bran + 125 c.c. water. B. 125 c.c. extract of bran + 125 c.c. water containing 2.5 gm. sodium glycerophosphate.

P_2O_5 in gm.			
	A	B	Difference
At commencement ...	0.0374	0.0389	0.0015
After 2 days	0.0389	0.0731	0.0342
„ 4 „	0.0389	0.0852	0.0463
„ 9 „	0.0393	0.1014	0.0621
Total	0.0406	0.1965	0.1559

The gradual increase in the amount of inorganic P_2O_5 , which occurred when the extracts of intestinal mucosa, kidney, castor oil seeds, yeast and bran were tested, is the best evidence that the hydrolysis of glycerophosphoric acid has been effected by an enzyme present in the tissues. The enzyme is not present in the pancreas and liver of animals. Though an increase in the amount of inorganic phosphate was found in the experiment with pancreatic juice activated by 4–5 drops of intestinal juice, the increase takes place very slowly; one-fifth is hydrolysed in 2 to 3 days, and after 36 days the hydrolysis is less than one-half of the total P_2O_5 in the solution. An explanation of

this increase is given by the experiments with intestinal extract, which contains the active enzyme; a small amount of the enzyme had therefore been added when activating the pancreatic juice. The absence of the enzyme from the extracts of pancreas and liver, which were prepared specially for lipase, and the presence of the enzyme in the extracts of the intestine and kidney, tissues not usually regarded as good sources of lipase, point to this enzyme being distinct from lipase. Proof of the difference of the enzymes is given by the experiment with castor oil seeds; lipase is insoluble in water but an aqueous extract contains glycerophosphatase. Its presence in bran, in which lipase has not yet been shown, is further proof that the two enzymes are not identical. A comparison of the actual amounts of the enzymes present in the several tissues is not possible, but it may be noticed that the amount of glycerophosphatase in the lung is very much less than in the intestine and kidney. The amount in yeast is also small and the amount contained in bran is smaller still.

II. PHYTIC ACID.

Phytin, the calcium magnesium salt of inositolphosphoric acid, or phytic acid, was first isolated by Posternak in 1903 from various seeds in which it is present as a reserve material containing phosphorus in organic combination. It also occurs in the bran of cereals [Hart and Andrews, 1903], that of rice being particularly rich in it [Suzuki and Yoshimura, 1907]. Phytin is thus the most important phosphorus-containing foodstuff for herbivora, and it has been administered to man to supply organically combined phosphorus. On this account more work has been done upon its metabolism than upon that of any of the other organic phosphorus compounds.

It has been shown by Scofone [1905], Giascosa [1905], Mendel and Underhill [1906], and also Horner [1907] that phytin, when given to animals by the mouth, is absorbed and that the phosphorus is excreted as inorganic phosphorus in the urine; but the absorption is never complete.

Suzuki and Takaishi [1907] have found that bran contains an enzyme, phytase, which completely hydrolyses phytin into inositol and phosphoric acid; Jordan, Hart and Patten [1906] have shown that the enzymes, pepsin and trypsin, of the animal body have no action on phytin, but according to McCollum and Hart [1908], the liver and also the blood contain a phytin-splitting enzyme.

The hydrolysis of glycerophosphoric acid by the enzymes of the intestinal mucosa suggested that phytic acid might also be split up by this tissue. Experiment, however, showed the contrary. Neither the mucous membrane

of the intestine of the carnivora, dog and cat, nor that of the herbivora, sheep, ox and rabbit, even when the latter animal was fed for some weeks on bran, contained an enzyme which was able to hydrolyse phytic acid. Phytic acid thus behaved very differently from glycerophosphoric acid. The difference in the behaviour of the two compounds does not extend to the enzymes of the liver. The hydrolysis of phytic acid by the liver observed by McCollum and Hart was very small and it has been impossible to regard this as a hydrolysis when compared with other results. Phytic acid is thus not hydrolysed by the enzymes of the digestive tract of animals.

The enzymes in an extract of zymine have no action upon phytin, but it is slowly hydrolysed by an acid extract of castor oil seeds. The only enzyme which readily hydrolyses phytin is contained in an extract of bran.

EXPERIMENTAL.

Commercial phytin was employed as the source of phytic acid in these experiments. This substance except for a small residue is easily soluble in water, to which it gives a distinctly acid reaction to litmus, and from which it is precipitated on rendering the solution alkaline with ammonia. It contains a small quantity of inorganic phosphate. The calcium was precipitated from the aqueous solution of phytin by adding the calculated quantity of oxalic acid, or of sodium or potassium oxalate. The solution of the free acid, or of the sodium or potassium salt, obtained on filtering was used as substrate in the experiments.

Extracts of the various tissues were prepared as described under glycerophosphoric acid; the mixture of enzyme solution and phytate was kept at 37° C. in the presence of toluene. Samples were removed at intervals and the inorganic P_2O_5 estimated.

In the first experiments the estimation of inorganic phosphate was carried out by precipitation with ammonium magnesium citrate in the presence of ammonia, but it was soon observed that the inorganic phosphate in the presence of phytic acid was not immediately precipitated but only came down slowly after standing for some time, and that the filtrate frequently gave a further precipitate on standing. The precipitate did not have the usual appearance of ammonium magnesium phosphate and it adhered very tenaciously to the sides of the vessel in which the precipitation took place. On converting these precipitates by heating into the pyrophosphate the weights obtained were also very variable. An examination into the cause of these difficulties revealed the fact that phytic acid inhibits the precipitation

of inorganic phosphate as ammonium magnesium phosphate and that under certain conditions phytic acid also separates as magnesium phytate from ammoniacal solution.

Hart and Andrews [1903], in their study of the relative amounts of inorganic and organic phosphate in foodstuffs, precipitated the inorganic phosphate as ammonium phosphomolybdate at 65° C. in the presence of dilute nitric acid. This method was therefore used but the precipitation was carried out at room temperature since it was thought that the phytic acid might be hydrolysed by the nitric acid at 65° C. A slight hydrolysis does occur at this temperature (see the following paper), but it is insufficient to vitiate the results of Hart and Andrews, or those of McCollum and Hart, who employed this method. The precipitation of the ammonium phosphomolybdate at the ordinary room temperature takes place slowly, but is generally complete in 1-2 days. The completion of the precipitation is shown by the colour of the solution; it is yellow when the reagents are added and becomes quite colourless when the deposition of the yellow precipitate is finished. In the presence of organic matter the solution may remain yellow, which is due to the action of nitric acid on protein in the enzyme extracts. At the same time protein matter is precipitated so that the estimation cannot be carried out by titration as in Neumann's method. When the precipitation was complete the precipitate was filtered off and washed with water and then dissolved in dilute caustic soda. Inorganic phosphate was then precipitated by ammonium magnesium citrate. The details of these experiments will be described in a later paper dealing with the analysis of phytin. Total P_2O_5 was estimated by Neumann's method.

Pancreas.

130 c.c. pancreatic juice + 130 c.c. sodium phytate solution.

P_2O_5 in gm.

At commencement	0.0025
After 14 days ...	0.0055
Total	0.0850

Liver.

A. 125 c.c. extract of dog's liver + 125 c.c. H_2O . B. 125 c.c. extract of dog's liver + 125 c.c. sodium phytate solution.

P_2O_5 in gm.

	A	B	Difference
At commencement ...	0.0348	0.0408	0.0060
After 1 day ...	0.0351	0.0454	0.0103
„ 5 days...	0.0379	0.0486	0.0107
„ 12 „ ...	0.0376	0.0516	0.0140
Total	0.0469	0.1407	0.0938

Intestine.

(1) 50 c.c. extract of cat's intestine (which hydrolysed trypsin digest of caseinogen) + 200 c.c. phytin solution.

P ₂ O ₅ in gm.	
At commencement	0·0126
After 1 day ...	0·0145
„ 2 days ...	0·0158
„ 4 „ ...	0·0180
Total	0·1154

(2) 100 c.c. extract of dog's intestine + 150 c.c. phytin solution.

P ₂ O ₅ in gm.	
At commencement	0·0190
After 5 days ...	0·0179
Total	0·1116

(3) 150 c.c. extract of dog's intestine + 100 c.c. sodium phytate solution.

P ₂ O ₅ in gm.	
At commencement	0·0256
After 1 day ...	0·0264
„ 2 days ...	0·0283
„ 9 „ ...	0·0306
Total	0·1154

(4) 200 c.c. extract of ox intestine + 100 c.c. sodium phytate solution.

P ₂ O ₅ in gm.	
At commencement	0·0198
After 1 day ...	0·0219
„ 3 days ...	0·0251
„ 5 „ ...	0·0264
„ 8 „ ...	0·0277
Total	0·0964

(5) 150 c.c. extract of sheep's intestine + 120 c.c. sodium phytate solution.

P ₂ O ₅ in gm.	
At commencement	0·0137
After 6 days ...	0·0139
Total	0·0913

(6) 100 c.c. extract of rabbit's intestine + 150 c.c. sodium phytate solution.

P ₂ O ₅ in gm.	
At commencement	0·0085
After 6 days ...	0·0106
Total	0·0964

(7) 150 c.c. extract of intestine of rabbit fed for 3 weeks on bran + 100 c.c. sodium phytate solution.

P ₂ O ₅ in gm.	
At commencement	0·0188
After 2 days ...	0·0267
„ 5 „ ...	0·0299
„ 8 „ ...	0·0301
Total	0·1065

Castor oil seeds.

(a) 150 c.c. acetic acid extract + 100 c.c. sodium phytate solution.

P ₂ O ₅ in gm.	
At commencement	0·0134
After 1 day ...	0·0190
„ 2 days ...	0·0230
„ 4 „ ...	0·0291
Total	0·1103

(b) 100 c.c. acetic acid extract + 150 c.c. sodium phytate solution.

P ₂ O ₅ in gm.	
At commencement	0·0188
After 1 day ...	0·0279
„ 4 days ...	0·0458
„ 8 „ ...	0·0554
„ 12 „ ...	0·0633
Total	0·1458

Yeast.

(1) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. sodium phytate solution.

P ₂ O ₅ in gm.			
	A	B	Difference
At commencement ...	0·0291	0·0362	0·0071
After 7 days...	0·0362	0·0412	0·0050
Total	0·0583	0·1509	0·0926

(2) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. sodium phytate solution.

P ₂ O ₅ in gm.			
	A	B	Difference
At commencement ...	0·0330	0·0373	0·0043
After 9 days...	0·0359	0·0411	0·0052
Total	0·0579	0·1420	0·0841

Bran.

A. 125 c.c. extract + 125 c.c. water. B. 125 c.c. extract + 125 c.c. sodium phytate solution.

	P ₂ O ₅ in gm.		
	A	B	Difference
At commencement	0·0513	0·0566	0·0053
After 1 day ...	0·0531	0·1087	0·0556
„ 4 days ...	0·0559	0·1354	0·0795
„ 10 „ ...	0·0559	0·1354	0·0795
Total	0·0609	0·1510	0·0901

These figures show such a marked difference in the extent of hydrolysis by the extract of bran and by the other enzymes that it can scarcely be concluded that phytase exists in any of the extracts except in that of castor oil seeds; the hydrolysis in this case is very slow; only a very slight increase in the amount of inorganic phosphate is noticeable with the intestinal and liver extracts, and none with pancreatic juice and extract of zymine.

III. HEXOSEPHOSPHORIC ACID.

Harden and Young [1908] have found that hexosephosphoric acid is formed during the fermentation of glucose and fructose by yeast or by zymase, and that it is subsequently hydrolysed. They attribute the hydrolysis to a special enzyme, hexosephosphatase. Harding [1912] has recently investigated the action of enzymes upon hexosephosphate and has observed that an enzyme present in castor oil seeds hydrolyses it, and that this enzyme is not present in ox pancreas. These data are so closely parallel to those obtained with glycerophosphoric acid that it was very important to test the action of the intestine and of bran upon this compound. Extracts of both these tissues were found to contain hexosephosphatase. The parallelism between the action of glycerophosphatase and hexosephosphatase is thus extremely close, and it would seem that the same phosphatase hydrolyses both these compounds.

EXPERIMENTAL.

The hexosephosphoric acid employed in these two experiments was most kindly supplied to me by Dr Young. The solution of hexosephosphoric acid was neutralised and mixed with a neutral extract of intestinal mucous membrane and extract of bran, both of which were prepared by the methods described under glycerophosphoric acid. Toluene was added and the mixture kept at 37° C. The inorganic P₂O₅ was estimated in samples by precipitation

with ammonium magnesium citrate and converted into magnesium pyrophosphate. Total P_2O_5 in the solution was estimated by Neumann's method with another sample.

(a) *Intestinal Extract.*

100 c.c. extract + 160 c.c. hexosephosphate solution.

P_2O_5 in gm.			
At commencement	...	...	0.0435
After 2 days	...	...	0.0815
„ 4 „	...	...	0.0819
Total 0.0860			

P_2O_5 in intestinal extract added per 50 c.c. sample = 0.0146 gm.

(b) *Bran Extract.*

A. 100 c.c. bran extract + 150 c.c. water. B. 100 c.c. hexosephosphate solution + 150 c.c. water. C. 100 c.c. bran extract + 100 c.c. hexosephosphate solution + 50 c.c. water.

	A Bran extract alone	B Hexosephosphate alone	C Hexosephosphate + bran extract	Difference between C and A + B
At commencement	0.0225	0.0092	0.0352	0.0035
After 1 day	0.0236	0.0093	0.1006	0.0677
„ 2 days	0.0240	0.0110	0.1115	0.0765
„ 4 „	0.0244	0.0121	0.1188	0.0823
Total	0.0279	0.1801	0.2054	—

Hydrolysis has occurred with both intestinal extract and bran extract; it is most marked in the case of intestinal extract where it was complete in two days. Bran extract caused a marked hydrolysis in one day but it was not complete in four days, in which time scarcely half of the compound was hydrolysed.

IV. ETHYL PHOSPHORIC ACID AND DIETHYL PHOSPHORIC ACID.

The action of enzymes upon the simple esters of phosphoric acid has never been investigated. Ethyl phosphoric acid and diethyl phosphoric acid were therefore tested against those extracts of tissues which had been found to hydrolyse glycerophosphoric acid, phytic acid and hexosephosphoric acid. The parallelism in the action of the enzyme upon glycerophosphoric and hexosephosphoric acid occurred again in their action upon ethyl phosphoric acid; diethyl phosphoric acid was not hydrolysed by intestinal extract nor by extract of castor oil seeds; unfortunately the quantity of diethyl phosphoric acid available was not sufficient to test against the other extracts.

The material used in these investigations was prepared either by the

action of phosphoric pentoxide or of phosphorus oxychloride upon absolute alcohol. The product of the reaction in each case was neutralised with excess of baryta; barium phosphate was filtered off and the clear filtrate precipitated by an equal volume of alcohol; barium ethyl phosphate was precipitated in crystalline form and recrystallised from water and alcohol. This was converted into the sodium salt by treatment with the calculated quantity of sodium sulphate and the solution so obtained was used in the experiments with the enzymes. A small quantity of barium diethyl phosphate was contained in the alcoholic filtrate obtained in the preparation by means of phosphorus pentoxide; it crystallised out on concentration of the alcoholic solution and was purified by recrystallisation from dilute alcohol. The solution of the sodium salt prepared by treatment of the barium salt with sodium sulphate was employed for the investigation of the action of the enzymes.

EXPERIMENTAL.

The enzyme solutions were prepared in the same way as described under glycerophosphoric acid. Inorganic phosphate was estimated by precipitation with ammonium magnesium citrate and conversion into magnesium pyrophosphate. The experiments were the following:—

A. *Sodium Ethyl Phosphate.**Pancreas.*

0.5 gm. trypsin (Fairchild) was dissolved in 110 c.c. decinormal sodium carbonate solution and filtered. 100 c.c. extract + 200 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0.0052
After 11 days ...	0.0046
Total	0.0659

Intestine.

(1) 125 c.c. neutral intestinal extract + 130 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0.0168
After 1 day ...	0.0492
„ 2 days ...	0.0572
„ 4 „ ...	0.0628
Total	0.0654

(2) 110 c.c. neutral intestinal extract + 150 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0.0121
After 1 day ...	0.0457
„ 2 days ...	0.0560
„ 4 „ ...	0.0626
Total	0.0672

Castor oil seeds.

(1) 125 c.c. acetic acid extract of seeds + 130 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0.0070
After 1 day ...	0.0235
„ 2 days ...	0.0307
„ 4 „ ...	0.0383
Total	0.0697

(2) 110 c.c. neutralised extract of castor oil seeds + 150 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0.0075
After 1 day ...	0.0135
„ 4 days ...	0.0177
Total	0.0735

Yeast.

60 c.c. extract of zymine + 200 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0.0111
After 1 day ...	0.0283
„ 5 days ...	0.0398
„ 12 „ ...	0.0491
Total	0.1052

Bran.

60 c.c. extract of bran + 200 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0.0148
After 1 day ...	0.0190
„ 2 days ...	0.0216
„ 5 „ ...	0.0259
„ 12 „ ...	0.0319
Total	0.0938

B. *Sodium Diethyl Phosphate.**Intestine.*

150 c.c. acid extract of intestinal mucosa + 100 c.c. sodium diethyl phosphate solution: after 3 days solution neutralised with a few drops of sodium carbonate solution.

P_2O_5 in gm.	
At commencement	0.0250
After 13 days ...	0.0250
Total	0.0888

Castor oil seeds.

150 c.c. neutralised acid extract + 100 c.c. sodium diethyl phosphate solution: after 4 days acidified with 2 drops of glacial acetic acid.

P_2O_5 in gm.	
At commencement	0.0138
After 11 days ...	0.0156
Total	0.0875

Sodium ethyl phosphate is hydrolysed most readily by the extract of the intestinal mucosa and it is hydrolysed by an acid extract of castor oil seeds; the hydrolysis also takes place where the extract is neutralised.

Extracts of yeast and of bran also contain an enzyme which can hydrolyse it; in the case of bran the hydrolysis is very slow.

Sodium diethyl phosphate was not hydrolysed by either intestinal extract or extract of castor oil seeds.

V. HYDROXYMETHYL-PHOSPHINIC ACID ($OH \cdot CH_2 \cdot PO_3 \cdot H$).

The previous results of the action of enzymes upon compounds which are esters of phosphoric acid led to an investigation of their action upon hydroxymethyl-phosphinic acid, a compound in which the phosphoric acid is linked to carbon instead of through oxygen as in the other compounds.

This compound was prepared by Mr H. J. Page [1912] in this laboratory and a quantity was kindly placed at my disposal for this purpose. No natural organic phosphorus compounds of this constitution are known; these experiments were consequently of further interest, since if hydroxymethyl-phosphinic acid were hydrolysed by enzymes, natural organic compounds of this constitution might be expected to occur in nature.

EXPERIMENTAL.

The solution of the free acid was exactly neutralised and mixed with the enzyme solution which was prepared as previously described; the analysis was carried out in the same way as with glycerophosphoric acid.

The results were:

(a) *Action of Trypsin.*

105 c.c. $\frac{N}{10}$ sodium carbonate extract of 0.5 gm. trypsin (Fairchild) + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P_2O_5 in gm.	
At commencement	0.0046
After 7 days ...	0.0055
Total	0.1344

(c) *Action of Extract of Castor oil seeds.*

100 c.c. acetic acid extract of castor oil seeds + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P_2O_5 in gm.	
At commencement	0.0009
After 4 days ...	0.0019
Total	0.2840

(e) *Action of Extract of Zymín.*

(1) 100 c.c. extract of zymín + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P_2O_5 in gm.	
At commencement	0.0393
After 1 day ...	0.0487
„ 2 days ...	0.0509
„ 4 „ ...	0.0528
„ 7 „ ...	0.0549
Total	0.2333

(b) *Action of Extract of Intestinal Mucosa.*

100 c.c. neutralised extract of dog's intestinal mucosa + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P_2O_5 in gm.	
At commencement	0.0134
After 4 days ...	0.0144
Total	0.2422

(d) *Action of Extract of Bran.*

100 c.c. extract of bran + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P_2O_5 in gm.	
At commencement	0.0508
After 4 days ...	0.0526
Total	0.2244

(2) A. 100 c.c. extract of zymín + 150 c.c. H_2O as control. B. 100 c.c. extract of zymín + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P_2O_5 in gm.			
	A	B	Difference
At commencement ...	0.0216	0.0227	0.0011
After 2 days...	0.0296	0.0362	0.0066
„ 5 „ ...	0.0310	0.0393	0.0083
„ 8 „ ...	0.0329	0.0424	0.0095
Total	0.0583	0.1977	0.1394

As was expected this compound was not hydrolysed by any of the extracts which contain glycerophosphatase even if we include the action of the extract of zymín; in this case an increase of inorganic P_2O_5 was observed at the commencement but the increase was very slight later on; it is probably due to a more rapid separation of P_2O_5 in the enzyme solution arising from a change in reaction when the mixture was made.

VI. NUCLEIC ACID.

The action of the enzymes of animal organs upon various nucleic acids has been studied by many investigators, but their attention has been confined almost entirely to the separation of the purine bases from the complex molecule, and little or no attention has been given to the separation of the phosphoric acid. Milroy [1896] found that 10–16 per cent. of the total phosphorus in a peptic digest of nuclein was inorganic phosphoric acid, and that in a trypsin digest the amount of inorganic phosphoric acid varied from 6–47 per cent. of the total phosphorus content. No estimation of the amount of the phosphoric acid present in the enzyme solution was made, so that a portion or the whole of the inorganic phosphate might have been so introduced into his solutions. Levene and Medigreceanu [1911] in some of their recent papers state that the enzymes of the intestinal mucosa hydrolyse nucleic acid with the liberation of phosphoric acid; no estimation of the amount of the inorganic phosphate was made. The formation of phosphoric acid during the autolysis of yeast, first observed by Béchamps in 1865, was attributed to the hydrolysis of nucleic acid; hexosephosphate and hexosephosphatase were unknown at that time and it is most probable that the phosphoric acid observed by Béchamps originated from this compound rather than from nucleic acid.

There is thus no satisfactory proof that inorganic phosphate is liberated by enzymes from nucleic acid though it is very probable. The following experiments were therefore carried out to test these earlier observations and to compare the hydrolysis of nucleic acid by the same enzymes which have been found to liberate phosphoric acid from the other organic phosphorus compounds.

It has not been possible to test all the various nucleic acids but the results show that an extract of intestinal mucosa slowly separates inorganic phosphoric acid from thymus nucleic acid and from wheat nuclein, but more rapidly and completely from the organic phosphorus compound (inosinic acid) in meat extract; trypsin does not hydrolyse thymus nucleic acid, nor the compound in meat extract. An extract of zymine showed no appreciable separation of phosphoric acid from the thymus nucleic acid, whereas an extract of bran hydrolysed it.

EXPERIMENTAL.

The thymus nucleic acid used in these experiments was prepared for me by Miss R. F. Skelton by Kossel's method [1894]. The preparation was almost pure white in colour and contained 8 per cent. of phosphorus and

17 per cent. of nitrogen. It dissolved readily in decinormal sodium carbonate giving a solution which was faintly brown in colour.

The meat extract was a solution of lemco, and the wheat nuclein was also kindly prepared for me by Miss Skelton from wheat germ by digestion with pepsin hydrochloric acid. The insoluble residue of nuclein was dissolved in dilute sodium carbonate, separated from starch by centrifugalisation and then reprecipitated by acid. After washing with water it was dissolved in decinormal sodium carbonate solution and the brown solution thus obtained was employed.

The enzyme extracts were prepared in the same way as those used in investigating the hydrolysis of glycerophosphoric acid; the extract was mixed with the solution of substrate and kept in the presence of toluene at 37° C. Samples were removed and inorganic P_2O_5 estimated by precipitation with ammonium magnesium citrate. Total P_2O_5 was estimated in another sample by Neumann's method.

(a) *Action of Extract of Pancreas.*

(1) 2 gm. trypsin (Fairchild) were dissolved in 260 c.c. $\frac{N}{10}$ sodium carbonate solution and filtered. A. 125 c.c. extract + 125 c.c. water. B. 125 c.c. extract + 125 c.c. solution containing 2.3 gm. thymus nucleic acid dissolved in $\frac{N}{10}$ sodium carbonate.

P_2O_5 in gm.		
	A	B
At commencement	0.0071	0.0070
After 1 day ...	0.0074	0.0087
„ 11 days ...	0.0074	0.0089
Total	0.0139	0.0951

(b) *Action of Extract of Intestine.*

(1) 100 c.c. neutralised extract of intestinal mucous membrane of dog + 150 c.c. solution containing 2 gm. thymus nucleic acid in $\frac{N}{10}$ sodium carbonate.

P_2O_5 in gm.	
At commencement	0.0152*
After 1 day ...	0.0254
„ 2 days ...	0.0277
„ 4 „ ...	0.0310
„ 7 „ ...	0.0327
Total	0.0938

* 0.0117 gm. P_2O_5 was contained in the intestinal extract for each sample.

(2) 0.5 gm. trypsin (Fairchild) dissolved in 110 c.c. $\frac{N}{10}$ sodium carbonate solution and filtered. 100 c.c. extract + 150 c.c. of a solution containing 10 gm. of lemco.

P_2O_5 in gm.	
At commencement	0.1189
After 7 days ...	0.1194
Total	0.1331

(2) 100 c.c. neutralised extract of intestinal mucous membrane of dog + 200 c.c. solution of wheat nuclein in sodium carbonate neutralised with acetic acid.

P_2O_5 in gm.	
At commencement	0.0147
After 1 day ...	0.0211
„ 2 days ...	0.0200
„ 4 „ ...	0.0228
Total	0.0596

(3) 60 c.c. neutralised extract of intestinal mucous membrane of a dog + 11 gm. lemco dissolved in 200 c.c. water.

P_2O_5 in gm.	
At commencement	0·1254
After 1 day ...	0·1458
„ 4 days ...	0·1470
Total	0·1521

(4) 100 c.c. neutralised extract of intestinal mucous membrane of cat + 10 gm. lemco dissolved in 160 c.c. water.

P_2O_5 in gm.	
At commencement	0·1409
After 1 day ...	0·1668
„ 5 days ...	0·1712
Total	0·1839

(c) *Action of Extract of Zymin.*

A. 100 c.c. extract of zymin + 150 c.c. H_2O .
 B. 100 c.c. extract of zymin + 150 c.c. of a solution containing 2 gm. thymus nucleic acid in $\frac{N}{10}$ sodium carbonate.

		P_2O_5 in gm.		Difference
		A	B	
At commencement	...	0·0216	0·0235	0·0019
After 2 days...		0·0296	0·0322	0·0026
„ 5 „ ...		0·0310	0·0353	0·0043
„ 8 „ ...		0·0329	0·0380	0·0051
Total		0·0583	0·1281	0·0698

(d) *Action of Extract of Bran.*

100 c.c. extract of bran + 150 c.c. solution containing 2 gm. thymus nucleic acid in decinormal sodium carbonate.

P_2O_5 in gm.	
At commencement	0·0297*
After 1 day ...	0·0432
„ 2 days ...	0·0498
„ 5 „ ...	0·0625
Total	0·1091

* 0·0279 gm. was contained in the bran extract for each sample.

Trypsin has no action upon nucleic acid; the action of intestinal extract upon thymus nucleic acid is very slight and is still less upon wheat nuclein; it readily hydrolyses the organic phosphorus compound contained in lemco. Extract of zymin has also a very slight hydrolysing action, but extract of bran is more active. No marked hydrolysis of nucleic acid is noticeable by any of the extracts.

VII. PHOSPHOPROTEIN.

The separation of the phosphorus of caseinogen by the action of enzymes and by alkali was investigated in 1906 by Plimmer and Bayliss [1906], who confirmed the earlier work of Biffi with regard to the action of trypsin. Pepsin slowly converted the phosphorus of caseinogen into a soluble form, but without the formation of phosphoric acid. Trypsin separated 35 per cent. of the phosphorus as phosphoric acid, the remaining 65 per cent. being present as a soluble organic compound. Papain also converted the phosphorus of caseinogen into a soluble form, but no investigation was made as to whether phosphoric acid was produced.

Complete separation of the phosphorus from caseinogen was thus not effected by trypsin. It seemed however very probable that the enzyme of

the intestinal mucosa, most probably erepsin, which according to Cohnheim hydrolyses caseinogen, would complete the separation as phosphoric acid. This was found to be the case. Papain also separates phosphoric acid from caseinogen in both acid and alkaline media but the separation is very slow. The vegetable proteoclastic enzyme "digestin" prepared by Yenjo in Japan from an *Aspergillus* behaves like papain. Neither an extract of zymin nor an extract of bran separates phosphoric acid from the organic phosphorus compound remaining after the action of trypsin upon caseinogen. It might have been expected that the extract of zymin would hydrolyse this compound and its inactivity is probably accounted for by its method of preparation with acetone, which coagulates the protein to which the proteoclastic enzyme is attached, so that the latter is not extracted by water.

EXPERIMENTAL.

A trypsin digest of caseinogen was prepared by digesting 200 gm. pure caseinogen dissolved in 2000 c.c. water containing 10 c.c. ammonia of sp. gr. 0.880 with 3 gm. trypsin (Fairchild) for two months at 37° in the presence of 20 c.c. of toluene. The solution was no longer alkaline to phenolphthalein after one month and ammonia was added till the solution became just red to this indicator. At the end of this time the tyrosine which had separated was filtered off. The clear brown solution contained 0.0456 gm. total P_2O_5 and 0.0209 gm. inorganic P_2O_5 per 50 c.c. 56 per cent. of the total P_2O_5 was thus present as organic P_2O_5 . A portion of this solution which was kept for another four months was found to contain 0.0418 gm. total P_2O_5 and 0.0228 gm. inorganic P_2O_5 per 50 c.c. A year later the amounts were 0.0430 gm. total P_2O_5 and 0.0242 gm. inorganic P_2O_5 , i.e. the solution contained 45 per cent. organic phosphorus.

Hydrolysis thus proceeds but even after 18 months it is not complete.

The action of the extracts of the intestinal mucosa, bran and zymin was investigated in the same way as described under glycerophosphoric acid. The action of papain and digestin was investigated with caseinogen which was dissolved in dilute caustic soda solution (20 gm. caseinogen + 40 c.c. semi-normal NaOH + 500 c.c. water): one half of this mixture was used for the experiment in alkaline solution and 200 c.c. of the remainder were acidified with 25 c.c. of semi-normal sulphuric acid and employed for the experiment in acid solution. Samples were removed at intervals and filtered for the estimation of the inorganic P_2O_5 and total P_2O_5 . The action of papain was also tested upon the above trypsin digest of caseinogen.

The experiments were:

Action of Intestinal Extract.

(a) 125 c.c. extract of cat's intestine + 125 c.c. trypsin digest of caseinogen.

P_2O_5 in gm.	
At commencement	0.0223
After 3 days ...	0.0500
„ 5 „ ...	0.0492
Total	0.0579

(b) 50 c.c. acid extract of cat's intestine + 200 c.c. trypsin digest of caseinogen.

P_2O_5 in gm.	
At commencement	0.0224
After 18 hours ...	0.0302
„ 24 „ ...	0.0313
„ 42 „ ...	0.0325
„ 4 days ...	0.0338

Total 0.0406

(c) 50 c.c. acid extract of dog's intestine + 200 c.c. trypsin digest of caseinogen.

P_2O_5 in gm.	
At commencement	0.0244
After 17 hours ...	0.0329
„ 65 „ ...	0.0357
„ 6 days ...	0.0367

Total 0.0431

Action of Papain.

(a) In acid solution on caseinogen.

P_2O_5 in gm.	
After 38 days	0.0037
„ 64 „	0.0052
Total	0.0203

(b) In alkaline solution on caseinogen.

P_2O_5 in gm.	
After 38 days	0.0075
„ 64 „	0.0092
Total	0.0349

(c) On trypsin digest. 200 c.c. trypsin digest + 55 c.c. extract of papain.

P_2O_5 in gm.	
At commencement	0.0166
After 1 day ...	0.0168
„ 2 days ...	0.0166
„ 3 „ ...	0.0168

Total 0.0368

Action of Digestin (Yenjo).

(a) In acid solution on caseinogen.

P_2O_5 in gm.	
After 4 months	0.0124
Total	0.0203

(b) In alkaline solution.

P_2O_5 in gm.	
After 4 months	0.0236
Total	0.0330

Action of Extract of Zymin.

A. 200 c.c. trypsin digest + 50 c.c. extract.
B. 200 c.c. water + 50 c.c. extract.

P_2O_5 in gm.			
	A	B	Difference
At commencement ...	0.0286	0.0085	0.0201
After 2 days...	0.0316	0.0131	0.0185
„ 4 „ ...	0.0282	0.0139	0.0143
Total	0.0634	0.0279	0.0355

Action of Extract of Bran.

A. 200 c.c. trypsin digest + 50 c.c. extract.
B. 200 c.c. water + 50 c.c. extract.

P_2O_5 in gm.			
	A	B	Difference
At commencement ...	0.0338	0.0142	0.0196
After 2 days...	0.0315	0.0148	0.0167
„ 4 „ ...	0.0327	0.0147	0.0180
Total	0.0494	0.0190	0.0304

In comparison with the other extracts that of the intestinal mucosa is the most active in separating phosphoric acid from caseinogen. The hydrolysis is almost complete within 24 hours; in all these experiments a small quantity remained in an organic form. This is most probably due to impurity and corresponds to the small quantity of organic phosphorus which always remains

when phosphoprotein is decomposed by alkali, as was found by Plimmer and Scott [1908].

Papain in alkaline solution separated one quarter of the total P_2O_5 in 35 days and this amount was slightly increased in 64 days; in acid solution in the same time one fifth was separated as inorganic phosphate, which amount was increased to one quarter in 64 days; further separation does not seem to occur as papain does not hydrolyse the organic P_2O_5 present in the trypsin digest of caseinogen. Digestin also never separated the whole of the phosphorus of caseinogen. Neither extract of bran nor extract of zymine separated phosphoric acid from the organic phosphorus in a trypsin digest.

SUMMARY AND CONCLUSIONS.

The action of enzymes upon the organic phosphorus compounds is best summarised by a tabulation of the results:

	Pancreas	Liver	Intestine	Castor oil seeds	Yeast (Zymine)	Bran
Glycerophosphoric acid ...	0	0	+	+	+	+
Hexosephosphoric „ ...	0	...	+	+	+	+
Ethyl phosphoric „ ...	0	...	+	+	+	+
Diethyl phosphoric „ ...	...	...	0	0	...	...
Phytic acid ...	0	0	0	+	0	+
Nucleic acid (Thymus) ...	0	...	+	...	+	+
„ „ (Wheat) ...	...	...	+	...	...	...
„ „ (Meat) ...	0	...	+	...	...	...
Hydroxymethylphosphinic acid	0	...	0	0	+	0
Phosphoprotein ...	2	...	+	...	0	0

The most active tissue in the hydrolysis of the organic phosphorus compounds is the intestinal mucosa; all the organic phosphoric compounds except phytic acid were hydrolysed by it. Diethyl phosphoric acid and hydroxymethyl-phosphinic acid must be excluded entirely as they were not hydrolysed by any extract. Phytic acid and phosphoprotein stand out in marked contrast with the other compounds. Phytic acid is attacked readily only by the enzyme in bran extract. Phosphoprotein is the only compound which is hydrolysed by the pancreas. The constitution of phosphoprotein is different from that of the other compounds; it is a protein and is hydrolysed by the proteoclastic enzymes and it seems most likely that the separation of the phosphoric acid is effected by the same enzyme which unlinks the amino-acids from one another. The other organic phosphorus compounds examined are esters of phosphoric acid. The inactivity of the animal tissues most rich in lipase and the activity of an extract of castor oil seeds which does not contain lipase shows that the lipoclastic enzymes have no action on these

esters and that they are hydrolysed by a special group of enzymes. Are we to consider that these esters of phosphoric acid are all hydrolysed by one enzyme, or are we to consider that each compound is hydrolysed by a specific phosphatase? If there were only one phosphatase then phytic acid should be hydrolysed by the enzyme of the intestine, but as this is not the case we must regard phytase as a specific enzyme. The varying extent of the hydrolysis of the other compound by the different extracts supports the supposition that there is a specific enzyme for each phosphoric ester. It can scarcely, however, be admitted that the specificity is really so great. If the chemical constitution of each of the compounds be considered it is seen that glycerophosphoric acid and ethyl phosphoric acid are simple esters, hexosephosphoric acid is regarded as hexosediphosphoric acid and nucleic acid is a complex made up of hexose or pentose mono-phosphoric acid.

One enzyme is supposed to hydrolyse the group of α -glucosides, another enzyme the group of β -glucosides, invertase separates fructose from sucrose and raffinose, trypsin attacks the polypeptide linking of the amino-acids; consequently it can scarcely be conceived that the specificity of the phosphatases extends to every ester and it would be better to group the phosphatases into several classes such as mono-phosphatases which attack mono-esters like glycerophosphoric acid and ethyl phosphoric acid, di-phosphatases which attack di-esters like hexosephosphoric acid, hexa-phosphatases which attack hexa-esters like phytic acid. Glycerophosphatase will hydrolyse both glycerophosphoric acid and ethyl phosphoric acid as well as other simple esters. Proof of such a supposition will only be given by further work when more simple esters have been examined and when the constitution of hexosephosphoric acid, phytic acid, and nucleic acid have been definitely established.

These experiments confirm the work of Fingerling and Gregersen which shows that the animal organism can synthesise its organic phosphorus compounds from inorganic phosphates. They are not in favour of the belief that organic phosphorus compounds are essential for the well-being of animals, a belief which has arisen from feeding experiments with inorganic and organic phosphorus. No proper control was ever made in such experiments. When phosphoprotein was compared with albumin as the substitute for the supply of protein the nutritive value of albumin was reckoned as equivalent to that of caseinogen, no attention being paid to the different composition of these proteins in amino-acids; when phytic acid, or phytin, was compared with inorganic phosphate the control was not made with inositol and phosphoric acid. Further, if assimilation of organically combined phosphorus occurred,

both phosphoprotein and phytic acid should be found in the blood and tissues. Phosphoprotein occurs only as the secretion of the mammary gland in mammals and of the yolk gland in birds; in these tissues it is synthesised. Phytin has never been found in animals but inositol has been found in most tissues.

The organic phosphorus compounds except phytic acid are all hydrolysed by the intestinal mucosa and their fate in assimilation is like that of all the foodstuffs:—the proteins are known to be completely broken down into amino-acids before they enter the circulation; the fats are hydrolysed as they pass through the intestinal wall; the disaccharides are broken down by enzymes in the mucous membrane of the small intestine. The organic phosphorus compounds are therefore almost certainly assimilated as inorganic phosphate and the organic radicle with which the phosphorus is combined.

The non-hydrolysis of phytic acid by the intestinal mucosa was at first attributed to the experiment being made with carnivora (dog and cat), to which animals vegetable food is not natural, but the same experiment with the herbivora (sheep, ox and rabbit) required some further explanation as to the manner of assimilation of this compound. The excretion of the phosphorus of phytic acid as inorganic phosphate, which was shown by Scofone, Giascosa, Mendel and Underhill and also Horner, pointed to the hydrolysis of the compound. If the absorption be not complete, as the above workers have pointed out, unchanged phytic acid should be present in the faeces. A rabbit was therefore fed for three weeks on food containing a large proportion of bran, and its faeces were examined at the end of this period for organic and inorganic phosphate. The faeces were extracted with dilute hydrochloric acid; inorganic and total P_2O_5 were estimated in the extract by precipitation with ammonium magnesium citrate and by Neumann's method. The phosphorus present in the extract was almost entirely inorganic P_2O_5 , as is shown by the following figures:

	H_2O	Total P_2O_5	Extract		Residue Total P_2O_5
			Total P_2O_5	Inorganic P_2O_5	
Nov. 30, 1911	14.8 per cent.	1.9 per cent.	1.5 per cent.	1.4 per cent.	0.2 per cent.
Dec. 1	27.7	1.7	1.6	1.4	—
„ 5	30.0	1.7	1.6	1.5	0.2
„ 6	25.6	2.0	2.0	1.9	—
„ 8	35.3	1.9	1.7	1.4	0.2

Hydrolysis of the phytic acid had occurred; this is not caused by the acid employed in extracting the faeces, for dilute acid at the ordinary temperature does not hydrolyse phytic acid (see following paper). Since the

animal was fed on bran the hydrolysis must be effected by the phytase in the foodstuff. The assimilation of phytic acid by the herbivora thus resembles that of cellulose, which was shown by Horace Brown [1892] to be hydrolysed by the cytase present in the vegetable food.

In carnivora and man, the assimilation of the phosphorus of phytic acid is also in the form of inorganic phosphate. Rogoginski [1910] has shown that in dogs the unabsorbed phytin is present as such in the faeces and that in man the phytic acid is hydrolysed by the bacteria in the large intestine. He also finds no beneficial effect on metabolism from the organic phosphorus compounds. Absorption of phytic acid by animals would require rapid dialysis through the semi-permeable membranes. The dialysis of phytic acid through parchment membranes is slow and is only complete after several days. This is shown by the following experiments:

	26 c.c. sodium phytate inside 50 c.c. water outside P ₂ O ₅ in 5 c.c.		30 c.c. sod. phyt. inside 100 c.c. water outside P ₂ O ₅ in 5 c.c.		70 c.c. sod. phyt. inside 200 c.c. water outside P ₂ O ₅ in 5 c.c.	
	Inside	Outside	Inside	Outside	Inside	Outside
	26.9 mgm.	0 mgm.	31.5 mgm.	0 mgm.	32.5 mgm.	0 mgm.
After 1 day	—	1.5	—	4.4	—	2.7
„ 2 days	—	5.3	—	7.0	—	5.1
„ 3 „	—	—	—	7.6	—	6.7
„ 4 „	11.5	9.3	8.0	8.0	20.0	6.6

In all probability the membrane of the intestine of animals behaves in the same way as the semi-permeable membrane of plant seeds, which does not allow acids to pass through, as was shown by Adrian Brown [1909].

There is thus no evidence of the absorption of phytic acid; it will be hydrolysed either by the enzyme in the foodstuffs, or by bacteria in the large intestine, and will enter the circulation of animals as inositol and phosphoric acid.

The value of the organic phosphorus compounds in nutrition entirely depends on the nature of the organic matter with which the phosphoric acid is combined.

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