CLII. ASCORBIC ACID AND PHOSPHATASE ACTIVITY

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In a recent communication Thannhauser *et al.* [1937] claim to have shown that ascorbic acid is an intense activator of serum phosphatase. We have been unable to confirm their claim.

In preliminary experiments, Thannhauser's published technique was adopted. The results of some typical experiments are shown in Table I. It will be seen that the presence of ascorbic acid produced no appreciable change in the amount of phosphatase hydrolysis.

Table I. *Phosphatase hydrolysis of phosphoric esters in the presence of ascorbic acid*

Enzyme	Units of phosphatase activity		Method
	Without ascorbic acid	With ascorbic acid	
β -Glycerophosphate, veronal buffer pH 8.9, Bodansky [1933] units			
Serum	6.4	8.4	Thannhauser <i>et al.</i> : 1 hr. at 37°, Briggs [1922] P-method—reducing agent quinol
Serum	7.0	7.0	
Serum	10.0	8.0	
Serum	14.0	17.0	
Serum	8.0	12.0	
Plasma	2.0	2.0	
Serum	2.0	2.0	(α -Glycerophosphate)
Serum	6.0	4.0	
Plasma	2.0	2.0	Ammon & Hinsberg [1936] P-method—reducing agent ascorbic acid
Plasma	4.9	4.7	
Plasma	5.0	5.0	P-precipitation method [Delory, 1938]
Serum	6.0	5.0	
Phenylphosphate, sodium carbonate—bicarbonate buffer pH 9.9, King-Armstrong units			
Serum	8.0	8.0	45 min. 37°, King's [1932] P-method—reducing agent aminonaphtholsulphonic acid
Serum + bile	44.0	47.0	
Serum + bile	40.0	38.0	
Serum + faeces	500.0	515.0	
Plasma	6.2	6.7	P-precipitation method [Delory, 1938]
Plasma	20.0	20.0	

The effect of using sodium α-glycerophosphate, sodium phenylphosphate, and sodium *p*-nitrophenylphosphate as substrate was also studied. In the case of sodium α-glycerophosphate, Thannhauser's technique was used, the α-glycerophosphate being substituted for the β-glycerophosphate.

EXPERIMENTAL

In view of Thannhauser's insistence on the necessity for speed to minimize the loss of ascorbic acid through oxidation, great care was taken to keep the time which elapsed between the addition of the several reagents (buffer, substrate etc.) down to a minimum. The ascorbic acid solution was prepared immediately before use.

Ascorbic acid reduces the phosphomolybdic acid formed during the course of the colorimetric phosphate determination to give a blue colour. A given amount of phosphate, in the Briggs [1922] procedure with quinol as the reducing agent, yields more blue colour in the presence of ascorbic acid than is obtained in its absence, the amount of extra colour depending on the amount of ascorbic acid present. As Thannhauser has shown, it is necessary to make a correction for this increase in colour. This procedure is not entirely satisfactory because the amount of ascorbic acid remaining in the tests (after 1 hr. at 37° and pH 8.9) is uncertain. Methods were therefore devised to overcome this difficulty. The phosphate was precipitated away from the remaining ascorbic acid solution in one set of experiments, while in another set, an excess of ascorbic acid was used as reducing agent, according to the colorimetric phosphate method of Ammon & Hinsberg [1936], no other reducing agent being added.

In the precipitation experiments, the hydrolysis was carried out in the usual way, and an amount of each of the trichloroacetic acid filtrates taken for the phosphate estimation according to the method described in the appended note [Delory, 1938]. Results of typical experiments are shown in Table I.

Attempts to use the King & Armstrong [1934] phenylphosphate method for phosphatase determination proved unsuccessful since the ascorbic acid interfered with the phenol reagent. It was therefore necessary to modify the method slightly and to determine the free phosphate produced instead of the phenol. Four tubes were set up as in the following scheme:

	ml. diluted serum or plasma (1 : 10)	ml. water	ml. ascorbic acid solution (5 mg. per ml.)	ml. N/20 NaOH
Tubes 1 and 2	1	1.45	1	0.55
Tubes 3 and 4	1	3.00	—	—

The tubes were placed in the water bath at 37°, and when the contents had attained the temperature of the bath (2 min.), 6 ml. of *M*/200 phenylphosphate in *M*/20 sodium carbonate-bicarbonate buffer (previously warmed at 37°) were added to each tube. The final pH in all the tubes was 9.9 (optimum for phenylphosphate hydrolysis). Immediately after addition of the substrate 2.5 ml. of 20% trichloroacetic acid were added to tubes 1 and 3 which served as controls. Tubes 2 and 4 were kept at 37° for $\frac{3}{4}$ hr. and were then treated with 2.5 ml. of trichloroacetic acid. The acid mixtures were kept at 0° for 30 min. to allow the precipitates to coagulate before filtering. 10 ml. of the trichloroacetic acid filtrates were used for colorimetric phosphate determination [King, 1932]. In this method, as in that of Briggs, the amount of blue colour was affected by the presence of ascorbic acid. The figures had, therefore, to be corrected in terms of the increased colour produced on adding known amounts of ascorbic acid to standard solutions. Experiments were also conducted with the precipitation procedure for liberated phosphate.

The results are expressed as units equal to mg. P liberated by 100 ml. of serum or plasma. The $\frac{3}{4}$ hr. incubation period was chosen because three times as much phenol as phosphorus by weight is liberated in the hydrolysis, and the King-Armstrong unit is defined in terms of the mg. phenol liberated in $\frac{1}{4}$ hr. at pH 9.9 [King *et al.* 1937].

Experiments were carried out to study the rate of oxidation of ascorbic acid under the conditions of the phosphatase hydrolysis. Two tubes were set up consisting of buffer, substrate, serum and ascorbic acid plus an equivalent amount of *N*/20 NaOH. To one of these tubes trichloroacetic acid was added

immediately, while to the other, the trichloroacetic acid was added after 45 min. at 37°. The filtrates were titrated with 2:6-dichlorophenolindophenol. In two typical experiments 5.1 and 5.4 mg. of ascorbic acid were recovered from the two tubes to which trichloroacetic acid was immediately added, and 4.1 and 3.8 mg. from those which were incubated for 45 min.

Experiments with dehydroascorbic acid produced by bubbling oxygen through a solution of ascorbic acid at pH 7, in the presence of a trace of ferrous sulphate [Thannhauser *et al.*] failed to affect the phosphatase activity.

The accurate determination of inorganic phosphate (in these experiments) was only possible by the use of a photometer or a photoelectric colorimeter. The colours produced in the Briggs method by the amounts of phosphate present in tests and controls were altogether too pale to allow of colorimetric comparison with an ordinary Duboscq type of instrument. The control in the Thannhauser procedure has only the amount of phosphorus represented in half the filtrate from 0.1 ml. of blood serum, i.e. about 1–2 μ g. inorganic P, and in a test representing 10 units of phosphatase activity the inorganic phosphate is increased by only 5 μ g. These very small amounts yield such pale colours in the final volume of 25 ml. used by Thannhauser in the Briggs method, that they can only be estimated by photometric measurement of the light absorbed by a considerable thickness (e.g. 3 cm.) of coloured solution. The measurements were made in a Zeiss Pulfrich photometer, a Millikan photoelectric colorimeter, and a Leitz "absolute colorimeter". But it was also desired to make the determinations of hydrolysed phosphate by ordinary colorimetric means. For this purpose the experiments were repeated using such larger amounts of serum or plasma as were capable of giving sufficient inorganic phosphate to be determined accurately in a Duboscq colorimeter. Appropriate buffer mixtures were used to give a final pH of 8.9 for the glycerophosphate substrates, and 9.9 for the phenylphosphate. The enzyme action was stopped and the proteins precipitated by amounts of trichloroacetic acid sufficient to give a final concentration of 4%. The results of several experiments are given in Table II.

Table II. *Effect of ascorbic acid on phosphatase activity*

(5 mg. ascorbic acid for each 0.1 ml. of serum or plasma used.)

Enzyme	Units of phosphatase activity		Phosphate method
	Without ascorbic acid	With ascorbic acid.	
	Bodansky units		
Serum (0.2 ml.)	10.0	9.0	Briggs [1922]
Serum (0.2 ml.)	5.0	5.0	"
Serum (0.2 ml.)	8.0	12.0	"
Plasma (0.5 ml.)	3.6	3.6	"
Plasma (0.4 ml.)	5.0	5.0	Ammon & Hinsberg
Plasma (0.4 ml.)	1.8	1.5	[1936]
Plasma (0.2 ml.)	12.0	13.0	Delory [1938]
	King-Armstrong units		
Serum (0.2 ml.)	4.0	3.0	King [1932]
Plasma (0.2 ml.)	4.3	4.2	"
Plasma (0.2 ml.)	5.0	5.0	Ammon & Hinsberg,
Plasma (0.4 ml.)	4.0	4.0	[1936]
Plasma (0.2 ml.)	10.0	10.0	Delory [1938]

The activity of plasma phosphatase with *p*-nitrophenylphosphate was studied in the presence and absence of ascorbic acid. Free *p*-nitrophenol is

much more highly coloured in alkaline solution than is its phosphoric ester. It was possible to follow the hydrolysis by studying the increase in yellow colour due to the liberation of the free phenol.

Equal amounts of substrate and buffer solution (pH 10) were placed in two similar tubes. 5 mg. of ascorbic acid were titrated with $N/10$ NaOH to phenolphthalein. The volume of $N/10$ NaOH required for neutralization was added to one of the tubes followed by 5 mg. of ascorbic acid. To the other tube was added enough water to produce the same total volume. To both tubes were added 0.1 ml. of phosphatase solution. The development of colour in the two tubes was then followed by taking measurements with the photoelectric colorimeter.

Reference to Table III shows that ascorbic acid had no effect on the phosphatase activity with this substrate.

Table III. *Hydrolysis of p-nitrophenylphosphate*

(5 ml. $M/1000$ substrate in carbonate-bicarbonate buffer.)

		Reading of photoelectric colorimeter (min.)			
		0	15	30	45
Plasma	Control	5	11	17	—
	+ 7 mg. ascorbic	5	11	17	—
Serum	Control	6	10	12	18
	+ 7 mg. ascorbic	6	9	11	18
Purified kidney phosphatase	Control	4	21	30	36
	+ 4 mg. ascorbic	5	21	29	35
"	Control	8	18	23	30
	+ 3 mg. ascorbic	8	17	20	28

In phosphatase determinations on the sera from cases of generalized bone disease and obstructive jaundice Thannhauser *et al.* could find little or no increase in activity in the presence of ascorbic acid. They concluded that the phosphatase in the blood of those cases was already optimally activated, and that the high phosphatase values characteristically encountered in these diseases are due to an increased enzyme activity rather than to an increased amount of enzyme. In the absence of any confirmation of the activation by ascorbic acid of the phosphatase in sera of normal persons, no support can be given to this contention.

SUMMARY

The influence of ascorbic acid on the enzymic hydrolysis of the phosphoric esters commonly used for phosphatase determinations has been studied. No appreciable effect on the activity of serum phosphatase with these substrates can be demonstrated. It is concluded that ascorbic acid does not activate phosphatase. No support is, therefore, found for the contention of Thannhauser *et al.* [1937] that the high serum phosphatase values encountered in certain diseases are due to the presence of an activator in the serum rather than to an accumulation of the enzyme itself.

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A NOTE ON THE DETERMINATION OF PHOSPHATE IN THE PRESENCE OF INTERFERING SUBSTANCES

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THE colorimetric determination of phosphate is often difficult or impossible in the presence of interfering substances. For this reason it has been found convenient to precipitate the phosphate away from the interfering substance and to determine it colorimetrically in the redissolved precipitate. Molybdic acid and an organic base are frequently used as precipitants. But in the presence of reducing substances such as ascorbic acid, this precipitation may be impossible, since these substances reduce the phosphomolybdate.

It has been found possible to precipitate completely the inorganic phosphate as $\text{Ca}_3(\text{PO}_4)_2$ in amounts as small as 0.01 mg. P by entrainment with light magnesium carbonate powder (MgCO_3). The centrifuged precipitate is washed, dissolved in perchloric acid, and the colorimetric determination carried out in the usual way.

EXPERIMENTAL

Measured amounts of phosphate solution are introduced into centrifuge tubes graduated at 15 ml. The contents of the tubes are neutralized (where necessary) to phenolphthalein by the dropwise addition of concentrated ammonia solution. An extra 0.2 ml. of the same solution is then added. After the addition of 1 ml. of 2.5% calcium chloride solution and 1 ml. of a 0.5% suspension of "light" magnesium carbonate in water, the tubes are shaken and allowed to stand for 30 min. They are shaken again and then centrifuged. The supernatant fluids are carefully poured off and discarded. The tubes are allowed to drain and the precipitates washed with 5 ml. of 2% ammonia solution (saturated with calcium phosphate). The tubes are centrifuged and drained as before. The precipitates are dissolved in 1.1 ml. of 60% perchloric acid. About 10 ml. of water are added, followed by 1 ml. of 5% ammonium molybdate solution and 0.5 ml. of 1:2:4-aminonaphtholsulphonic acid solution. Water is added to bring the total volume to 15 ml. After standing for 10 min., the blue solutions are read in the colorimeter against standard solutions prepared from suitable amounts of a dilute standard phosphate solution together with 1 ml. of 60% perchloric acid, etc., in 15 ml. volumetric flasks [King, 1932].

The table shows recovery of phosphorus from (1) simple solution; (2) 2.5 % trichloroacetic acid; (3) solutions containing 5 mg. ascorbic acid; (4) 2.5 % trichloroacetic acid solutions containing 5 mg. ascorbic acid; and (5) solutions containing 5 mg. ascorbic acid, trichloroacetic acid and 1 ml. of 5 % sodium β -glycerophosphate.

If the amount of phosphate to be recovered is very small, it may be desirable to concentrate the colour produced by making the solution of the precipitate up to a smaller final volume. For this purpose the precipitate is dissolved in 0.25 ml. perchloric acid followed by 2.5 ml. water, 0.2 ml. ammonium molybdate and 0.1 ml. aminonaphtholsulphonic acid, making a total volume of 3 ml.

Table I. *Recovery of inorganic phosphate by precipitation with calcium*

(Figures represent mg. P.)

(1) Phosphate precipitated from simple solution					
P present	0.025	0.050	0.075	0.100	
P recovered	0.025	0.048	0.080	0.100	
(2) Phosphate precipitated from trichloroacetic acid solution					
P present	0.010	0.020	0.030	0.040	
P recovered	0.010	0.018	0.026	0.037	
(3) Phosphate precipitated from ascorbic acid solution					
P present	0.010	0.050	0.070	0.100	
P recovered	0.010	0.048	0.067	0.096	
(4) Phosphate precipitated from trichloroacetic and ascorbic acid solution					
P present	0.020	0.050	0.070	0.100	
P recovered	0.018	0.050	0.071	0.100	
(5) Phosphate precipitated from solution containing β -glycerophosphate trichloroacetic acid and ascorbic acid					
P present	0.020	0.050	0.070	0.100	
P recovered	0.020	0.049	0.070	0.099	

REFERENCE

King (1932). *Biochem. J.* 26, 292.