CLIII. THE INFLUENCE OF VITAMIN C (ASCORBIC ACID) ON PLANT AND ANIMAL AMYLASES.

By ARNULF PURR.

From the Cancer Research Laboratories, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.

(*Received April 4th, 1934.*)

In a recent publication from this laboratory Purr [1933] reported on the effect of ascorbic acid on the action of the proteolytic enzymes. The present paper deals with the influence of ascorbic acid on plant and animal amylases which are important in cancer research on account of their relation to carbohydrate metabolism.

Kuhn [1925], in a fundamental work, studied the mutarotation of the products formed in the first stages of starch hydrolysis by amylases. He found that one group of amylases yields primarily α-maltose, which mutarotates downward (α-amylase), while another group yields β-maltose, which mutarotates upward (β-amylase). The amylases in pancreas and saliva belong to the α- type, while those of sprouted and unsprouted grains are of the β- type. These findings were later confirmed by Reichel [1932]. By studying the relation between the iodine reaction and the mutarotation of the starch decomposition products, it was found that the two types of amylase differ in their mode of action on the starch complex from the first point of contact. Thus, α-amylases are believed to break down first those groups in the starch complex which are responsible for the blue colour with iodine. Characteristically, the blue colour disappears with very low saccharification. On the other hand, β-amylases break down the starch in such a way that saccharification is almost complete while the blue colour remains unaltered. Through the discovery of this relation between saccharification and coloration with iodine, a simple method became available for distinguishing the amylases found in nature. It is merely necessary to allow the hydrolysis to proceed until a very definite colour tone, preferably the change from ultramarine blue to blue-violet, is reached and to determine the saccharification at that point. This is known as the transition point, and it expresses the amount of saccharification (in percentage maltose formed) at the point of colour change.

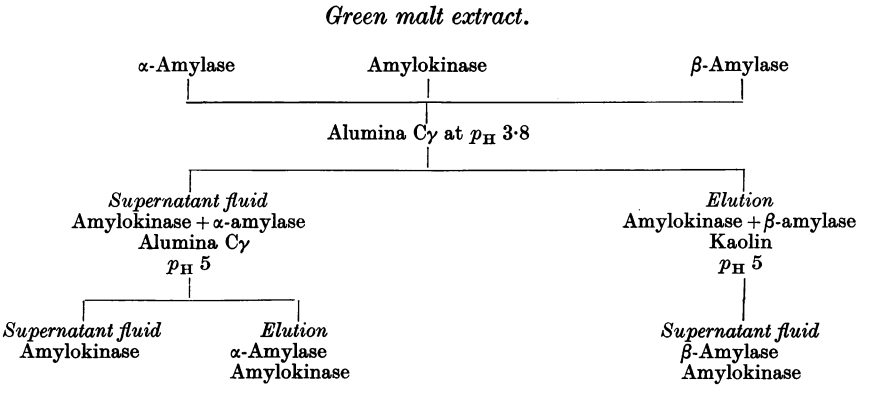
The transition points for several different types of amylase are as follows:

Transition point (percentage maltose formed).

Plant amylases		Animal amylases		
Barley	Green malt	Pancreas	Liver	Saliva
102 %	84 %	20 %	15 %	11 %
β-Amylase		α-Amylase		
Mutarotation upward		Mutarotation downward		
High saccharification		Low saccharification		
Iodine colour, blue-violet		Iodine colour, blue-violet		

By means of adsorption methods, Waldschmidt-Leitz, Reichel and Purr [1932] were able to isolate two amylases, α and β, from barley and green malt. While both amylases extracted from green malt were fully active, in barley the α-form was totally inactive and the β-form partially active. The difference between the barley enzyme system and the malt system lies in the formation of an activator of organic nature, "amylokinase," which arises during the process of sprouting.

The following scheme represents the process of separating the amylases of green malt.



This method, when applied to animal amylase systems, was unfortunately not so successful. However, the transition point of pancreas amylase indicates the presence of an animal β-type. As a result of the discovery, reported in this paper, of the specific activating effect of vitamin C on animal β-amylase, it has become possible to demonstrate the existence in pancreas of a β-type of amylase.

Vitamin C and animal amylases.

The effect of vitamin C on the activity of various animal amylases was investigated, using as source of enzyme the following: human saliva (0·2 ml. with 0·015 amylase units), acetone-ether-dried pig pancreas (0·1 ml. of a 1 : 10 aqueous extract with 0·035 amylase units), and rat-liver (1·5 ml. of a 1 : 5 aqueous extract with 0·0014 amylase units). To separate portions of the designated quantities of each of these were added (*a*) 8 mg. of ascorbic acid in 1 ml. of H_2O, (*b*) 8 mg. oxidised ascorbic acid in 1 ml. of H_2O, or (*c*) 1 ml. of H_2O. These mixtures were allowed to stand at 0° and p_H 7 for 5 minutes, after which 50 ml. of the stock solution were added. This contained 250 mg. of soluble starch (Lintner), 10 ml. of $M/15$ phosphate buffer of p_H 6·8 (or 10 ml. of $M/10$ citrate buffer of p_H 5·1), and 2·0 ml. of $M/5$ NaCl solution, in a total volume of 50 ml. The mixtures were incubated at 37° for the indicated periods of time, after which the enzyme action was stopped by addition of 2 ml. of N HCl. The amount of saccharification was determined by the method of Willstätter and Schudel [1918], the results being expressed in percentage maltose formed. The oxidised ascorbic acid was prepared by bubbling oxygen for 4 hours through a solution of 8 mg. of ascorbic acid in 1 ml. of H_2O at p_H 7. A trace of $FeSO_4,7H_2O$ was added to hasten the oxidation, the completion of which was determined by iodine titration.

Table I. *Ascorbic acid and animal amylases. Effect of* p_H.

Additions	p_H	Saliva Time mins.	Saliva Maltose %	Pancreas Time mins.	Pancreas Maltose %	Liver Time mins.	Liver Maltose %
Water	6·8	10	22·6	8	37·04	60	13·72
Ascorbic acid	6·8	10	21·95	8	48·00	60	24·00
Oxidised ascorbic acid	6·8	10	22·00	8	46·64	—	—
Water	5·1	20	15·5	25	20·58	—	—
Ascorbic acid	5·1	20	15·9	25	19·20	—	—

From the figures obtained it can be seen that of the animal amylases tested, only the salivary amylase remained inactivated at both p_H 6·8 and 5·1. Pancreas and liver amylases, on the other hand, undergo considerable activation, but only at the optimum p_H for the enzyme reaction (6·8). In this respect the activation differs from that obtained with calcium salts, which occurs only at p_H 5·1, but not at 6·8.

The salivary amylase, as has been mentioned, has the lowest transition point found as yet, and therefore represents the purest α-type. The higher transition points of pancreas and liver amylases indicate the presence of mixtures of both α- and β-types. It was of interest to determine, therefore, whether these facts could be correlated with the non-activation of salivary amylase and the activation of pancreas and liver amylases by ascorbic acid. The investigations carried out in this direction, as shown in Table II, indicate that the activations obtained with ascorbic acid are related to a specific activation of the animal β-amylase.

Table II. *The effect of ascorbic acid on the transition point of pancreatic amylase.*

	Without ascorbic acid			With ascorbic acid			
	Saccharification				Saccharification		
Time mins.	Maltose mg.	Maltose %	Iodine colour	Iodine colour	Maltose mg.	Maltose %	
0	0·0	0·0	Ultramarine-blue	Ultramarine-blue	0·0	0·0	
4	20·0	8·0	,,	,,	37·5	15·0	
Transition point 10	45·0	18·0	Blue-violet	Blue-violet	70·0	28·0	Transition point
15	63·8	25·5	Violet	Violet	86·3	34·5	
20	80·0	32·0	Violet-red	Violet-red	102·5	41·0	
25	95·0	38·0	Red-violet	Red-violet	120·0	48·0	
30	112·5	45·0	Red-brown	Red-brown	136·3	54·5	

A large volume of a reaction mixture was prepared, containing 250 mg. of soluble starch, 10 ml. of $M/15$ phosphate buffer of p_H 6·8, 2 ml. of $M/5$ NaCl solution, and 1 ml. of a 1 : 10 aqueous extract of pancreas containing 0·015 amylase units (or the same amount of enzyme previously activated by 8 mg. of ascorbic acid), in a total volume of 50 ml. The mixture was incubated at 37°. The course of the reaction towards the transition point was followed by the method to be described later. At definite intervals, 50 ml. portions of the reaction mixture were removed and treated with 2 ml. of N HCl, and the amount of maltose formed was determined.

As shown in Table II, the activating effect of ascorbic acid is linked with an increase in the transition point. This indicates that ascorbic acid has a specific

activating effect on the β-amylase, and that the latter is present in pancreas and liver in an almost inactive state naturally.

Waldschmidt-Leitz and Purr [1932] were able to report an activating effect on animal amylase by calcium salts. As previously mentioned, this form of activation is sharply differentiated from that obtained with ascorbic acid in the conditions of p_H necessary. The same conclusion is reached by a study of the transition point, as shown in Table III.

Table III. *Activation of pancreatic amylase by $CaCl_2$ at p_H 5·1. Transition point.*

	Without $CaCl_2$				With $CaCl_2$			
		Saccharification				Saccharification		
	Time mins.	Maltose mg.	Maltose %	Iodine colour	Iodine colour	Maltose mg.	Maltose %	
	0	0·0	0·0	Ultramarine-blue	Ultramarine-blue	0·0	0·0	
	10	6·3	2·50	,,	,,	30·00	12·00	
	15	18·75	7·50	,,	Blue-violet	47·00	18·80	Transition point
	20	30·0	12·0	,,	Violet	62·50	25·00	
Transition point	25	47·5	19·0	Blue-violet	Violet-red	82·5	33·00	
	35	65·0	26·0	Violet	Red-violet	100·0	40·0	

Additions and procedure were the same as those for Table II, except that 10 ml. of $M/10$ citrate buffer of p_H 5·1 were used instead of the phosphate and 12 mg. of $CaCl_2$ instead of the ascorbic acid.

Calcium salts do not change the transition point, but only hasten its appearance, while ascorbic acid increases it. It is evident from these results that the effect of calcium salts is fundamentally different from that of ascorbic acid.

Procedure for the determination of the transition point.

Each of a series of test-tubes contained 2 ml. of distilled water and 3 drops of $N/100$ iodine solution. At definite time intervals, 1 ml. of the enzyme reaction mixture was added, until the first definite blue-violet colour could be recognised. Immediately, 50 ml. of the hydrolysing mixture were removed, 2 ml. of N HCl were added to stop the enzyme reaction, and the sugar formed was estimated by the Willstätter-Schudel method.

It should be mentioned that the method can be used for quantitative comparisons only when no substances are present which take up iodine in neutral or acid solution. When these conditions exist, the method is in all cases inapplicable; for the presence of large quantities of such reducing substances (cell residue, tissue suspensions, thiosulphate, *etc.*), leads to the formation of hydrogen iodide or its salts. The latter compounds, as is known, weaken the colour of the starch-iodine complex, and therefore interfere with the colour comparison. These substances affect the starch-iodine colour in a manner similar to that of amylase itself and may easily lead to erroneous conclusions.

In order to eliminate this source of error when using ascorbic acid, which takes up iodine very readily, it is necessary, in the determination of the transition point, to compensate for its presence. The method of procedure was the same as previously described, except that to those test-tubes in which the

transition point of pancreatic amylase alone was to be determined, 0·16 mg. of ascorbic acid was previously added. This amount corresponds to that present in 1 ml. of the enzyme mixture which is being activated by ascorbic acid. In each case, then, equivalent amounts of ascorbic acid were present, so that the error due to formation of hydrogen iodide was eliminated. Because of the consumption of iodine which occurs in the presence of ascorbic acid, it was necessary to add 10 instead of 3 drops of $N/100$ iodine to each test-tube, in order to have the necessary excess for the determination and for stopping the enzyme reaction.

The next series of experiments (Table IV) shows the effect of several amino-acids and of insulin on the activity of pancreatic amylase at p_H 6·8 and 5·1. The procedure was exactly the same as that described in Table I, except for the substitution of the indicated amounts of amino-acids and insulin for the ascorbic acid.

Table IV. *The effect of amino-acids on the activity of pancreatic amylase.*

	p_H 6·8			p_H 5·1		
Additions	Time mins.	Saccharification Maltose %	Activation Maltose %	Time mins.	Saccharification Maltose %	Activation Maltose %
None	7	14·7	0	20	12·00	0
Cysteine (20 mg.)	7	14·9	0	20	18·00	6·0
Cystine (20 mg.)	7	14·7	0	20	21·50	9·50
Alanine (20 mg.)	7	14·7	0	20	12·00	0
Histidine monohydrochloride (20 mg.)	7	14·8	0	20	23·00	11·0
Proline (20 mg.)	7	14·5	0	20	12·00	0
Insulin—132 units (9·5 mg.)	7	14·8	0	20	22·00	10·00

It is clear from this table that the p_H of the amino-acid activation agrees with that observed in the case of calcium activation. The agreement also can be seen in a study of the transition points (these figures are not presented in this paper). This activation therefore also differs sharply from that obtained with ascorbic acid.

The extent to which the activating influence of ascorbic acid is exerted on the complicated mechanism of glycolysis will be reported in a later communication.

Vitamin C and plant amylases.

The significance of ascorbic acid as an enzyme regulator is not limited to the amylases of animal origin, but as the following experiments show, it must also be credited with a similar function with regard to plant amylolytic processes.

The plant amylases investigated were the following: barley (0·5 ml. of a 1 : 5 aqueous extract, with 0·030 amylase unit), green malt (0·25 ml. of a 1 : 5 aqueous extract with 0·030 amylase unit), barley amylase purified by alumina Cγ, β-form (1·0 ml. with 0·015 amylase unit), and the green malt amylase purified by alumina Cγ, α-form (3·0 ml. with 0·015 amylase unit). To the designated amounts of each of these were added (*a*) 8 mg. of ascorbic acid in 1 ml. of H_2O, (*b*) 8 mg. of oxidised ascorbic acid in 1 ml. of H_2O or (*c*) 1 ml. of H_2O. The mixtures were allowed to stand 10 minutes at 37°, after which 50 ml. of the stock solution of p_H 5·1 previously described were added. The incubation and sugar determinations were carried out exactly as previously described. The purified β-amylase from barley was prepared by treating 10 ml. of 1 : 5 barley extract containing 1·5 amylase units with alumina Cγ (8 ml. = 60 mg. Al_2O_3) at

p_H 3·8 (2 ml. of *M* acetate buffer). The adsorbate was eluted with 10 ml. of *M*/15 secondary phosphate and brought to p_H 7 by the addition of *N*/10 HCl. The purified α-amylase was prepared by adsorbing 10 ml. of 1 : 5 green malt extract eight times with alumina Cγ (145 mg. × 8 of Al_2O_3) at p_H 3·8 (2 ml. of *M* acetate buffer). Compare the schema, p. 1142.

Table V. *Effect of ascorbic acid on plant amylases.*

	No additions		Ascorbic acid		Oxidised ascorbic acid	
Enzyme	Time mins.	Maltose %	Time mins.	Maltose %	Time mins.	Maltose %
Barley (unpurified)	9	35·68	7	12·34	7	36·36
Malt (unpurified)	10	37·04	10	19·90	10	28·81
β-Amylase (purified from barley)	20	37·70	20	10·37	10	36·36
α-Amylase (purified from malt)	9	19·90	10	16·46	10	11·90

While the animal amylases are either activated or else not affected at all by ascorbic acid, the plant amylases thus far investigated are inhibited. This inhibition is dependent not only on whether reduced or oxidised ascorbic acid is used, but also on the type of amylase, α or β. As shown in Table V, the β-type of plant amylase is very strongly inhibited by reduced ascorbic acid, but is unaffected by the oxidised form. This behaviour is shown both by the purified enzyme, and by the crude green malt extract which contains amylokinase. The opposite picture appears in the case of plant α-amylase, whose activity, as has been previously mentioned, is dependent on the presence of amylokinase. The activity of this form is inhibited only by the oxidised form of ascorbic acid, while it is scarcely affected by the reduced form. The slight inhibition which may be observed can be explained by the presence of a small amount of β-amylase which remains after the purification.

These findings extend the knowledge of the essential differences between the plant and animal amylases which are summarised in Table VI.

Table VI. *Comparative behaviour of plant and animal amylases toward various reagents.*

+ = Activation; − = Inhibition; 0 = Indifferent.

		Plant amylases		Animal amylases	
Reagent	p_H	β	α	β	α
Amylokinase	5·1	+	+	0	0
,,	6·8	+	+	0	0
Vitamin C	5·1	−	0	0	0
,,	6·8	−	0	+	0
Oxidised vitamin C	5·1	0	−	0	0
,,	6·8	0	−	+	0
NaCl	5·1	0	0	0	+
,,	6·8	0	0	0	+
$CaCl_2$	5·1	0	0	0	+
,,	6·8	0	0	0	0
p_H optimum		5·1	5·1	6·8	6·8

Table VII shows the effect of oxygen on the plant amylase complex from barley in the presence and absence of ascorbic acid. Oxygen was bubbled through a barley amylase extract for 4 hours in the presence and absence of ascorbic

acid. A trace of $FeSO_4$ was added as an oxidation catalyst. The determinations of amylase activity after the oxygenation were carried out exactly as previously described. The results obtained indicate that ascorbic acid may also exert an effect in protecting the amylase from oxidation.

Table VII. *Effect of oxygen on barley amylase in the presence and absence of ascorbic acid.*

	Barley amylase without vitamin Maltose (%)	Barley amylase with 8 mg. vitamin Maltose (%)
4 hour treatment with oxygen	12·3	20·6

Recently Virtanen *et al.* [1933] found a relation between growth of plants and vitamin C content. In view of the connection between vitamin C and the amylases reported in the present paper, it is of interest to present the results of an investigation[1] of the changes occurring in the activities of the several kinds of amylases during the ripening of various grains.

The crushed grains at different stages of ripening were extracted for 24 hours with 5 parts of water, toluene being added as antiseptic, and then filtered by suction. The reaction mixtures consisted of 140 mg. of amylo-amylase (prepared according to Samec), 10 ml. of citrate buffer of p_H 5·1, and aliquot parts of the grain extracts containing 0·01 amylase unit, in a total volume of 50 ml. The mixtures were incubated at 37°, and the course of the reaction towards the transition point was followed as previously described. The mutarotation was determined by the method of Kuhn [1925]. A 2 dm. polarimeter-tube was filled with the reaction mixture, and the rotatory power of the solution was determined at intervals, first without carbonate, and again after ½ hour standing with 0·5 g. of anhydrous sodium carbonate. The difference between the two determinations expresses the mutarotation, the magnitude of which is dependent on the experimental procedure. The decisive point is the direction of the mutarotation (− for α-; + for β-amylase).

Table VIII. *Relation between amylase activity and the ripening process of various grains.*

Grain	State of grain content	Transition point Maltose %	Mutarotation at transition point	Remarks
Barley	Milky fluid	77·5	+0·07°	For comparison, green malt has a mutarotation of +0·15 at the transition point (84 % maltose)
	Soft mass	67·3	+0·06	
	Mealy mass	82·1	+0·08	
	Ripe grain	96·1	+0·10	
Rye	Milky fluid	88·2	+0·13	
	Soft mass	68·0	+0·08	
	Mealy mass	76·0	+0·09	
	Ripe grain	83·3	+0·05	
Oats	Milky fluid	80·85	+0·15	
	Soft mass	60·7	+0·08	
	Mealy mass	75·0	+0·06	
	Ripe grain	90·0	+0·10	

On the basis of these results, it can be seen that in the first stages of the development of grains, the relationships between the different amylase types

[1] This investigation was carried out at the Biochemical Institute of the Deutsche Technische Hochschule, Prague. The various kinds of grains used in each stage of the ripening process were obtained from J. Purr, Grabschuetz, Czechoslovakia.

(α or β) are similar to those existing in sprouting grains such as green malt. The nearer the grain approaches to ripening, the higher is the transition point, until it finally reaches the value for fully ripened, dormant grain. The increase in the transition point indicates that the α-amylase is gradually becoming inactive, and from this it may be concluded that the α-enzyme in growing (including sprouting) plant cells is involved as an energy-supplying factor in the enzymic intermediate processes. It is probable that the decrease in vitamin C during the ripening of grains, as found by Virtanen *et al.* [1933], is related to the inactivation of the α-amylase. This working hypothesis is supported by the finding that oxidised vitamin C inhibits plant α-amylase. Whether amylokinase arises during the ripening process, and its relation to vitamin C, must be determined by further experiment.

Summary.

1. It has been shown that vitamin C (ascorbic acid) is a specific activator for the β-type of animal amylase. β-Amylase occurs in animal organs in a practically inactive state.

2. Vitamin C exerts an inhibiting effect on the β-type of plant amylase but has no effect on the α-type. The oxidised form of ascorbic acid has no effect on the β-form but inhibits α-amylase of plants.

3. A study of the relation between amylase activity and the growth of grains leads to the conclusion that in the first stages of growth the same amylase relationships exist as in the sprouting grain. The fact that the α-components become inactive at the end of the growth period is in agreement with the findings of Virtanen on the vitamin C changes which occur, and with the fact that the oxidised form of the latter inhibits α-plant amylase.

The author wishes to acknowledge the encouragement and support received from Dr Ellice McDonald, Director, during the course of this work.

REFERENCES.

Kuhn (1925). *Liebig's Ann.* **443**, 1.
Purr (1933). *Biochem. J.* **27**, 1703.
Reichel (1932). Dissertation. Deutsche technische Hochschule, Prag.
Virtanen, Hausen and Saastamoinen (1933). *Biochem. Z.* **267**, 179.
Waldschmidt-Leitz and Purr (1932). *Z. physiol. Chem.* **213**, 63.
—— Reichel and Purr (1932). *Naturwiss.* **20**, 254.
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.