CLXXIX. RESEARCHES ON THE PHOSPHATASES.

III. ON THE MECHANISM OF THE INACTIVATING ACTION OF SODIUM OXALATE AND OF PHOS- PHATES ON THE "ALKALINE" PHOSPHATASES OF ANIMAL TISSUE.

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IN our first report [Belfanti *et al.*, 1935, 1] we dealt with the fact that, in many extracts of animal organs, the existence may be noted of a second "optimum" of phosphatase activity in an acid medium besides that optimum (which has been known for a long time) which exists in an alkaline medium¹.

Without prejudging the question as to whether the phenomenon is due to differentiation between the active groups of the enzymes themselves or to a diversity of colloidal carriers, we adopted, for the sake of greater clearness, the terms acid² phosphatases and alkaline phosphatases and we studied the different effects exercised by several inhibitory agents on these two forms of phospho-esterase activity.

In the present work, we have devoted our attention exclusively to the study of the effect of sodium oxalate on the alkaline phosphatases in order to decide whether or not the alkaline phosphatases of various origins studied up to the present may be considered as identical, and to find an explanation for several divergencies observed between the experimental data obtained by us and by Munemura [1933].

THE ACTION OF SODIUM OXALATE ON THE ACTIVITY OF THE ALKALINE PHOSPHATASES OF LIVER AND BONE EXTRACTS.

The methods employed were the same as those previously described [Belfanti *et al.*, 1935, 1, 2].

The extract of liver was prepared as previously described. The extract of bone was obtained from the tibiae and femora of an adult rabbit: the bones

¹ The following should be added to the references cited in our first report: Bamann and Riedel [1934].

² Until recently, the Japanese authors of Akamatsu's school divided the phosphatases into phosphomonoesterases, phosphodiesterases, pyrophosphatases and phosphoamidases and claimed to have demonstrated the specificity of the several enzymes. As the whole of our experiments were made with sodium β -glycerophosphate as substrate, the enzymes studied by us should be called, according to the terminology of the Japanese authors, phosphomonoesterases. However, in a recent work by Hotta [1934] it is shown that the specificity of the phosphatases is not determined only by the nature of the bond which unites phosphoric acid to the alcoholic residue but also by the chemical nature of the alcoholic residue itself. Until such time as the question is settled, we prefer to retain the generic denomination of phosphatases.

were freed from the marrow and separate extracts were made of the epiphyses and metaphyses. The phosphatase activity of both extracts was slightly less than that of the corresponding extracts prepared by us for the experiments described in our first paper.

Figs. 1 and 2 show the hydrolysis caused by one and the same extract of rabbit liver at different p_H values and at different times in the presence and absence of $M/50$ sodium oxalate respectively.

Fig. 1.

Fig. 2.

Fig. 1. The hydrolysis of β -glycerophosphate in the absence of added oxalate at different p_H values by rabbit liver extracts. — Hydrolysis in presence of veronal-acetate buffer; ---- hydrolysis in presence of glycine buffer.

Fig. 2. The hydrolysis of β -glycerophosphate in the presence of added $M/50$ oxalate at different p_H values by rabbit liver extracts. — Hydrolysis in presence of veronal-acetate buffer; ---- hydrolysis in presence of glycine buffer.

Whilst the curves in Fig. 1 (in the absence of oxalate) show a fairly regular course, those of Fig. 2 (in the presence of oxalate) show a curious phenomenon: at first (from the beginning up to the 3rd hour), the phosphatase activity is seen to be considerably inhibited as compared with the activity developed in the absence of oxalate; later (from the 3rd to the 6th hour) the inhibitory action of the oxalate is no longer manifest and moreover at certain p_H values the rate of hydrolysis in the presence of sodium oxalate is higher than in its absence. Still later (from the 6th to the 14th hour) the rate of hydrolysis is equal to that observed in the absence of sodium oxalate.

The bone extracts, as may be seen in Fig. 3 (hydrolysis in the absence of oxalate) and Fig. 4 (hydrolysis in the presence of $M/50$ oxalate), do not show this phenomenon: in the presence of sodium oxalate the rate of hydrolysis decreases more rapidly than in its absence.

In Figs. 1, 2, 3 and 4 it may be seen from the course of the curves in presence of glycine buffer, that in the case of liver extracts the use of glycine buffer leads

to an hydrolysis which is slightly less than that which may be obtained at the same p_H value when using veronal buffer both in the presence and in the absence of sodium oxalate. On the other hand, in the case of bone extracts, the use of glycine buffer in both cases leads to a much higher hydrolysis.

Fig. 3.

Fig. 4.

Fig. 3. The hydrolysis of β -glycerophosphate in the absence of oxalate at different p_H values by rabbit bone extracts. — Hydrolysis in presence of veronal-acetate buffer; ---- hydrolysis in presence of glycine buffer.

Fig. 4. The hydrolysis of β -glycerophosphate in the presence of added $M/50$ oxalate at different p_H values by rabbit bone extracts. — Hydrolysis in presence of veronal-acetate buffer; ---- hydrolysis in presence of glycine buffer.

*The kinetics of the alkaline phosphatases of liver and bone
in the absence and in the presence of sodium oxalate.*

Martland and Robison [1927] studied the course of the hydrolysis of sodium glycerophosphate by bone phosphatase in the absence and in the presence of inorganic phosphates. They found that, even without the addition of phosphates, the values of the velocity constant, calculated according to the equation of unimolecular reactions, decreased more rapidly than could be accounted for by the thermal inactivation of the enzyme; they advanced the hypothesis that this phenomenon was due to the process of resynthesis between phosphoric acid and glycerol. They demonstrated the retarding influence of phosphates on the enzymic hydrolysis of glycerophosphate; but, working with phosphate and

glycerol in the concentrations employed in the previous experiments, they did not succeed in demonstrating the possibility of a resynthesis.

Erdtman [1927], using an enzymic preparation of hog kidney, also found that the values of the velocity constant, in the absence of activating agents, rapidly decreased. He attributed this fact, at least in part, to a destruction of the enzyme and to an inactivating action of the products of hydrolysis.

Fig. 5.

Fig. 6.

Fig. 5. The hydrolysis of β -glycerophosphate in the presence and absence of added oxalate by rabbit liver extracts. $\bullet$ — $\bullet$ Hydrolysis in absence of added oxalate; $\bullet$ — $\bullet$ — $\bullet$ hydrolysis in presence of added oxalate.

Fig. 6. The hydrolysis of β -glycerophosphate in the presence and absence of added oxalate by rabbit bone extracts. $\bullet$ — $\bullet$ Hydrolysis in absence of added oxalate; $\bullet$ — $\bullet$ — $\bullet$ hydrolysis in presence of added oxalate.

Under the experimental conditions habitually adopted by us, we also have found that there is a rapid fall in the values of the velocity constant. However, in agreement with the observations of Němec [1923] when studying the action of soya beans on glycerophosphate, Schütz's law was approximately followed (naturally in the absence of sodium oxalate) with the alkaline phosphatases of both liver and bone.

In Tables I (liver) and II (bone) we give the values of $k = \frac{1}{t} \log \frac{a}{a-x}$ and of $k_1 = \frac{x}{\sqrt{t}}$ obtained for a hydrolysis at 37° of sodium β -glycerophosphate, concentration about $M/100$, in a glycine buffer in the absence of sodium oxalate, and the corresponding k' and k_1' values under the same conditions but in the presence of sodium oxalate.

Table I (liver extract) shows that the values of k' which at the beginning are very small, tend with time to equal the corresponding k values and that, while the k_1 values remain constant, those of k_1' increase greatly at the beginning and then decrease slowly. In Table II (bone extract), on the other hand, the values of both k' and k_1' show a progressive and marked decrease with time as compared with the corresponding values of k and k_1 .

Table I. *Hydrolysis of sodium β -glycerophosphate 0.009344 M by the alkaline phosphatase of liver in glycine buffer.*

10 ml. of extract in 50 ml. of total solution.

In absence of sodium oxalate $p_H = 8.89$				In presence of $M/50$ sodium oxalate $p_H = 8.90$		
Time hours	P hydro- lysed %	$k = \frac{1}{t} \log \frac{a}{a-x}$	$k_1 = \frac{x}{\sqrt{t}}$	P hydro- lysed %	$k' = \frac{1}{t} \log \frac{a}{a-x}$	$k_1' = \frac{x}{\sqrt{t}}$
3	7.80	0.0118	0.0130	3.69	0.0055	0.0062
6	12.33	0.0095	0.0146	11.71	0.0090	0.0139
9	14.36	0.0075	0.0138	13.16	0.0068	0.0127
14	18.99	0.0064	0.0147	15.92	0.0054	0.0124

Table II. *Hydrolysis of sodium β -glycerophosphate 0.009344 M by the alkaline phosphatase of bone in glycine buffer.*

10 ml. of extract in 50 ml. of total solution.

In absence of sodium oxalate $p_H = 8.92$				In presence of $M/50$ sodium oxalate $p_H = 8.90$		
Time hours	P hydro- lysed %	$k = \frac{1}{t} \log \frac{a}{a-x}$	$k_1 = \frac{x}{\sqrt{t}}$	P hydro- lysed %	$k' = \frac{1}{t} \log \frac{a}{a-x}$	$k_1' = \frac{x}{\sqrt{t}}$
2	12.12	0.0280	0.0248	7.39	0.0111	0.0151
4	18.44	0.0222	0.0267	9.25	0.0070	0.0134
8½	26.90	0.0173	0.0267	11.67	0.0063	0.0116
14	33.25	0.0125	0.0257	13.91	0.0046	0.0107

From this it is concluded that the alkaline phosphatase of liver and that of bone differ, in the presence of oxalate, entirely in their kinetic behaviour, whilst the simple examination of the kinetic behaviour of the two extracts in the absence of inhibitory agents might have led one erroneously to conclude in favour of the identity of the two phosphatases.

On increasing the concentration of the substrate, the values of the hydrolysis effected by the phosphatases of liver and of bone no longer conform (even in the absence of sodium oxalate) to Schütz's law. In Table III are given the values of k_1 relative to the hydrolysis of sodium β -glycerophosphate, in concentrations 5 and 10 times that of the previous experiments, for the alkaline phosphatase of the liver and that of bone. In the latter case, there is a decrease in the values of k_1' which is even more sharply marked.

Table III. *Hydrolysis of sodium β -glycerophosphate in varying concentrations by the alkaline phosphatases of liver and of bone in glycine buffer.*

10 ml. of extract in 50 ml. of total solution.

Extract of rabbit liver values of $k_1 = \frac{x}{\sqrt{t}}$			Extract of rabbit bone values of $k_1 = \frac{x}{\sqrt{t}}$		
Time hours	Glycerophosphate			Glycerophosphate	
	$M/20$	$M/10$		$M/20$	$M/10$
½	0.0236	0.0286		0.0137	0.0193
1	0.0221	0.0234		0.0127	0.0164
2	0.0204	0.0250		0.0112	0.0135
3	0.0224	0.0243		0.0114	0.0132

In all probability, by suitably decreasing the concentration of the substrate, the inverse effect may be obtained and therefore also a greater constancy of the k values.

The attempts made to establish to which of the two types of inactivation described by Michaelis and Rona [1914] that exercised by sodium oxalate on the alkaline phosphatases belongs, *i.e.* whether the inactivation is due to an affinity of the enzyme to the inactivating agent or to an alteration of the "nature of the solvent", did not lead to conclusive results either in the case of the alkaline phosphatase of the liver or that of bone.

Action of sodium oxalate on the alkaline phosphatase of liver with various concentrations of substrate. Influence of the addition of inorganic phosphates on the hydrolysis of glycerophosphate in the presence of sodium oxalate.

In order to explain the strange behaviour of the alkaline phosphatase of liver (and, as we shall see later, of kidney) in the presence of sodium oxalate wherein at the beginning there is a strongly marked fall in the hydrolysing power and immediately afterwards the phosphatase activity recovers as if the oxalate were no longer present, two hypotheses could be advanced: (1) that in the extracts of such organs there exist enzymes which are capable of demolishing the oxalate ions; (2) that the reactivating action is due to the products of hydrolysis themselves, that is to say, to phosphoric acid and glycerol.

Determinations of oxalic acid carried out in the course of the hydrolysis led us to discard the first hypothesis. On the other hand, examination of the course of the hydrolysis in the presence of sodium oxalate at varying concentrations of substrate brought important evidence in support of the second hypothesis.

The previous experiments were carried out with solutions of about $M/100$ sodium glycerophosphate (see Table I); we then experimented with concentrations of glycerophosphate of 5 and 10 times that strength and the results are shown in Table IV.

Table IV. *Influence of sodium oxalate on the alkaline phosphatase of liver with high degrees of concentration of substrate.*

Liver extract 10 ml. in 50 ml. of total solution. Glycine buffer.								
Glycerophosphate 0.04672 <i>M</i>					Glycerophosphate 0.09344 <i>M</i>			
Time mins.	Without oxalate		With oxalate <i>M</i> /50		Without oxalate		With oxalate <i>M</i> /50	
	<i>p</i> _H 9.36		<i>p</i> _H 9.36		<i>p</i> _H 9.36		<i>p</i> _H 9.21	
	P hydrolysed		P hydrolysed		P hydrolysed		P hydrolysed	
	mg.	%	mg.	%	mg.	%	mg.	%
30	0.0167	1.15	0.0084	0.58	0.0202	0.70	0.0047	0.16
60	0.0221	1.53	0.0089	0.61	0.0234	0.81	0.0056	0.19
120	0.0289	2.00	0.0264	1.82	0.0354	1.22	0.0345	1.19
180	0.0386	2.67	0.0344	2.38	0.0422	1.46	0.0381	1.33

The hypothesis that an enzyme capable of destroying the oxalate ions is to be found in liver extracts was once again shown to be inaccurate by the experiments made with varying concentrations of substrate. If this were the case, seeing that the quantity of enzymic solution and that of the sodium oxalate used were always the same, the inactivating action of the oxalate should have disappeared in equal times in all cases. Tables I and IV show instead that this period varies from 6 hours to 60 minutes.

Moreover, on comparing the data of Table I (concentration of the substrate about $M/100$) with those of Table IV (concentration of the substrate about

$M/20$ and $M/10$), it will be seen that the inactivating action of the sodium oxalate may be considered as having ceased when the hydrolysis of the substrate has reached percentages of 12, 2 and 1.20 respectively; that is to say, when the phosphorus freed corresponds roughly in all three cases to 0.03 mg. per ml.

The phenomenon of the disappearance of the inactivating action of the oxalate does not therefore depend on the percentage of hydrolysis of the substrate, but corresponds to the freeing of a certain quantity of inorganic phosphorus (or of glycerol), which, for a given enzymic solution used in the same proportions at all times, must be considered as being practically constant.

This quantity of inorganic phosphorus varies directly with the activity of the enzyme and therefore with the "mass" of the enzyme itself.

Table V shows the results obtained when the concentration of the substrate is maintained constant but the quantity of enzymic solution is varied.

The extract of rabbit liver used in this experiment as well as in the succeeding one was different from that used in the previous experiments and was a little less active; consequently the values in Table V and Fig. 7 are not comparable, numerically, with the previous ones.

It will be seen, from Table V, that on using 10 ml. of liver extract the inhibitory action of the oxalate disappears after from 0.0234 to 0.0292 mg. of P per ml. has separated in the corresponding test without oxalate. On using 20 ml. of extract the disappearance of the inhibitory action of the oxalate occurs when from 0.0357 to 0.0414 mg. of P is freed in the corresponding test without oxalate.

Table V. *Hydrolysis of β -glycerophosphate in the presence of sodium oxalate by various quantities of rabbit liver extract.*

Time mins.	Glycerophosphate 0.09344 <i>M</i> . Glycine buffer.			
	10 ml. of liver extract in 50 ml. of total solution		20 ml. of liver extract in 50 ml. of total solution	
	With oxalate	Without	With oxalate	Without
	$M/50$	oxalate	$M/50$	oxalate
	p_H 9.38	p_H 9.30	p_H 9.21	p_H 9.25
	mg. P hydrolysed per ml.			
30	0.0045	0.0165	0.0030	0.0232
60	0.0048	0.0202	0.0040	0.0300
90	0.0094	0.0234	0.0045	0.0357
120	0.0295	0.0292	0.0461	0.0414
180	0.0343	0.0348	0.0585	0.0582
240	0.0396	0.0408	0.0686	0.0600

The proof that the disappearance of the inhibitory action of sodium oxalate is due to the inorganic phosphate set free during the hydrolysis is given by the experiment illustrated in Fig. 7.

The four parallel tests were made simultaneously with the same quantity of rabbit liver extract and under the same conditions of concentration of the substrate and of acidity.

Curve *A* represents the hydrolysis of the glycerophosphate in the presence of sodium oxalate $M/50$ and of disodium hydrogen phosphate added to the extent of 0.03 mg. of P per ml. and, as may be seen, it no longer shows the characteristic course of inactivation with oxalate (*cf.* Fig. 2). Curve *B* represents hydrolysis in presence of the same quantity of inorganic phosphate but in the absence of sodium oxalate.

The two curves practically coincide, curve *A* showing a course which is parallel with and only slightly below that of curve *B*. This means that in

the presence of a suitable quantity of inorganic phosphate the action of sodium oxalate on the alkaline phosphatase of liver is practically nil. Curves *C* and *D* represent the hydrolysis without the addition of inorganic phosphate but in the presence and absence respectively of sodium oxalate. It will be noted

Fig. 7. The hydrolysis of β -glycerophosphate (0.09344 *M*) in the presence and absence of added oxalate and phosphate by a rabbit liver extract (glycine buffer).

Curve A $\bigcirc$ — — $\bigcirc$ Hydrolysis in presence of added *M*/50 oxalate and in presence of added *M*/1000 sodium phosphate; B $\bigcirc$ — — $\bigcirc$ hydrolysis in absence of added oxalate and in presence of added *M*/1000 sodium phosphate; C $\times$ — — $\times$ hydrolysis in presence of added *M*/50 oxalate and in absence of added sodium phosphate; D $\times$ — — $\times$ hydrolysis in absence of added oxalate and in absence of added sodium phosphate.

that curve *C* keeps at a much lower level, for the first 90 minutes, than that of *A* and *B*. This means that in the absence of inorganic phosphates the sodium oxalate fully develops its inhibitory activity and then, in the space of 30 minutes, curve *C* rapidly rises until it reaches and almost merges into curve *D* which runs parallel with the two curves *A* and *B* but at a higher level. The difference between the various points of curve *D* and the corresponding points of curve *B* represents the inhibition exercised on the normal hydrolysis of the glycerophosphate, in the absence of other inactivating agents, by the addition of an excess of inorganic phosphate. It is natural therefore that in the test represented by curve *C*, in which this addition was not made, after a certain time the curve itself merges into curve *D* and not, for example, into curve *A*.

It is obvious that the points marked " $\bigcirc$ " in Fig. 7 represent the values of inorganic P determined in tests *A* and *B* less the inorganic phosphate previously added (0.03 mg. per ml.).

Influence of sodium oxalate on the alkaline phosphatase activity of extracts of hog kidney.

As the statements of Munemura [1933] regarding the influence of sodium oxalate on Type III phosphomonoesterase were made as the result of experiments with an extract of hog kidney, we prepared an extract of hog kidney by following exactly, as Munemura did, Asakawa's prescriptions [1928].

Moreover, a part of this extract was treated with MgCO_3 and sodium acetate according to the method described by Kurata [1931] in order to separate the

alkaline phosphatase of the kidney from the acid phosphatase. Several experiments were made in parallel and the two extracts, that prepared according to Asakawa's method and that further purified according to Kurata's method were compared.

Exp. 1. Date: November 19th, 1934. Glycine buffer.

Extract of hog kidney according to Asakawa

Without oxalate	p_H 8.45	P hydrolysed after $5\frac{1}{2}$ hours	0.1000 mg.
With oxalate $M/50$	p_H 8.67	" "	0.0354 mg.

Extract of hog kidney according to Kurata

Without oxalate	p_H 8.68	P hydrolysed after $5\frac{1}{2}$ hours	0.1023 mg.
With oxalate $M/50$	p_H 8.61	" "	0.0366 mg.

It will be seen that not even the alkaline phosphatase of hog kidney is wholly inactivated by the sodium oxalate.

We would also point out that the extract prepared according to Kurata had not completely lost the acid phosphatase; in fact, on testing the phosphatase activity of this extract on β -glycerophosphate in an acid medium, we obtained the following results:

Extract of kidney according to Kurata (veronal buffer)	p_H 4.75	P hydrolysed after $5\frac{1}{2}$ hours	0.0310 mg.
Extract of kidney according to Asakawa (veronal buffer)	p_H 4.72	" "	0.0762 mg.

In the process of separating the alkaline from the acid phosphatase, Kurata kept the enzyme in the presence of magnesium carbonate in an alkaline medium at 37° for 1 hour. In our previous paper we showed that keeping an alkaline phosphatase at 37° in an acid medium, without any other addition, caused a marked inactivation of the phosphatase. Contardi and Ercoli [1933] have shown that keeping an acid phosphatase (from rice husks) in an alkaline medium causes an inactivation of that phosphatase; so much so in fact that when preparing enzymic extracts from rice husk dephosphorylated with barium hydroxide it is necessary to limit exposure in the alkaline medium to the shortest possible time.

We believe therefore that the mechanism of the separation of the two phosphatases, acid and alkaline, according to Kurata's method, is not exclusively that of adsorption by the magnesium carbonate or the aluminium hydroxide respectively, but that it consists, above all, of an inactivation due to the effect of a p_H which is unsuitable for the conservation of the enzyme. The recent work of Bamann, Riedel and Diederichs [1934] and of Bamann and Diederichs [1934], who separated the two types of enzymes by keeping the phosphatases themselves in an acid or an alkaline medium for a suitable time confirms this interpretation.

Exp. 2. Date: November 22nd, 1934. Glycine buffer.

Extract of hog kidney according to Asakawa.

		mg. of P hydrolysed per ml. after				
	p_H	$1\frac{1}{2}$	$3\frac{1}{2}$	$5\frac{1}{2}$	$7\frac{1}{2}$	12 hours
Without oxalate	8.70	0.0892	0.1667	0.2202	0.2275	0.2342
With oxalate $M/50$	8.55	0.0333	0.0721	0.1935	0.2096	0.2308

The sodium oxalate first slows down the speed of hydrolysis to a marked degree. Later on, however, the enzyme recovers its activity as though the inhibiting agent were no longer present.

This experiment again confirms what we said regarding the experiments summarised in Table V, that on increasing the quantity of enzyme, the quantity of inorganic phosphate to be set free before the inactivating action of the oxalate disappears also increases. In the present case that quantity is very large, corresponding with the activity of the extract.

Moreover, the phosphatase activity of this extract, which in the meantime had been kept in the ice-chest at p_H 7.6, had undergone a notable increase as compared with that displayed in the previous experiment¹.

Exp. 3. Date: November 26th, 1934.

The results given in Table VI show that similarly to what occurs with rabbit liver extracts but not with bone extracts (Figs. 1, 2, 3, 4), hydrolysis in the presence of glycine buffer is a little less rapid than in the presence of veronal buffer. It seems moreover that the inhibitory influence of sodium oxalate is greater in the presence of glycine buffer than in that of veronal buffer.

Table VI. *Influence of glycine buffer as compared with veronal buffer on the phosphatase activity of kidney extracts in the presence and in the absence of sodium oxalate.*

Extract of hog kidney prepared according to Asakawa's method. mg. P hydrolysed.						
Veronal buffer				Glycine buffer		
Time hours	Without oxalate p_H 8.75	Oxalate $M/50$ p_H 8.72	Difference	Without oxalate p_H 8.52	Oxalate $M/50$ p_H 8.72	Difference
1	0.1764	0.1519	0.0245	0.1538	0.1200	0.0338
3½	0.2189	0.1987	0.0202	0.2189	0.1666	0.0523
8	0.2400	0.2222	0.0178	0.2362	0.2069	0.0293

The phosphatase activity of the hog kidney extract during the interval between the second and the third experiments (7 days) was again increased to a most marked degree.

Exp. 4. Date: November 28th, 1934.

In all the experiments on hog kidney extract described so far the final concentration of sodium glycerophosphate was about $M/100$. Munemura instead worked with a $M/500$ sodium glycerophosphate. A test made with a concentration of substrate equal to that used by the Japanese author did not confirm his (Table VII) results.

Table VII. *Influence of sodium oxalate on the alkaline phosphatase of hog kidney with $M/500$ glycerophosphate.*

Extract of hog kidney (the same as that used in the previous experiments but diluted from 1 to 4).

Sodium glycerophosphate $M/500$. Glycine buffer. mg. P hydrolysed.					
Without oxalate		p_H 8.26	p_H 8.70	p_H 8.93	p_H 9.31
After 1 hour		0.0320	0.0461	0.0453	0.0453
After 2½ hours		0.0444	0.0502	0.0508	0.0500
With oxalate $M/50$		p_H 8.20	p_H 8.82	p_H 9.07	p_H 9.40
After 1 hour		0.0139	0.0250	0.0266	0.0247
After 2½ hours		0.0222	0.0282	0.0306	0.0315

¹ A similar case was recently observed by Bamann, Riedel and Diederichs [1934] for a liver extract. Bodansky [1932] also found an increase of about 10 % in the original phosphatase activity of serum after preservation for 24 hours in the ice-chest. A similar activation of phosphatase was observed by Euler and Ohlsén [1911].

Influence of the addition of inorganic phosphates on the hydrolysis of glycerophosphate in the presence of sodium oxalate by bone phosphatase.

Fig. 8 represents an experiment carried out in the same way as that described in Fig. 7 but with an extract of bone of young rabbit.

Fig. 8. The hydrolysis of β -glycerophosphate (0.09344 M) by a rabbit bone extract in the presence and absence of added oxalate and phosphate.

Curve A $\circ$ — $\circ$ — $\circ$ Hydrolysis in presence of added $M/50$ oxalate and in presence of added $M/1000$ sodium phosphate; B $\circ$ — $\circ$ — $\circ$ hydrolysis in absence of added oxalate and in presence of added $M/1000$ sodium phosphate; C $\times$ — $\times$ — $\times$ hydrolysis in presence of added $M/50$ oxalate and in absence of added sodium phosphate; D $\times$ — $\times$ — $\times$ hydrolysis in absence of added oxalate and in absence of added sodium phosphate.

In the presence of sodium phosphate added simultaneously with the oxalate, the behaviour is analogous to that of the alkaline phosphatase of the liver.

Influence on the bone phosphatase of time of contact between enzymic solution and sodium oxalate before the reactivation with inorganic phosphate.

In order to demonstrate that the oxalate tends permanently to inactivate the bone phosphatase, we placed in three 50 ml. flasks:

	I	II	III
	ml.	ml.	ml.
Glycine $M/10$	14	14	14
NaOH $N/10$	6	6	6
Sodium oxalate $M/5$	5	5	5
Disodium hydrogen phosphate $M/20$	1	—	—
Bone extract	10	10	10

After 3 hours' digestion at 37° we added:

Disodium hydrogen phosphate $M/20$	—	1	—
Sodium glycerophosphate 10 % (anhydrous salt)	10	10	10

The contents of the flasks were brought to the mark with distilled water and they were then placed in the thermostat in order to proceed in the usual manner to determine the phosphatase activity. The results, corrected for the quantity of phosphorus added in the form of sodium phosphate, are given in Table VIII.

Table VIII.

	I	II	III
	The oxalate acted for 3 hours at 37° in the presence of added inorganic phosphate	The oxalate acted for 3 hours at 37° in the absence of inorganic phosphate, which was added only after the third hour	In the presence of oxalate but without any addition whatsoever of inorganic phosphate
Time mins.			
30	0.0211	0.0178	0.0078
45	0.0267	0.0202	0.0091
60	0.0308	0.0239	0.0296
90	0.0414	0.0315	0.0368
120	0.0489	0.0366	0.0400
180	0.0557	0.0416	0.0494

In the absence of added sodium phosphate the oxalate tends permanently to inactivate the bone phosphatase as time proceeds.

DISCUSSION.

In our previous work we arrived at the conclusion that aqueous extracts of liver and kidney contain a system of phosphatase enzymes which is different from that of bone extracts and of blood serum. Among the differences encountered was the behaviour in presence of sodium oxalate of the alkaline phosphatase of bone and of serum as compared with that of the alkaline phosphatase of liver and of kidney. In the present work we have carried the study of this behaviour as regards the oxalate ions further and have also arrived at interesting conclusions regarding the general mechanism of phosphatase scission.

Jacobsen [1932], from the work of Martland and Robison [1927] and of Erdtman [1927; 1928] and above all from the results of his own accurate work on the inhibitory action of inorganic phosphates on kidney phosphatase, deduces that in a phosphatase + glycerophosphate system an enzyme-phosphate complex must be formed in addition to the enzyme-substrate complex. The enzyme-phosphate complex, generated by the phosphates freed during the course of the hydrolysis, is, according to Jacobsen himself, inactive. We however demonstrate that this enzyme-phosphate complex has a hydrolytic action on glycerophosphoric acid.

In the experiments described in Tables I, IV and V hydrolysis in the absence of oxalate occurs, at the very beginning of the reaction, according to the now universally accepted general scheme of enzymic actions:

From the tables in question, it may be seen that, in the presence of sodium oxalate, the phosphate freed by the alkaline phosphatase of liver at the beginning of the reaction (0.0055, 0.0084, 0.0047, 0.0045, 0.0038 mg. *etc.*) is much less than that freed contemporaneously in comparative tests in absence of oxalate (respectively 0.0226, 0.0167, 0.0202, 0.0165, 0.0232 mg.). If this fact is interpreted in the sense that the enzyme, besides having affinity for the substrate

has also affinity for sodium oxalate, we must deduce from these data that the division of the enzyme between substrate and oxalate occurs predominantly in favour of the latter, that is to say, in the formation of the two complexes, the first active, the second inactive

the balance is clearly displaced from left to right.

As soon as a small quantity of inorganic phosphate has been freed, we must admit with Jacobsen [1932] the formation of the new complex, enzyme-phosphate, in equilibrium with the previous ones.

Now, in Fig. 7, we have seen that in the presence of sodium phosphate, previously added in suitable quantity, the hydrolysis occurs as if the oxalate were no longer present (curves *A* and *B*), that is to say, the inorganic phosphate impedes the formation of the inactive enzyme-oxalate complex.

This antagonistic action of the phosphate can only be explained by admitting that the enzyme may combine not only with the substrate, but also through a second bond, with other electrolytes. When oxalate unites with the enzyme, there results such a modification in the properties of the enzyme itself that the enzyme-oxalate complex thus formed can no longer be bound to the substrate and is therefore inactive; when instead the phosphate is so bound, the enzyme conserves its property of uniting itself with the other bond to the substrate and of hydrolysing it.

The fact that, as in our case, the inactive enzyme-oxalate complex cannot form in the presence of inorganic phosphates artificially added means that the affinity between enzyme and phosphate is greater than that between enzyme and oxalate and that therefore, out of the two complexes possible, the enzyme-phosphate complex tends to be formed predominantly.

On again examining the action of the alkaline phosphatase of liver on glycerophosphate in the presence of sodium oxalate, it follows from the considerations made that among the complexes which we have admitted exist in the system:

the balance will tend to move in favour of the formation of enzyme-phosphate-substrate. Therefore, as and when, during the course of hydrolysis, the inorganic phosphate is set free, the latter displaces the oxalate from the inactive enzyme-oxalate complex and gives rise to the formation of the active enzyme-phosphate complex¹.

The demonstration of this process is given by the experiments described in the experimental part (see Figs. 2 and 7, curve *C*) in which the hydrolysis in the presence of oxalate is accelerated as the inorganic phosphate is freed. This acceleration, seeing that the destruction of the oxalate ions was experimentally excluded, can only be attributed to the progressive formation of the active enzyme-phosphate complex at the expense of the inactive enzyme-oxalate complex.

The experiments with hog kidney, made in order to confirm Munemura's statement [1933], allow us to conclude that the mechanism of the action of that phosphatase is identical with that of the phosphatase of rabbit liver. In this case too, therefore, the enzymic action develops at the beginning directly

¹ The fact that the oxalate ions may be easily and completely displaced from the enzyme-oxalate complex has already been demonstrated by us [Belfanti *et al.*, 1935, 2].

between enzyme and substrate; with the gradual formation of the enzyme-phosphate complex, the hydrolysis caused by the latter also comes into play.

The relations existing at the beginning of the reaction between the concentration of the substrate and that of the enzyme will favour in greater or less degree and with greater or less rapidity the formation of the enzyme-phosphate-substrate complex at the expense of the enzyme-substrate complex and according to circumstances the course of the reaction will be more or less removed from that of a unimolecular reaction. In no case however can perfect concordance with this law be expected as the hydrolysis is caused simultaneously by two agents which possess different properties and whose quantitative relations vary continuously with time. In fact, one has to deduce from the experiments represented in Fig. 7 (curves *B* and *D*) that the value of the dissociation constant of the enzyme-phosphate-substrate complex is notably less than that of the constant of dissociation of the enzyme-substrate complex. It is not probable that this is the only difference existing between the properties of the two complexes produced; for example, the complexes may have different acidity optima. In this way one may easily explain several anomalies which are fairly frequently observed in the ordinary processes of enzymic hydrolysis of the phosphoric esters, such as the variation of the optimum of phosphatase activity with the concentration of the substrate [Asakawa, 1928], or during the course of the hydrolysis (Fig. 3).

The considerations discussed in connection with the phosphatase of liver and kidney may perhaps be extended also to other enzymes. One may suppose in fact that the accelerations met with in certain enzymic processes and the abbreviation of the so-called induction period, which may be obtained by adding to the solutions under examination one of the products of the reaction (*e.g.* acetaldehyde in the case of alcoholic fermentation), may be explained by the formation, between enzyme and product of hydrolysis, of a complex which is more active than the free enzyme.

Returning to the phosphatase of liver and kidney, it is probable that the activating action of magnesium, as follows from Erdtmann's work, consists in the formation of a magnesium-enzyme complex which is still capable of uniting with the substrate to hydrolyse it and in which the magnesium is not displaceable by phosphate.

Phosphatase of bone. As pointed out by us in the experimental part (Figs. 3, 4 and 6), the phosphatase of bone in the presence of sodium oxalate behaves in a different way from the phosphatase of liver and of kidney. The paralysing action of the oxalate is not limited only to the beginning of the hydrolysis but is manifest during the whole course of the same. In our previous note [Belfanti *et al.*, 1935, 2] we demonstrated that this phosphatase, kept at 37° in an acid medium (p_H 4.5), loses its activity much more rapidly than the alkaline phosphatase of the liver and kidney. Analogously, one may suppose that it is also more sensitive to the action of sodium oxalate and that therefore the formation of the enzyme-oxalate complex is accompanied by a profound alteration of the enzyme, an alteration which occurs gradually during a relatively long period of time and which may lead to the permanent disappearance of the phosphatase activity. One may thus easily explain the fact that the phosphates set free during the course of hydrolysis do not, of themselves, succeed in determining complete reactivation such as occurs in the case of liver and of kidney.

This interpretation of the behaviour of the phosphatase of bone to sodium oxalate is confirmed by the experiment recorded in Fig. 8. For this experiment we purposely used an extract of bone of a young rabbit, recently prepared and

very concentrated, the activity of which was therefore very marked. Hence the time needed in order to free a quantity of inorganic phosphate sufficient to reactivate the enzyme was much shorter and, correspondingly, it became less probable that the enzyme would become permanently inactive. One could hope therefore for at least a partial reactivation of the enzyme by the inorganic phosphate freed during hydrolysis.

In fact, on observing the course of curve *C* (in the presence and in the absence of added inorganic phosphate) one notes at a certain point (between the 45th and 60th minutes) a notable rise in hydrolysing power. Spontaneous reactivation is however very far from being complete. In the case of hydrolysis in the presence of sodium phosphate added at the same time as the oxalate (curve *A*), the behaviour is analogous to that met with in the case of the alkaline phosphatase of liver: in fact, the corresponding curve practically coincides with that of hydrolysis in the presence of phosphate but in the absence of oxalate (curve *B*). This fact may easily be explained because, according to our views, in this case the active enzyme-phosphate complex must be predominantly formed, so that the enzyme is removed from the alterative action of the oxalate.

In order to confirm that the oxalate tends to inactivate the phosphatase of bone in a permanent way, we carried out the experiment recorded in Table VIII (p. 1502). As may be seen, the values in column I are all higher than those of column II. This means that in the first case the formation of the enzyme-phosphate complex has at least in part impeded the permanent inactivation of the enzyme itself by the oxalate. In test II, as the greater part of the enzyme was permanently inactivated, successive additions of inorganic phosphate did not suffice to restore the phosphatase activity of the extract: in fact, the values obtained in this test, except the first two, are markedly inferior to those of test III in which no addition of inorganic phosphate was made. This is natural seeing that the addition of inorganic phosphate slowed down the hydrolysis caused by the phosphatase which was still unaltered.

As a partial conclusion of the work described in this and the previous notes, it may be well to recapitulate our present knowledge of the question of the identity of the alkaline phosphatases.

Robison and Soames [1924], studying the activity of the phosphatases of various animal tissues at different p_H values, concluded in favour of the hypothesis that the spleen, liver and pancreas contain a phosphatase which is different from that of the kidney and bone. Kay [1928; 1931] from a series of comparisons deduced that the phosphatases of the bones, kidneys, intestinal mucosa and blood plasma are identical. More recently, several Japanese authors of Akamatsu's school [Usawa, 1932; Hori, 1932; Munemura, 1933], after a series of valuable researches, came to the conclusion that the phosphomonoesterases existing in nature may be divided into three types having their optima of activity at p_H 3, 5.5 and 9-10 respectively. The affirmation that all the phosphatases which we have agreed to call alkaline, that is to say, those phosphatases which are capable of hydrolysing the monoesters of phosphoric acid at a p_H optimum between 9 and 10 inclusive, are identical is implicit in this classification.

From the experiments carried out in the present work, it follows that:

(1) With the phosphatase of bone a hydrolysis is obtained which is much more rapid in the presence of glycine buffer than in the presence of veronal buffer; the contrary occurs with the phosphatase of liver and kidney.

(2) The inhibition caused by oxalate of the phosphatase of liver and kidney is annulled by inorganic phosphate whether this be added artificially or formed in the course of hydrolysis. On the other hand, the inactivation of the phos-

phatase of bone by means of oxalate tends to become permanent so that the phosphates freed during the process of hydrolysis only partially reactivate the enzyme.

In our previous work [Belfanti *et al.*, 1935, 2] we demonstrated that the phosphatase of bone, in contrast with the alkaline phosphatase of the liver and kidneys, is extremely labile in an acid medium.

In correlation with these results, one may recall the fact observed by Hommerberg [1929] that the phosphatase of bone, in contrast with the alkaline phosphatase of the kidneys [Erdtman, 1928] and of the liver [Bamann and Riedel, 1934], is not activated by magnesium¹. Moreover, according to Hommerberg, the phosphatase of the bone differs from that of the kidney by reason of its greater solubility in alcohol.

The knowledge in our possession is therefore sufficient to allow one to affirm that these are different phosphatases.

The experiments made in the present work were not extended to the phosphatases of the blood and therefore it is not possible for us to make definite assertions on this subject; however, on the basis of the preliminary experiments described in our first report, one may deduce that the phosphatase of blood serum is analogous to that of the bones. The alkaline phosphatases examined by us may therefore be divided into two distinct groups: those of liver and of kidney belong to the first group whilst those of bone and serum belong to the second.

SUMMARY.

1. When an extract of liver or of kidney is allowed to act in an alkaline medium on glycerophosphate in the presence of sodium oxalate, the hydrolysis at first proceeds very slowly, then little by little it accelerates and finally proceeds as if the oxalate were no longer present, until the substrate is completely hydrolysed. In the presence of inorganic phosphate added artificially, sodium oxalate does not manifest any inhibitory action whatsoever on the alkaline phosphatase of liver and kidney.

2. It is suggested that the explanation of these phenomena is that the inorganic phosphate set free from the glycerophosphoric acid gradually displaces the oxalate ions from the inactive enzyme-oxalate complex giving rise to an active enzyme-phosphate complex capable, like the free enzyme, of uniting with the substrate and of hydrolysing it.

3. Inorganic phosphates, if previously added, manifest their antagonistic action to sodium oxalate even in the case of the phosphatase of bone. The latter, however, in contrast to the alkaline phosphatase of liver and kidney, when allowed to act on glycerophosphate in the presence of sodium oxalate is incapable of spontaneous reactivation. The probable explanation of this phenomenon is discussed.

4. The kinetic behaviour of the phosphatases of liver and bone was studied.

5. Kurata's process for the separation of the acid phosphatase from the alkaline phosphatase in extracts of hog kidney was examined.

6. The experiments of Munemura regarding the inactivation of the alkaline phosphatases by means of sodium oxalate were not confirmed and the statements of several authors relative to the identity of the alkaline phosphatases of different origin have been discussed. These phosphatases should be divided, for the reasons given in this and our previous papers, into two distinct groups: that of the liver and kidneys and that of bone and probably also of serum.

¹ Kay and Jenner [1931] maintain that the phosphatase of bone is also susceptible to the activating action of magnesium, but only when the latter is present in concentrations of $M/1000-M/100$.

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