XCIX. RESEARCHES ON THE PHOSPHATASES.

II. INACTIVATION AND REACTIVATION OF THE PHOSPHATASES OF ANIMAL ORGANS.

By SERAFINO BELFANTI, ANGELO CONTARDI and ALBERTO ERCOLI.

From the Istituto Sieroterapico Milanese and R. Istituto Superiore Agrario, Milan.

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Recently Robison and Rosenheim [1934] made the interesting observation that sodium fluoride, even in extremely small quantities, impedes *in vitro* the deposition of calcium in hypertrophic cartilage; however, even in much higher degrees of concentration, it does not alter the extent of hydrolysis brought about by a purified preparation of bone phosphatase on sodium β-glycerophosphate.

In a recent paper Belfanti *et al.* [1935], after having examined the inhibitory action of sodium fluoride on the "acid" phosphatases of extracts of animal tissues, mentioned, in connection with the probable importance of the fluorides in the phenomena of calcification and decalcification of bone, the fact that in certain circumstances sodium fluoride may also inhibit the activity of the "alkaline" phosphatases.

In the present work, we have attempted to find under what conditions that phenomenon may occur.

It seemed moreover that it would be of interest to find out if the inactivation effected by sodium fluoride was permanent in character or was reversible.

Inouye [1929], when experimenting with the glycerophosphatase of *Aspergillus oryzae*, found that on eliminating the fluorine ions by the addition of an excess of calcium chloride, the phosphatase activity was not restored, whereas the inhibitory effects of oxalate ions and sulphate ions could be eliminated by the addition of $CaCl_2$.

Experimental.

As regards the preparation of the enzymic extracts and the method used to estimate the phosphatase activity, we refer the reader to our previous work and when necessary discuss any modifications that have been introduced.

Reactivation of phosphatases inactivated with sodium oxalate.

(1) *Reactivation by the addition of $CaCl_2$.* Experiments made with an extract of rabbit liver agree in the results with those made by Inouye [1929] on the phosphatase of *A. oryzae*: we obtained, in other words, the restoration of the phosphatase activity of the extract. In order to ascertain if the time of contact between the oxalate and the enzyme before treating with calcium chloride had a

sensible influence on the inactivation, a series of five numbered test-tubes each containing 7 ml. of the undermentioned solution was prepared.

Acetate-veronal solution	...		15 ml.
8·5 % solution of NaCl	...		15 ,,
Distilled water	...	...	12 ,,
Rabbit liver extract	...	...	30 ,,
HCl $N/5$	...	...	36 ,,

$M/5$ sodium oxalate solution (1 ml.) was added to test-tubes 1, 2, 3 and 4. In test-tube No. 1, this addition was made immediately, whereas in test-tube No. 2 it was made after 30 mins., in test-tube No. 3 after 85 mins. and in test-tube No. 4 after 90 mins. 1 ml. of distilled water was added to test-tube No. 5. 1 ml. of $N/2$ $CaCl_2$ solution was then added to test-tubes Nos. 1, 2, 3 and 5 whilst 1 ml. of distilled water was added to test-tube No. 4. Finally, 1 ml. of 5 % (anhydrous salt) solution of sodium glycerophosphate was added to all five test-tubes, together with two drops of toluene. The five test-tubes were then placed at the same time in a thermostat at 37° and 16 hours later the inorganic phosphorus contents were estimated. Table I shows that, even when the sodium oxalate is allowed to act for one hour and a half and at p_H less than 5 on an enzymic solution obtained from rabbit liver, there is no notable alteration in the activity of its acid phosphatase.

Table I. *Influence on the acid phosphatase of rabbit liver of the time of contact between enzymic solution and sodium oxalate before reactivation with* $CaCl_2$.

Test-tube No.	Time of contact before the addition of $CaCl_2$	p_H of the solutions under examination	P hydrolysed mg.
1	90 mins.	4·87	0·0407
2	30 ,,	4·91	0·0414
3	5 ,,	4·88	0·0402
4	Control without oxalate	4·92	0·0398
5	Control without $CaCl_2$	5·02	0·0125

(2) *Reactivation by means of dialysis.* 25 ml. of $M/10$ sodium oxalate solution (portion A) were added to 25 ml. of a rabbit liver extract, whilst another 25 ml. of the same extract were diluted with 25 ml. of distilled water (portion B). The two portions were subjected to dialysis through cellophane against distilled water for 24 hours. When the dialysis was terminated, both liquids were brought to a volume of 60 ml. with distilled water. Three 25 ml. flasks were then prepared, each of which contained: solution of sodium glycerophosphate (5 % anhydrous salt), 1 ml.; acetate-veronal solution, 2 ml.; NaCl solution, 2 ml.; N HCl, 1·5 ml. 10 ml. of the enzymic extract (A) were added to flask No. 1, 10 ml. of extract (B) were added to flask No. 2 and 10 ml. of extract (B) *plus* 5 ml. of $M/5$ sodium oxalate were added to flask No. 3. All three flasks were then filled up to the mark with distilled water. Their p_H was about 3·2. After the addition of toluene and digestion in the thermostat for 16 hours, the following results were obtained:

Flask No.		P hydrolysed per ml. mg.
1	Dialysed after the addition of sodium oxalate	0·0289
2	Dialysed without the addition of sodium oxalate	0·0277
3	Control in the presence of $M/25$ oxalate added after dialysis	0·0074

The enzymic solution inhibited with sodium oxalate had reacquired its activity during dialysis.

Reactivation of phosphatases inactivated with sodium fluoride.

(1) *Reactivation by means of addition of $CaCl_2$.* Experiments carried out both with rabbit liver extract and with rabbit kidney extract demonstrate the possibility of reactivating, at least partially, by means of addition of $CaCl_2$, an enzyme which has been previously rendered inactive with NaF. The extent of this reactivation was, however, unlike that which was found in the case of sodium oxalate, in inverse relation to the time during which the fluorine ions had acted on the enzyme of the solution before being precipitated in the form of calcium salts. The experiment was carried out in exactly the same way as those done with sodium oxalate (see Table I). Table II shows the results obtained in two experiments with rabbit liver extract and rabbit kidney extract respectively.

Table II. *Influence on the acid phosphatase of rabbit liver and of rabbit kidney of the time of contact between enzymic solution and sodium fluoride before the addition of $CaCl_2$.*

Test-tube No.	Time of contact before the addition of $CaCl_2$	p_H of the solutions under examination	P hydrolysed mg.	Reacquired activity as % of the original activity
	Rabbit liver extract			
1	90 mins.	4·94	0·0136	15·4
2	30 ,,	4·86	0·0286	29·1
3	5 ,,	4·93	0·0296	59·3
4	Control without NaF	4·86	0·0364	—
5	Control without $CaCl_2$	4·89	0·0080	—
	Rabbit kidney extract			
1	90 mins.	4·9	0·0142	37·7
2	30 ,,	4·9	0·0207	65·8
3	5 ,,	4·8	0·0226	74·0
4	Control without NaF	4·92	0·0231	—
5	Control without $CaCl_2$	5·07	0·0055	—

As may be seen from Table II, under similar conditions, the activity of the acid phosphatase of kidney extract is reacquired in a greater measure than that of the acid phosphatase of liver extract.

(2) *Reactivation by means of dialysis.* 40 ml. of rabbit liver extract were added to 8 ml. of a solution of NaF, acidified with acetic acid to p_H 4·4. The whole was allowed to stand at room temperature for three hours. Afterwards, the solution was divided into two equal parts: the first was dialysed for three days against distilled water (extract A) whilst the second was kept in the ice-chest (extract B). Simultaneously with A, 20 ml. of liver extract with the addition of 4 ml. of distilled water (extract C) were dialysed. The phosphatase activities of the three extracts as regards sodium glycerophosphate and in the presence of veronal buffer were then estimated in the usual way.

An experiment was also carried out, by the same method, on an extract of hog kidney prepared according to Asakawa [1929].

The following results were obtained:

		Rabbit kidney		Hog kidney	
Extract		p_H of the solution	P hydrolysed per ml. mg.	p_H of the solutions	P hydrolysed per ml. mg.
A	Dialysed after adding NaF	5·4	0·0243	4·77	0·0818
B	Not dialysed after adding NaF	5·2	0·0040	4·60	0·0225
C	Dialysed without adding NaF	5·4	0·0389	4·72	0·0803

These experiments show that dialysis leads to the partial reactivation of the acid phosphatases of extracts of rabbit and hog kidney previously inactivated with sodium fluoride. We wish to point out, however, that the contact between fluoride and enzyme, although protracted over a very long period of time, took place at room temperature instead of at 37° as was the case in the experiments on reactivation with calcium chloride.

Inactivation with sodium fluoride of the "alkaline" phosphatase of the liver and kidney. Lability of bone phosphatase in an acid medium.

In each of four 25 ml. flasks, numbered from 1 to 4, were placed:

	No. 1 ml.	No. 2 ml.	No. 3 ml.	No. 4 ml.
Liver extract	5	5	5	5
Acetate-veronal solution	4·5	4·5	4·5	4·5
N/5 HCl	6	6	—	—
2 % NaF	1	—	1	—
H_2O	—	1	6	7

The four flasks were left in the water-bath at 37° for 2 hours and then to each of them were added:

N/5 NaOH	5·5	5·5	—	—
5 % glycerophosphate	1	1	1	1

and the whole brought up to the mark with distilled water. After determination of the p_H, addition of toluene and digestion in the thermostat for 16 hours, we proceeded to estimate the P hydrolysed. The following is the result of the four tests:

No. 1	p_H 8·65	P hydrolysed per ml. mg.	0·0235
No. 2	p_H 8·80	,, ,,	0·0484
No. 3	p_H 8·62	,, ,,	0·0605
No. 4	p_H 8·90	,, ,,	0·0610

A second experiment, carried out with another extract of liver of the rabbit, much more active than the previous one, gave the following results:

No. 1	p_H 9·27	P hydrolysed per ml. mg.	0·1000
No. 2	p_H 9·32	,, ,,	0·1935
No. 3	p_H 8·70	,, ,,	0·1818

The data given above show that exposure to 37° for two hours at p_H about 4·5 partially inactivates the "alkaline" phosphatase of rabbit liver extract. In presence of sodium fluoride, the resultant inactivation is much higher.

The alkaline phosphatase of the kidney behaves in an analogous manner, as is shown by the results of an experiment made with an extract of hog kidney prepared according to Asakawa's method [1929], in which exposure to 18° in an acid medium at p_H 4·5 was protracted for 17 hours.

No. 1	p_H 8·71	P hydrolysed per ml. mg.	0·0182
No. 2	p_H 8·75	,, ,,	0·1066
No. 3	p_H 8·85	,, ,,	0·2286

Similar experiments were carried out with an extract of bone (metaphysis and epiphysis) from the adult rabbit and the results obtained were as follows:

No. 1	p_H 8·65	P hydrolysed per ml. mg.	Traces
No. 2	p_H 8·70	,, ,,	Traces
No. 3	p_H 8·95	,, ,,	0·0631
No. 4	p_H 8·90	,, ,,	0·0727

As exposure for two hours to 37° in an acid medium had of itself completely inactivated the alkaline phosphatase, the experiment was repeated for shorter

periods of time. In a second test at p_H 4·5 the mixture was heated for only 20 mins.:

No. 1	p_H 8·66	P hydrolysed per ml. mg.	Traces
No. 2	p_H 8·67	,, ,,	Traces
No. 3	p_H 9·06	,, ,,	0·0789
No. 4	p_H 9·05	,, ,,	0·0789

In a third experiment the mixture was heated for 5 mins. at p_H 4·5 and at 37°:

No. 1	p_H 8·83	P hydrolysed per ml. mg.	0·0125
No. 2	p_H 8·80	,, ,,	0·0130
No. 3	p_H 8·84	,, ,,	0·0670
No. 4	p_H 8·84	,, ,,	0·0720

These experiments show that the "alkaline" phosphatases may be inactivated by sodium fluoride when the latter acts for a certain period of time in an acid medium. The results also show that the alkaline phosphatase of bone is much more sensitive to the inactivating action of the acid medium than the alkaline phosphatases of the liver and of the kidney.

SUMMARY.

1. The "acid" phosphatases of extracts of liver and of kidney are reversibly inactivated by sodium oxalate and can be reactivated without loss by precipitating the oxalate ions by means of calcium salts or removing them by dialysis.

2. Inactivation of the "acid" phosphatases of the liver and of the kidney by sodium fluoride is of a different nature from that produced by oxalate. In the latter case, there is probably a formation of an inactive enzyme-oxalate complex which can easily be split up, restoring the unaltered enzyme. The formation of the inactive enzyme-fluoride complex on the other hand is accompanied by a profound alteration of the enzyme, which occurs in a relatively long period of time and may lead to the permanent disappearance of the phosphatase activity. Eliminating the fluorine ions from the system more or less rapidly restores the enzymic activity in a greater or less degree. Permanent inactivation of the "acid" phosphatase by sodium fluoride occurs more rapidly at 37° than at room temperature.

3. If the "alkaline" phosphatases of the extracts of kidney and of liver at 37° are kept at p_H about 4·5 and then brought back to an alkaline p_H, there occurs an inactivation of greater or less degree according to the duration of the treatment in an acid medium.

4. Sodium fluoride also inactivates the "alkaline" phosphatases of extracts of liver and of kidney when it is allowed to act for some time on these extracts brought to p_H about 4·5. This is probably due to the fact that the formation of the inactive fluoride-alkaline phosphatase complex occurs only in an acid medium. By this process the inactivation of the alkaline phosphatase occurs in a much more rapid and complete manner than occurs with a simple acid treatment.

5. The "alkaline" phosphatase of bone is extremely sensitive to the acid reaction of the medium and in this is different from the "alkaline" phosphatase of the liver and of the kidney as the latter is much less sensitive. This provides evidence that the "alkaline" phosphatase of the liver and of the kidney is not identical with that of bone, a subject which will be further discussed in our next paper.

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