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A Study of Enzymatic Relationships
in some Parasitic Organisms.

by

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ABSTRACT

I. A STUDY OF THE ENZYMATIC RELATIONSHIPS IN SOME PARASITIC ORGANISMS

A comparison was made of two organisms, Botrytis cinerea and Bacillus carotovorus, both of which cause a soft rot in the tissue which they parasitise. Enzymic extracts capable of producing a soft rot were prepared from both organisms. The physiological behaviour of the extracts was examined and also their enzymic constitution. It was found that soft rotting enzymes of considerably different constitution had the same effect of causing a soft rot of a piece of potato tissue.

II. OBSERVATIONS ON THE OCCURRENCE AND SPREAD OF THE RING SPOT OR ANTHRACNOSE DISEASE OF LETTUCE CAUSED BY MARSSONINA PANATTONIANA

Outbreaks of the disease were first observed in the field on a number of market gardens. Information was collected concerning the variety of lettuce attacked, the source of the seed, conditions favouring attack, the extent of previous attacks and whether lettuce or diseased lettuce had been grown on the same ground before. Seed was raised from clean and infected plants and sown in succeeding seasons. It was found that seed from infected plants produced infected seedlings while the clean seed produced clean seedlings. Experiments were run to see if the disease carried over in the soil; the indications from these were that infection lasted a short time in the soil but that after longer periods it was not present.

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I. INTRODUCTION.

Earlier work on the physiology of parasitism has indicated that, where rotting is a feature of invasion, the problem is bound up with the production and activity of an enzyme which brings about the dissolution of the host plant tissues. Enzymic extracts producing rotting were seen to act primarily by dissolving certain constituents of cell walls, more particularly the middle lamellae. The enzyme concerned was referred to as cytase by Brown (1), and as pectinase by Harter and Weimer (7), Fernando (4) and others. Willaman (11) and Davison and Willaman (3) drew attention to three different enzymes of this type, viz., protopectinase which dissolves middle lamella substance, i.e., turns insoluble pectin into soluble pectin and so causes a loss of coherence of plant tissue ; pectase, which partially hydrolyses pectin to pectic acid, and pectinase, which completely hydrolyses pectin or pectic acid to galactose, etc. These three enzymes were found to have characteristic pH optima and points of heat inactivation. The enzyme called cytase or pectinase by the earlier workers was considered by Davison and Willaman to be identical with their protopectinase.

Tissue rotting enzymes show marked sensitivity to the acidity or alkalinity of the medium. Fernando (4) found that two typical rotting parasites, Botrytis cinerea and Bacillus carotovorus gave enzymes which had a similar effect in disintegrating tissue but which had markedly different pH optima. The fungal and bacterial enzymes were active in acid

and alkaline media respectively. The test used for the rotting activity was the same as for the protopectinase of Willaman and Davison but, whereas these authors considered the pH optimum to be a constant feature of protopectinase and one of the criteria for distinguishing it from the other enzymes, it is clear that protopectinase preparations from different sources may vary with respect to their pH optima.

Interesting points have arisen from the comparison of a number of parasites with weakly or non-parasitic relatives. Under identical conditions the non-parasites may produce more active enzyme than the parasites, as was shown by Harter and Weimer (7) for various species of *Rhizopus*. Again an organism like *Bacterium malvacearum*, which is incapable of parasitising potato, can be grown successfully on potato extract with the continuous production of an enzyme capable of rotting potato tissue. (9).

Some plant juices when expressed and added to enzyme preparations have a retarding effect on activity, an effect which is not attributable to alteration in the pH value. As recorded by Menon (8) and Chona (2) this effect is in some cases made less if there has been previous treatment of the enzyme with the juice, e.g. by incorporating the latter in the medium on which the parasite is cultured. Most of the enzymes and the parasites examined attacked potato tissue but potato juice was found to have a distinctly retarding action on many rotting enzymes.

Fernando found that if an enzyme with an optimum on the alkaline side were stored at a more acid pH, the optimum pH for activity moved towards the acid side. This change is comparable to the effect of the

juice added to the medium outlined above, where the enzyme becomes in some way accommodated to conditions which would ordinarily be unfavourable. The successful parasite must not only produce active enzyme but its growth must result in conditions favourable for the continued activity of the enzyme.

It was found by Hall (6) and confirmed by Fernando (5) that when spores or mycelium of B.cinerea were placed in a moist atmosphere on the cut surface of potato tuber tissue the inoculum dried up and gave no attack, but if a film of water was maintained round the inoculum a rotting resulted from the growth of the fungus. A purified enzyme preparation from B.cinerea behaved in the same way. When a small piece of potato tissue was immersed in the enzyme a rapid rotting followed but if a few drops of such an active enzyme were placed on the surface of a cut potato they were rapidly absorbed and the tuber remained uninjured. On the other hand an inoculum of B.carotovorus similarly placed attacked the tissue and a purified enzyme preparation from this parasite caused a rotting equally well whether the potato tissue were immersed in the enzyme or whether a few drops were placed on a block.

B.cinerea was found to be capable of growing as a parasite on potato tuber and producing active rotting enzyme only under conditions of high water content. B.carotovorus, although it was no more active as a parasite and although the enzyme it produced could act no more rapidly than that of B.cinerea under wet conditions, was yet capable of causing a rot under conditions where B.cinerea failed to act. The B.cinerea and the

B.carotovorus enzymes behaved in a closely similar manner under one set of conditions but differently under other conditions. The purpose of this paper was to institute a closer comparison between the rotting enzymes of these two organisms.

II. MATERIAL AND METHODS.

^{Two}~~Five~~ strains of Bacillus carotovorus, Jones, were used, one isolated from tomato and one, an ~~Acroideae~~ strain from hyacinth, both supplied by the Bacteriology Dept. of the Imperial College of Science and Technology. The culture of Botrytis cinerea, Pers., first used was from the collection of the Plant Pathology Dept., but this was later replaced by two new strains isolated one from lettuce and the other from Zinnia. All fungal cultures were kept on malt agar slopes. Quantities of B.cinerea spores were prepared from plates of malt agar which was inoculated before pouring with a spore suspension. Bacterial cultures were kept on potato mush agar slopes. When required for inoculation purposes these were used when one day old, suspensions being prepared by shaking up about 5 cc. of sterile water in the culture tube.

The various vegetable extracts were prepared from fresh tissue cut into small chips, boiled till soft and squeezed through muslin. The standard amount used was 200 grms. to 1 litre of water. For bacterial cultures the nutrient extract was autoclaved in 20 cc. lots in medicine

bottles which were seeded with 0.5 cc. each of bacterial suspension of an approximately standard turbidity. The bottles were shaken and then laid flat in a 25°C. incubator for 8-10 days. For enzymic tests the culture solution was either used as such, ^{with toluene} i.e. as crude external enzyme, or it was partially purified by precipitation with four times its bulk of 95% alcohol. The precipitate was centrifuged or allowed to settle, drained and dried in shallow dishes in a current of air until the alcohol was evaporated. It was then finally dried in a desiccator over calcium chloride.

Fungal enzyme was prepared after the manner of Brown (l.c.). The dry products from both the fungus and the bacterium were ground with an equal weight of silver sand and the resulting powder stored dry until required. A weighed amount of this, usually 0.5 grms. to 50 cc., was extracted in distilled water, the debris centrifuged off and the resulting solution used as 'purified' enzyme.

Vegetable juices were prepared by mincing fresh tissues finely, squeezing through a muslin bag and clarifying by centrifuging.

The rotting activity was measured in two ways. Firstly by the time, called the Reaction Time (R.T.) taken by the preparation to cause a complete loss of coherence of discs of potato, turnip, etc., 1.8 cm. in diameter, and .5 mm. thick for potato and .25 mm. thick for other tissues. The discs which for any one experiment were all cut from the medulla of one potato tuber were immersed in the enzyme and tested from time to time by pulling gently with forceps. With any one disc of tissue an R.T. less

than $\frac{1}{2}$ hr. was accurate to approximately one minute ; above $\frac{1}{2}$ hr. the possible error increased. On the other hand the variation between discs from different tubers was considerable, so that only general comparisons were possible between readings obtained with different tissue. By the second method, the activity was measured by the amount of rotting caused by a known small quantity of enzyme, usually 0.4 cc., placed on the surface of a relatively large piece of potato tissue. This method was thought to approximate more closely to the natural conditions of attack. Blocks for this purpose were cut from tubers with a No.14 cork borer, the cylinders so prepared being divided into slices approximately .8 cm. thick. King Edward potatoes were used throughout and the blocks were selected at random into lots of ten except where otherwise stated. These were put in petri dishes with the film of enzyme solution on top of them for 16-18 hours. Any rotted tissue was then scraped off with a horn spatula and weighed.

Other enzymes were tested for as follow :-

Catalase : Twelve-inch lengths of glass tubing of 5 mm. bore were sealed at one end with paraffin wax. This could be broken off easily and the tubes washed for use again. Into the top of each 0.2 cc. or 0.5 cc. of enzyme was run and the tube filled with "ten-volume" hydrogen peroxide and quickly inverted over a dish of the same liquid. The action of the catalase was to liberate oxygen gas from the peroxide and the length of the column of oxygen gave an approximate measure of catalase activity.

Diastrase : To 5 cc. lots of 1% Lintner soluble starch was added 4 cc, 3 cc, 2 cc, 1 cc, 0.5 cc. and 0.25 cc. of enzymic solution and the tubes, with a drop of toluene added to each, were incubated at 38°C. for 12 hours.

They were tested with iodine to show any remaining starch. The amount of enzyme which was just sufficient to digest all the starch gave an inverse measure of the diastatic activity.

Oxidase : 1. To 3 cc. of enzymic solution were added 1 cc. of 1% α -naphthol in 50% alcohol and 1 cc. of 1% aqueous p-phenylene-diamine hydrochloride. The time required for a blue colour to appear was taken as an approximate inverse measure of oxidase activity.

2. To 3 cc. of enzymic solution was added 1 cc. of a 10% aqueous solution of a 10% tincture of gum guaiacum : the time taken for a blue colour to appear gave a second rough inverse measure of oxidase activity.

Peroxidase : The presence of this enzyme was shown by a positive oxidase reaction in the presence of hydrogen peroxide.

Reductase : To 3 cc. of enzymic solution was added 0.5 cc. of a 0.001% aqueous solution of methylene blue and the time for decolouration taken as an approximate inverse measurement of reductase activity. In some cases where a negative result was obtained by this method the test was repeated in vacuo in the presence of a reducing sugar.

Standard colour indicators and Clark and Lubs' buffer solutions were used for pH determinations. In one or two cases where the solution to be tested was coloured, determinations were made with a quinhydrone electrode.

III. EXPERIMENTAL RESULTS.

Fernando found that Bot. cinerea and Bac. carotovorus enzymes which had the same R.T. behaved in a very different way on the cut surface of a potato tuber where the Botrytis enzyme was absorbed and disappeared while the Bac. carotovorus preparation caused a considerable amount of rotting. He at first thought that there might be some difference in the osmotic activities of the two enzymes but experiments which he carried out did not indicate this. The following section of this paper gives an account of a repetition of Fernando's experiments carried out on a larger scale.

1. Preliminary.

Fernando used Bot. cinerea purified enzyme which he found usually dried up on potato tissue but in the experiment illustrated below a crude, fungal, enzymic extract which would attack fresh potato tissue was used. The enzyme preparations of Bot. cinerea and Bac. carotovorus were adjusted to contain the same impurities and their activities then compared. The results are presented in Table I.

These results confirm Fernando's general conclusion that there is a difference between the enzymes which is not overcome by adjusting both so that they contain the same impurities. The addition of boiled Bac. carotovorus enzyme to active enzyme of Bot. cinerea significantly* increased

* Any difference for which the value of P calculated according to Fisher was .05 or less was considered to be significant.

Table I.

1.	<u>Bot.cinerea</u>	crude enzyme + equal vol. water					R.T. 20 min.
2.	"	"	"	"	"	boiled <u>Bot.cinerea</u> crude enzyme	" 20 "
3.	"	"	"	"	"	" <u>Bac.carotovorus</u> crude enzyme	" 20 "
4.	<u>Bac.carotovorus</u>	"	"	"	"	water	" 20 "
5.	"	"	"	"	"	boiled <u>Bot.cinerea</u> crude enzyme	" 20 "
6.	"	"	"	"	"	" <u>Bac.carotovorus</u> crude enzyme	" 20 "
.4 cc. of these enzyme mixtures on potato block 18 hours.							
Enzyme	1	2	3	4	5	6	
Grms. rot	.21 $\pm$.05	.19 $\pm$.02	.37 $\pm$.04	1.29 $\pm$.07	1.0 $\pm$.12	1.16 $\pm$.09	

the amount of rotting as compared with the crude Bot. cinerea extract alone, but there was still a significant difference between this value and that for any of the preparations containing active Bac. carotovorus enzyme. Addition of other material was thus seen to assist a Bot. cinerea enzyme which tended to dry up on solid potato blocks, but even when a Bot. cinerea extract contained exactly the same amount of impurities as a Bac. carotovorus enzyme of the same R.T., there was still a marked difference in the amount of rotting which equal volumes of them would produce on solid potato tuber tissue.

Fernando suggested that the addition of a sugar to a Bot. cinerea enzyme which tended to be absorbed would increase the amount of rotting caused by the latter on fresh potato blocks by osmotically restraining the drying up of the enzymic liquid. This effect has been investigated, the results being given in Table II, the figures of which show the average amounts of rotting produced on potato blocks when .2 cc. of Botrytis purified enzyme (R.T. 20 min.) diluted with .1 cc. of the liquid indicated (water, sucrose and glucose solutions) is placed on each block.

Table II.

Water	Sucrose		Glucose	
	10%	20%	10%	20%
On fresh potato blocks 74.2% water				
.13 ± .003	.47 ± .03	.50 ± .04	.56 ± .04	.60 ± .03
On injected blocks 77.6% water				
1.38 ± .14	1.52 ± .11	1.93 ± .12	1.76 ± .16	1.94 ± .14

The points brought out in the above table are as follows :-

(1) With fresh tissue the addition of either sugar to the enzyme gives a highly significant increase in the amount of rotting, the 10% solution being as effective as the 20%. With injected tissue only the 20% concentration gives a significant increase.

(2) Injecting the tissue with water is much more effective in increasing the rotting action than is the addition of either sugar to the enzyme.

(3) There is an indication that glucose is more potent than sucrose in increasing enzymic activity on the solid blocks of tissue.

From this last conclusion it followed that a possible factor of importance in the different behaviour of the two enzymes might be the effect of oxygen on the enzymic reaction. It was thought that the injection of potato tissue with water might assist the enzyme in some way by excluding air as well as by accelerating the movement of the enzyme into the tissue.

2. Oxidising and Reducing Enzymes present in Rotting Enzymic Extracts.

It was thought possible that the presence of oxidising and reducing enzymes in the enzymic extracts might influence their behaviour. A comparative series of tests was made therefore on the extracts of Bot. cinerea and Bac. carotovorus for the presence of other enzymes, the results being given in the following table.

From these figures it is seen that the crude enzyme of Bot. cinerea shows more oxidase activity than the crude Bac. carotovorus extract which, on its side, shows more catalase activity. The purified enzymes of both organisms are free from these subsidiary enzymes. It did not seem likely therefore that the presence or absence of these could be in any way an explanation of the differences in behaviour found between the fungal and bacterial enzymes because, in the tests of Hall and Fernando, purified extracts in the main had been used. It was desirable, however, to compare

Table III.

<u>Bot. cinerea</u> enzymes from cultures on a potato medium							
Activity	Crude enzymic extracts					Purified extracts	
R.T. (minutes)	20	45	90	45	60	35	45
pH	6	6.6	6.8	6.6	6.8	7	7
Guaiacum oxidase *	180	1	1.5	6	-	0	0
Phenolic oxidase *	-	-	-	4	14.5	0	0
Peroxidase *	-	-	-	-	-	0	0
Reductase *	0	36	36	6	6	0	0
Catalase (mm.)	125	8	0	54	53	0	0
<u>Bac. carotovorus</u> enzymes from cultures on a potato medium							
Activity	Crude enzymic extracts					Purified extract	
R.T. (minutes)	30	90	60	75	30	30	75
pH	8.4	7.5	8	8	8.5	8.2	7
Guaiacum oxidase	0	0	-	-	0.8	-	0
Phenolic oxidase	0	0	0	0	1	2.6	0
Peroxidase	0	25	25	0	-	-	0
Reductase	0	3	72	72	12	3	0
Catalase (mm.)	125	50	20	250	0	9	0

0 = negative test ; - = no test made.

*activity = reciprocal of time in minutes x 360.

a wider range of both Bot. cinerea and Bac. carotovorus crude and purified enzymic extracts on solid blocks of tissue, both to decide how much effect the presence or absence of these other enzymes had on the ability to attack potato blocks and, also, to examine the extent of the difference in behaviour between them.

3. Activity of Different Enzymes on Solid Blocks of Potato Tuber Tissue.

Over a period a series of experiments were carried out each comparing the action of a Bot. cinerea and a Bac. carotovorus rotting enzyme on blocks of potato tissue, both fresh and injected. Table IV is a collection of the results of all these experiments. Each horizontal line represents one experiment for which the same test tissue was used.

The general conclusions to be drawn from the table can be summarised as follows :-

(1) On blocks of fresh tissue both the purified and crude enzymic extracts of Bac. carotovorus are shown, on the whole, to be more active in proportion to their R.T. than the enzymes of Bot. cinerea. The latter show, however, considerable variation. In five examples the fungal enzyme is absorbed and causes no rotting, in three examples, 7, 8 and 13, there is markedly less rotting due to Bot. cinerea while in examples 1, 2, 3, 4 and 6, there is scarcely a significant difference between the amount of rotting due to Bot. cinerea and that caused by Bac. carotovorus.

(2) On injected tissue the position is substantially the same although the

Table IV.

	Bot. cinerea enzymic extracts						Bac. carotovorus enzymic extracts			
	R.T. min.	Grms. rot of fresh tissue	% water of fresh tissue	Grms. rot of infect. tissue	% water of infect. tissue	R.T. min.	Grms. rot of fresh tissue	Value of significant difference between columns 3 and 8	Grms. rot of infect. tissue	Value of significant difference between columns 5 and 10
Purified	1 45	.5 ± .12	79.2	2.4 ± .31	81.2	50	.63 ± .05	.29	2.1 ± .31	1.9
	2 45	.38 ± .04	79.6	.99 ± .07	83.0	50	.48 ± .04	.12	1.22 ± .05	.19
	3 30	.82 ± .03	-	1.53 ± .13	-	60	.48 ± .11	.26	.61 ± .02	.30
	4 20	.55 ± .05	80.2	3.0 ± .05	85.0	50	.42 ± .03	.13	1.6 ± .15	.36
	5 50	0	79	-	-	50	.60 ± .07	-	-	-
	6 30	.20 ± .08	-	1.12 ± .09	-	45	.19 ± .05	.21	1.57 ± .09	.29
	7 40	.11 ± .02	76	1.03 ± .02	81	45	.44 ± .04	.10	1.9 ± .01	.05
	8 40	.27 ± .04	75	1.93 ± .20	81	40	.87 ± .06	.16	2.6 ± .15	.57
	W 9 30	0	-	.57 ± .07	-	45	.60 ± .05	-	1.06 ± .16	.40
	W 10 40	0	77	1.18 ± .15	78	45	.50 ± .10	-	-	-
	W 11 40	0	77	.95 ± .20	79	40	.29 ± .07	-	2.9 ± .10	.50
	12 35	0	74	.73 ± .04	80.5	30	.34 ± .05	-	1.52 ± .02	.10
	13 30	.24 ± .03	73	.56 ± .04	80	30	.47 ± .05	.13	1.7 ± .10	.24
Crude										

W : a white skinned variety of potato was used in these 3 tests.

amounts of rotting are considerably increased for all enzymes. Where there was a marked difference shown between the enzymes on fresh tissue there is also a distinct difference on blocks of the same tissue injected with water and where the two enzymes cause nearly the same amounts of rotting on fresh tissue the difference between the figures for injected tissue is also not so great.

(3) The difference between the two enzymes seems to be greatest on tissue of low water content where the Bot. cinerea enzymic extracts tend to be rapidly absorbed. On tissue of high water content where the tendency to dry up is not so great, the Bot. cinerea enzyme may act as efficiently as the Bac. carotovorus. It must be remembered however that when the tendency to dry up on a particular tissue of low water content is removed by injecting the tissue with water there is still a characteristic difference in the amount of rotting due to each enzyme.

4. Further Methods of separating the rotting Enzymes from Oxidising and Reducing Enzymes.

In section III, 2, it was shown that various enzymic preparations of Bot. cinerea and Bac. carotovorus differ with regard to their content of oxidising and reducing enzymes. As the latter could be removed by purification without affecting the characteristic differences shown by the enzymes in rotting plant tissue, it did not appear that these differences could be

related to oxygen conditions prevailing in the enzymic media. Nevertheless it was considered advisable to explore the matter further in order to examine the effect of other methods of separation. The following is an account of the results obtained.

(a) Differential Effect of pH.

The writer found the optimum point for the rotting action of *Botrytis* enzyme to be near pH⁶ and for *Bac. carotovorus* enzyme near pH 8, which agrees with Fernando's statements on this matter. The oxidase, reductase and catalase enzymes in the extracts from both organisms behave similarly, inasmuch as they all show highest activity in alkaline media (Figs. 1 - 5).

The effect of pH on the rotting of potato blocks by *Bot. cinerea* enzyme is shown in the following table.

Table V.

<u><i>Bot. cinerea</i></u> crude enzyme R.T. 45 min.	
pH	Grms. rotting of potato blocks.
8.5	0
7.5	0
6.5	.13 ± .04
5.5	.24 ± .04
4.5	.21 ± .02
3.5	0

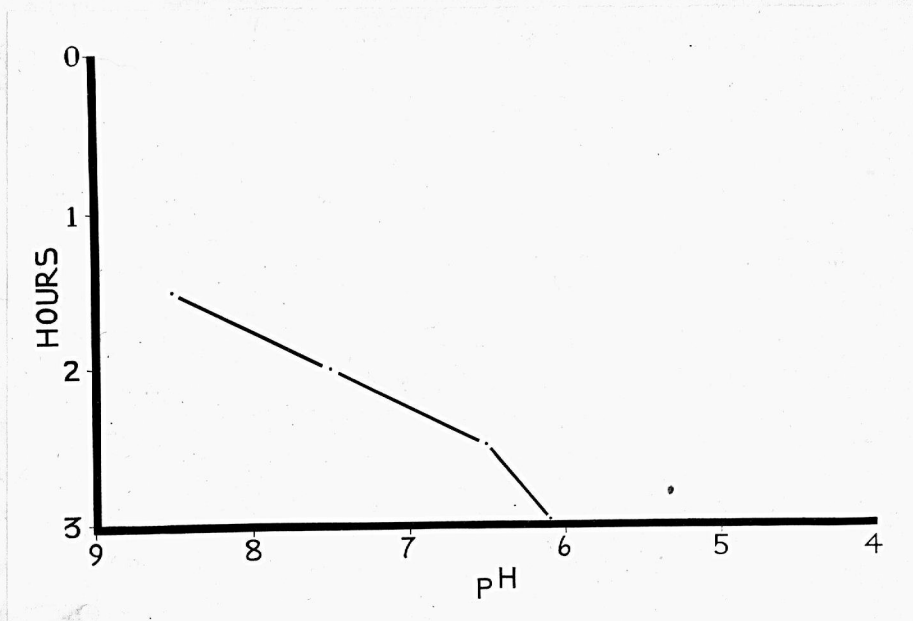


Fig.1. Reductase activity (time for decolouration in hours) at different pH values of Bac. carotovorus crude enzyme, R.T. $\frac{1}{2}$ hour at pH 8.5.

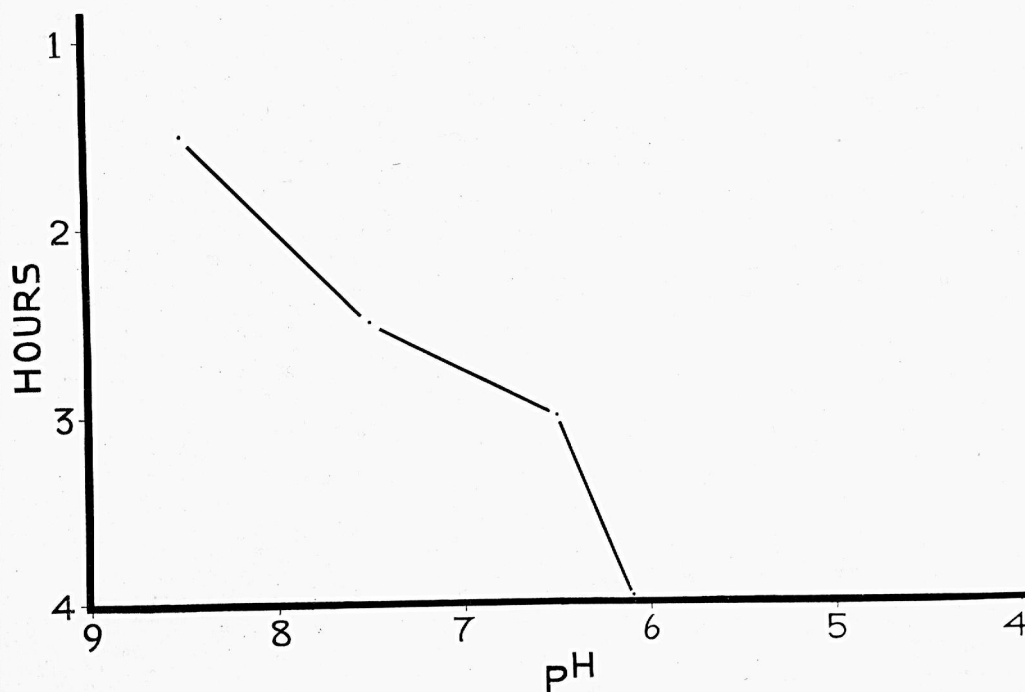


Fig.2. Oxidase activity (time for decolouration in hours) at different pH values of Bac. carotovorus crude enzyme, R.T. $\frac{1}{2}$ hour at pH 8.5.

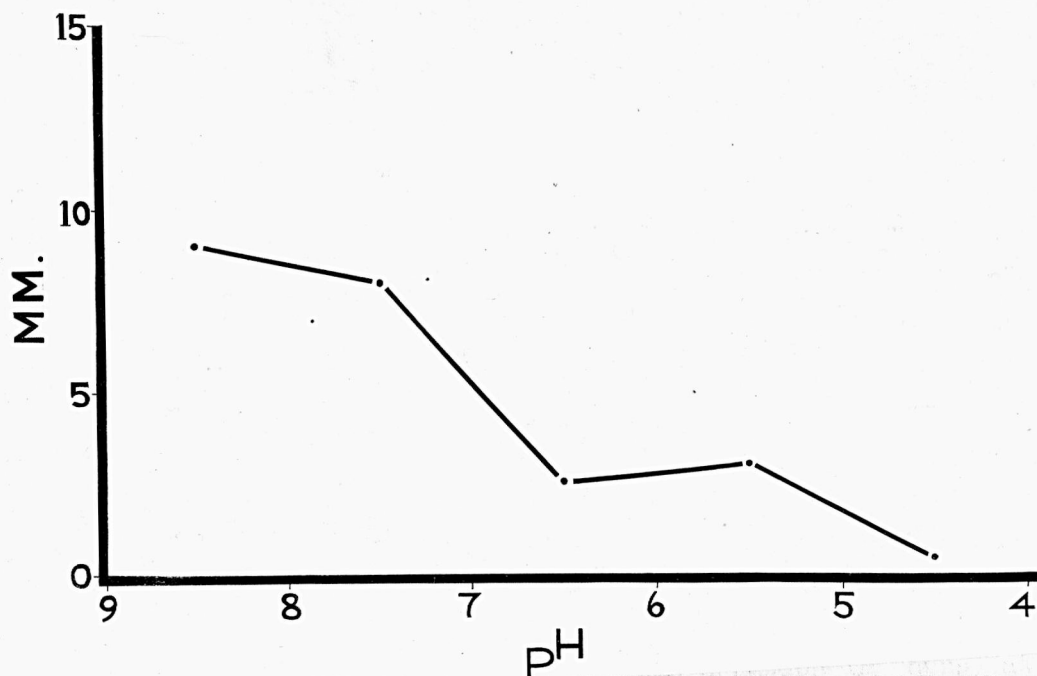


Fig.3. Catalase activity (mm. of oxygen) at different pH values of

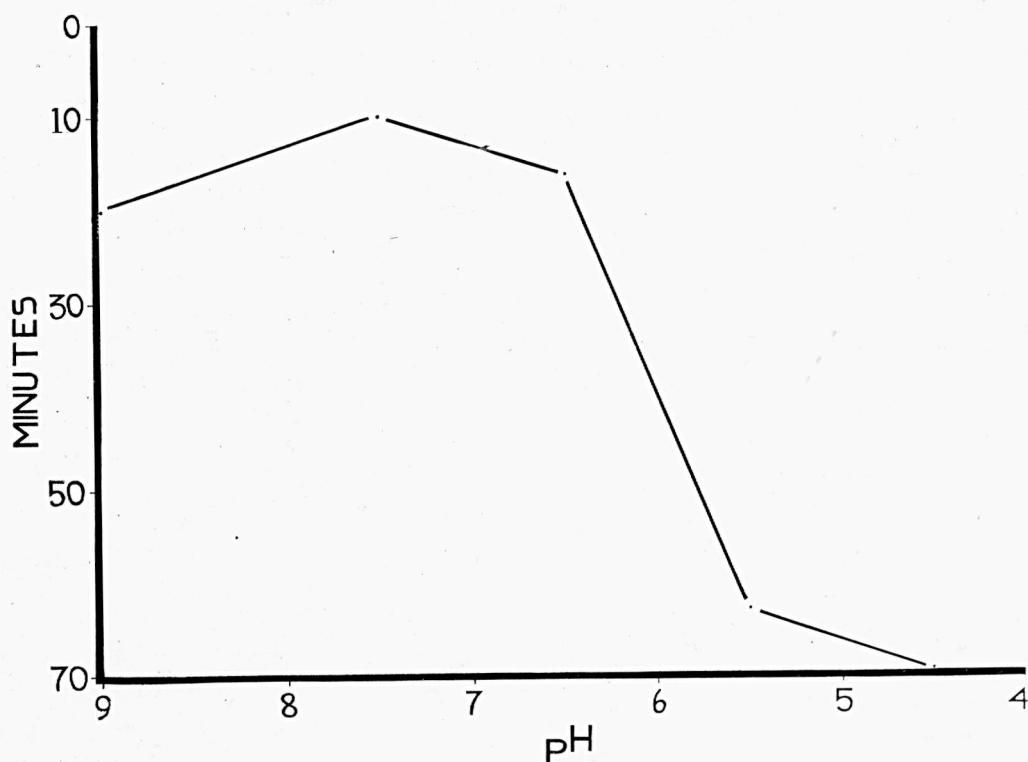


Fig.4. Reductase activity (time for decolouration in minutes) at different pH values of Bot. cinerea crude enzyme, R.T. $\frac{1}{2}$ hour at pH 6.5.

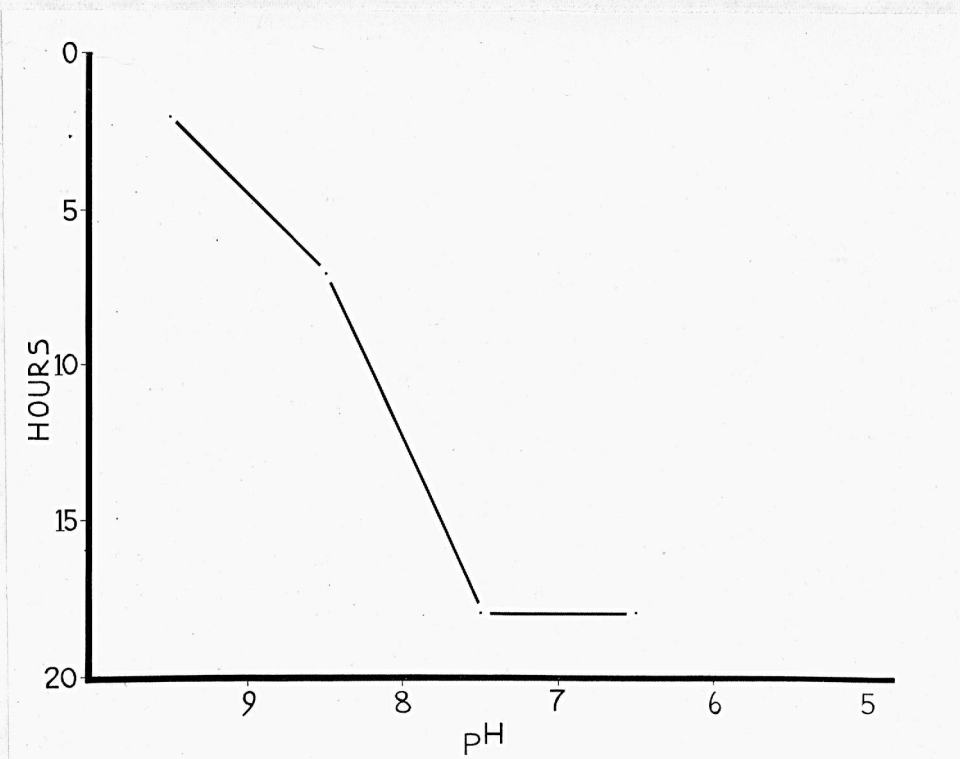


Fig.5. Oxidase activity (time for decolouration in hours) at different pH values of Bot. cinerea crude enzyme, R.T. $\frac{1}{2}$ hour at pH 6.5.

The corresponding behaviour of Bac. carotovorus enzyme is illustrated in the next table.

Table VI.

Bac. carotovorus crude enzyme R.T. 45 min.

pH	Grms. rotting of potato blocks.
8.8	.91 $\pm$.09
7.8	.84 $\pm$.09
6.8	1.26 $\pm$.21
5.8	1.06 $\pm$.21
4.8	1.02 $\pm$.19
3.8	0

Since pH affects the activity of rotting enzymes as well as of the oxidising and reducing enzymes the data of the above two tables do not give critical evidence on any effect resulting from the presence or absence of oxidising and reducing enzymes, as the effect of pH on the rotting activity alone is sufficient to explain them. It will be noticed that the optimum is not clearly defined by this method of measurement. When the different enzymes begin to attack the potato blocks the juice oozing out from the rotted tissue quickly brings them all to the same pH but for the enzymes which are too weak to cause any rotting the pH remains at the unfavourable value.

In Table V it is shown that a Bot. cinerea enzyme which attacks blocks of potato tissue at a favourable pH is unable to do so at a very low or a very high pH. It was also found in experiments of this type that a preparation of Bot. cinerea which was absorbed without causing any rotting at pH 6, was not absorbed at the extremes of pH 7.5 or 3.5. There was, however, no rotting presumably due to the depressing effect of these extreme pH values on enzymic activity.

(b) Deactivation by Heat.

The different enzymes present in the rotting extracts were examined for their resistance to heating. Brown (1) had found that the internal Bot. cinerea enzyme with which he worked lost its ability to attack potato tissue instantaneously at 65°C., and Davison and Willaman used low temperature deactivation as a method for separating their protopectinase from the

other pectic enzymes. In the tests illustrated in Figs. 6 and 7 however, a much wider range of tolerance to heat was found. Bot. cinerea crude enzyme was able to withstand five minutes heating at 70°C. and in a series of Bac. carotovorus enzymes there was considerable variation in the reaction to heating, the weaker and more purified enzymes being, on the whole, more readily injured. It is apparent from these data that any method for separating protopectinase from other enzymes by heating would not be complete with extracts of this kind. All the subsidiary enzymes except catalase were

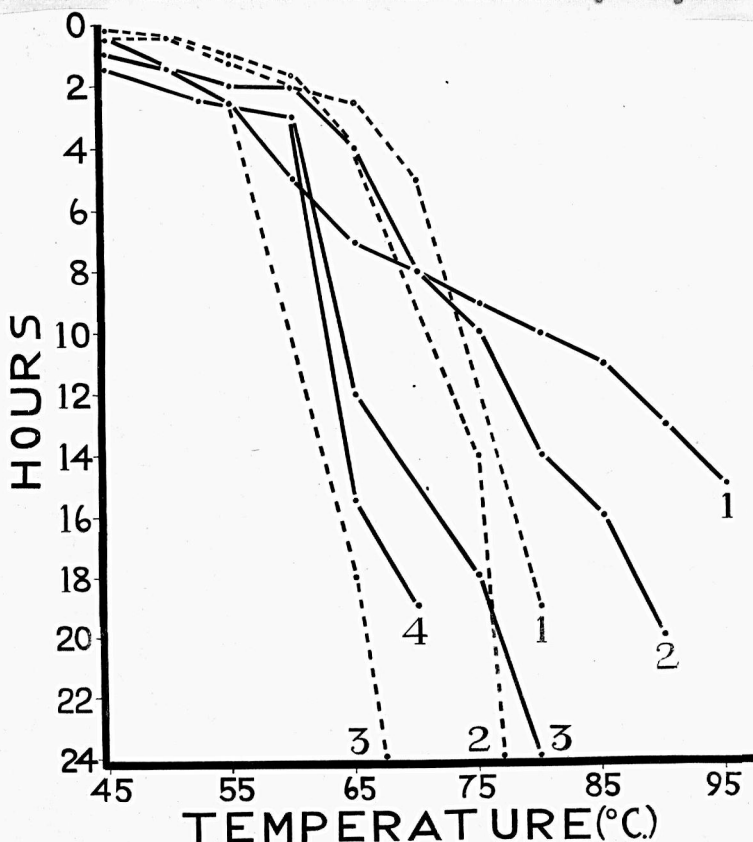


Fig.6. Heat inactivation of Bac. carotovorus enzymes, R.T. in hours after 2 mins. heating at different temperatures in °C. Continuous lines = crude enzymic extracts, 1, pH 8 ; 2, pH 8.5 ; 3, pH 7.5 ; 4, pH 8.4. Broken lines = purified enzyme, 1, pH 7 ; 2, pH 7 ; 3, pH 7.2.

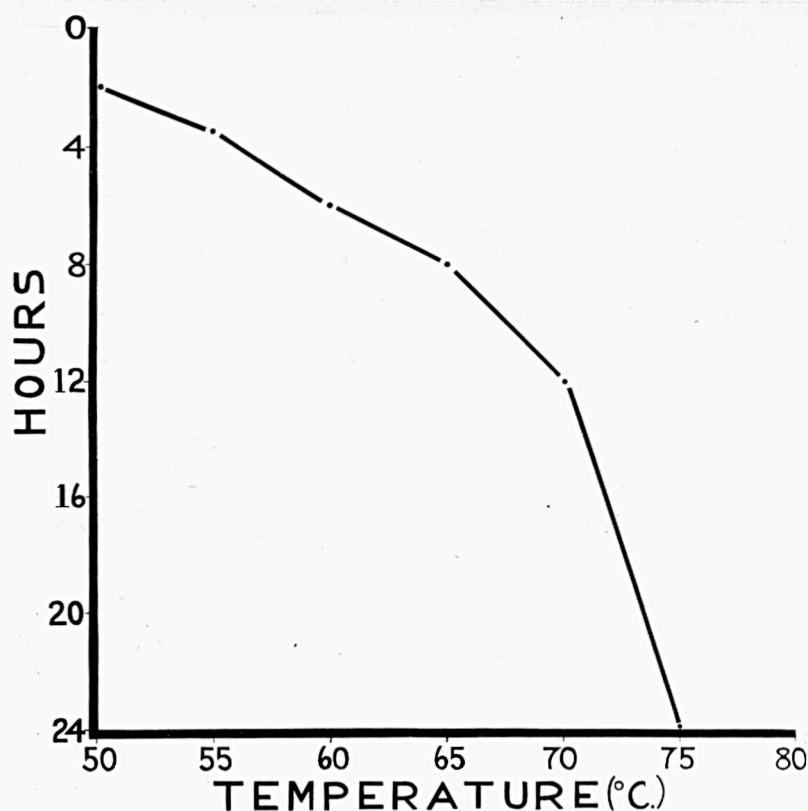


Fig.7. Heat inactivation of *Bot. cinerea* crude enzyme pH 6.8, R.T. in hours after 2 mins. heating at different temperatures in °C.

destroyed within the same range of heating as the rotting activity (Figs.8, 9, 10 and 11).

(c) Adsorption.

Different methods of adsorption were tried as a means of purifying the enzyme without success. The difficulty was to elute enough enzyme to give a satisfactory rotting of potato tissue. Aluminium hydroxides and ferric hydroxide prepared according to the methods of Waldschmidt Leitz and

Purr (10) and Willstatter and Kraut (12) were tried as adsorbents.

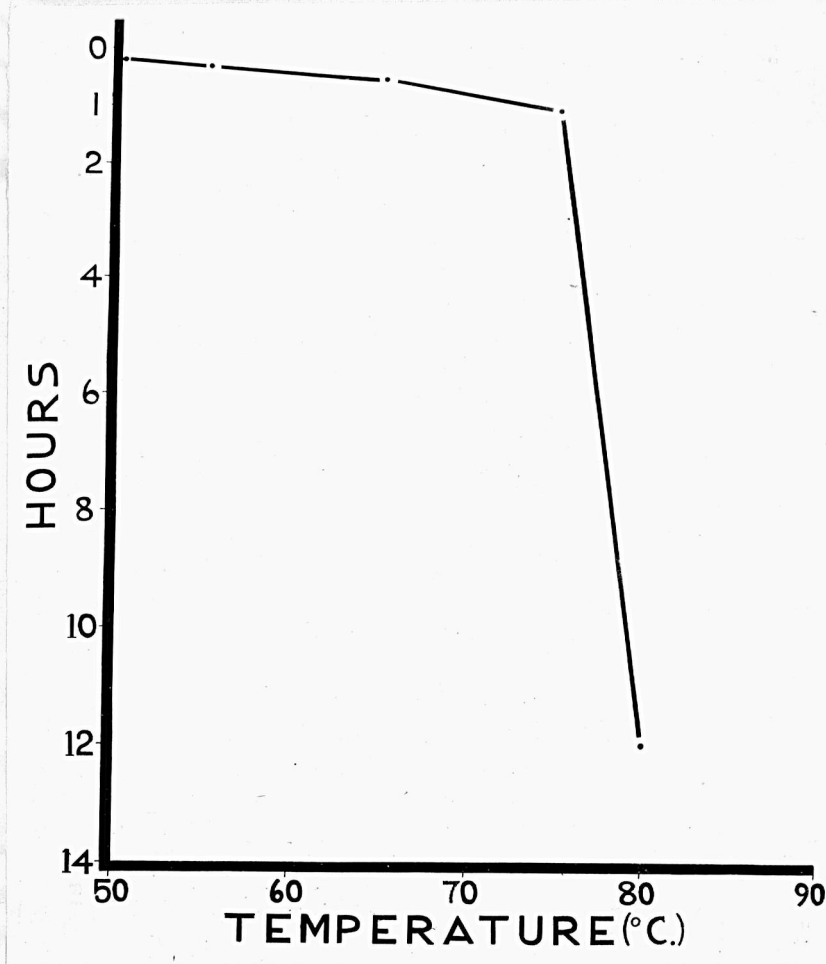


Fig.8. Reductase heat inactivation of Bac. carotovorus crude enzyme (R.T. 45 mins., pH 8.5), decolouration time in hours after 2 mins. heating at different temperatures in °C.

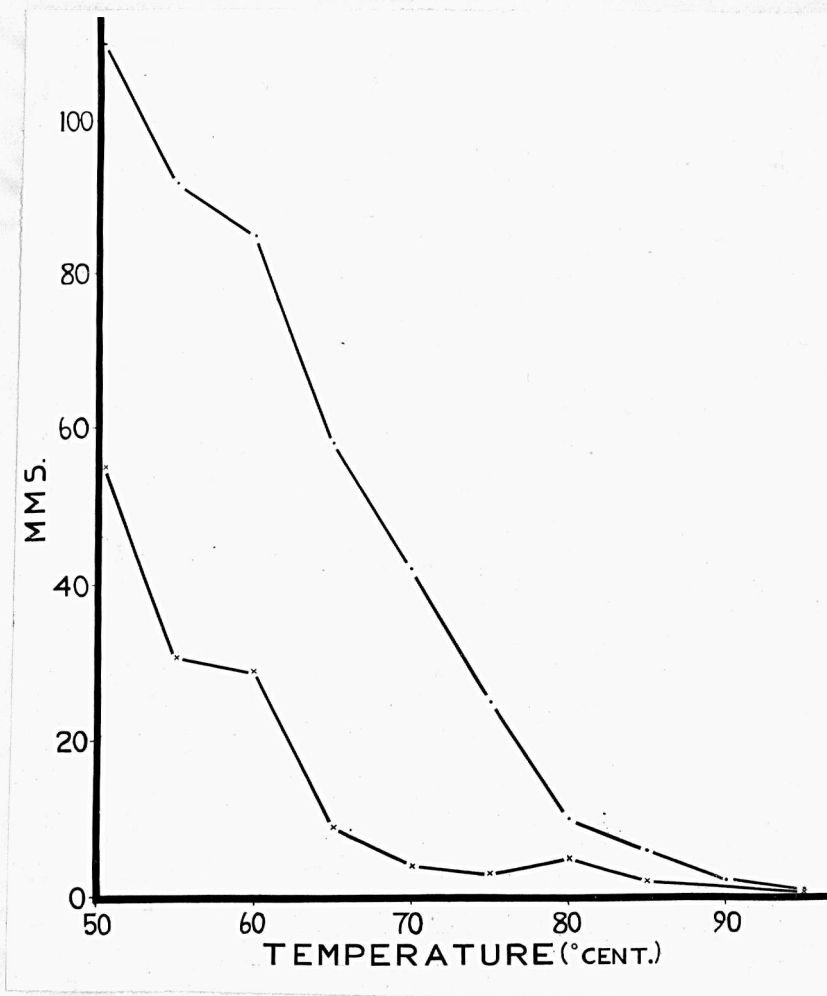


Fig.9. Catalase heat inactivation of Bac. carotovorus crude enzyme, mm. of oxygen evolved by enzymes heated for 2 min. at different temperatures in °C.

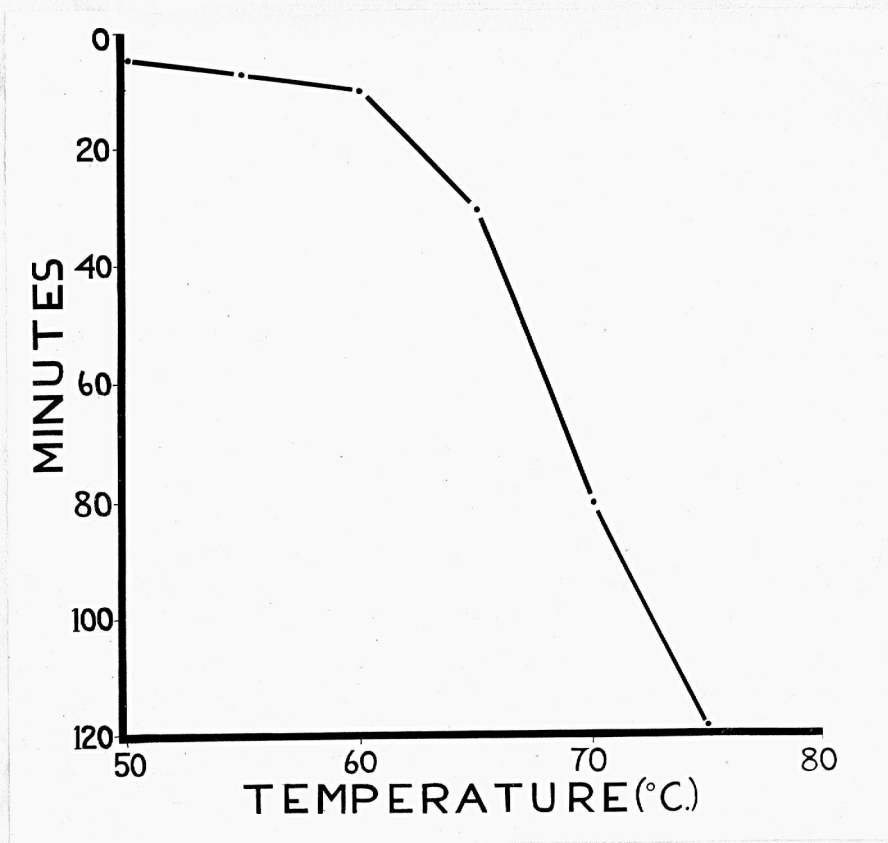


Fig.10. Reductase heat inactivation of Bot. cinerea crude enzyme, decolouration time in mins. after 2 min. heating at different temperatures in °C.

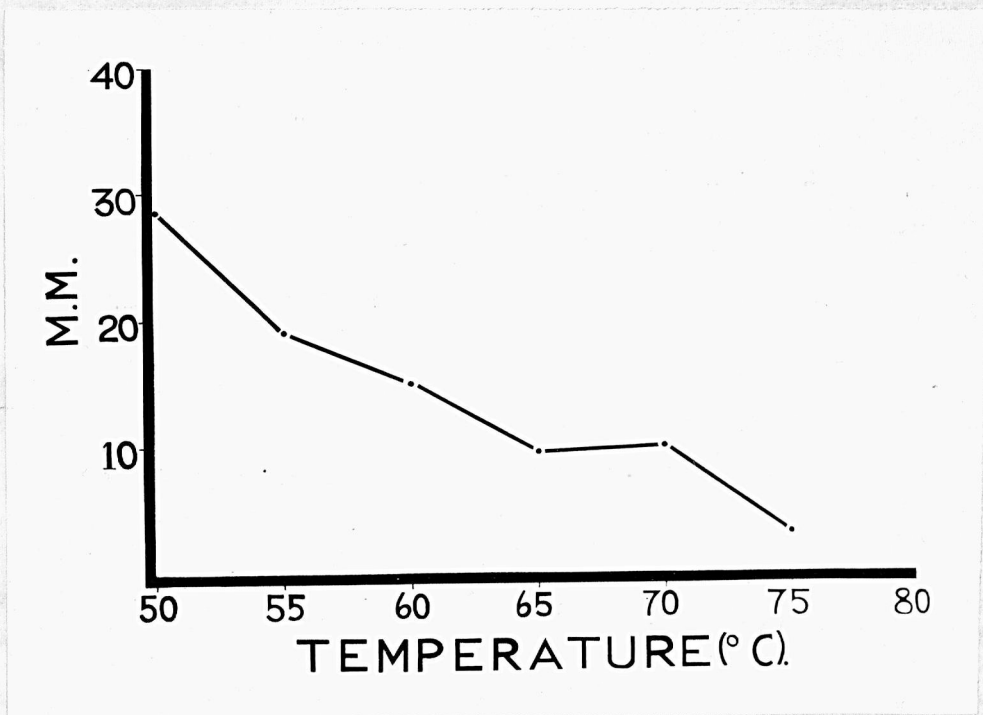


Fig.11. Catalase heat inactivation of Bot. cinerea crude enzyme, mm. of oxygen evolved by enzyme heated for 2 mins. at different temperatures in °C.

(d) Irradiation.

Irradiation with ultraviolet light was found to destroy the rotting enzyme while other enzymes present in the extracts were unaffected or gained in activity. A typical example is shown in the Table below.

Table VII.

<u>Bac. carotovorus</u> crude enzyme.		
	Fresh enzyme	Irradiated 10 mins.
Phenolic oxidase	10 hrs.	1 hr.
Catalase	30 mm.	30 mm.
Reductase	20 min.	10 min.
R.T.	60 min.	120 min.

Ultraviolet light was destructive of the rotting activity of both enzymes, e.g. Bac. carotovorus purified enzyme with an R.T. of 60 minutes, after irradiation for 45 minutes was found to have an R.T. of 10 hours and Bot. cinerea purified enzyme with an R.T. of 45 minutes after irradiation for the same time had an R.T. of 12 hours.

(e) Freezing.

Tubes of crude enzyme were stood in a mixture of ice and salt and the whole contents of the tube frozen solid at a temperature not lower than -3°C . On thawing a precipitate settled out and a clear enzymic solution was left. In most cases the rotting enzyme only or mainly was recoverable from the precipitate while the clear fraction still contained the other enzymes present in the crude extract. A Bac. carotovorus enzyme frozen for four hours gave a precipitate which was filtered, washed once and then shaken up with an equal volume of distilled water ; the fractions gave the

following tests :-

Table VIII.

Activity	Crude <u>Bac.carotovorus</u> enzyme	Clear frozen fraction	Precipitate
R.T.	30 min.	30 min.	30 min.
pH	7.5	7.5	7.5
Guaiacum oxidase	3 min.	15 min.	0
Reductase	20 "	20 "	720 min.
Catalase	20 mm.	16 mm.	4 mm.
Grms. rot new potatoes	.75 $\pm$.03	.72 $\pm$.06	.64 $\pm$.02
Grms. rot old potatoes	1.44 $\pm$.23	1.37 $\pm$.18	1.43 $\pm$.12

The two fractions of the frozen enzyme have the same R.T. and the same pH but the precipitate has very much less of the oxidase and reductase activities than the clear portion. There is however, no difference in the amounts of rotting of solid potato tissue brought about by the two fractions. It is noticeable that there is a significant increase in the amount of attack on old potatoes over that on new potatoes.

The results from the similar treatment of Bot. cinerea enzyme are given in the table below. The precipitate in this case was less in amount and finely divided so that it was separated by centrifuging.

Table IX.

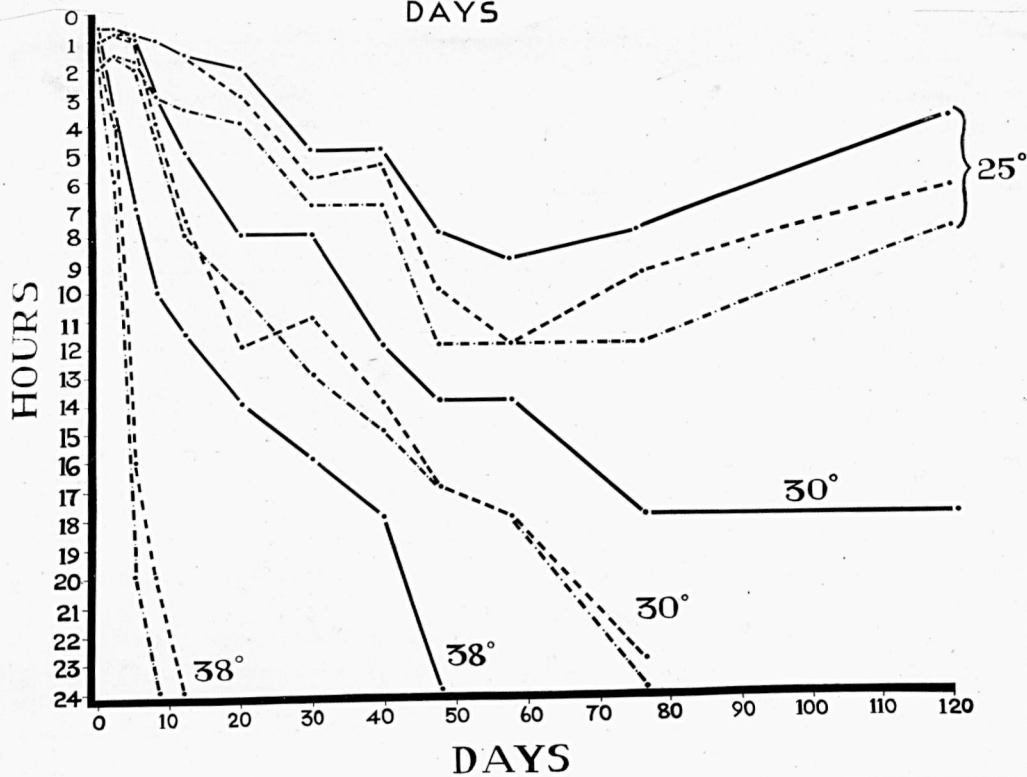
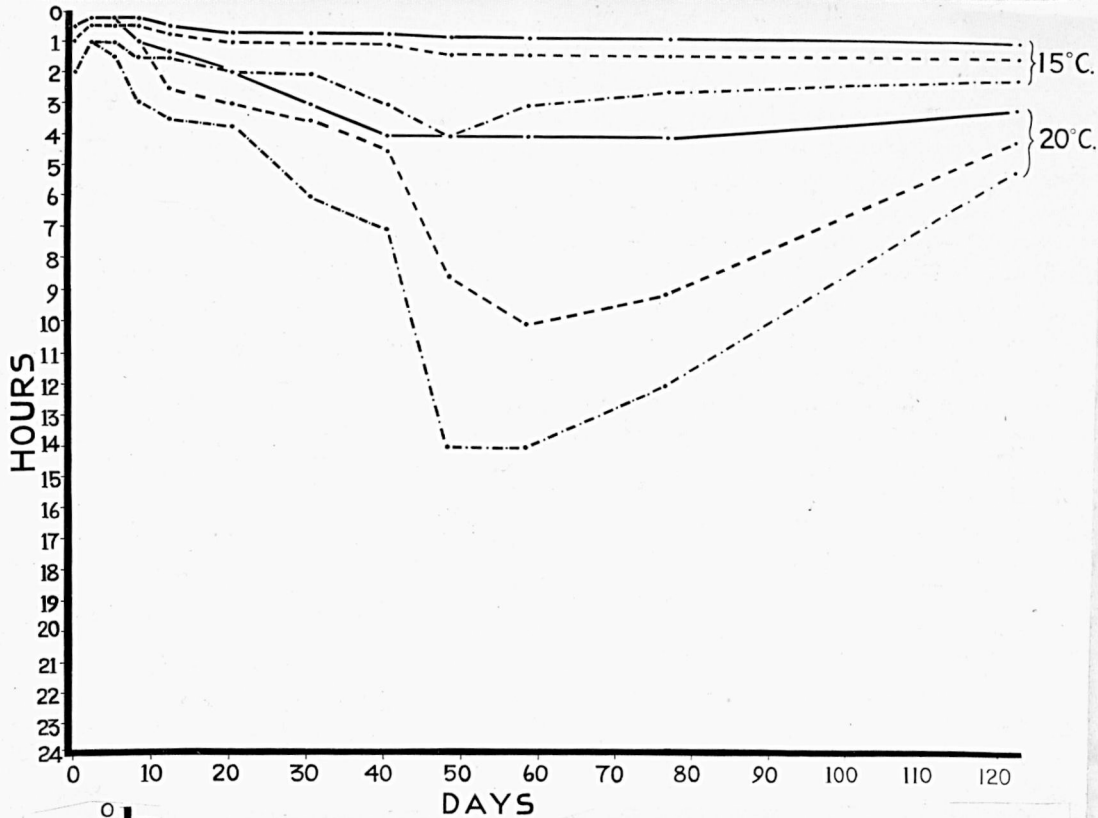
Activity	<u>Bot.cinerea</u> crude enzyme	Clear frozen fraction	Precipitate
R.T.	45 min.	45 min.	60 min.
pH	6.2	6.8	7.2
Guaiacum oxidase	1 $\frac{1}{2}$ min.	2 min.	0
Reductase	15 "	30 "	0
Catalase	54 mm.	25 mm.	15 mm.
Grms. rot	all lots dried up		

From these experiments it is concluded that the removing of most of the subsidiary activities by this method does not affect the behaviour of the rotting enzymes.

5. Stability of Rotting Enzymes of Bot. cinerea and Bac. carotovorus.

(a) Stability in aqueous solution in presence of toluene.

When enzyme extracts were kept in aqueous solution with toluene it was found that the rotting activity declined very slowly but that the other enzymes deteriorated comparatively quickly, no reactions for them being given after 7 to 10 days. Figs. 12 and 13 show the activity of crude Bac. carotovorus enzyme kept at different temperatures in solutions of different pH. At a favourable pH of 8.3 and a low temperature of 15°C. the



Figs.12 and 13. R.T. in hours of *Bac. carotovorus* crude enzyme after storage for different periods of days in aqueous solution in presence of toluene at different pH and different temperature. Continuous line = pH 8.3 ; discontinuous line = pH 7 ; dash and dotted line = pH 5.3.

extract has lost but little of its activity after three months. At higher temperatures and less favourable pH the activity fell off more quickly. The initial rise in activity shown in these tables is probably due to the lethal action of the toluene on the bacteria which liberated amounts of enzyme in the first few days. The later dip and recovery in the curves is attributed to seasonal variation in the test tissue.

The enzyme of Bot. cinerea was slowly deactivated in storage in aqueous solution with access of air but not to the same extent in an atmosphere devoid of oxygen. The following table gives the figures for the loss of activity of a Bot. cinerea external enzyme stored at a temperature varying within 2° of 18°C.

Table X.

<u>Bot. cinerea</u> crude enzyme	R.T.
Initial activity	20 minutes
After 2½ months with access of air	150 "
" " " over pyrogallol	60 "

This Bot. cinerea crude enzyme both before and after storage was tested by the block method in an atmosphere without oxygen as well as in air but in no case was there any attack.

(b) Stability after Drying.

A striking feature of the behaviour of a Bot. cinerea enzyme and to a

much less extent of a Bac. carotovorus enzyme is that, when placed on a block of ordinary potato tuber tissue it becomes absorbed and causes no rotting. If water is added within one hour after drying to a potato block on which has dried up an active Bot. cinerea enzyme with an R.T. of 30 mins. or less, rotting proceeds normally. At a later stage the Bot. cinerea enzyme is not recoverable. Rotting can be induced by the further addition of water to potato blocks on which Bac. carotovorus enzyme has dried down within 18 hours of the time of its drying. For experiments of this type the enzymes were induced to dry up on the potato tissue by drying the blocks with blotting paper before the enzyme was run on and then passing a current of air over them. The drying of the enzyme was brought about mainly by withdrawal of water from it by the cells of the potato tissue and to a lesser extent by evaporation and by sinking into the intercellular spaces. It is impossible to say whether the enzyme in such cases is deactivated by coming in contact with some principle present in the plant cells or merely by a certain degree of desiccation brought about mainly by withdrawal of water from it by the living cells. In this connection it appeared desirable to test whether the same differences were shown between the enzymes of the two organisms in relation to desiccation on glass. The following table gives the results of drying the enzymes on glass plates and keeping these under laboratory conditions for various times.

Table XI.

Enzymes dried on glass and rewashed in equivalent volumes of distilled water				
Enzyme	Initial R.T.	After 1½ days	4 days	40 days
<u>Bot. cinerea</u> purified	45 mins.	60 mins.	300 mins.	> 48 hrs.
" crude	60 "	180 "	240 "	"
<u>Bac. carotovorus</u> purified	60 "	90 "	120 "	240 mins.

In the case of drying on glass as well as of drying on the potato blocks the Bac. carotovorus enzyme was the more easily recoverable. The indication from these experiments and also from those of the previous section of the paper is that the Bot. cinerea enzyme is more readily oxidised than that of Bac. carotovorus.

6. Effect of Plant Extracts.

As pointed out above (p.2) earlier workers have shown that the addition of certain plant extracts to pectinase preparations retards the activity of the latter. This line of investigation has been extended. Whereas Chona and Menon (l.c.) restricted themselves to potato discs as test material the writer has used discs of potato, swede, carrot, parsnip and beet in parallel

tests. Furthermore the enzyme solutions have been prepared on a variety of media and a number of plant extracts have been used as diluents. Lastly, the tests have been carried out according to the R.T. method, i.e. with thin discs of tissue immersed in an excess of the solution, and by the block method, i.e. with a relatively small amount of the active solution laid on the surface of a large mass of tissue. The data are set out in Tables XXV - XXVII. In each table the figures in the same horizontal line refer to test tissue cut from the same part of the same tuber or root. Double horizontal lines separate different experiments.

A study of these tables shows a certain amount of irregularity in detail but three well defined conclusions can be drawn :

(a) The retarding action of plant extracts on enzymic activity as measured by the R.T. method is most prominently shown by potato extract and especially when the test tissue is also potato. To quote one illustration from the tables, (Table XXVII, 2nd experiment) :- Addition of potato extract to an enzymic solution slowed down the R.T. from 30' to 90' when the test discs were of potato ; on the other hand there was no effect when the test discs were of swede, carrot or parsnip. The effect is therefore to a large extent peculiar to discs of potato tissue.

Table XXV.

Activity of Bot. cinerea purified enzyme with added juice.

Enzyme prepared on	Enzyme tested on	Enzyme diluted with				
		Water	Potato juice	Swede juice	Carrot juice	Parsnip juice
Potato extract	Potato discs	150'	150'	180'	150'	150'
	Swede "	150'	150'	150'	150'	150'
	Carrot "	150'	150'	180'	150'	150'
	Potato blocks	0 g.	0 g.	0 g.	0 g.	0 g.
Potato extract	Potato discs	100'	100'	100'	100'	
	Swede "	90'	90'	90'	90'	
	Carrot "	90'	90'	90'	90'	
Swede extract	Potato discs	90'	150'	120'	90'	120'
	Swede "	45'	45'	60'	45'	45'
	Carrot "	60'	60'	60'	60'	60'
	Parsnip "	60'	60'	60'	60'	60'
	Potato blocks	.40±.06g	.27±.06g	.10±.06g	.40±.05g	.19±.06g
Parsnip extract	Potato discs	50'	120'	20'	50'	90'
	Swede "	60'	60'	60'	60'	60'
	Carrot "	50'	50'	30'	50'	40'
	Potato blocks	0 g.	0 g.	0 g.	0 g.	0 g.
Parsnip extract	Potato discs	105'	135'	105'		
	Swede "	135'	135'	135'		
	Potato blocks	0 g.	0 g.	0 g.		

Table XXVI.

Activity of Bot.cinerea external enzyme with added juice.

Enzyme prepared on	Enzyme tested on	Enzyme diluted with					
		Water	Potato juice	Swede juice	Carrot juice	Parsnip juice	Beet juice
Potato extract	Potato discs	45'	210'	120'	60'	60'	
	Swede "	30'	40'	30'	30'	30'	
	Carrot "	30'	50'	60'	45'	45'	
	Parsnip "	30'	50'	30'	45'	45'	
	Potato blocks	.30±.04g	.41±.06g	.37±.01g	.36±.01g	.39±.01g	
Swede extract	Potato discs	20'	25'	25'			
	Swede "	20'	25'	25'			
	Potato blocks	.25±.08g	.48±.11g	.08±.003g			
Swede extract	Potato discs	45'	120'	60'			120'
	Swede "	30'	30'	30'			30'
	Beet "	> 24 hrs.	> 24 hrs.	> 24 hrs.			> 24 hrs
	Potato blocks	.55±0.6g	.58±.04g	-			
Carrot extract	Potato discs	90'	100'	90'	90'		
	Swede "	90'	90'	90'	90'		
	Carrot "	90'	90'	90'	90'		
	Potato blocks	0 g.	0 g.	0 g.	0 g.		
Parsnip extract	Potato discs	60'	75'	75'	75'	60'	
	Swede "	60'	75'	75'	75'	60'	
	Carrot "	60'	75'	75'	75'	60'	
	Potato blocks	0 g.	0 g.	0 g.	0 g.	0 g.	

Table XXVII.

Activity of Bac. carotovorus enzyme with added juice.

Enzyme prepared on	Enzyme tested on	Enzyme diluted with					
		Water	Potato juice	Swede juice	Carrot juice	Parsnip juice	Beet juice
Potato extract	Potato discs	45'	120'	60'			120'
	Swede "	30'	30'	30'			30'
	Potato blocks	1.66 .11g	1.15 .05g	1.28 .06g			.65 .03g
Potato extract	Potato discs	30'	90'	60'	30'	30'	
	Swede "	30'	30'	45'	30'	30'	
	Carrot "	45'	45'	60'	45'	45'	
	Parsnip "	45'	45'	60'	45'	45'	
	Potato blocks	1.14 .28g	1.18 .13g	1.02 .09g	1.04 .11g	.94 .11g	
Potato extract	Potato discs	45'	90'	75'	75'		
	Swede "	75'	75'	75'	90'		
	Carrot "	75'	75'	75'	75'		
	Potato blocks	1.09 .09g	1.04 .09g	.71 .03g	1.05 .07g		
Swede extract	Potato discs	30'	45'	30'	30'		
	Swede "	30'	30'	30'	30'		
	Carrot "	30'	30'	30'	30'		
	Potato blocks	1.29 .06g	1.51 .07g	1.16 .07g	1.62 .06g	1.33 .07g	
Carrot extract	Potato discs	25'	50'	50'	50'	50'	
	Swede "	60'	60'	60'	60'	60'	
	Carrot "	25'	50'	30'	50'	50'	
Parsnip extract	Potato discs	20'	30'	20'	25'	25'	
	Swede "	60'	50'	60'	60'	60'	
	Carrot "	20'	20'	20'	20'	20'	
Crude enzyme	Potato "	50'	120'	50'	50'	50'	
	Swede "	60'	90'	90'	60'	90'	
	Carrot "	50'	60'	60'	45'	60'	

(b) The retarding effect of plant juices on enzyme activity which has been noted under (a), is not shown when tests are made on blocks of tissue. Thus in the experiment indicated under (a) the activity shown by the "block" method is as great when the diluent is potato extract as when it is water.

(c) The suggested rule which appeared from Chona's and Menon's work, viz. that enzymic solutions are less sensitive to plant extracts when prepared on these extracts is only partly borne out by the data of Tables XXV-XXVII. The data of Table XXV support it, those of Tables XXVI and XXVII are distinctly against it. Obviously relationships are more complex than was envisaged by these earlier workers. The effect of plant extracts on the activity of pectinase enzyme was considered by Chona and Menon to be a direct one to the enzyme. The fact that different tissues react differently as shown in (a) above disfavours this simple view. It is known that certain plant extracts have effects upon the tissues used for testing purposes but subsidiary experiments have shown that this effect does not appear soon enough to explain the results obtained. What the basis of the retarding action is, is therefore not clear and in the light of (b) above the details do not appear to be of direct interest to the problem of pathogenicity.

It will be noted that Tables XXV-XXVII fully confirm the differences previously noted between the enzymes of Bot. cinerea and Bac. carotovorus. Enzymic preparations of Bot. cinerea of a high activity as measured by the R.T. method either failed to act or gave relatively little effect when

tested by the block method (Tables XXV and XXVI). On the other hand preparations of Bac. carotovorus of a comparable R.T. produced much greater rotting when tested on blocks of tissue (Table XXVII).

7. Bot. cinerea and Bac. carotovorus on potato tissue.

From time to time tests were made of the virulence of the parasites in attacking fresh potato. Although old potatoes were more readily attacked by both organisms than new ones the results of these periodic tests always showed the same difference between the two parasites, namely, that Bac. carotovorus required much less water present for growth and successful rotting than did Bot. cinerea. Thus an inoculum of 1 c.c. of a bacterial spore suspension on the surface of a 0.8 cms. thick slice of a medium sized potato tuber in a petri dish was always sufficient to start rotting. This amount of a Bot. cinerea spore suspension practically always dried up but if the amount were increased to 5-10 ccs. growth and rotting followed.

In the absence of free water an inoculum of Bot. cinerea and to a less extent one of Bac. carotovorus tended to dry up even in a saturated atmosphere, with resultant failure of attack. If the region of the inoculation were moistened by sufficient water attack was facilitated up to a certain point. The following is an account of an experiment in this connection. A number of potato slices in petri dishes were seeded with 2 ccs. of a dense Bot. cinerea spore suspension. Two were kept wet by the frequent addition

of sterile water and the others were allowed to stand. At intervals of 2, 4, 6, 18, 24 and 48 hours some of the latter were rewet and kept wet. Microscopic observations showed that in all cases the spores germinated. On slices which were rewet and kept wet within 18 hours after inoculation the fungus continued to grow and some rotting was demonstrable after 48 hours. On the other hand those which received no more water until after 18 hours had elapsed showed no attack. The spores had presumably been killed by desiccation. In contrast to the preceding an inoculum of Bac. carotovorus had already produced a small amount of rotting within 18 hours.

Tissues rotted by both enzymes were examined microscopically after staining with ruthenium red. Old potato tubers with not too many starch grains in the cells and cucumber were the most easily examined in this way. Material was taken from blocks which had been rotted by a small amount of enzyme on the surface and also of discs which had been immersed in enzymic extracts for varying lengths of time before and after the loss of coherence of the discs. In fresh tissues the whole cell wall stained brightly but it could not be established that the middle lamella stained any more intensely. In rotted tissue where the cells had lost coherence the cell walls still stained pink throughout though not so deeply as in fresh tissue. If these separated cells were exposed to further enzyme action they lost the ability to stain with ruthenium red. Bot. cinerea and Bac. carotovorus enzymes which brought about loss of coherence of discs in the same time also brought about the inability to stain red in approximately the same time. Thus by

this staining technique no distinction could be drawn between the two enzymes.

On the other hand potato discs when subjected to the action of Botrytis enzyme show a distinct physical difference from similar ones subjected to the enzyme of Bac. carotovorus. At the time when the former have lost nearly all their mechanical coherence, the discs would be described as brittle, that is when bent they crumble with great ease. The suggestion is that the cells are alive and turgid but the cementing matrix between them has been very seriously weakened. Similar discs immersed in Bac. carotovorus enzyme, when at the stage at which they have negligible mechanical resistance to a pull, are limp. One might describe them as having a soft "slimy" feel in contrast to a "granular" feel in the others. Whether the difference consists in an earlier killing of the protoplasts by Bac. carotovorus than by Bot. cinerea enzyme is not known, since potato tissue is difficult material for a study of this type.

IV. DISCUSSION.

The work described above has confirmed the general conclusion of Fernando that there is a substantial difference between the tissue rotting type of enzyme as prepared from Bot. cinerea and Bac. carotovorus. Apart from their different reactions to pH (optimum for Bot. cinerea, near pH 6 ; for Bac. carotovorus near pH 8) there is the striking difference in activity when tested on blocks of potato tissue. Preparations from the two organisms

which show the same Reaction Time when discs of potato tissue are immersed in them, are strikingly unequal when tested by the former method, the preparation from Bac. carotovorus being the more active under these conditions. Raising the water content of the blocks increases the amount of rotting caused by both enzymes without obliterating the characteristic quantitative difference between them.

The suggestion that this difference is associated with oxidising or reducing enzymes has not been borne out by experiment. So far as can be seen the tissue rotting activity of preparations of either organism is not associated in any way with the presence or absence of oxidising and reducing systems. This result thus also discounts the suggestion that lack of air in the tissue rather than a high water content is the factor which stimulates the process of rotting.

A possible difference between the two enzyme systems in question is to be found in their constituent enzymes. The delimitation of pectin attacking enzymes offers great difficulties arising from the unsatisfactory position with regard to the chemistry of pectic substances. It is not clear for example how far middle lamella substance is similar to or different from pectic constituents of the cell wall proper. It can be suggested therefore that the tissue rotting type of enzyme consists of two constituents one acting mainly upon pectic constituents of the cell wall proper and the other upon middle lamella substance. On this basis the enzyme system of Bac. carotovorus would be presumed to contain a larger proportion of the enzyme

which attacks the cell wall matrix while the *Botrytis* enzyme acts more readily upon the middle lamella substance.

The effect of plant juices upon the rotting activity of various enzymic preparations as reported by Chona (l.c.) and Menon (l.c.) have been confirmed in part. It is doubtful however, if one can attach much significance to these effects because they disappear to a large extent when the tests are carried out under conditions which approximate more closely to those prevailing in actual parasitism.

The choice of the two organisms *Bot. cinerea* and *Bac. carotovorus* for purposes of a physiological comparison was made in the first instance on purely technical grounds ; namely, that equally active, tissue rotting preparations could be obtained readily from both. A satisfactory enzymological comparison could therefore be made. The main result of this is that the enzyme of the potato attacking organism (*Bac. carotovorus*) is more effective in rotting potato tissue under conditions which approximate to the normal. Thus one might plausibly correlate the relative pathogenicity of the two organisms on potato with this difference but such a conclusion is questionable in the light of the findings of this paper. The non attack of potato tissue by *Bot. cinerea* is not due to shortcomings of its enzyme but arises from failure of the fungus to continue growing under conditions which are favourable to the growth and enzyme secretion of *Bac. carotovorus*.

The dominant factor concerned is the water content of the host tissue. With tissue of a certain water deficit, an inoculum of *Bot. cinerea* is

robbed of necessary water so that any growth which may have taken place is arrested and no attack can therefore follow. Under the same conditions Bac. carotovorus may develop and attack. How far this is due in the latter case to more rapid secretion of enzyme, or to a smaller water requirement or to the ability of bacteria to infiltrate the intercellular space system and therefore to escape the effects of drying, is unknown. It is possible that fuller information on these points would be obtained by contrasting strains of Bac. carotovorus of different virulence than has been possible with two organisms of habit so different as Bot. cinerea and Bac. carotovorus.

V. SUMMARY.

1. Tissue rotting enzymes of Bot. cinerea and Bac. carotovorus which are found to be equally active when tested with small discs of potato tissue immersed in the preparations are found to behave differently on solid blocks of potato tuber. Under these conditions the enzyme of Bac. carotovorus is found to cause considerably more rotting than an equal amount of that of Bot. cinerea.
2. Oxidase and reductase enzymes are present to a variable extent in crude enzyme preparations but their elimination by various methods does not alter this essential difference between the tissue rotting enzymes of the

two organisms.

3. The addition of certain plant juices to the enzymic preparations has an effect on the activity as measured by the disc method but is not found to have any effect on the action of small amounts of enzyme on blocks of potato tissue.
4. The two parasites under comparison are found to differ in the amounts of free water they require for growth and enzyme production. Bac.carotovorus is able to continue growing and secreting enzyme under conditions which check the growth of Bot. cinerea and thus prevent its parasitising the tissue. The failure of the fungus to attack potato tubers is thus not due to any defect in the activity of its enzyme.
5. It is suggested that the enzyme systems of Bot. cinerea and Bac.carotovorus may both comprise more than one enzyme each of which acts on a different constituent of the cell wall. It is further suggested that the fungal enzyme consists mainly of a middle lamella attacking element while the bacterial enzyme includes other elements as well capable of attacking the cell wall matrix.

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