CI. KIDNEY PHOSPHATASE.

By HERBERT DAVENPORT KAY (*Beit Memorial Research Fellow*).

From the Medical Unit, The London Hospital.

(*Received July 1st, 1926.*)

During the past few years much attention has been directed towards the acid-soluble phosphorus compounds present in muscle and in the blood, and to the related synthetic and hydrolytic systems. There are present in each of these tissues at least two forms of organic phosphorus compounds which are not precipitated on acidification, and which are in equilibrium, *in vivo*, with the inorganic phosphate present. This equilibrium is controlled by balanced synthetic and hydrolytic systems, both of which, by suitable means, can be shown to exist. The synthetic system, which brings about the esterification of inorganic phosphoric acid is, in each case, more closely associated with the living tissue, though not with the integrity of the cells, than is the hydrolytic, and is much more readily destroyed. Embden and his fellow workers found that in the presence of certain salts such as sodium fluoride and calcium chloride, the synthetic activity of fresh muscle, or muscle juice, outweighs the hydrolytic, whilst Lawaczeck [1924] and Martland, Hansman and Robison [1924] have demonstrated the presence of a synthetic system in recently shed blood. Martland [1925] has shown how delicately balanced is the equilibrium between organic and inorganic phosphorus in blood, slight alterations from the normal hydrogen ion concentration of the blood bringing about either synthesis or hydrolysis. Alkalinity favours the former, and acidity the latter.

In only a few tissues of the body has the phosphorus partition been studied closely. It has been recognised for a long time that phosphorus in organic combination is universally present in the cells of the body. Both "lecithin" and nucleic acid, which do not come into the acid-soluble category, have been detected in most of the organs. Some form of acid-soluble organic phosphorus compound also occurs in most tissues and body fluids which have been examined, namely, muscle, blood, bone, liver, kidney, milk, gastric and intestinal mucosa, spleen, mammary and suprarenal gland. It was shown by Kay and Robison [1924] that the acid-soluble phosphorus in red corpuscles was present in at least two forms, one hydrolysable by bone phosphatase, the other stable to this enzyme. Both forms appeared to be esters of orthophosphoric acid. Goodwin and Robison in subsequent work [1924] showed that they were probably not simple esters. Milk also contains organic acid-soluble phosphorus in at least two forms [Kay, 1925] and hitherto unpublished work

by this author has shown that the same is true for freshly excised liver, kidney, spleen, mammary and suprarenal gland. All these tissues contain phosphatases in greater or less amounts. In fact it may be said that in the tissues of the body and probably in every cell (though not necessarily in the body fluids), wherever there is acid-soluble organic phosphorus, there also is a phosphatase. Any comprehensive picture of the cell's activity must therefore find a place for both enzyme and substrate.

The problem of the physiological significance of the body phosphatases is a fascinating one, and several suggestions have been made with regard to it. Robison [1923] thought that the presence of such an enzyme in growing bone might be associated with the deposition of calcium salts in this tissue, and he and his collaborators have since supported this suggestion with a large amount of experimental evidence. The phosphatase is present, however, not only in the bones and in teeth, but also in considerable quantities in the intestinal mucosa and in the kidney. If it is indeed associated with bone formation, there is undoubtedly some other local factor which determines calcium phosphate deposition, otherwise one would expect calcification to begin, if not in the intestinal wall, then in the kidney. Possibly in the kidney (if, *in vivo*, kidney phosphatase is hydrolytically active) the inorganic phosphate, once liberated, is rapidly excreted and so safely removed from the sphere of the reaction, before it can combine with calcium.

Eichholtz, Robison and Brull [1925] have recently put forward the idea that "normally a considerable part, if not all, of the urinary phosphates is derived from the organic phosphates of the serum (plasma?) by a process of hydrolysis in the cells by the kidney enzyme."

It was decided to study a typical tissue phosphatase more closely, since this seemed to be a point of attack which might yield clues to the main problem of the part played by the organic phosphorus compounds in the functional activity of the tissues. In the present communication, work on kidney phosphatase, mainly of an exploratory nature, is described. At the same time, certain aspects of both the endeavours quoted above to find functions for these very active agents, known to be present in most of the essential tissues of the body, have received attention.

The acid-soluble phosphorus compounds in the normal kidney.

Before dealing with kidney phosphatase itself, a few quantitative observations on the acid-soluble phosphorus of kidney tissue (which contains a substrate upon which, presumably, kidney phosphatase acts in the living animal) are of interest.

The inorganic phosphate of the whole kidney increases rapidly if the fresh tissue is kept for a short time before determinations are made. In order, therefore, to obtain as nearly as possible the distribution of acid-soluble phosphorus in kidney tissue *in vivo*, the kidneys of rabbits which had been killed by bleeding were rapidly dissected out, put on an ice-cold plate, de-

capsulated, opened and freed from fat, connective tissue and traces of blood and urine, weighed and ground up as soon as possible with cooled 3 % trichloroacetic acid. After standing 10 minutes, the mass was made up to a definite volume, filtered, and aliquot portions of the filtrate taken for determinations of free, total acid-soluble, and enzyme-hydrolysable organic phosphorus. Bone phosphatase prepared from rat bone was used as the hydrolytic agent in the majority of the experiments. In three of these, kidney phosphatase was also employed and, on the whole, hydrolysed slightly more of the organic phosphorus than did the bone enzyme. In all cases there was sufficient excess of enzyme to bring about equilibrium in 18 hours. The results for normal rabbit kidney tissue are shown in the first part of Table I. In view of the difficulty of completely removing the last traces of fat, etc. from the pelvis of the kidney in the short time available, the results are surprisingly consistent. Both the organic and acid-soluble inorganic phosphorus of normal, adult kidney tissue are maintained during health within fairly narrow limits

Table I. *Acid-soluble phosphorus in the kidney.*

Normal kidney.

mg. P per 100 g. fresh tissue.

Exp.	Inorg. P	Total P	Hydr. P	Non-hydr. P	Remarks
5	29·5	110·0	60·0	20·5	Three weeks old rabbit
			65·3*	15·2*	
6	22·4	88·7	50·3	16·0	Adult
9	23·5	93·2	53·0	16·7	
			51·8*	17·9*	Adult
10 A	22·2	103·2	42·8	38·2	Adult
11 A	23·5	100·8	48·9	28·4	
			59·0*	18·3*	Adult
20	21·8	89·7	46·6	21·3	Adult
Average	23·8	97·6	50·3	23·4	

Effect of autolysis.

7	43·8	97·6	29·8	24·0	Young adult—kidney removed ¾ hour post-mortem
8	57·0	92·8	11·7	24·1	Adult—kidney removed 2·5 hours post-mortem
10 B	60·4	113·6	19·0	34·2	Adult—other kidney of 10 A above. Removed at once, but kept 3 hours at 35° before analysis
11 B	56·3	106·4	30·3	19·8	Adult—other kidney of 11 A above. Removed at once but kept 3 hours at 35° before analysis
			31·6*	18·5	

Effect of previous injury on rate of autolysis.

51 Normal	58·0	98·8	20·3	20·5	
Injured	62·4	96·4	16·2	17·8	See text
54 Normal	69·0	110·0	27·5	13·5	
Injured	79·8	104·0	18·5	5·3	See text
122 Normal	60·4	107·0	39·5*	7·1	
Injured	65·0	110·0	34·5*	10·5	See text

* Kidney phosphatase used as hydrolytic agent. These figures are not included in the averages.

of concentration, as has been found for the corresponding phosphorus values in the blood.

The organic phosphorus hydrolysable by phosphatase, when the greater experimental errors are taken into consideration, is also maintained at a very constant level in the adult kidney[1].

Effect of autolysis and of injury on the acid-soluble phosphorus of the kidney, As with muscle and with liver [Sevringhaus, 1923], but not with unlaked blood the inorganic phosphoric acid increases rapidly on leaving the fresh kidney to stand for a short time, either within or without the body, after death, and that even at room temperatures. The rate of autolysis of organic phosphorus compounds at 37° is distinctly increased in the kidney by post-mortem injury (second part of Table I).

In Exp. 51 (Table I) two kidneys were taken from the same rabbit as aseptically as possible and weighed. One was injured by striking several times with the sterile scissors. Both were put into an incubator at 37° and allowed to remain in an atmosphere saturated with water vapour for 3 hours, when they were taken out, ground up simultaneously with 3 % trichloroacetic acid, and the acid-soluble phosphorus partition determined.

In Exp. 54 a kidney was divided into two approximately equal halves and each weighed. One half was then cut up with sterile scissors into pieces approximately 2 mm. cube and both halves were put into the incubator, with the same precautions against evaporation, for 3 hours at 37° and analysed as before.

Exp. 122 was a repetition of 54, except that the injured half was damaged by a fairly heavy blow from a pestle.

From these experiments it may be concluded that the rapid production of inorganic phosphorus by the autolysing kidney takes place, as would be expected, principally at the expense of the hydrolysable organic phosphorus, and that there is also a small increase in inorganic phosphorus from non-acid-soluble sources (possibly from lecithin). The effect of injury is to accelerate the rate of inorganic phosphorus production at the expense of the hydrolysable organic phosphorus. It is clear that stoppage of the blood supply to the kidney, or mechanical injury of this organ, throws over the equilibrium (which, in the living animal, is maintained at a very constant level of inorganic phosphorus) to the side of rapid hydrolysis.

Is the equilibrium maintained in the kidney, as in the muscle, by a balanced synthetic and hydrolytic enzyme system? So far, in four experiments, although it has been found that sodium fluoride in effective concentrations of from $N/20$ to $N/10$ markedly hinders the hydrolysis of organic phosphorus com-

[1] In the kidney cells, as in muscle tissue, leucocytes and liver cells, there exists some mechanism by which the inorganic phosphorus is maintained well above that in the circulating blood. The resting-level of inorganic phosphorus in 100 g. rabbit's tissue is as follows: blood 5–6 mg., kidney 23·8 mg., liver 40 mg., muscle 95 mg. The resting-level of organic acid-soluble phosphorus is, however, in the rabbit, less variable from one tissue to another. In mg. per 100 g. it is for red cells 87·4, for kidney 73·8, for liver about 100 and for muscle 99.

pounds in fresh, macerated kidney at room temperatures, no definite evidence of synthesis, such as that described by Embden and his collaborators as occurring in the case of muscle, has been observed. This question must therefore be left open for the present.

KIDNEY PHOSPHATASE.

That there is an active phosphatase in the kidney was shown by Plimmer [1913]. Recent work of Tomita [1922], Forrai [1923, 1924], Robison [1923] and Takahashi [1924] has confirmed and extended these findings, and endeavours have been made by these authors to arrange the various tissues of the body in the order of their phosphatase activity. As Demuth [1925, 1] points out, unless care has been taken to examine the phosphatase activity at the optimum c_H for the enzyme, any series of this kind may be misleading. He finds an optimum c_H for the action of kidney phosphatase on sodium hexosediphosphate in the region of p_H 6. This I have not been able to confirm for the action of kidney phosphatase on either hexosediphosphate or glycerophosphate.

A fairly satisfactory separation of kidney phosphatase from the inactive residue may be carried out as follows. The kidney is ground up very thoroughly with sand and chloroform water and the mass allowed to stand overnight at room temperature. The following morning the mixture is again thoroughly ground up and water added until the total volume is 20 cc. for each g. of kidney tissue. It is then filtered slowly through cotton wool and the opalescent filtrate brought to a p_H of 8·9 with a few drops of 10 % sodium hydroxide. (The volume added in this way is negligible.) If the residual tissue, on the cotton wool, is freed from liquid, as far as possible, by suction, again ground up with water, made up to the same volume as before and again filtered, the filtrate still contains a very small amount of enzyme. In one experiment the second extract had between 6 and 7 % of the activity of the first. The residual kidney had a still smaller but definite phosphatase activity. A single extraction removes at least 90 % of the total phosphatase from the kidney. Using the same method of extraction throughout, results obtained, though quantitatively all some 10 % low, are comparable.

Optimum c_H for kidney phosphatase. Using a preparation of pig's kidney, made as described, it has been found that the optimum c_H, with either sodium glycerophosphate, sodium hexosediphosphate, the organic acid-soluble phosphoric ester of kidney tissue, or the similar ester of blood as substrate, is in the neighbourhood of p_H 9·0, usually between p_H 8·8 and 9·2. Phosphatase from rabbit, rat or human kidney gives the same results. It is, of course, necessary to employ buffer solutions, either borate[1] or glycine buffers (Sørensen) cover the range well. This remarkably alkaline optimum, the most alkaline

[1] Although the buffer is present in excess in all the determinations I have made, there is a theoretical objection to the use of borate buffers where glycerophosphate is the substrate, since the formation of the strong glyceroboric acid, which occurs as soon as glycerol is set free during the enzymic actions, might interfere with the p_H.

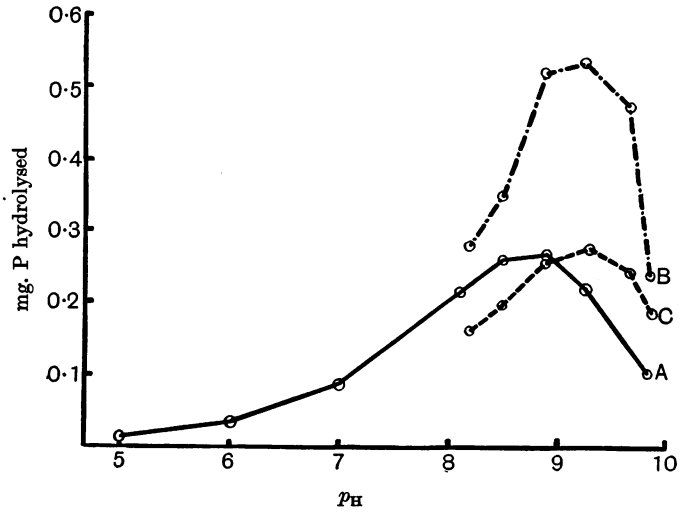

Fig. 1.

Curve A. Action of kidney phosphatase (pig) at differing c_H on sodium hexosediphosphate at 38° (borate buffers).

Curves B and C. Action of kidney phosphatase (pig) at differing c_H on sodium hexosediphosphate (B) and sodium glycerophosphate (C) at 38° (glycine buffers).

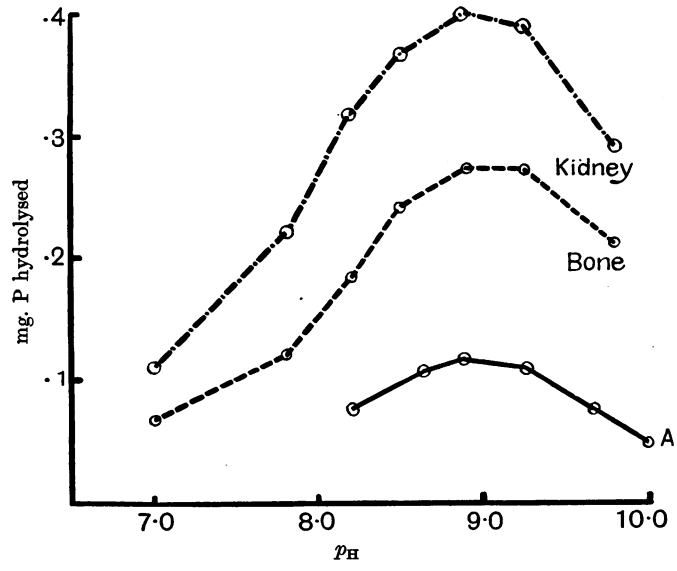

Fig. 2.

Curve A. Action of kidney phosphatase (rabbit) at differing c_H on the acid-soluble organic phosphorus compounds of rabbit's kidney at 38° (glycine buffers).

The other two curves show the action of bone and of kidney phosphatase from the same rat on sodium glycerophosphate at 38° at differing c_H (borate buffers).

yet recorded for any enzyme, agrees with that of bone phosphatase. The significance of this high optimum p_H is by no means clear.

The results of a few experiments which are typical of many others are shown in Figs. 1 and 2.

Using human or rabbit *plasma* phosphatase as the hydrolytic agent, and either glycerophosphate or hexosediphosphate as the substrate, the few experiments made point to the same optimum for this phosphatase also.

Method of estimating phosphatase activity of a tissue. The existence of a fairly broad optimum c_H for both bone and kidney phosphatase renders it possible to estimate with some accuracy the phosphatase content of a preparation of this enzyme. A unit of phosphatase is taken as the amount of enzyme which, at 38° and in Sørensen's glycine-NaOH buffer solution at p_H 8·9, will liberate 1 mg. of phosphorus from excess of sodium β-glycerophosphate solution in 2 hours. By preparing the enzyme solution from tissues in a standard way, one can obtain information as to the relative phosphatase contents of the latter. By this method, the relative activity of bone and kidney of the same animal at various stages of development, the distribution of enzyme in bone and kidney, and the phosphatase content of the kidney in certain cases of disease, have been investigated.

The relative phosphatase content of bone and of kidney. Tissue extracts were carefully prepared, in the standard way just described, from the kidneys and from the bones of a variety of animals, and their phosphatase value determined by the above method. With healthy adult animals, the phosphatase content per g. of whole kidney appears to be fairly constant for each species, but in the young animal the amount of enzyme present varies a good deal with the age. The tissues of the smallest animal investigated—the rat—appear to be most active, but the activity is not inversely proportional to the size of the animal, since the adult pig's kidney is more active than that of the adult rabbit.

Table II. *The phosphatase content of the kidney in adult animals.*

Animal	Phosphatase content (units per g. tissue)	Remarks
Rat	19·2	Average of 4
Rabbit	8·3	,, 2
Pig	12·9	,, 4
Man	About 5	—

Table III shows the change in kidney and bone phosphatase activity with the age of the animal in the case of the rabbit. The same general type of change in the case of kidney phosphatase is true for man; the kidney of the six months foetus is practically inactive, but at term the activity has increased and after birth rises rapidly, probably reaching a maximum before 10 years of age.

Table III. *Development of kidney and bone phosphatase in the rabbit.*

Age of animal	Units per g. tissue: Kidney phosphatase	Units per g. tissue: Bone phosphatase
2–3 days from term	2·6	18·0
New born	4·1	13·7
New born	3·4	15·6
10 days	8·2	15·0
24 days	9·6	11·0
10 weeks	8·2	8·8
Adult (1) 26 weeks	9·4	—
(2) ?	8·2	6·7
Old adult	7·6	1·4

The above results, which are supported by a few figures obtained from the rat, lead to the conclusion that the phosphatase of the kidney, which is low per g. of tissue in the foetal stage, and which increases rapidly just before, and in the few days immediately after, birth, is to be correlated with kidney *function*, and not with the rate of kidney growth. The amount per g. of bone phosphatase on the other hand appears to be a function of the *rate of growth* of this tissue. It is to be expected that as the proportion of inert, inorganic salts in the bone increases, the amount of enzyme present per g. of whole bone will diminish.

In the bone, however, as Robison and Soames [1924] first demonstrated, the enzyme is not equally distributed, and it has been found by the present author that if care is taken to separate the zone lying between the cartilage and shaft in growing bone, where ossification is rapidly proceeding, the activity of this zone is much greater than that of the whole kidney, or the kidney cortex, at any stage of development. The great concentration of phosphatase in this ossifying layer of cartilage has been strikingly shown in the case of a femur from a $6\frac{1}{2}$ months human foetus, confirming the results of Martland and Robison [1924]. In this femur, although the ossifying zone was not completely separated from the cartilage (which was quite inactive) before determining its phosphatase content, it was far more active, weight for weight, than the kidney of the same foetus, or than any human kidney which has been examined (see Table IV).

Table IV. *Relative phosphatase content of bone and kidney.*

Tissue	Phosphatase content (units per g. of tissue)
Foetal kidney	1·0
,, cartilage	nil
,, shaft	6·2
,, ossifying zone (some inactive cartilage present)	12·6
Most active human kidney	5·8

As concerns its phosphatase the ossifying zone in foetal cartilage is far more active than the kidney, and is in this respect probably the most active tissue in the entire foetal body.

The distribution of phosphatase in the kidney. The enzyme was found to be present in much larger amount in the cortex than in the medulla, in all cases examined.

Pig's kidney. Two portions of cortex were taken, one from the peripheral portion, the other from the inner portion adjoining the medulla. A portion of the medulla, mainly consisting of pyramids, was also worked up. The phosphatase content of each portion was as follows in phosphatase units per g. of tissue:

Outer cortex	14·2
Inner cortex	14·2
Medulla	2·1

The enzyme is thus very unequally distributed in the kidney. When hexosediphosphate was used as substrate, the enzyme preparation from the two portions showed the same relative hydrolytic activity as with glycerophosphate.

In another experiment with pig's kidney, the enzyme derived from the cortex and from the medulla (pyramids excluded as far as possible) was allowed to act, under parallel conditions, on solutions of sodium glycerophosphate and sodium hexosediphosphate, containing the same amount of organic phosphorus per cc. (see Table V).

Table V. *Distribution of phosphatase in pig's kidney.*

	mg. phosphorus hydrolysed in 2 hours	
Enzyme	from glycerophosphate	from hexosediphosphate
Cortex	0·234	0·318
Medulla	0·112	0·156
Ratio $\frac{\text{cortex}}{\text{medulla}}$	2·09 : 1	2·04 : 1

This experiment is quoted since it also provides some evidence that the same enzyme is responsible for the hydrolysis of both glycerophosphate and hexosediphosphate, or, if each is hydrolysed by a separate enzyme, then the relative amount of the two enzymes is the same in each portion of the kidney.

Rabbit's kidney. The cortex and medulla were separated as completely as possible with a scalpel, and each ground up with sand and chloroform water as usual. With such a small kidney separation was not complete, but the relative phosphatase content was found to be: cortex : medulla = 2 : 1.

Human kidney. In one experiment with a normal kidney the phosphatase content of the cortex was about four times that of the medulla.

Stability of kidney phosphatase. Preparation of solid phosphatase.

Temperature of inactivation. The enzyme is comparatively thermolabile. After exposure to a temperature of 60° for 5 minutes, there is 90 % inactivation, exposure to 55° for 5 minutes gives 22 % inactivation, slight inactivation after 10 minutes at 50–51°, no noticeable inactivation after 10 minutes at 47·5°. The experiments were made with the enzyme at p_H 7·0.

Stability on keeping. Kidney phosphatase kept at 0° and at 38° for some days gave the following figures in the usual phosphatase units.

Table VI. *Stability of phosphatase on keeping.*

Time kept (hours)	Phosphatase units	
	Kept at 0°	Kept at 38°
0	11·5	11·5
24	11·3	9·3
96	—	7·7
260	11·1	4·2

The enzyme is thus very stable at 0°.

Effect of autolysis of kidney tissue on its phosphatase content. Pig's kidney was finely minced and an equal volume of chloroform water added. Toluene was added to cover the surface of the mixture. Samples were taken for phosphatase determination as follows: at once, activity 15·6 units; after a week at 38°, 5·3 units; after 3 further days at 45°, activity 0·5 unit. Thus autolysis at 38° slowly inactivates kidney phosphatase. In quantitative work with this enzyme, *e.g.* determination of the optimum c_H for its activity, or determination of the phosphatase content of a tissue, experiments of short duration should therefore be made.

Effect of leaving kidney in the body after death. In order to find out whether material from the post-mortem room may be used without serious error as to initial phosphatase content, a few experiments have been carried out with rabbit's kidneys. It was found in preliminary experiments that the two kidneys of a normal rabbit were about equal in phosphatase content. A rabbit was killed, one kidney taken out at once, and its phosphatase content determined. The other kidney remained in the body for at least 24 hours, was then taken out and the determination repeated on this. The results of three experiments, using glycerophosphate as substrate were:

Rabbit (*a*). 12 % diminution in second kidney after 24 hours.
,, (*b*). 9 % diminution after 30 hours.
,, (*c*). 10 % diminution after 30 hours.

In another experiment, a fresh pig's kidney was divided into two parts, the phosphatase activity determined at once in one, and in the other part after keeping for 60 hours at room temperature (slight smell of putrefaction). The diminution in the second part was again 10 %. In the first 30 hours, therefore, post-mortem changes appear to effect a diminution of some 10 % in the phosphatase content of the kidney.

Preparation of a dried phosphatase. If kidney tissue is finely minced, rapidly dried *in vacuo* and ground up in a mortar to a fine powder, the latter is found to possess considerable phosphatase activity. In one experiment with pig's kidney, the activity per g. of *dried* tissue was 19·2 units, whilst the activity of the same kidney tissue as determined by the standard method, was 12·9 units per g. of *wet* weight, or over 40 units per g. dry weight. There

is thus a loss of activity on drying. Once dried, however, the powder retains its activity almost unimpaired for 2 months, and is thus a convenient source of enzyme of definite strength.

Although fractional precipitation of standard kidney enzyme preparations by alcohol or acetone did not yield portions having increased activity, it was observed that after leaving a kidney enzyme preparation at − 4° for a month, on warming to 0° a precipitate settled out which could be filtered off and washed free from salts and soluble proteins and was found to be highly active. The filtrate was almost inactive. The dried solid was found to have an activity of 21·6 units per g., which was retained in the desiccator for 6 weeks at least. The almost inactive filtrate was found to have no adjuvant action on the dried, washed enzyme, which points to the absence of a co-enzyme for kidney phosphatase. A second preparation of solid phosphatase gave the same result. A portion of this second preparation was dried with alcohol and ether. Its activity was found to have fallen from 19·7 units per g. when dried *in vacuo* to 11·4 units per g. when dried with alcohol and ether.

Action of kidney phosphatase on various organic phosphorus compounds.

Hexosephosphate and glycerophosphate. An extract of kidney tissue hydrolyses hexosediphosphate, hexosemonophosphate [Robison, 1923], saccharosephosphate [Neuberg and Behrens, 1926] and glycerophosphate, all phosphoric esters of polyhydroxy-alcohols, and, at least in the case of sodium hexosediphosphate and sodium β-glycerophosphate, the same enzyme appears

Table VII. *Relative rate of hydrolysis of hexosephosphate and glycerophosphate by kidney phosphatase.*

Exp.	Source of phosphatase	Ratio $\frac{\text{Inorg. P liberated from glycerophosphate}}{\text{Inorg. P liberated from hexosediphosphate}}$
5	Pig's kidney—cortex	0·74 : 1
	,, medulla	0·72 : 1
11	Normal human kidney	0·69 : 1
12	Rabbit's kidney—fresh	0·64 : 1
	,, after standing 24 hours in body	0·71 : 1
88	Rabbit's kidney—fresh	0·73 : 1
30	Rat kidney—fresh	0·78 : 1
33	10 days old rabbit—kidney	0·70 : 1
	,, bone	0·74 : 1
	Same bone enzyme after standing 5 days at 0°	0·65 : 1
40	10 weeks old rabbit—kidney	0·75 : 1
	,, bone	0·76 : 1
40 (a)	24 days old rabbit—kidney	0·70 : 1
	,, bone	0·78 : 1
	Mean	0·72 : 1

to be responsible for these hydrolyses. This is shown by the following considerations:

1. The optimum c_H for the action of kidney phosphatase on both hexosediphosphate and glycerophosphate is the same (Fig. 1).

2. Whether the enzyme is derived from the kidney of the rat, the pig, the rabbit, or man, or even from rabbit or rat bone, the relative speeds at which hexosediphosphate and glycerophosphate are hydrolysed, at the optimum p_H of about 8·9 and in presence of excess of the substrate, is, within experimental error, the same. If hexosediphosphate and glycerophosphate were hydrolysed by different enzymes, one would not expect the ratio of the amount of the two enzymes present to be so constant (Table VII).

3. If a preparation of kidney phosphatase is allowed to act on (*a*) excess of glycerophosphate, (*b*) excess of hexosediphosphate, (*c*) excess of both present together for the same length of time, the amount hydrolysed in (*c*) is not equal to the sum of (*a*) and (*b*), as would be expected if two separate enzymes, a hexosediphosphatase and a glycerophosphatase, were both present in the solution, but is some intermediate figure. The same is true for bone phosphatase. Thus:

	mg. P in 15 cc.	
Hydrolysed in 2 hours from excess of	Kidney phosphatase	Bone phosphatase
Glycerophosphate	0·120	0·169
Hexosediphosphate	0·188	0·246
Both present together	0·127	0·180

α- and β-Glycerophosphate. For the determination of phosphatase activity, pure commercial "sodium glycerophosphate crystals" was employed. This consists of the sodium salt of β-glycerophosphoric acid. If the two pure salts, sodium α- and β-glycerophosphate are compared, it is found that they are not hydrolysed at the same rate by kidney phosphatase, although the optimum c_H remains the same. β-Glycerophosphate, the form incapable of optical activity, is hydrolysed somewhat more readily than the α-form (Table VIII).

Table VIII. *Relative rates of hydrolysis of α- and β-glycerophosphate by kidney phosphatase.*

	mg. P liberated from 10 cc. solution in 2 hours	
p_H	α	β
8·2	0·316	0·320
8·5	0·384	0·425
8·9	0·425	0·494
9·25	0·422	0·478
9·66	0·262	0·353
10·0	0·119	0·218
Total P in 10 cc. (mg.)	2·25	2·23

Caseinogen. Pig's kidney phosphatase was allowed to act on caseinogen solution for 24 hours at 38°. Under the same conditions, but using sodium glycerophosphate or hexosephosphate of the same content of organic phosphorus per cc. of reaction mixture, at least 95 % of this phosphorus would

have been hydrolysed. With caseinogen as substrate in two experiments with two different preparations, only 5 % and 6·2 % of the organic phosphorus of the protein was liberated as free phosphate. The maximum amount of hydrolysis took place at p_H 6·0. Using phosphatase from rabbit's kidney the hydrolysis of caseinogen was slightly greater, being 11 % after 24 hours at p_H 8·9.

Between p_H 6·0 and 9·66 caseinogen phosphorus is only very slowly affected by kidney extract. The small hydrolysis observed is probably due in each case to the presence of small quantities of proteolytic enzymes in the phosphatase preparation (the action of kidney phosphatase on certain of the hydrolysis products of caseinogen is dealt with by Rimington and Kay [1926]).

Nucleic acids and nucleotides. When compared with the rate at which glycerophosphates and hexosephosphates are hydrolysed by kidney phosphatase, the organic phosphorus of yeast nucleic acid, under the same conditions, is hydrolysed slowly, but definitely, and that of thymus nucleic acid very slowly. In conditions in which sodium glycerophosphate would be almost completely hydrolysed in 6 hours by kidney phosphatase, 64 % of yeast nucleic acid containing the same amount of organic phosphorus was hydrolysed in 6 hours, and 83 % in 18 hours at 38°. Only 13 % of thymus nucleic acid under the same conditions was hydrolysed in 18 hours.

The optimum p_H for the action of kidney extract on yeast nucleic acid is on the acid side of 8·5. It would appear that both a "nuclease" and a "nucleotidase" are present in this extract. It is worthy of note that the nucleic acid of plant origin is more easily attacked by animal phosphatase than is that of animal origin.

Table IX. *Hydrolysis of nucleic acids by kidney phosphatase.*

	mg. P liberated per 10 cc. solution	
Time (hours)	Yeast nucleic acid	Thymus nucleic acid
0	0	0
6	0·372	0·025
18	0·480	0·075
Total P	0·580	0·580

Nucleotides. A mixture of nucleotides prepared from yeast nucleic acid by Jones' [1920] method was found to be hydrolysed by kidney phosphatase only slightly more rapidly than yeast nucleic acid of the same concentration in organic P and of the same c_H. As with yeast nucleic acid, equilibrium was reached at about 80 % hydrolysis.

Presence in blood of substrate for kidney phosphatase. Kay and Robison [1924] showed that in mammalian blood filtrates only a portion of the acid-soluble organic phosphorus was hydrolysed by bone phosphatase. The present author has found that pig's kidney phosphatase hydrolyses these phosphoric esters to about the same extent as the bone enzyme, whereas kidney phosphatase from young rabbits brings about almost complete hydrolysis.

Table X. *Hydrolysis of blood phosphoric esters by phosphatases.*

mg. P per 100 cc. blood.

Exp.	Substrate	Free P	Total P	Org. P	Hydrolysis by pig's kidney phosphatase	Hydrolysis by bone phosphatase	Hydrolysis by rabbit's kidney phosphatase
	Rabbit's blood filtrate	4·8	37·5	32·7	13·4	14·0	—
56	Human blood filtrate (man)	3·1	25·4	22·3	6·6	6·5	21·5
53	Human blood filtrate (woman)	2·8	25·7	22·9	—	6·6	20·3
51	Human blood filtrate (man)	3·2	25·5	22·3	6·6	6·6	—
38	Human blood filtrate (diabetic)	3·7	—	—	—	7·7	16·5

Presence in plasma of substrate for kidney phosphatase. Before one can accept the suggestion of Eichholtz, Robison and Brull [1925] as to the physiological function of kidney phosphatase, namely, that it is, by bringing about the continuous hydrolysis of the organic phosphoric esters of the blood, the agent responsible for the presence of the greater part, if not all, of the inorganic phosphates in the urine, it has first to be demonstrated either (*a*) that phosphoric esters capable of hydrolysis by kidney phosphatase are present in the circulating plasma, or (*b*) that the phosphoric esters known to be present in the formed elements of blood are able by some means [cf. the suggestion of the present author, 1925, p. 443] to come into contact with kidney phosphatase. In this connection, it must not be overlooked that both leucocytes and platelets are rich in organic acid-soluble phosphorus (*i.e.* in addition to lecithin and nucleic acid phosphorus). In experiments in conjunction with Dr F. B. Byrom it has been shown that in the white cells, and also in the platelets, the organic acid-soluble phosphorus is partly of a type readily hydrolysable by tissue phosphatases. The leucocytes have perhaps a more direct metabolic relationship to the tissues than the erythrocytes, and it is conceivable that they take a direct part in phosphorus interchange [*vide* Kay, 1926].

Although the amount of acid-soluble phosphorus in plasma is very small, it has been found possible to show that it is partly hydrolysable by kidney phosphatase, and therefore that the liquid bathing the phosphatase-containing cells contains the substrate for their activity. Both rabbit and human blood plasma have been employed, prepared from oxalated or heparinised blood by centrifuging for 20 minutes to remove all cells (including platelets). The plasma is then precipitated with the minimum quantity of trichloroacetic acid, neutralised and the filtrate concentrated to small bulk and freed from inorganic phosphate by magnesia mixture, then neutralised, diluted and treated with kidney phosphatase, with the usual controls. In two quantitative experiments with rabbit's plasma, one heparinised, the other oxalated, it has been found that the hydrolysable phosphorus of the plasma is 0·39 and 0·47 mg. per 100 cc., *i.e.* about 10 % of the inorganic phosphorus. Three similar experi-

ments with human plasma have given results which show that hydrolysable organic phosphorus is present in still smaller, but quite definite quantities.

Action of kidney phosphatase on whole blood. If kidney phosphatase in 0·85 % sodium chloride solution is added in excess to whole blood, there is only very slight hydrolysis, such as might be expected from the small amount of phosphoric ester in the plasma. The method of experiment is as follows. In each of four 50 cc. flasks are placed 3 cc. of whole blood (heparinised and collected under aseptic precautions) + 3 cc. of kidney enzyme in normal saline. To two of these, 20 cc. of water are added, and at once 4 cc. of 25 % trichloroacetic acid, making the volume up to 30 cc. An aliquot portion of filtrate is then taken for the phosphorus determination. The other two flasks (without additional water) are placed in the incubator at 38° for 2–3 hours, then taken out, cooled, and water and trichloroacetic acid added in the same way. Controls with blood and boiled enzyme, with active enzyme alone and with blood and saline alone are carried out under the same conditions. The results are shown in Table XI.

Table XI. *Action of kidney phosphatase on whole blood.*

			mg. P per 100 cc. blood.			
			After period with active enzyme at 38°		After same period with boiled enzyme at 38°	
Exp. no.	At start Free P	Hours	Free P	P hydrolysed	Free P	P synthesised
52	2·6	2½	3·0	0·4	2·1	0·5
26	2·9	3	3·3	0·4	0·7	2·2
28	2·7	3	3·8	1·1	2·3	0·4
20	3·1	3	11·0	7·9	6·8	3·7*

* Hydrolysed.

In Exp. 28 there was very slight laking in the active enzyme flasks, but not in the boiled enzyme flasks, after 3 hours. In Exp. 20 there was marked laking, both in the active and boiled enzyme flasks. In the other two experiments there was no trace of laking. With blood and saline alone there was also a small but definite amount of synthesis.

It is evident from the above experiments that in absence of haemolysis there is:

1. a slight but definite amount of hydrolysis of the phosphoric esters of the whole unlaked blood in presence of kidney phosphatase;

2. in absence of the active enzyme, under the conditions of these experiments, synthesis of organic phosphorus compounds from inorganic phosphate. If there is laking of the corpuscles, the presence of kidney phosphatase markedly increases the rate of hydrolysis.

Effects of certain agents on the rate of hydrolysis of sodium glycerophosphate by kidney phosphatase.

Concentration of substrate. (*a*) With small amounts of enzyme and excess of substrate, the hydrolysis is directly proportional to the amount of the enzyme.

Table XII. *Hydrolysis of excess of glycerophosphate by kidney phosphatase.*

Enzyme added (cc.)	mg. P liberated per 10 cc.	
	Found	Calculated
0·1	0·102	0·096
0·2	0·192	0·192
0·4	0·374	0·384
0·5	0·486	0·480

(*b*) If substrate is not present in excess, the usual type of enzyme curve is obtained:

Table XIII. *Hydrolysis of glycerophosphate by excess of kidney phosphatase.*

Time (hours)	mg. P liberated per 10 cc.
0	0·000
1	0·136
2	0·202
3	0·250
4	0·278
6	0·310
24	0·330
Total P	0·338

(*c*) With constant small amounts of enzyme there is an optimal concentration of glycerophosphate for hydrolysis at about $M/40$.

Table XIV. *Hydrolysis of glycerophosphate by kidney phosphatase.*

Concentration of glycerophosphate in reaction mixture (M)	Relative rate of hydrolysis in 2 hours
0·2	74
0·13	79
0·10	84
0·05	94
0·033	97
0·025	100
0·016	98·5
0·004	74

Concentration of glycerol. With constant amount of enzyme, increasing amounts of glycerol in the reaction mixture above about 0·8 M diminish the rate of hydrolysis. There is some evidence of a not very marked optimum at about 0·8 M concentration of glycerol.

Concentration of other alcohols. With a constant amount of enzyme present, the addition of increasing amounts of alcohol to the reaction mixture increasingly inhibits the hydrolysis of glycerophosphate. This is true of all the lower alcohols up to butyl and *iso*-butyl. Amyl alcohol is not sufficiently soluble in the reaction fluid to show much inhibition. The percentage inhibition with methyl, ethyl and *n*-propyl alcohol in concentrations from 4 % to 16 % by volume of the reaction mixture is roughly proportional to the concentration of alcohol present. Below 4 % of alcohol by volume the inhibition is very small. Above 16 % analytical difficulties begin to interfere. The inhibitory effect increases as follows: ethyl, methyl, propyl, *iso*-butyl, butyl alcohol.

With the butyl alcohols, insolubility prevents the use of high concentrations.

Action of hormones. Insulin, pituitrin and adrenaline have been investigated and no effect with any of these agents has been observed on the rate of hydrolysis of hexosediphosphate or glycerophosphate by kidney phosphatase. This agrees with the findings of Demuth [1925, 2] and supports the view that hormones which influence carbohydrate equilibrium do not bring about their characteristic effect by directly influencing the activity of enzymes which hydrolyse hexosediphosphate. The optimum c_H of kidney phosphatase is not affected by any of these agents [cf. Langfeldt, 1921].

Dialysis. Preparations of kidney phosphatase have been dialysed in collodion sacs against water. The membranes were impermeable to proteins, but permeable to methylene blue. When dialysed for 18 hours against an equal volume of water, there is no change in the activity of the enzyme solution, and the dialysate remains inactive. On dialysing for a further 18 hours against running water, there is a 5 % loss of activity of the enzyme within the sac.

It may be concluded that kidney phosphatase does not dialyse, nor does it lose its activity on losing the salts and substances of low molecular weight associated with it. The enzyme solution after 18 hours' dialysis against tap water was free from phosphates and contained only as much chloride as tap water. No dialysable co-enzyme is necessary for phosphatase action.

Kidney lecithinase.

There is in kidney extracts, and therefore in kidney tissue itself, a weak lecithinase, which appears to be a different enzyme from kidney phosphatase.

The action of such an enzyme on lecithin is more complex than that of kidney phosphatase on an ester like glycerophosphoric ester, since in order to liberate the free phosphoric acid, both the glycerol residue and choline must be separated from it. This reaction has not been studied in detail. The optimum c_H for kidney lecithinase has been determined, and some idea of the amount present in kidney extracts obtained.

"Lecithin" (Duncan) (0·5 g.) was dissolved in alcohol (20 cc.) and the boiling solution poured slowly into 180 cc. of boiling water. This gives a stable emulsion of lecithin, containing about 0·9 mg. P per 10 cc., less than 1 % of this phosphorus being in inorganic form.

Preliminary experiments showed that the optimal p_H for the lecithinase action of kidney tissue extracts was between 7·0 and 8·0. On repeating the determination (using phthalate and borate buffers) it was found that the optimum was between 7·0 and 7·4, *i.e.* about the normal reaction of the tissue.

The amount of kidney extract which set free 0·3 mg. P from lecithin at the optimum p_H of 7·4 liberated 17·1 mg. from excess of sodium glycerophosphate at the optimum p_H, for the latter hydrolysis, of 8·9. The standard kidney extract contains, therefore, only a relatively weak lecithinase.

Table XV. *Optimum c_H for kidney lecithinase.*

2 cc. kidney phosphatase (prepared in the standard way) to each 10 cc. of lecithin emulsion. mg. P per 10 cc. lecithin emulsion after 60 hours' hydrolysis

p_H	Free P Active enzyme and lecithin	Free P Boiled enzyme and lecithin	Hydrolysed
6·0	0·206	0·159	0·047
7·0	0·291	0·161	0·130
7·4	0·299	0·157	0·142
7·8	0·278	0·161	0·117
8·2	0·272	0·163	0·109
9·25	0·242	0·163	0·079

Total phosphorus in 10 cc. of lecithin emulsion 0·900 mg.

The source of urinary phosphates.

It is possible, in view of certain of the results of the paper, to examine in a roughly quantitative way the view of Eichholtz, Robison and Brull [1925] that the organic phosphoric esters of the plasma may provide a large part of the phosphate of the urine.

The daily excretion of urinary phosphorus by the normal adult is about 1·2 g. From the activity of human kidney phosphatase (approximately 5 phosphatase units per g. kidney at p_H 8·9) and assuming the active tissue to be 300 g. it would be possible to liberate 5×300 mg. P from glycerophosphate in 2 hours or $5 \times 300 \times 12$ in 24 hours $= 18$ g. and considerably more from hexosephosphate. The rate of hydrolysis of the blood esters is, under similar conditions, not far short of that of glycerophosphate. But we have no reason to suppose that the phosphatase of the kidney is working, *in vivo*, at other than the p_H of the blood, *i.e.* 7·4. At this p_H its phosphatase activity is from one-third to one-half of its activity at the optimum p_H. However, taking the lower value of one-third, which would bring the excretion down to 6 g. of phosphorus from glycerophosphate and somewhat less from the blood esters, there is quantitatively a large excess of phosphatase.

From the experiments quoted in this paper, the limiting factor would appear to be not the amount of phosphatase, but the quantity of substrate in the *plasma*. (We cannot assume that the phosphoric esters in the corpuscles diffuse into the plasma *in vivo* until it has been shown to be possible *in vitro*, and so far every endeavour to get the phosphoric ester out of the corpuscles, short of laking them, has been unsuccessful.) It has been shown that the greater portion, at least of the organic acid-soluble phosphorus of the plasma, is hydrolysed by kidney phosphatase *in vitro*. If we assume this amount to be 0·3 mg. per 100 cc. of blood (probably an over-estimate), it seems at first that the normal amount of phosphorus excreted in the urine in 24 hours—1200 mg.—is very large compared with this concentration. If all the plasma organic phosphorus present in the entering blood were hydrolysed during each passage through the kidney, and if the plasma level were maintained by some synthetic mechanism in another part of the organism, 16 litres of blood per hour would have to be completely relieved of its plasma organic phosphorus

by the kidney. It has been computed that some 900 litres of blood pass through the human kidneys in 24 hours, an average of just under 40 litres per hour, so that it is quantitatively possible for the whole of the inorganic phosphate of the urine to come from the organic phosphoric ester of the plasma.

The close correlation between the level of inorganic P in blood and in urine [cf. Wigglesworth and Woodrow, 1924; Addis, Meyers and Bayer, 1925; Adolph, 1925] could perhaps be explained, on the enzyme theory of phosphate excretion, by the additional hypothesis that increase of inorganic phosphate in the plasma leads, possibly by mass action, to increased synthesis of phosphoric esters and this to the greatly increased excretion of inorganic phosphate. Up to the present, there is no direct evidence available on this point. In a few experiments reported by Wigglesworth and Woodrow no increase, but rather a decrease in the organic acid-soluble phosphorus of *whole blood* was noted, though a simultaneous rise in the organic phosphorus of the plasma is not thereby excluded. The extreme rapidity of the excretion of inorganic phosphate, described by Addis and his collaborators [1925] as following the injection of massive doses of neutral phosphate solutions, is difficult to explain even with the help of the additional hypothesis. It appears on the whole that, before a theory postulating the dependence of inorganic phosphate excretion by the kidney on the level of phosphoric esters in the plasma can be accepted, much more evidence is required.

The occurrence in a particular tissue of an enzyme capable of decomposing a substrate present in the same locality in the body, is not, in itself, sufficient evidence that the two are directly physiologically related. But the presence and peculiar properties of this very active phosphatase in such an important and efficient organ as the kidney, if we reject the hypothesis of Eichholtz, Robison and Brull, demands an explanation. It may be urged that phosphatase activity, not only in the kidney but also in other tissues, is merely the fortuitous result of a particular type of molecular configuration of fairly frequent occurrence in the cell. There is also the view that phosphatase, *in vivo*, acts as a synthetic enzyme, and is connected with the inner life of the cell, in the maintenance of the essential nuclear phosphorus compounds, and is not involved directly in the functional activity of the organ in which it occurs. But as regards the phosphatase in intestinal juice and the mucosa of the gut wall, this is clearly not the case; with bone phosphatase also the correlation between the rate of deposition of calcium in the growing bone and the phosphatase content of the active zone bears strongly against this possibility; further, the correspondence between the functional activity of, and the amount of phosphatase present in the kidney at various stages in the development of the animal, is against its being true for this organ.

SUMMARY.

1. The inorganic phosphate, and to a less degree the acid-soluble organic phosphorus in the kidney remain constant during the adult life of the animal.

2. The rate of production of inorganic phosphate during the autolysis of the whole kidney is increased by mechanical injury to the organ.

3. Kidney extracts contain a very active phosphatase which is the main agent concerned in post-mortem phosphatolysis. It has an optimum p_H of 8·8–9·2, the most alkaline optimum yet recorded for any enzyme.

4. A simple method has been worked out for determining the phosphatase content of a tissue extract, and has been applied to the determination of the phosphatase content of bone and kidney tissue at various stages in the life of an animal.

5. It is concluded that bone phosphatase is correlated with bone *growth*, and kidney phosphatase with the functional activity and not with the growth of this organ.

6. Phosphatase is not uniformly distributed in the kidney, being present in greatest quantity in the cortex.

7. A short autolysis reduces the phosphatase content of the kidney very little. At 0° in presence of antiseptic kidney phosphatase remains for weeks without serious loss of activity.

8. An insoluble phosphatase preparation has been obtained, which, when dry, is stable at room temperature.

9. Hexosediphosphate and glycerophosphate are hydrolysed by the same enzyme. Sodium β-glycerophosphate is hydrolysed more readily than the α-salt. Caseinogen is hardly attacked by kidney phosphatase. Yeast nucleic acid and nucleotides are hydrolysed readily, but thymus nucleic acid only slowly, by this enzyme.

10. Preliminary experiments have been carried out on the effect of certain agents on the rate of hydrolysis by kidney phosphatase.

11. Kidney extracts contain a feeble lecithinase which has an optimum p_H of 7·0–7·4.

12. Both red cells and blood plasma contain a substrate for kidney phosphatase.

13. The kidney phosphatases from different animals vary in their ability to hydrolyse the organic phosphoric esters of the blood.

14. Kidney phosphatase acting on whole blood will hydrolyse the organic phosphorus in the plasma, but is not able to hydrolyse the organic esters within the red cells themselves.

15. The possible physiological significance of kidney phosphatase is discussed.

I am indebted to Dr J. H. Quastel for pure specimens of sodium α- and β-glycerophosphate, and to Mr P. G. Marshall for assistance in some of the later experiments.

REFERENCES.

Addis, Meyers and Bayer (1925). *Amer. J. Physiol.* **72**, 125.
Adolph (1925). *Amer. J. Physiol.* **74**, 93.
Demuth (1925, 1). *Biochem. Z.* **159**, 415.
—— (1925, 2). *Biochem. Z.* **166**, 162.
Eichholtz, Robison and Brull (1925). *Proc. Roy. Soc. Lond.* B, **99**, 91.
Forrai (1923). *Biochem. Z.* **142**, 282.
—— (1924). *Biochem. Z.* **145**, 54.
Goodwin and Robison (1924). *Biochem. J.* **18**, 1161.
Jones (1920). Nucleic Acids (Longmans, London).
Kay (1925). *Biochem. J.* **19**, 433.
—— (1926). *J. Exp. Path.* **7** (in the press).
Kay and Robison (1924). *Biochem. J.* **18**, 755.
Langfeldt (1921). *J. Biol. Chem.* **46**, 391.
Lawaczeck (1924). *Biochem. Z.* **145**, 351.
Martland (1925). *Biochem. J.* **19**, 117.
Martland, Hansman and Robison (1924). *Biochem. J.* **18**, 1152.
Martland and Robison (1924). *Biochem. J.* **18**, 1354.
Neuberg and Behrens (1926). *Biochem. Z.* **170**, 254.
Plimmer (1913). *Biochem. J.* **7**, 43.
Rimington and Kay (1926). *Biochem. J.* **20**, 786.
Robison (1923). *Biochem. J.* **17**, 286.
Robison and Soames (1924). *Biochem. J.* **18**, 740.
Sevringhaus (1923). *J. Biol. Chem.* **57**, 181.
Takahashi (1924). *Biochem. Z.* **146**, 161.
Tomita (1922). *Biochem. Z.* **131**, 170.
Wigglesworth and Woodrow (1924). *Proc. Roy. Soc. Lond.* B, **95**, 96.