

LX. STUDIES ON THE PHOSPHATASES.  
THE INFLUENCE OF SOME ELECTROLYTES ON THE  
PHOSPHATASES OF ANIMAL TISSUE. PHOSPHATASES  
OF THE LIVER, KIDNEY, SERUM AND BONES OF  
THE RABBIT.

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IN recent work Davies [1934] found that the autolysed extracts of liver and spleen hydrolyse sodium  $\beta$ -glycerophosphate at two different optima of acidity, namely  $p_H$  4.5-5 and  $p_H$  8.9, and he expressed the opinion that there were two different phosphatases; however, it was not possible for him to purify the extracts in such a way as to obtain from them two fractions each containing one of the two distinctzymes.

An analogous fact had been observed for the phosphatases of hog kidney by Kurata [1931] who had succeeded in obtaining, by adsorption processes and from the same starting material, two different enzymic solutions, one having an optimum of phosphatase activity at  $p_H$  3, the other at  $p_H$  9.

Recently some Japanese authors of Akamatsu's school [Usawa, 1932; Munemura, 1933, *etc.*] came to the conclusion that the enzymes which hydrolyse the monoesters of phosphoric acid belong to three types:

(a) A phosphomonoesterase type I,  $p_H$  optimum 3, extracted by Manaka [1931] from takadiastase.

(b) A phosphomonoesterase type II,  $p_H$  optimum 5.4-5.6, extracted by Usawa [1932] from rice husk and purified by an adsorption process.

(c) A phosphomonoesterase type III,  $p_H$  optimum 9-10, studied by Asakawa [1928; 1929] in kidney extracts.

Hori [1932], confirming the results obtained by Roche [1931], reported the presence of a phosphomonoesterase of type II in erythrocytes. In this way, according to the Japanese authors quoted, phosphomonoesterases of all three types are to be found in animal tissues, whereas type III phosphomonoesterase would appear always to be absent from vegetable tissues.

A study of the behaviour of the three phosphomonoesterases in the presence of different electrolytes has been made by Munemura [1933], who made his experiments on purified solutions of extracts of takadiastase, of rice husks and of hog kidney, chosen as being representative of the three types of phosphomonoesterase. Starting from the presupposition that all the enzymes which hydrolyse the monoesters of phosphoric acid and which exist in nature are included in these three types, he generalised the results thus obtained.

We wished to study the influence of some inactivating agents and, in particular, of sodium fluoride and oxalate, on the phosphatase activity of extracts

of liver, kidney, bone and serum of the rabbit. It seems to us that the results we obtained lead to deductions which are somewhat different from those arrived at by the Japanese authors.

#### EXPERIMENTAL.

##### *Technique.*

The general method of research was as follows. In a series of 25 ml. flasks were placed: 1 ml. of a solution of sodium glycerophosphate (5 % of the anhydrous salt); 2.5 ml. of a solution of sodium acetate and sodium veronal in concentration double that prescribed by Michaelis [1930], containing 38.856 g. of  $\text{CH}_3\text{COONa}$ ,  $3\text{H}_2\text{O}$  and 58.856 g. of sodium veronal per litre of solution; 2 ml. of an 8.5 % solution of pure NaCl. To each of the flasks was added, in order to obtain the desired  $p_{\text{H}}$  value, that quantity of HCl prescribed by Michaelis for the purpose; when it was desired to examine the action of some inhibiting agent a measured quantity of a concentrated solution of that agent was also added. Finally, 5 ml. of the enzymic solution under examination were placed in each flask and the liquid brought to the mark. In view of the fact that the enzymic extracts, like the solutions of oxalate and of fluoride, often notably displace the  $p_{\text{H}}$  of the buffer solutions, the  $p_{\text{H}}$  was controlled electrometrically on an aliquot part of the solution. The remainder was distributed in portions of 5 or 10 ml. in thick-walled test-tubes which, after the addition of a drop of toluene, were tightly closed and left in the thermostat at  $37^\circ$  for 16 hours. At the end of that time, a measured quantity of 10 % trichloroacetic acid was added to each test-tube. The contents were filtered and inorganic phosphorus was determined in the filtrate using Lohmann and Jendrassik's [1926] modification of Fiske and Subbarow's [1925] method. The values thus ascertained were then calculated to 1 ml. of the solution under examination.

We used the acetate-veronal buffer according to Michaelis, notwithstanding its weak buffering powers, because it offers the advantage of an extremely wide range of acidity, between  $p_{\text{H}}$  9.64 and 2.62, simply by adding different amounts of hydrochloric acid. When it was necessary to use still higher  $p_{\text{H}}$  values (10 to 11), we added a few drops of dilute solution of sodium hydroxide to the veronal buffer, counting also on the buffering power of the enzymic solutions themselves. In this way we excluded the possibility of the enzymes being diversely influenced by the different nature of the buffer.

The crystallised sodium glycerophosphate used by us, and placed on the market by Messrs Carlo Erba of Milan, was composed exclusively of the  $\beta$ -form [Carrara, 1932].

##### *Phosphatases of liver and kidney extracts.*

The tissues used in these experiments were taken from adult and normal rabbits. The technique employed for the preparation of the extracts had many points of similarity with that adopted by Asakawa [1928]. The animals were killed by cutting the carotid. The abdominal cavity was then immediately opened, a cannula needle was introduced into the vena cava and, after ligaturing the portal vein, the blood vessels were washed with physiological salt solution at  $37^\circ$ . The organs were then removed and, after being freed from the residues of surrounding tissues, were rapidly washed with physiological salt solution, weighed, triturated with sand in a mortar and placed in an Erlenmeyer flask together with ten times their weight of sterile distilled water and a few ml. of toluene. The whole was then placed in a thermostat at  $37^\circ$  and allowed to autolyse for 48 hours. It was afterwards filtered through a folded filter. The clear filtrate was

dialysed for 36 hours in small bags of collodion placed in distilled water. The dialysed extracts were again filtered, a small quantity of toluene was added and the whole kept in an ice-chest. The extracts thus prepared do not contain enzymes from the blood when the operation of washing with physiological salt solution has been conducted properly. They are, moreover, extremely poor in albuminous substances and keep their phosphatase activity for a fair length of time. Again, possible sources of error, including those due to the hydrolytic action of phosphodiesterases and pyrophosphatases, are avoided because the phosphorylated organic compounds pre-existing in the tissues are decomposed during the digestion in the thermostat and the inorganic phosphates are eliminated wholly by means of the dialysis. The determinations themselves thus become less laborious because controls are not necessary.

Table I shows that, within very wide limits, the effect of sodium fluoride on the phosphatase activity of a liver extract does not change with variations in the degree of concentration of this salt.

Table I. *Inhibitory action of sodium fluoride on the phosphatase activity of rabbit liver extracts.*

$p_H$ of the buffer solution	mg. P hydrolysed in the presence of NaF				mg. P hydrolysed in the absence of NaF
	M/50	M/7	M/4	M/2	
3.6	—	0.006	—	—	0.034
5.5	0.003	—	0.005	0.007	0.067
7.5	0.040	—	0.039	0.040	0.050
8.3	0.067	0.066	0.067	0.065	0.065

From Fig. 1, which represents the results of an experiment carried out by acting contemporaneously with an extract of rabbit liver on sodium glycerophosphate in the absence of inhibitory agents (Curve A), in the presence of M/50 NaF (Curve B), and in the presence of sodium M/50 oxalate (Curve C), it may be seen that, analogously with the results obtained by Davies [1934], the

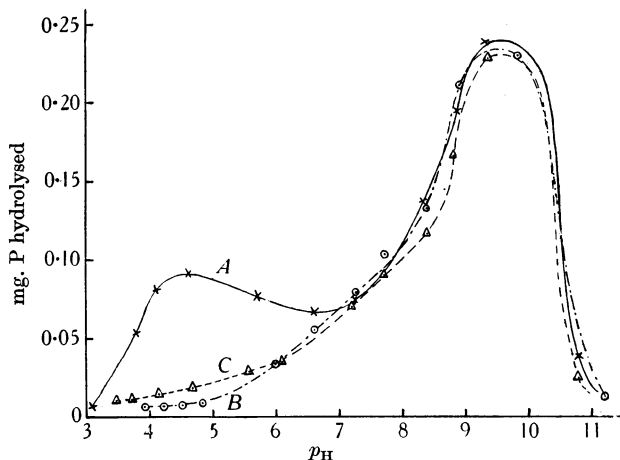


Fig. 1. The hydrolysis of  $\beta$ -glycerophosphate in the absence and presence of added fluoride and oxalate by rabbit liver extracts under varying conditions of hydrogen ion concentration.

Curve A.  $\times$  —  $\times$  Hydrolysis in absence of inactivating agents.  
 Curve B.  $\circ$  - - -  $\circ$  Hydrolysis in presence of added fluoride.  
 Curve C.  $\Delta$  - - -  $\Delta$  Hydrolysis in presence of added oxalate.

phosphatase of the liver acts at two different  $p_{\text{H}}$  optima, one in an alkaline zone and the other in an acid zone. It is also clearly shown that the two inhibitory agents examined leave the "alkaline" phosphatase activity almost unaltered and that they affect only the "acid" phosphatase activity. As this inactivation is very profound and the residual activity from  $p_{\text{H}}$  3 to 6 is almost negligible, it may be held that the problem set by Davies [1934] to separate the two phosphatases has been solved at least in part.

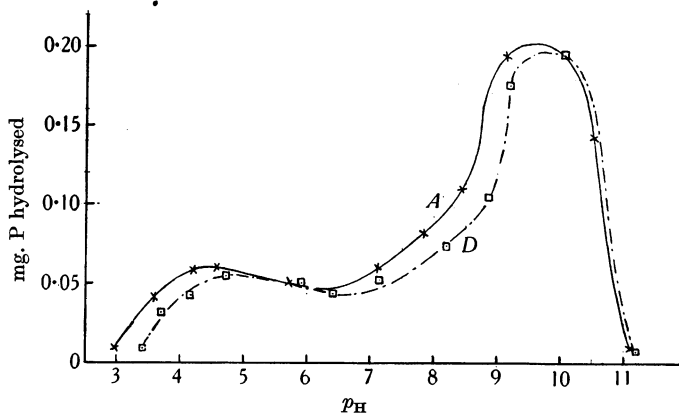


Fig. 2. The hydrolysis of  $\beta$ -glycerophosphate in the absence and presence of added iodoacetate by liver extracts (rabbit) under varying conditions of hydrogen ion concentration.

Curve A.  $\times$  —  $\times$  Hydrolysis in absence of added iodoacetate.  
 Curve D.  $\square$  - - -  $\square$  Hydrolysis in presence of added iodoacetate.

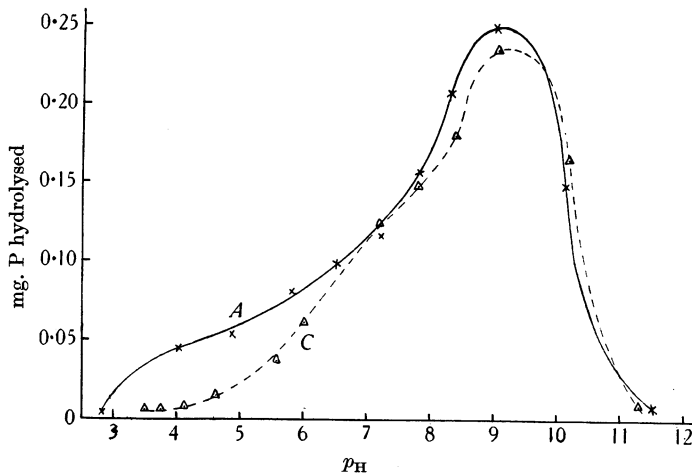


Fig. 3. The hydrolysis of  $\beta$ -glycerophosphate in the absence and presence of added oxalate by kidney extracts (rabbit) under varying conditions of hydrogen ion concentration.

Curve A.  $\times$  —  $\times$  Hydrolysis in absence of added oxalate.  
 Curve C.  $\triangle$  - - -  $\triangle$  Hydrolysis in presence of added oxalate.

The results of this experiment are not in agreement with the views of some of the Japanese authors mentioned above. In fact, according to Munemura [1933], sodium oxalate completely inhibits the activity of the phosphatase type

III (alkaline) whereas this did not occur in our case. Again, according to the same author the oxalate should not have a very noticeable action on type II phosphatase at a  $p_H$  optimum of 5.5. It should be observed, however, that, in analogy with what was found by Kobayashi [1927] and Inouye [1928] in the case of the phosphatase of *A. oryzae*, our acid phosphatase might also be a type I phosphatase modified in its properties by the presence of a substance X.

Table II. *Inhibitory action of sodium trichloroacetate on the phosphatase activity on extracts of rabbit liver.*

$p_H$ of the buffer solution	mg. P hydrolysed		
	In the presence of $CCl_3COONa$		In the absence of $CCl_3COONa$
	$M/50$	$M/20$	
3.2	0.0089	0.0067	0.0338
5.3	0.0674	0.0686	0.0649
8.9	0.0828	0.0805	0.0828

From Table II it may be seen that sodium trichloroacetate has a similar action to those of sodium fluoride and oxalate on the phosphoesterase of the liver.

Fig. 2 shows the behaviour of the same extract of liver on sodium  $\beta$ -glycerophosphate in the presence and in the absence of  $M/50$  sodium iodoacetate. This liver extract was the same as that used for the experiment illustrated in Fig. 1. The experiment was, however, carried out 6 days later with the same enzymic solution which had been kept in the ice-chest in the meantime. As may be seen, whilst the "alkaline" phosphatase activity remained almost unaltered, the "acid" phosphatase underwent a much more noteworthy inactivation. The iodoacetate acts extremely weakly on the acid phosphatase, and it appears to have only the same weak influence on the alkaline phosphatase as have oxalate and fluoride. The determinations of the  $p_H$  in the presence of iodoacetate under alkaline conditions were carried out by means of the colorimeter because the iodoacetate poisons the hydrogen electrode.

Fig. 3 shows the action of the phosphatase of a rabbit kidney extract on  $\beta$ -glycerophosphate in the absence (Curve A) and in the presence (Curve C) of  $M/50$  sodium oxalate. In Curve A, besides the optimum of activity at  $p_H$  9.2, the existence of a second optimum in the acid zone is not to be found. However, in the presence of sodium oxalate under acid conditions there is a marked inhibition of the phosphatase activity, analogous to that which occurs in the case of liver extracts. Therefore, in this case also one may suppose the presence of an "acid" phosphatase. The considerations already set forth in the matter of the "acid" phosphatase of the liver hold good also in this case and are supported by the experiments, of which we have already made mention, carried out by Kurata [1931] on the phosphatases of hog kidney.

As regards the "alkaline" phosphatase, we were not able to find inactivation by sodium oxalate in extracts of rabbit kidney prepared by the method used by Asakawa and by Munemura. This is the contrary of what Munemura found when experimenting with extracts of hog kidney.

On the whole, the behaviour of the phosphatases of the liver and those of the kidney, in the presence of inactivating agents, is such as to cause one to admit the qualitative identity of the two enzymic systems.

*Phosphatases of rabbit and horse blood serum.*

We used the serum from 8 rabbits which were kept without food for 24 hours. The blood was taken from the carotid. The horse serum was obtained from an animal which had been kept without food for 24 hours. In this case, the blood was taken by opening the external jugular vein.

The sera thus obtained were dialysed directly, without predigestion in the thermostat, in small bags of collodion placed in distilled water for 36 hours. Fig. 4 shows the hydrolysis of  $\beta$ -glycerophosphate produced at different  $p_H$  values in the absence of inhibitory agents (Curve A), in the presence of  $M/50$  NaF (Curve B), in the presence of  $M/50$  sodium oxalate (Curve C) and in the presence of  $M/100$  sodium iodoacetate (Curve D).

Fig. 5 shows the hydrolysis produced by dialysed horse serum in the absence of inhibitory agents (Curve A), in the presence of  $M/50$  NaF (Curve B), and in the presence of  $M/50$  sodium oxalate (Curve C).

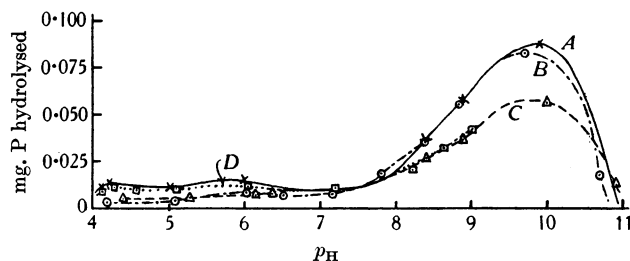


Fig. 4. The hydrolysis of  $\beta$ -glycerophosphate in the absence and presence of added fluoride, oxalate and iodoacetate by rabbit serum under varying conditions of hydrogen ion concentration.

Curve A.  $\times$  ———  $\times$  Hydrolysis in absence of inactivating agents.  
 Curve B.  $\circ$  — · — ·  $\circ$  Hydrolysis in presence of added fluoride.  
 Curve C.  $\triangle$  - - - -  $\triangle$  Hydrolysis in presence of added oxalate.  
 Curve D.  $\square$  · · · ·  $\square$  Hydrolysis in presence of added iodoacetate.

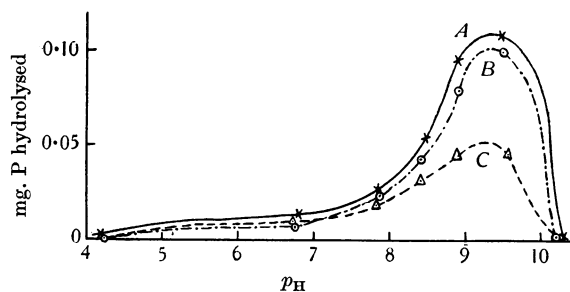


Fig. 5. The hydrolysis of  $\beta$ -glycerophosphate in the absence and presence of added fluoride and oxalate by horse serum under varying conditions of hydrogen ion concentration.

Curve A.  $\times$  ———  $\times$  Hydrolysis in absence of inactivating agents.  
 Curve B.  $\circ$  — · — ·  $\circ$  Hydrolysis in presence of added fluoride.  
 Curve C.  $\triangle$  - - - -  $\triangle$  Hydrolysis in presence of added oxalate.

In serum one also observes the presence of an acid phosphatase, although very weak. In the presence of inhibitory agents, it behaves in the same way as the extracts of liver and kidney. As regards the alkaline phosphatase, this is not inactivated by sodium fluoride but is greatly inhibited by the action of sodium

oxalate. As in the case of the liver extract, iodoacetate does not much affect the acid phosphatase; it acts on the alkaline phosphatase, at least up to the  $p_H$  values employed in the same way as sodium oxalate.

*The phosphatases of bone.*

The femora, tibiae and humeri of a 25-day old rabbit were carefully freed from their soft parts. The epiphyses were detached, the bones split along their

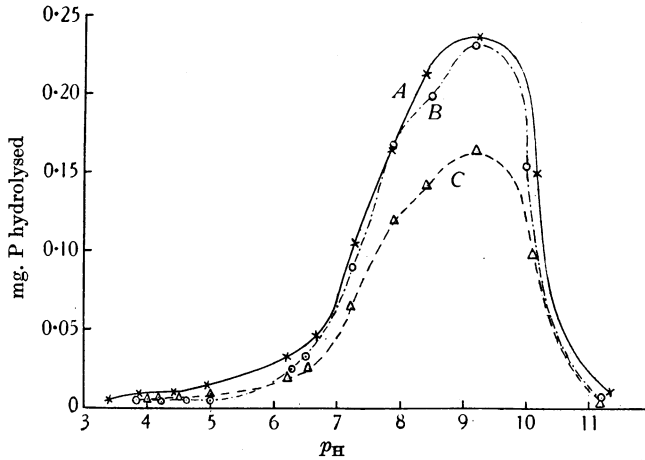


Fig. 6. The hydrolysis of  $\beta$ -glycerophosphate in the absence and presence of added fluoride and oxalate by rabbit bone (shaft) extracts under varying conditions of hydrogen ion concentration. Curve A.  $\times$ — $\times$  Hydrolysis in absence of inactivating agents. Curve B.  $\circ$ — $\circ$  Hydrolysis in presence of added fluoride. Curve C.  $\Delta$ — $\Delta$  Hydrolysis in presence of added oxalate.

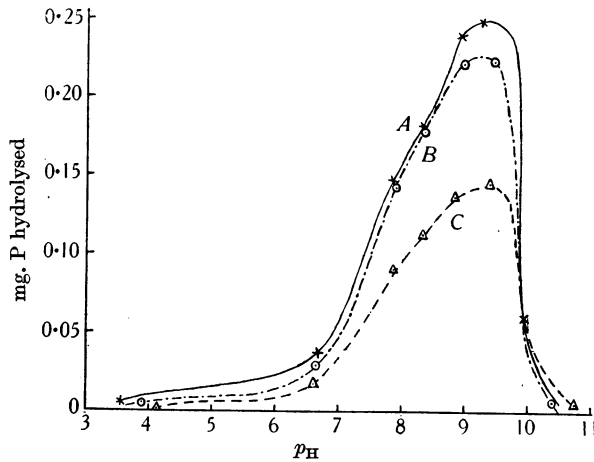


Fig. 7. The hydrolysis of  $\beta$ -glycerophosphate in the absence and presence of added fluoride and oxalate by rabbit bone (epiphysis) phosphatase under varying conditions of hydrogen ion concentration. Curve A.  $\times$ — $\times$  Hydrolysis in absence of inactivating agents. Curve B.  $\circ$ — $\circ$  Hydrolysis in presence of added fluoride. Curve C.  $\Delta$ — $\Delta$  Hydrolysis in presence of added oxalate.

length and the marrow carefully removed. They were then rapidly washed with physiological salt solution. The shafts and the epiphyses, after having been broken up separately in a mortar with quartz sand, were subjected to the treatment described in connection with the extracts of kidney and liver.

Figs. 6 and 7 show the hydrolyses produced respectively by the extracts of shaft and of epiphysis at various degrees of acidity in the absence and in the presence of fluoride and of oxalate. As may be seen, the two extracts behave in an analogous manner.

Examination of the two diagrams shows that the considerations set forth in the case of the phosphatases of serum may be repeated in the case of bone. The two systems behave in a markedly similar manner towards oxalate and fluoride. In young animals the phosphatase of the bone is much more active than that of the serum.

#### DISCUSSION.

Examination of the diagrams previously explained shows the following facts.

(1) Extracts of liver and kidney contain, besides the alkaline phosphatase which is that which up to the present has most attracted the attention of research workers, an "acid" phosphatase which has some activity but in any case is not negligible. It seems probable to us that this "acid" phosphatase has a physiological function.

(2) In bones (shaft and epiphyses) and in blood serum, the activity of the "acid" phosphatase is extremely slight.

(3) In extracts of bone and in serum, the "alkaline" phosphatase is sharply differentiated from the corresponding one present in extracts of liver and kidney by the fact that sodium oxalate reduces its activity in greater measure.

(4) Near neutrality, the influence of the inactivating agents which we have considered is negligible. One might therefore distinguish three different systems of phosphatase enzymes in animal tissues: that of the liver and of the kidney (and probably also of the spleen [Davies, 1934]) characterised by the presence of an "acid" and an "alkaline" phosphatase, the latter not being inhibited by sodium oxalate; that of serum and of bone characterised (the acid phosphatase being nearly negligible) by the presence of an "alkaline" phosphatase which is partially inhibited by sodium oxalate,<sup>1</sup> and lastly, that of the erythrocytes characterised, according to the statements of Roche [1931] and Hori [1932], by the presence of the acid phosphatase only. Moreover, according to Hori, this phosphatase of the erythrocytes ( $p_H$  optimum 5.5) is different from that of the kidney ( $p_H$  optimum 3).

In order to explain the negligible activity of the "acid" phosphatase of bone extract, one might put forward the hypothesis that the accumulation in the bones of notable quantities of fluoride which, as we have seen, inhibits the activity of this phosphatase, contributes to this fact.

We are working on the influence of fluorides on phosphatases; the results of our experiments will be given in one of our next communications.

We believe in fact that fluorine plays an important part in the phenomena of calcification and decalcification of bone and that by carrying research work still farther in this direction, it may be possible to obtain some light on this complicated question.

<sup>1</sup> We wish to observe in this connection that most authors, on the basis of Kay's work [1930], hold that the phosphatase of serum is of osseous origin: latterly however the work of Bodansky [1934] and of Bodansky and Jaffe [1934] tends to show that, except in cases of disease of the bone, the phosphatase of serum is of different origin.



Many considerations confirm this point of view.

(a) The fixation of fluorine by the bone, a phenomenon which in itself must surely have some physiological meaning.

(b) The phenomena consequent on chronic fluoride poisoning. These phenomena, on which a vast literature already exists [McClure, 1933], may easily be associated with a degenerated phosphatase activity of the osseous tissues.

(c) The alterations of the phosphatase activity of the plasma recently observed by Phillips [1934] in cows suffering from fluorosis. In this connection we have already referred to the analogy which exists between the phosphatase system of serum and that of bone.

(d) The fact, discovered by Robison and Rosenheim [1934],<sup>1</sup> that the addition of sodium fluoride, even in extremely small quantities, impedes *in vitro* the deposition of calcium on the ossifying cartilage.

To these considerations it may be objected that, in all the cases observed up to the present, the fluoride acts only on the acid phosphatases and not on the alkaline ones which appear, as regards the process of calcification of the bones and having regard to the reaction of the media in which they must operate, to be the most important. Robison and Rosenheim [1934] have observed that fluoride does not depress the activity of the bone phosphatases (alkaline). Furthermore, it may be objected that, in the phenomena of calcification, the two mechanisms admitted by Robison (scission of the phosphoric ester and precipitation of the calcium phosphate) may occur even at different hydrogen ion concentrations. Experiments not yet completed demonstrate that in certain conditions fluorides may also inhibit the activity of the alkaline phosphatases.

At this point we wish to recall attention to the classification of the phosphatases proposed by the Japanese authors. It has already been said that Akamatsu and his collaborators distinguish three types of phosphomonoesterase corresponding to the three different degrees of dissociation of a monoester of phosphoric acid (the hydrogen of both hydroxyls not replaced,  $p_H$  2.8-3; that of one hydroxyl replaced,  $p_H$  5.5; that of both hydroxyls replaced,  $p_H$  9-10) and analogous to the three types of proteinase: pepsin, papain and trypsin-kinase. From several points of view, this classification appears to be most suggestive, but it is difficult to accept entirely the views of the Japanese school on the behaviour of these three types of phosphatase as regards inhibitors and chemical activators. For example, we have seen how, according to Munemura [1933], the activity of the alkaline phosphatase should be completely inhibited in the presence of sodium oxalate. We found instead, in some of the cases examined, only partial inactivation, whilst in other cases the inactivation was practically nil. From this, ought one to deduce that there exists a fourth type of phosphatase having an optimum of activity at the same  $p_H$  as the third type, but which can be distinguished from the latter because it maintains its activity even in the presence of sodium oxalate? It appears to us that it is more probable that the inactivation of the enzymes by inhibitory agents as commonly used may be exerted, at least in certain cases, not on the haptophore groups but on the colloidal carriers of the enzyme itself. In these cases, therefore, one and the same enzyme changes its properties towards activating and inactivating agents when there are changes in the colloidal carrier. Objections might be raised to the

<sup>1</sup> For the sake of brevity, we have omitted to cite the other works of Robison to whom, together with Kay, belongs the merit of having commenced and of having given great impulse to the study of the phosphatases. On the other hand, the works of both these authors and of their collaborators, published for the greater part in this *Journal* since 1923, are well known to all those who are interested in this subject.

technique employed by us because the determinations were made after a somewhat lengthy period of digestion in the thermostat (16 hours) so that the hydrolysis of the substrate, at the optimum  $p_H$ , in some cases reached 80 %. Moreover, the experiments were made with a veronal buffer. The determinations carried out by Munemura [1933], using a glycine buffer with an extract of hog kidney in the presence and in the absence of sodium oxalate, were made when the hydrolysis of the glycerophosphate, in the absence of oxalate and at the optimum  $p_H$ , was about 50 %. It may be suspected that this is the cause of the notable difference between our results and those of the Japanese author.

Preliminary experiments have however shown us that the conclusions are not substantially changed either when the hydrolysis is interrupted at 50 % or when it is still further prolonged, and this both in the presence of veronal buffer and in that of glycine buffer.

This preliminary examination led, among other things, to interesting observations on which we will report in our next communication.

On the other hand, when carrying out the inorganic phosphorus determinations we found it possible to obtain, after a sufficiently long space of time, detectable quantities of phosphorus even at acid  $p_H$  values and thus to have the possibility of a simultaneous and accurate measure of the activities of both acid and alkaline phosphatases.

We feel that it is opportune to make a further remark in connection with the use of sodium oxalate as an anticoagulant for the taking of blood for research work on the phosphatases of the plasma. It is clear, from what we have explained, that by operating in this manner, the action of the phosphatases present is greatly inhibited. Bodansky [1933] in fact observed that the phosphatase activity of oxalated plasma was about 10 % lower than that of heparin plasma. He, however, attributed this to the increase in osmotic pressure due to the potassium oxalate and to the consequent dilution of the plasma.

For the same reasons it is inadvisable to employ trichloroacetate which some authors have used when studying the scissions of the phosphorylated compounds of the blood.

#### SUMMARY.

1. Extracts of liver and kidney contain, besides an "alkaline" phosphatase, an "acid" phosphatase, the activity of which is not negligible.
2. The activity of the "acid" phosphatase of liver and kidney extracts is inhibited by the fluoride, oxalate and trichloroacetate of sodium, that of the "alkaline" phosphatase of the same extracts is not influenced by these electrolytes. In this connection the two enzymic systems are qualitatively similar.
3. The activity of the "acid" phosphatase is extremely low in bone and serum.
4. In serum and extracts of bone, the "alkaline" phosphatase is partially inhibited by sodium oxalate. From this point of view these two enzymic systems may be considered to be qualitatively identical.
5. Near neutrality the influence of the inactivating agents which we have considered is negligible.
6. The influence of fluorides on the processes of calcification and decalcification of bone is discussed.

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