THE PRODUCTION AND PROPERTIES OF PECTOLYTIC AND MACERATING ENZYMES SECRETED BY FUSARIUM MONILIFORME SHELDON.

A Thesis presented

by

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"The production and properties of pectolytic and macerating enzymes secreted by Fusarium moniliforme Sheldon".

Fusarium moniliforme is a pathogen of sugarcane, paddy, maize and other members of the Gramineae. It has also been found pathogenic to various vegetables e.g., tomatoes, potatoes, apples etc.

The fungus produces pectolytic enzymes on various natural media but does not do so on apple extract or juice. It also produces these enzymes on various synthetic media when a proportion of certain natural extracts or citrus pectin is added to them. An active enzyme preparation was obtained on a synthetic medium containing: Glucose, 0.5%; Asparagine, 0.4%; Pectin, 1.0%; KH_2PO_4, 0.1%; $MgSO_4$, 0.05% and NaCl 0.1%. Several other pectic compounds and D-galacturonic acid have been found to induce enzyme production in the above medium. A number of N and C sources have been found suitable for enzyme production. These enzymes are present in the mycelium and also in parasitised tissues of apples and tomatoes.

The study of the properties of the enzyme preparations reveal the presence of Protopectinase and Polygalacturonase (PG) in them. Some of the important features of the enzymes are: a high pH optimum in the range 8.0 - 9.0; not easily inactivated by heat; increasing its activity on dialysis and activity inhibited by phosphate.

Pathogenicity studies reveal that although it produces active pectolytic enzymes in culture it is pathogenic only to apples and tomatoes out of a variety of storage tissues tested. There is therefore no definite correlation between the secretion of enzyme on plant tissue decoctions and pathogenicity to the parent tissue.

Evidence is produced to suggest that the fungus produces pectolytic enzymes with properties different from those described from other sources.

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I. INTRODUCTION.

The fungus Fusarium Moniliforme Sheldon is an economically important parasite upon a wide range of tropical and sub-tropical plants. It is best known as attacking various grasses and in particular sugarcane in which it causes the disease known as pokkah-bŏeng or top-rot. It also occurs on rice, maize, many other Gramineae, and on such miscellaneous hosts as potato tubers, apples, tomato and chillie fruits and cotton. Because of its wide host range and the ease with which it is cultured, this fungus was considered a promising subject for physiological investigations and in particular for enzymic studies since the typical effect on plant tissues is to cause a soft rot. The work to be described is chiefly concerned with an analysis of the nutritional factors affecting production of pectic enzymes by F.moniliforme and a study of the properties of these enzymes. Preliminary work on the pathogenicity of the fungus to a variety of plant tissues under various conditions is also reported.

II. HISTORICAL

Systematic work on enzymes macerating plant tissues was started by Brown (1915) using Botrytis cinerea. Methods were introduced for obtaining preparations from young hyphae which were much more active than those used in earlier and similar work.

A number of workers have since studied the conditions under which pectic enzymes are secreted by micro-organisms. Since many pathogens are believed to attack the pectic materials of plant tissue the influence of such substances on production of pectic enzymes in pure culture has received a good deal of attention. Thus Harter and Weimer (1921-23) found that pectinase is produced by Rhizopus tritici, causing a soft rot of sweet-potatoes, only on vegetable extracts and in synthetic media containing pectin as a carbon source. They failed to produce the enzyme on synthetic media containing glucose as the sole source of carbon and concluded that the production of pectinase enzyme on natural media is stimulated by the pectin in them.

Willaman and Kertesz (1931) grew Penicillium glaucum Link., in media containing, in addition to a standard salt supplement, various combinations of sucrose, starch, peptone and pectin. Qualitative tests based on the time to clarify grape juice did not indicate any stimulatory action of pectin, but showed sucrose to be essential. In most cases,

the amount of exo- and endo- pectic enzymes produced were found to be approximately equal.

Proskuriakov and Ossipov (1939) used dried ground mycelium of Aspergillus oryzae, Aspergillus niger, Mucor sp., Botrytis cinerea, Sclerotinia fructigena and Rhizopus nigricans as sources of pectinase. They showed that addition of pectin to the medium markedly increased the formation of pectic enzymes. Similar results were obtained by Phaff (1947) working with Penicillium chrysogenum who found that the fungus is adaptive as regards the production of both pectinase (polygalacturonase) and pectinesterase. He found only traces of endo-cellular enzyme in the mycelium and observed that pectin, pectic acid, D - galacturonic acid, mucic acid, L - galactonic acid and gum tragacanth stimulated production of both enzymes. No other of some fifty carbon compounds tested showed this stimulatory effect.

Gaumann and Bohni (1947) reported that in cultures of Botrytis cinerea pectinase was produced constitutively. Pectinesterase however was formed adaptively.

Ashour (1949) working with Pythium deBaryanum found that pectin was a poor source of carbon for growth and that it was inhibitory in a medium otherwise suitable for enzyme production.

It is seen from the above that in the production of the two enzymes pectinase and pectinesterase, fungi do not react

uniformly to the presence of pectic materials in culture media.

There is much further information on the effect of other factors on enzyme production in various media. This will not be described in detail. It may however be said that while some organisms are relatively exacting in their requirements for active enzyme production as for example Pythium deBaryanum, others such as Bacterium aroideae are much less so. The carbon source (other than pectic substances) have generally less influence than the nitrogen source. Here, asparagine seems particularly suitable for some organisms. Enzyme production often does not parallel growth as measured by weight of mycelium produced, while the period of incubation is frequently of significance.

The literature on the properties and activities of pectic enzymes is now very extensive and will not be dealt with here systematically. References to relevant work will be made later. It will however be necessary to give briefly the terminology of these enzymes as now generally accepted (Kertesz 1951).

Protopectinase - hydrolyses protopectin of plant tissues causing a separation of the cells which is commonly called "maceration". The existence of this enzyme is still a matter of dispute.

Pectin-polygalacturonase or Polygalacturonase (For brevity referred to as PG; also known as Pectinase or Pectolase) - hydrolyses the 1, 4 glycosidic linkages in the polygalacturonic acid framework of pectin or pectic acid with the formation of polygalacturonic acid polymers of lower molecular size and finally mono-galacturonic acid.

Pectin-methylesterase (Pectase; abbreviation PE) - catalyses the hydrolysis of the methyl ester groups in pectinic acids and pectin.

Pectic acid "Depolymerase" - There is evidence for the existence of this enzyme which only partially hydrolyses polygalacturonic acid. The action of this enzyme does not proceed beyond the formation of quite high molecular weight compounds and no galacturonic acid is produced. This terminology will be used in the following account.

III MATERIALS AND GENERAL METHODS.

The fungi used in this work are as follows:

(1) Fusarium moniliforme.

(2) Fusarium avenaceum.

(3) Fusarium coeruleum.

The cultures of Fusarium moniliforme was obtained from the Indian Agricultural Research Institute, New Delhi by courtesy of Dr. R.S. Vasudeva and had been originally isolated from sugarcane with "top-rot" disease. The cultures of Fusarium avenaceum and Fusarium coeruleum were obtained from the Agricultural Research Council Potato Storage Investigation, Loughborough, Leics., by courtesy of Dr. R.K. McKee. Most of the work was carried out with Fusarium moniliforme, the other two fungi being brought in only for pathogenicity tests on potato tubers and for comparative studies of pectolytic enzyme secretion.

Stock cultures were maintained on potato extract agar or soil-glucose medium. They were kept in the deep-freeze ($-18^{\circ}C$), in a refrigerator ($+5^{\circ}C$) and at $25^{\circ}C$. Those at $25^{\circ}C$. were recultured about once a month. The successive recultures remained constant and were satisfactory for the work on hand so that the reserve cultures stored in the frozen or chilled condition were in fact not required. It may be noted however that both remained viable after 18 months storage at these temperatures. In preliminary tests,

Fusarium moniliforme was grown on 12 different media such as potato extract agar, potato dextrose agar, oatmeal agar, malt agar, Czapek's Dox agar, etc. to observe the general growth habit. Potato extract agar was finally selected, because it gave reasonably good growth and good sporulation. The optimum temperature for linear growth was determined as 28°C.

The potato extract had the composition:

Peeled potatoes - 200 gm.

Water to 1 litre.

The general method of producing enzyme was as follows. Cultures were set up in 8 oz. medicine flats, 20 ml. of medium being added to each. When synthetic media were used, the phosphate was autoclaved separately from the remainder of the medium in order to minimize precipitation of magnesium phosphate. The flats were sterilised at 15 lbs. pressure for 15 minutes. For inoculation purposes, a dense spore-suspension was made up in sterile distilled water and 1 ml. added to each flat. These were then incubated at 25°C. for 4-5 days. After this period, the culture solution was poured off, strained through muslin, centrifuged and used either directly as a source of pectolytic enzyme or, more often, stored at -18°C until a bulk supply had been accumulated. In some experiments, the crude enzyme preparation was partially purified by precipitation or by dialysis.

To a limited extent, enzyme was also extracted from the mycelial mat and from certain parasitised fruit tissues. The details of these particular experiments will be given later in the appropriate sections.

Considerable attention was paid to the effect of pectic compounds in the culture media on the production of pectolytic enzymes. Three such compounds were used, viz.,

(1) Pectin. Citrus pectin, as supplied by the Californian Fruit Growers Exchange, was purified by washing thrice with acidified alcohol (5% HCl in 60% alcohol), followed by washing in 60% alcohol until free of chloride (as tested with 1% $AgNO_3$) and finally in 90% alcohol. It was then air-dried and stored at 5°C.

(2) "Protopectin" - prepared from turnip roots, by Mr. W. Jarvis in this laboratory. The chopped turnip roots were homogenised in a Waring blendor and then extracted five times with cold water and five times with 90% alcohol. The final product which was mostly crude fibre was dried and stored at 5°C. 1.0 gm. of this material was suspended in 100 ml. of distilled water and kept overnight at 5°C before preparing the medium.

(3) Sodium pectate - 1.0 gm. of pectic acid (Californian Fruit Growers Exchange) was suspended in 50 ml. of water and 0.5 N NaOH added until the pectic acid dissolved. It was then made up to 100 ml. so as to give a 1% solution of sodium pectate.

Enzyme activity was determined by maceration of standard tissue slices or by the viscometric methods. The latter method will be described in Section V, B.

For maceration tests, discs 0.5 mm. thick were prepared with a sliding microtome from turgid cylinders of potato tubers cut with a cork-borer (diameter 8 mm.). Three such discs were placed in the enzyme solution and the average time (reaction time = R.T.) for loss of coherence (as tested by hand) determined. These tests were made at a definite pH and temperature. The pH was adjusted by means of phosphate or veronal acetate buffer; and most of the tests were carried out in the neighbourhood of pH 8 which is optimum for enzyme activity. In each such test 2.5 ml. of enzyme solution was added to 0.5 ml. of the buffer, and the pH of the mixture determined by indicators, in some cases both before and after maceration of the discs.

Dry weight of mycelium. The mycelium was taken out from the medicine flats, washed thoroughly with water and pressed to get rid of surplus water. It was then placed in the folds of weighed filter paper. They were kept at 70°C for 24 hours. They were then taken out and kept in a desiccator to cool and the final weight determined.

IV PRODUCTION OF ENZYME BY FUSARIUM MONILIFORME.

A. On Natural Media.

The behaviour of F.moniliforme on a variety of natural media, after 5 days' growth at 25°C, is set out in Table I. The media were prepared as follows:

Tomato, Potato, Turnip and Apple extracts; 200 gm. of the respective tissue boiled, strained through muslin and made up to 1 litre.

Tomato puree (Elvea, Italian) - 5 or 10 gm. diluted to 100 ml.

Culture fluids were adjusted to pH 8.0 with vernonal/acetate buffer before testing for enzyme activity by the maceration method. This was the practice throughout unless otherwise stated.

Table I.

Production of Enzyme on various natural Media.

Medium	pH Before	pH After	R.T.(min.)	Drywt. of mycl. (mg.)
Tomato ext., 20 ml.	5.1	8.7	40 - 45	212
5% Tomato puree, 20 ml.	4.8	8.6	110 - 120	118
10% " " " "	4.6	8.8	40 - 50	210
Potato ext., 20 ml.	7.1	8.6	80 - 90	165
" " , 10 ml.	7.1	8.6	50 - 60	110
Turnip ext., 20 ml.	6.4	8.2	130 - 150	138
" " , 10 ml.	6.4	8.2	80 - 90	115
Apple ext., 20 ml.	3.8	6.2	>20 hrs.	10

All media, except apple extract, were suitable for enzyme production. The latter remained acid throughout, whereas all the others became alkaline. The unsuitability of apple extract was not due to failure of the fungus to grow as it grew reasonably well on it. Potato extract was superior to turnip extracts, and both of them somewhat inferior to tomato extract. With potato and turnip extracts where different volumes of media were used the concentration of enzyme was higher with the lower volume (10 ml.) of nutrient medium was used. There was no clear relationship between amount of mycelium produced and enzyme activity.

As extracts from tomatoes seemed to be a particularly suitable medium, the effect of various concentrations was examined. Tomatoes were pulped by means of a Waring blendor, giving a puree (unfiltered) which was used as such or in various dilutions, as recorded in Table II.

Table II.

Effect of Concentration of Tomato Juice on Enzyme Production.

Medium	pH		R.T. (min.)	Dry wt. of mycl. (mg.)
	Before	After		
Full strength	4.7	8.3	25 - 30	328
80%	4.8	8.3	25 - 35	295
60%	4.8	8.3	30 - 35	272
40%	4.7	8.2	30 - 38	219
20%	5.2	8.4	45 - 50	189
10%	5.8	8.4	60 - 70	136

Under the conditions of the experiment enzyme activity and weight of mycelium diminished steadily with dilution of the culture medium, very active preparations being obtained at thehigher concentrations.

A similar series of determinations was made with a similar set of media after clarifying by high speed centrifuging (8-10,000 r.p.m. for 10 minutes). These were equally suitable for growth as the turbid media of Table II, and the pH changes occurring during growth were much the same, but enzyme activity in all was very low, being barely detectable after 20 hours. It should be noted that the filtered tomato extract used in the experiment of Table I was boiled, so that certain constituents were probably taken up in solution,

whereas in the experiment under consideration, they probably were removed by centrifuging before the medium was heated.

The corresponding behaviour of potato juice and turnip juice is shown in Table III and IV respectively. The materials were frozen by keeping them at -18°C overnight. They were then thawed, pressed and strained through muslin. The liquid thus obtained was used as such or in various dilutions.

Table III.

Effect of Concentration of Potato Juice on Enzyme Production.

Medium	pH		R.T. (min.)	Dry wt. of mycl. (mg.)
	Before	After		
Undiluted	6.3	9.2	30 - 35	184
Diluted to 1/2	6.4	9.3	45 - 50	159
" 1/4	6.5	9.5	65 - 70	115
" 1/8	6.7	9.2	100 -110	89

It is evident that the activity of enzyme and weight of mycelium decreased with dilutions of culture medium. There was not much variation in the initial and final pH's of the various media. In all cases, the final pH was distinctly alkaline and again active preparations were obtained at the higher concentrations.

Table IV.

Effect of Concentration of Turnip Juice on Enzyme Production.

Medium	pH Before	pH After	R.T.	Dry wt. of mycl. (mg.)
Undiluted	5.3	4.8	20 hours	113
Diluted to 1/2	5.4	4.8	20 "	125
" to 1/4	5.7	8.2	5-6 "	138
" 1/8	5.9	7.8	>20 "	150

Enzyme activity was low in all cases, specially in the undiluted medium. Fungal growth was also poorer the richer the medium, probably because of the low unfavourable pH values which persisted at these concentrations.

The behaviour of apple juice at various dilutions is shown in Table V. This medium was prepared in the manner described under Tables III and IV.

Table V.

Effect of Concentration of Apple Juice on Enzyme Production.

Medium	pH Before	pH After	R.T.	Dry wt. of mycl. (mg.)
Undiluted	3.3	3.2	>20 hours	78
Diluted to 1/2	3.3	3.4	"	86
" 1/4	3.4	3.6	"	91
" 1/8	3.5	3.7	"	83

The initial and final pH's of the media were very low and there was no enzyme activity on any medium. The growth of the fungus was poor, probably on account of the low pH. The high acidity was eliminated by adding 1% $CaCO_3$ to apple extract and to apple juice at full strength and at 1/4 dilution. The effect of this is given in Table VI.

Table VI.

Effect of buffering Apple Juice on Enzyme Production.

Medium	pH Before	pH After	R.T.	Dry wt. of mycl.(mg.)
Apple extract	3.9	4.6	>20 hours	155
" + 1% $CaCO_3$	7.6	7.2	"	163
Apple juice (undiluted)	3.3	3.2	"	78
" " + 1% $CaCO_3$	7.5	7.2	"	132
Apple juice (diluted to 1/4)	3.4	3.6	"	91
" " + 1% $CaCO_3$	7.6	7.3	"	140

The addition of $CaCO_3$ maintained the pH of the solutions within the range 7.2-7.6 but again there was no enzyme production in any case though the growth of the fungus was improved and comparable with that on the undiluted potato juice which gave active preparations. As a further modification of apple media, 0.5% glucose or 0.5% asparagine was added, with or without 1% $CaCO_3$, with the results shown in Table VII.

Table VII.

Effect of Glucose and Asparagine on Enzyme Production in Apple Juice.

Medium	pH Before	pH After	R.T.	Dry wt.of mycl.(mg.)
Apple extract	3.9	4.6	>20 hours	158
" + 0.5% glucose	3.9	4.4	"	132
" + " + 1% $CaCO_3$	7.6	7.3	"	165
" + 0.5% asparagine	4.2	7.8	"	162
" + " + 1% $CaCO_3$	7.6	7.4	"	167

None of the additions induced production of enzyme.

From Tables V - VII, it is clear that apple extract is highly unsuitable for the production of pectinase by F.moniliforme. This unsuitability is not removed by neutralising the high acidity of the medium nor by increasing its carbon or nitrogen content. Whatever the factor which inhibits enzyme formation it is not readily eliminated by dialysis as is shown by the results given in Table VIII. The apple juice used was first dialysed through a cellophane membrane, first for 6 hours against tap water at laboratory temperature and then for 48 hours against distilled water at 4°C.

Table VIII.

Effect of Dialysis on Enzyme Production in Apple Juice.

Medium	pH Before	pH After	R.T.	Dry wt. of mycl. (mg.)
Dialysed Apple juice diluted to 1/2	3.3	3.6	>20 hours	50
" " + 0.5% asparagine	3.8	8.5	"	85
" " + " + 1% $CaCO_3$	7.7	8.2	"	188
" "diluted to 1/4	3.6	4.8	"	44
" " + 0.5% asparagine	4.0	8.8	"	75
" " + " + 1% $CaCO_3$	7.8	8.5	"	113

B. On Synthetic Media.

The behaviour of F.moniliforme on a variety of synthetic media, after 4 days' growth at 25°C, is given in Table IX. The media used were as follows; in gm. per litre.

1. Glucose - asparagine medium: Glucose, 10.0; Asparagine, 0.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5.
2. Ashour's medium: Glucose, 5.0; starch, 25.0; asparagine, 4.5; K_3PO_4, 3.4; $MgSO_4.7H_2O$, 1.9 (1949).
3. Czapek's Dox medium: Sucrose, 30.0; $NaNO_3$, 2.0; KCl, 0.5; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01.
4. Glucose-peptone medium: Glucose, 10.0; peptone, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5.
5. Synthetic potato medium: Glucose, 2.0; potato starch, 40.0; asparagine, 1.8; peptone, 1.8; KCl, 0.15; K_3PO_4, 1.35; $MgSO_4.7H_2O$, 0.75; $FeCl_3.6H_2O$, a trace (Brown, 1925).
6. Simplified form of 5: Glucose, 2.0; asparagine, 2.0; K_3PO_4, 1.25; $MgSO_4.7H_2O$, 0.75.

Table IX.

Production of Enzyme on various Synthetic Media.

Medium	pH		R.T. (hours)	Dry wt. of mycl. (mg.)
	Before	After		
1	5.4	8.2	>20	189
2	7.4	7.8	4½ - 5	153
3	7.2	7.9	>20	212
4	5.8	7.8	"	272
5	7.6	7.9	20	214
6	7.7	8.2	"	80

On nearly all these media growth was good and there was no development of unfavourable pH reactions. Nevertheless enzyme production was poor in all.

Two of the media in Table IX, viz., 2 and 5 contained starch and showed some enzyme activity. It was thought worthwhile to test the effect of starch on media 1 and 4, which contained glucose and had asparagine and peptone respectively as source of nitrogen. The results are given in Table X.

Table X.

Effect of Starch on Enzyme Production.

Medium	pH Before	pH After	R.T.	Dry wt. of mycl.(mg.)
Glucose-asparagine	5.4	8.2	>20 hours	150
" + 5% starch	4.8	8.0	"	175
Glucose-peptone	5.8	7.8	"	215
" + 5% starch	5.6	8.0	"	243

The addition of starch in the medium did not induce the production of enzyme.

Since enzyme was produced in all the natural media tested except apple extract, andthere was no or very poor activity in synthetic media, the effect of adding a proportion of natural extract to a synthetic medium was studied. For this purpose, various combinations of Glucose-asparagine medium and 10% tomato puree were tried. The results are given in Table XI.

Combinations of Glucose-asparagine medium (A) and 10% Tomato puree (B):

A. 100 ml. A.

B. 100 ml. B.

C. 50 ml. A + 50 ml. B.

D. 50 ml. A + 50 ml. H_2O.

E. 50 ml. B + 50 ml. H_2O.

F. 80 ml. A + 20 ml. B.

G. 80 ml. H_2O + 20 ml. B.

H. 90 ml. A + 10 ml. B.

I. 90 ml. H_2O + 10 ml. B.

Table XI.

Effect of Tomato Puree in Glucose-Asparagine Medium on Enzyme Production.

Medium	pH		R.T. (mins.)	Dry wt. of mycl. (mg.)
	Before	After		
A	4.9	8.6	>20 hours	128
B	4.3	7.9	40-45	188
C	4.3	8.4	45-50	156
D	5.2	8.5	>20 hours	72
E	4.3	7.5	70-80	88
F	4.5	8.6	50-60	128
G	4.5	7.5	110-120	69
H	4.8	8.6	70-80	125
I	4.6	7.7	$2\frac{3}{4}$ - 3 hours	38

The addition of tomato puree induced the production of enzyme in the synthetic medium in all cases, but there was no enzyme production in its absence.

C. Effect of Addition of Pectin to Media.

The production of enzyme on the various natural extracts and also on synthetic media to which tomato puree was added led to the conclusion that some natural product induces enzyme formation under these conditions. Since many micro-organisms have been shown to produce pectic enzymes adaptively the effect of adding 1% citrus pectin (C.F.G.) to various synthetic media was studied. The results are given in Table XII.

Table XII.

Effect of Citrus Pectin on Enzyme Production.

Medium	pH		R.T.	Dry wt. of mycl. (mg.)
	Before	After		
Glucose-asparagine	5.4	8.2	>20 hours	153
" + 1% pectin	3.5	8.6	20-25 min.	196
Glucose-peptone	5.8	7.8	>20 hours	208
" + 1% pectin	3.6	3.2	5-6 "	209
Ashour's medium	7.4	7.8	4½-5 "	272
" + 1% pectin	6.2	5.4	100-110min.	292
Czapek's Dox medium	7.2	7.8	>20 hours	159
" + 1% pectin	3.8	5.3	4 "	181

The addition of pectin to the media considerably

increased enzyme activity in each case. The most active preparation was obtained with glucose-asparagine medium. The addition of pectin lowered the pH of the media and a low pH reaction persisted in all media containing pectin except the one with glucose-asparagine. The wide tolerance of acidity shown by the fungus with respect to growth is illustrated in the Table, but it is suggested that good enzyme formation takes place only when pectin is present and when an alkaline reaction develops.

Since active enzyme preparations were obtained by adding pectin to the synthetic media, its effect on natural media was also studied. 0.5% citrus pectin (C.F.G.) was added to natural media, the fungus grown for 5 days and the extract tested for enzyme activity. The results are set out in Tables XIII - XV for potato, turnip and apple respectively.

Table XIII.

Effect of Pectin in Potato Juice on Enzyme Production.

Medium	pH		R.T.(min.)	Dry wt. of mycl. (mg.)
	Before	After		
Undiluted	6.4	8.9	30-35	184
" + 0.5% pectin	6.0	8.8	"	209
Diluted to 1/2	6.4	9.0	45-50	157
" + 0.5% pectin	6.0	8.9	50-55	159
Diluted to 1/4	6.4	9.1	65-70	115
" + 0.5% pectin	6.2	9.0	70-75	121
Diluted to 1/8	6.6	9.2	110-120	89
" + 0.5% pectin	6.3	9.0	"	101

Table XIV.

Effect of Pectin in Turnip Juice on Enzyme Production.

Medium	pH		R.T.(hours)	Dry wt. of mycl.(mg.)
	Before	After		
Undiluted	5.3	4.8	>20	113
" + 5% pectin	4.9	4.7	"	119
Diluted to 1/2	5.4	4.8	20	125
" + 0.5% pectin	5.0	4.5	"	131
Diluted to 1/4	5.7	8.2	2½ - 3	138
" + 0.5% pectin	5.1	7.9	"	144
Diluted to 1/8	5.9	7.6	20	131
" + 0.5% pectin	5.2	7.3	"	138

Table XV.

Effect of Pectin in Apple Juice on Enzyme Production.

Medium	pH		R.T.	Dry wt. of mycl. (mg.)
	Before	After		
Undiluted	3.0	3.1	>20 hours	78
" + 0.5% pectin	2.8	2.8	"	63
" + " + 1% $CaCO_3$	6.6	6.7	"	132
Diluted to 1/2	3.0	3.2	"	86
" + 0.5% pectin	2.8	2.9	"	75
" + " + 1% $CaCO_3$	7.0	7.8	"	136
Diluted to 1/4	3.1	3.4	"	91
" + 0.5% pectin	3.2	3.3	"	83
" + " + 1% $CaCO_3$	7.1	7.9	"	140
Diluted to 1/8	3.4	3.7	"	83
" + 0.5% pectin	3.2	3.3	"	65
" + " + 1% $CaCO_3$	7.3	7.9	"	112

The addition of pectin to potato or turnip media slightly but regularly increased growth but had no material effect on enzyme production. On apple media the factor of high acidity which was somewhat intensified by addition of pectin was associated with poor growth of the fungus, but when the media contained $CaCO_3$, growth was much improved but

there was still no detectable enzyme. Summarising therefore - with potato, turnip and apple media, which are respectively good, poor and useless for the production of pectinase, the addition of pectin has no appreciable effect on their performance. As all three media , no doubt, already contain a certain amount of pectin, this result might well be expected; the relative unsuitability of turnip and apple media presumably not being due to lack of pectin but to some other factors of their composition. This is confirmed by the following experiment in which apple juice was added to a synthetic medium highly suitable for enzyme production. The results shown in Table XVI clearly demonstrate the inhibitory effect of apple juice on enzyme secretion.

Table XVI.

Effect of Apple Juice on Synthetic Media containing Pectin.

Medium	pH		R.T.(min.)	Dry wt. of mycl.(mg.)
	Before	After		
Synthetic only	3.2	8.4	12- 15	122
3 parts synthetic + 1 part apple juice.	3.3	3.5	120-130	125

From Table XII, it is seen that a glucose-asparagine medium which gave no pectinase enzyme under the cultural conditions adopted, produced an active preparation when 1% citrus pectin was added. A further slight improvement

in this respect was obtained when 0.1% NaCl was also added, as illustrated in Table XVII. The possible significance of NaCl was suggested by a chance observation that unwashed pectin gave a greater response than washed pectin preparations.

Table XVII

Effect of Sodium Chloride on Enzyme Production.

Medium	pH Before	pH After	R.T.(min)	Dry wt. of mycl (mg.)
Glucose-asp.med. + 1% pectin	4.0	8.3	20-25	131
" + " + 0.1% NaCl	3.8	8.2	15-20	125

The following medium:

Glucose - 1.0%
Pectin - 1.0%
Asparagine - 0.5%
KH_2PO_4 - 0.1%
$MgSO_4.7H_2O$ - 0.05%
NaCl - 0.1%

was therefore studied in detail with regard to the effect of concentration of its various constituents on enzyme production.

D. Effect of Concentration of Constituents on Enzyme Production.

The effect of omitting each constituent of the medium given on page was examined, with the results set on in Table XVIII.

Table XVIII.

Effect of each Constituent on Enzyme Production.

Medium	pH		R.T.(min.)	Dry wt. of mycl. (mg.)
	Before	After		
Full medium	3.8	8.2	15-20	125
" (- glucose)	3.8	7.8	40-45	78
" (- pectin)	5.0	8.8	>20 hours	69
" (- asparagine)	3.7	2.9	"	25
" (- KH_2PO_4)	3.9	5.5	"	negligible
" (- $MgSO_4.7H_2O$)	4.0	3.7	"	"
" (- NaCl)	4.0	8.3	20-25	122
" (- NaCl & glucose)	3.8	8.0	60-70	65

Removal of asparagine, KH_2PO_4 or $MgSO_4.7H_2O$ reduced growth and enzyme secretion almost completely. The latter effect was produced also when pectin was omitted but without any comparable depression of growth. Absence of glucose somewhat reduced growth and enzyme production, but not to an equal extent as there were other sources of carbon present.

The effect of glucose concentration is shown in Table XIX.

Table XIX.

Effect of Glucose Concentration on Enzyme Production.

% Glucose	pH		R.T.(min.)	Dry wt. of mycl. (mg.)
	Before	After		
1.5	3.5	3.8	50-60	175
1.0	3.5	7.2	15-20	163
0.5	3.5	8.3	10-15	119
0.25	3.5	8.3	15-20	88
0.1	3.5	8.2	25-30	78
0.0	3.6	8.0	45-50	63

With diminishing glucose content, the amount of growth also falls. Optimum enzyme activity is found in the range 1-0.25% glucose, on both sides of which it falls off. The higher concentrations of glucose have a tendency to maintain an acid reaction in the medium, and the falling off of enzyme activity at these concentrations is probably caused by this.

Table XX shows the effect of varying pectin content in the medium.

Table XX.

Effect of Concentration of Pectin on Enzyme Production.

% Pectin	pH		R.T.(min.)	Dry wt. of mycl. (mg.)
	Before	After		
2.0	3.5	8.2	10-15	138
1.5	3.6	8.4	"	131
1.0	3.8	8.5	"	125
0.5	4.4	8.6	20-25	100
0.25	5.1	8.5	60-70	75
0.0	4.9	8.8	>20 hours	69

There was a gradual increase in mycelial growth when the pectin content was raised. The lowest R.T. value was obtained with 1.0% pectin.

With diminishing asparagine content the amount of growth and enzyme activity steadily decreased as shown in Table XXI.

Table XXI.

Effect of Asparagine Concentration on Enzyme Production.

% Asparagine	pH Before	pH After	R.T.(min.)	Dry wt. of mycl. (mg.)
0.5	3.4	8.5	10-15	125
0.4	3.4	8.2	"	119
0.3	3.3	4.1	25-30	110
0.2	3.3	3.8	40-45	75
0.1	3.1	3.0	3-4 hours	53
0.0	3.0	2.9	>20 "	13

The effect of the concentration of KH_2PO_4 or $MgSO_4$ on growth or on enzyme production is slight until low values are reached. The results are given in Tables XXII and XXIII.

Table XXII.

Effect of KH_2PO_4 Concentration on Enzyme Production.

% KH_3PO_4	pH Before	pH After	R.T.(min.)	Dry wt. of mycl. (mg.)
0.2	3.5	8.0	10-15	119
0.1	"	8.2	"	119
0.05	3.4	8.3	15-20	113
0.025	"	8.1	20-25	100
0.01	"	7.9	30-35	69
0.0	"	4.4	>20 hours	13

Table XXIII.

Effect of $MgSO_4.7H_2O$ Concentration on Enzyme Production.

% $MgSO_4.7H_2O$	pH Before	pH After	R.T.(min.)	Dry wt. of mycl. (mg.)
0.2	3.3	8.5	10-15	131
0.1	3.3	8.4	"	131
0.05	3.4	8.4	"	125
0.025	"	8.1	20-25	113
0.01	"	7.9	30-35	106
0.0	"	3.7	>20 hours	16

The effect of NaCl concentration was also tested when it was found that over the range 0.5% to nil there was no measurable effect on growth. Maximum enzyme activity was shown at about 0.05% NaCl. At 0.5% NaCl, the R.T. of pectinase enzyme was 40-45 min. as against 10-15 min. at 0.05%.

On the basis of the foregoing results the composition of the medium used for enzyme preparation was modified to the following:

Glucose - 0.5%

Pectin - 1.0%

Asparagine - 0.4%

KH_2PO_4 - 0.1%

$MgSO_4.7H_2O$ - 0.05%

NaCl - 0.05%

This medium was used subsequently for the routine preparation of enzyme solutions in this work. It will be called "Standard medium".

E. Effect of Substitutions on Enzyme Production.

The effect of replacing glucose of the standard medium in equivalent amounts by other carbohydrates is set out in Table XXIV.

Table XXIV.

Effect of various Carbohydrates on Enzyme Production.

Carbohydrate	pH		R.T.(min.)	Dry wt. of mycl. (mg.)
	Before	After		
Glucose	3.5	8.1	10 - 15	119
Fructose	3.5	8.3	8 - 12	144
Sucrose	3.4	8.2	15 - 20	119
Lactose	3.5	8.2	30 - 35	131
Maltose	3.6	8.4	15 - 20	125
Starch	3.4	8.4	25 - 30	119
Cellulose	3.4	8.6	30 - 35	131
None.	3.5	8.2	45 - 50	69

Each of the seven carbohydrates tested produced good growth and similar changes in the pH of the media. Enzyme production was good in each case; glucose, fructose, sucrose

and maltose being somewhat superior to lactose, starch and cellulose.

The effect of replacing pectin in the standard medium by other pectic or allied substances at 1% concentration is shown in Table XXV.

Table XXV.

Effect of Various Carbon Compounds on Enzyme Production.

C Source	pH Before	pH After	R.T.(min.)	Dry wt. of mycl. (mg.)
Pectin	3.8	8.3	10-15	122
Protopectin	4.7	8.4	15-20	94
Na- pectate	4.8	8.9	35-40	131
Na- polypectate	5.2	8.9	45-50	121
D- galacturonic acid* (buffered)	6.2	8.4	35-40	125
" (unbuffered)	3.1	3.8	5-6 hours	87
Galactose	7.8	8.8	>20 hours	210
Gum arabic	7.7	9.0	"	138
Gum tragacanth	4.9	8.8	"	100
Alginic acid	7.1	8.9	"	56
Mucic acid	5.8	9.1	"	81

* N.B. This medium contained 1% $CaCO_3$.

It is apparent that only pectic materials or one of their breakdown product, D- galacturonic acid, are suitable

for enzyme production and that with D- galacturonic acid enzyme is not produced unless the medium is buffered at relatively high pH values.

The effect of D- galacturonic acid as a replacement for pectin on enzyme production was studied in greater detail using various proportions of it and glucose. The solutions were buffered by adding 1% $CaCO_3$. The results are given in Table XXVI.

Table XXVI.

Effect of D- galacturonic Acid in Standard Medium on Enzyme Production.

Medium		pH		R.T. (min.)	Dry wt. of mycl. (m.g.)
% D- galacturonic acid	% glucose	Before	After		
1.5	0.0	6.0	8.4	70-80	100
1.25	0.25	6.1	8.4	55-60	119
1.0	0.5	6.2	8.4	35-40	125
0.75	0.75	6.2	8.2	60-70	131
0.5	1.0	6.5	8.3	5-6 hours	138
0.0	1.5	6.9	8.6	>20 hours	150

Maximum activity was obtained with 1% D- galacturonic acid and 0.5% glucose. With other proportions the activity diminished and there was little or no enzyme production in the absence of D- galacturonic acid. The weight of mycelium

increased steadily with increase in glucose concentration but this was not correlated with increased enzyme production.

The effect of replacing asparagine of the standard medium by the nitrogen sources listed below at equivalent % N is shown in Table XXVII.

N- sources	% N	% Used.
Peptone	15.0	0.56
NH_4NO_3	35.0	0.24
NH_4Cl	26.0	0.32
$NaNO_3$	16.5	0.51
KNO_3	14.0	0.6
Asparagine (as in standard med.)	21.2	0.4

The experiments were set up in two series, with and without 1% $CaCO_3$.

Table XXVII (a)

Effect of Nitrogen Sources on Enzyme Production (unbuffered).

N Source	pH Before	pH After	R.T.	Dry wt. of mycl. (mg.)
Asparagine	3.5	8.2	10-15 min.	119
Peptone	3.4	3.3	6 hours	125
NH_4NO_3	2.7	2.8	>20 hours	22
NH_4Cl	2.7	2.3	"	16
$NaNO_3$	2.6	2.7	"	25
KNO_3	2.8	2.8	"	31
Nil	2.8	2.8	"	22

Table XXVII (b)

Effect of Nitrogen Sources on Enzyme Production (buffered).

N Source	pH Before	pH After	R.T.(min.)	Dry wt. of mycl. (mg.)
Asparagine	7.1	8.5	15-20	122
Peptone	6.7	8.2	"	134
NH_4NO_3	7.0	7.6	40-45	116
NH_4Cl	6.1	7.5	35-40	103
$NaNO_3$	6.1	8.0	60-70	116
KNO_3	7.0	8.1	30-35	100
Nil	7.1	6.5	>20 hours	25

These results show that a variety of N sources are suitable for enzyme production if the pH of the medium during growth is sufficiently high. Asparagine and peptone are best under these conditions. In unbuffered media only asparagine produces an alkaline drift and is the only N source producing active enzyme solution.

F. Extraction of Endo-enzyme from the Mycelium.

Mycelium from active exo-enzyme producing cultures were collected. It was washed thoroughly with distilled water and pressed gently to remove the surplus. It was then placed in acetone at -18°C for 30 minutes. The acetone was removed and the mycelial mat dried in a vacuum desiccator. 1.5 gm. of this was ground with sand and 50 ml. water in the cold room and the suspension kept overnight at 5°C. The suspension was then centrifuged at 8000 r.p.m. for 5 minutes and the clear liquid decanted and tested for enzyme on 0.5 mm. thick potato discs. The pH of the extract obtained was 6.8 and the reaction time 18-22 mins. After adjusting to pH 8.0 the reaction time was 15-20 mins. A reasonably active endo-enzyme preparation was therefore obtained by this treatment.

G. Extraction of Enzyme from Rotted Tissues.

Rotted tissue obtained from apples or tomatoes was collected, ground into a paste, and then pressed through muslin. The liquid obtained was centrifuged at 8000 r.p.m. for 5 minutes. The clear supernatant liquid was decanted and tested at theinitial and optimum pH values. In the controls, healthy tissue of apples or tomatoes was similarly treated.

Table XXVIII.

Enzyme Activity of Extracts from Rotted Tissue.

Extracts	pH	R.T.
Apple rot	4.4	5 hours
Control	3.5	>20 hours
Apple rot	8.0	150 mins.
Control	8.0	>20 hours
Tomato rot	4.8	130-140 mins.
Control	4.4	>20 hours
Tomato rot	8.0	70-80 mins.
Control	8.0	>20 hours

The extracts from the rotted tissue showed some enzyme activity in both cases, the activity in terms of R.T. increasing as the pH of the extract was increased from its initial low value to 8.0.

V. PROPERTIES OF THE ENZYME.

A. By Maceration Methods.

(a) Effect of dilution.

An active enzyme preparation was diluted with varying amounts of an autoclaved enzyme solution and tested for enzyme activity in the usual way. Activity, expressed as $1000 \times \frac{1}{R.T.(min.)}$, was plotted against enzyme units to give the dilution curve shown in Fig. 1. This curve was subsequently used to estimate the number of enzyme units in solutions of unknown activity e.g., a solution having a R.T. of 20 mins. andactivity $\frac{1000}{R.T.} = 50$, contains 60 enzyme units (E.U.).

(b) Thermal inactivation.

5.0 ml. samples of an active enzyme solution were transferred to thin glass vials in water-baths at various temperatures. After 2 or 5 minutes at a particular temperature, they were placed in ice-water. When all samples were ready, they were tested for enzyme activity in the usual way. The activity expressed as enzyme units was plotted against temperature to give the temperature inactivation curves shown in Fig. 2.

With 5 minutes exposure, inactivation was rapid over the range 40-60°C then more gradual until at 90°C only

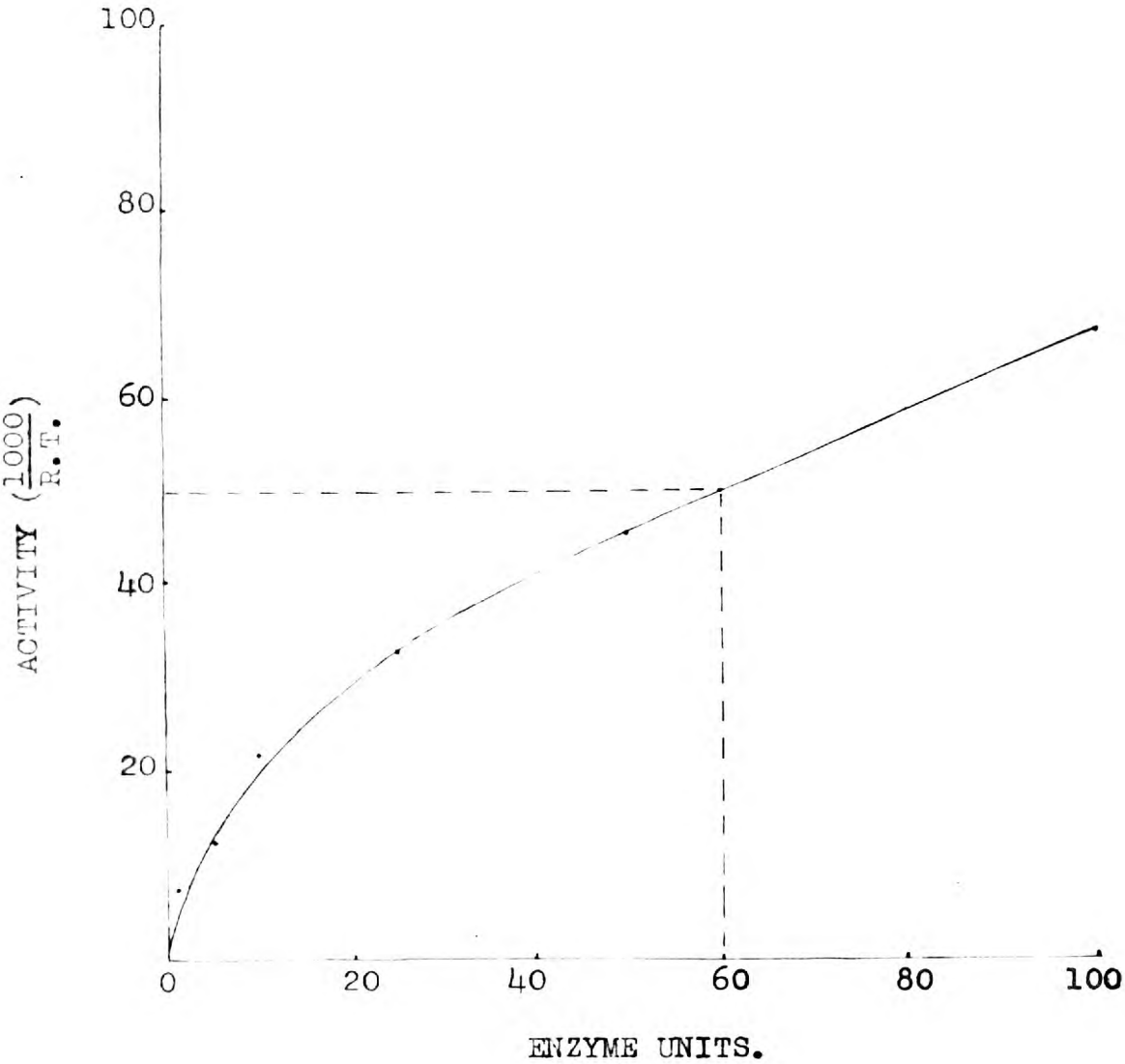


Fig. 1. **Effect of dilution on the activity of crude <u>F.moniliforme</u> enzyme preparation.**

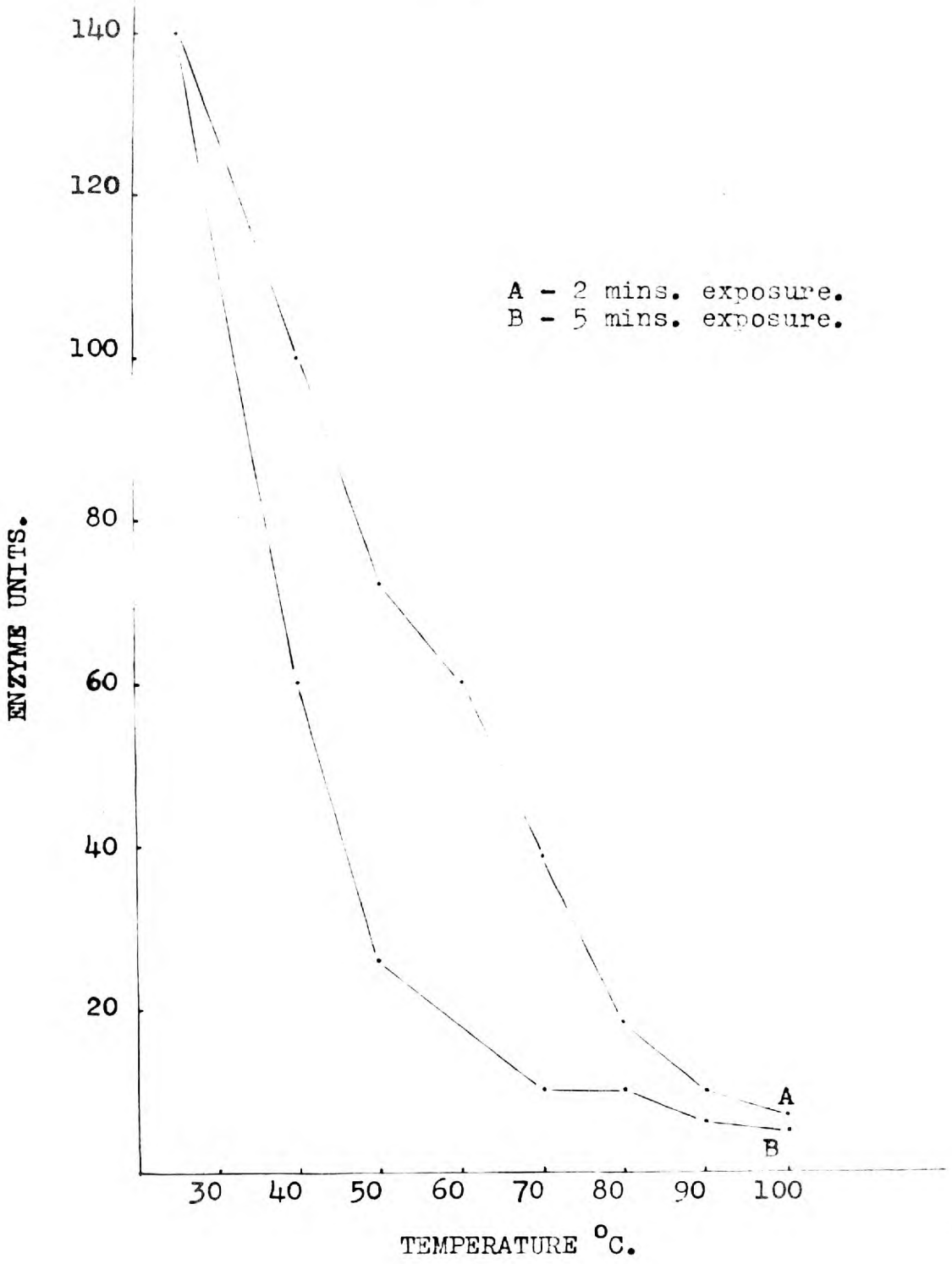


Fig. 2. Effect of heating on the activity of F.moniliforme enzyme preparation.

some 6% of the original activity remained. With 2 minutes exposure there was considerably less inactivation at temperatures up to 70°C. Above this temperature the curves come together. Complete inactivation of the enzyme was effected either by heating it for 20 minutes at 100°C or by autoclaving at 120°C for 15 minutes.

(c) Effect of pH.

The activity was determined of samples of an active enzyme solution adjusted to various pH values in the range 2.0 - 10.0. The pH of the samples was taken at intervals during these determinations and also when maceration of the discs was complete. No significant variations from the initial values were noted. Activity expressed as enzyme units was plotted against pH to give the curve shown in Fig. 3.

The optimum pH was in the range 8.0 - 9.0 and activity fell rapidly on either side. There was little or no activity at pH 5.0 and no activity was detected at pH 2.0 in 24 hours. All discs lost their turgidity at lower pH values i.e., below 5.0.

(d) Effect of dialysis.

The enzyme was dialysed using cellophane tubes first in running tap-water for 6 hours and then in distilled water for

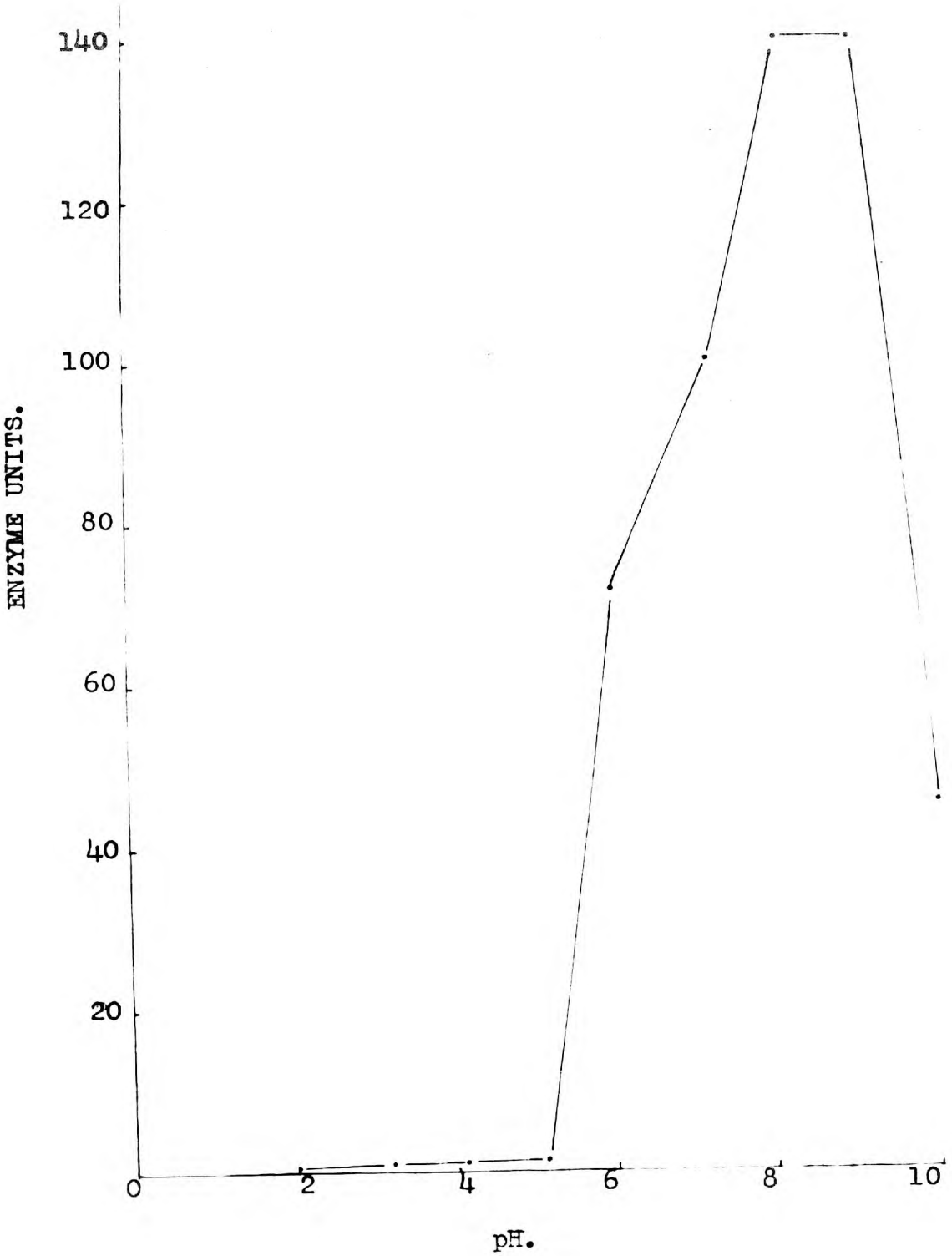


Fig. 3. Effect of pH on the activity of F.moniliforme enzyme preparation.

18 hours at 4°C. The procedure outlined in (a) was then followed using active solutions before and after dialysis. The results are given in Table XXIX.

Table XXIX.

Effect of Dialysis on Enzyme Activity.

Dilutions.	Undialysed		Dialysed	
	R.T.(min.)	E.U.	R.T.(min.)	E.U.
Undiluted	10-15	66.6	10-15	66.6
50%	18-22	45.5	18-22	45.5
25%	25-30	33.3	25-28	35.7
10%	40-45	22.2	35-40	25.0
5%	70-80	12.5	65-70	14.3
1%	135-150	6.6	120-135	7.4

The dialysed preparation was a little more active than the untreated solution at the higher dilutions.

(e) Effect of concentration of phosphate.

The effect of various concentrations of phosphate buffer at pH 8.0 on enzyme activity was tested with the results illustrated in Fig. 4.

There is some inhibition above M/50 and a rapid increase at M/30 and almost complete loss of activity at M/10.

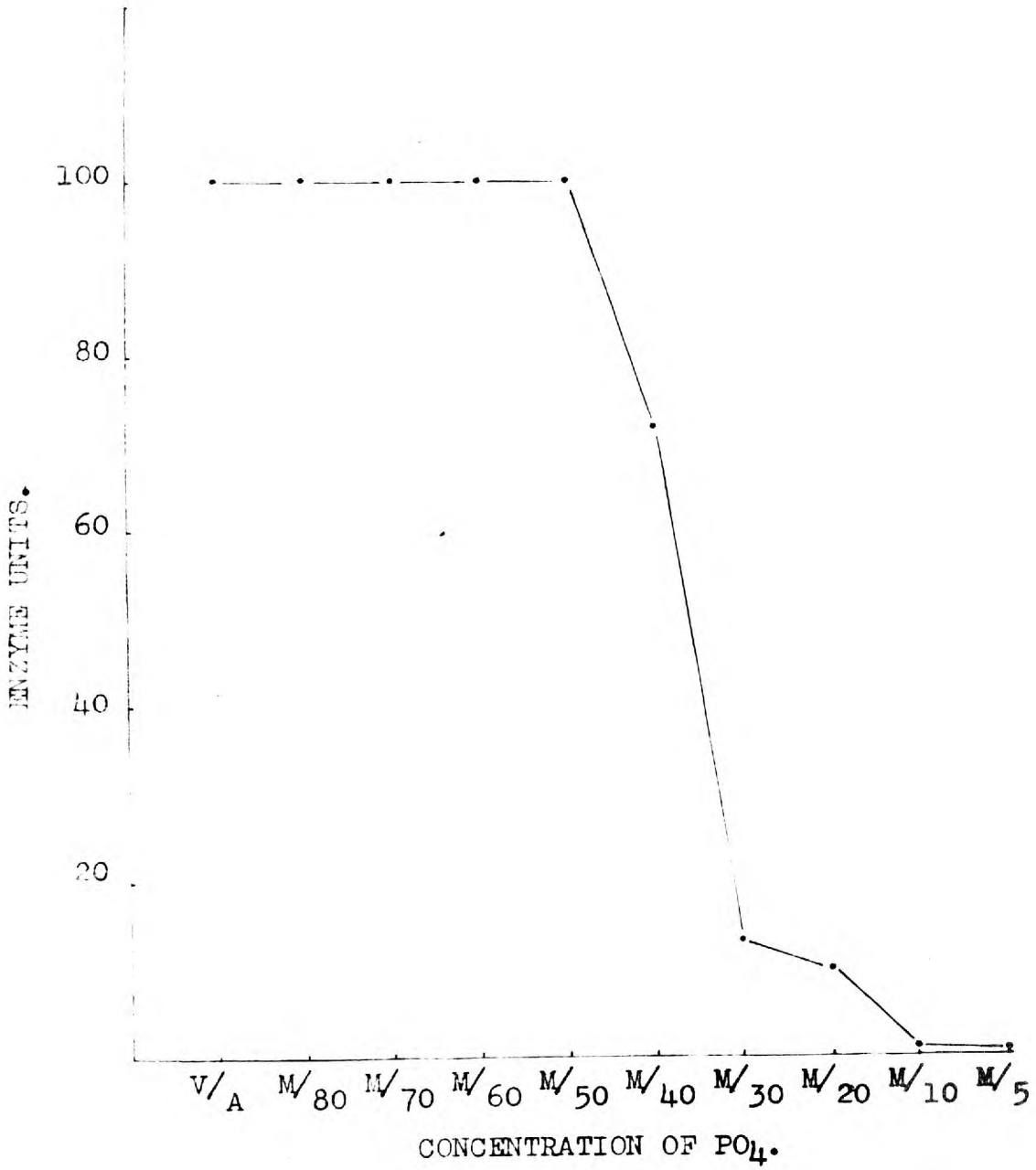


Fig. 4. Effect of various concentrations of phosphate (KH_2PO_4 - Na_2HPO_4) on the activity of F.moniliforme enzyme preparation.

(f) Effect of temperature

While conducting the maceration tests with enzyme solutions, it was observed that the R.T. was affected by seasonal variation in the room temperature. In order to assès its importance, the effect of temperature on the R.T. was studied. Two enzyme samples of somewhat different activities at 25°C were used in this case. Fig. 5 illustrates the results obtained.

Q 10 values of 3.6-4.0 were obtained over the ranges 5-15 and 15-20°C and 2.6-3.0 over the range 15-25°C. These high values emphasise the desirability of conducting maceration tests at constant temperatures particularly with relatively inactive preparations.

(g) Effect of storage.

Samples of an enzyme solution were stored at various temperatures under toluene. The activity of these samples was tested at intervals of 1, 5 and 10 days. The results are illustrated in Fig. 6.

Loss in activity at 20°C and 25°C was relatively rapid. It was slight at 5°C and not detectable at -18°C.

(h) Effect of precipitation.

(i) Effect of pH.

5.0 ml. samples of an enzyme solution were adjusted to various pH values with veronal/acetate buffers. Equal

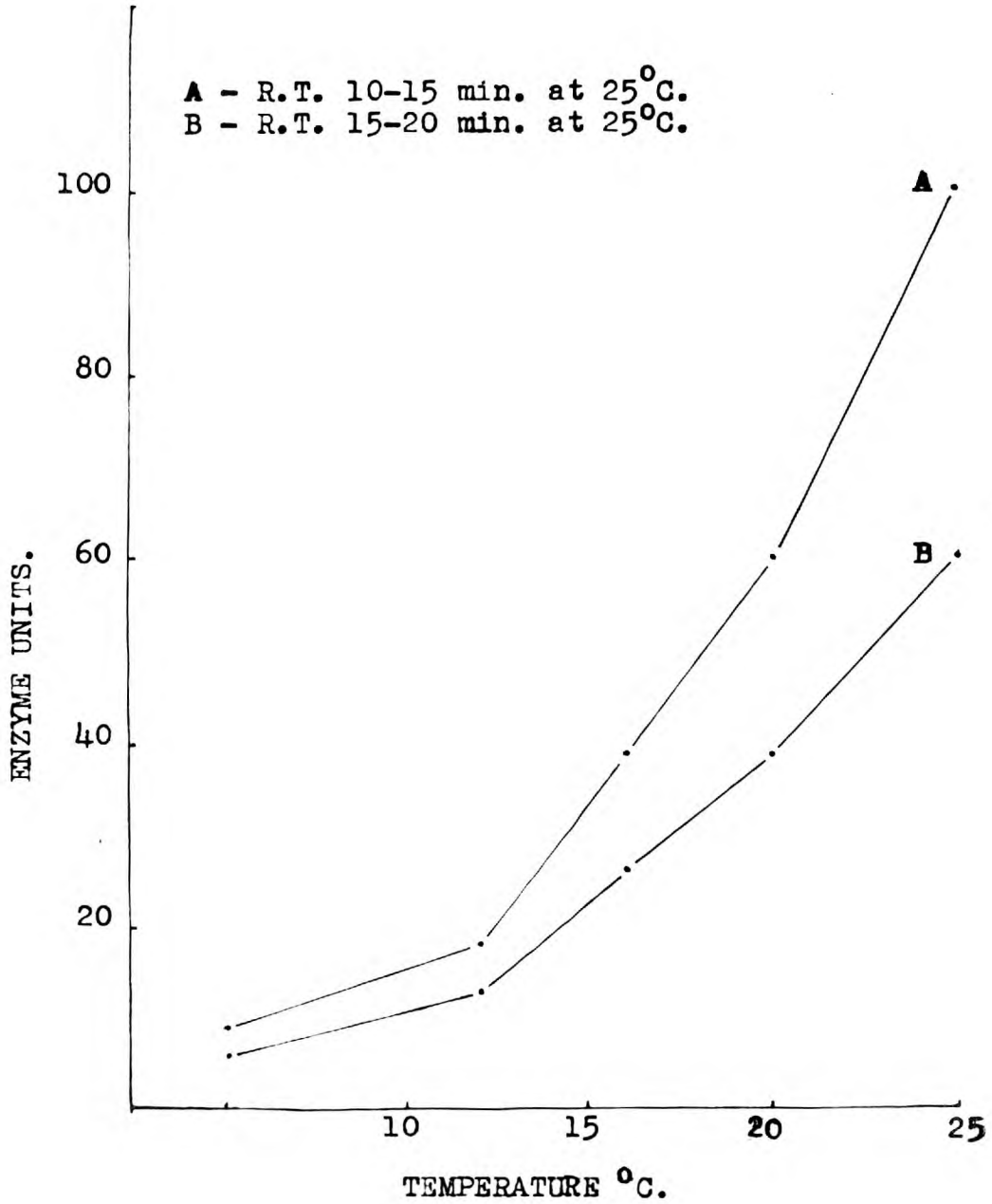


Fig. 5. Effect of temperature on the activity of F.moniliforme enzyme preparation.

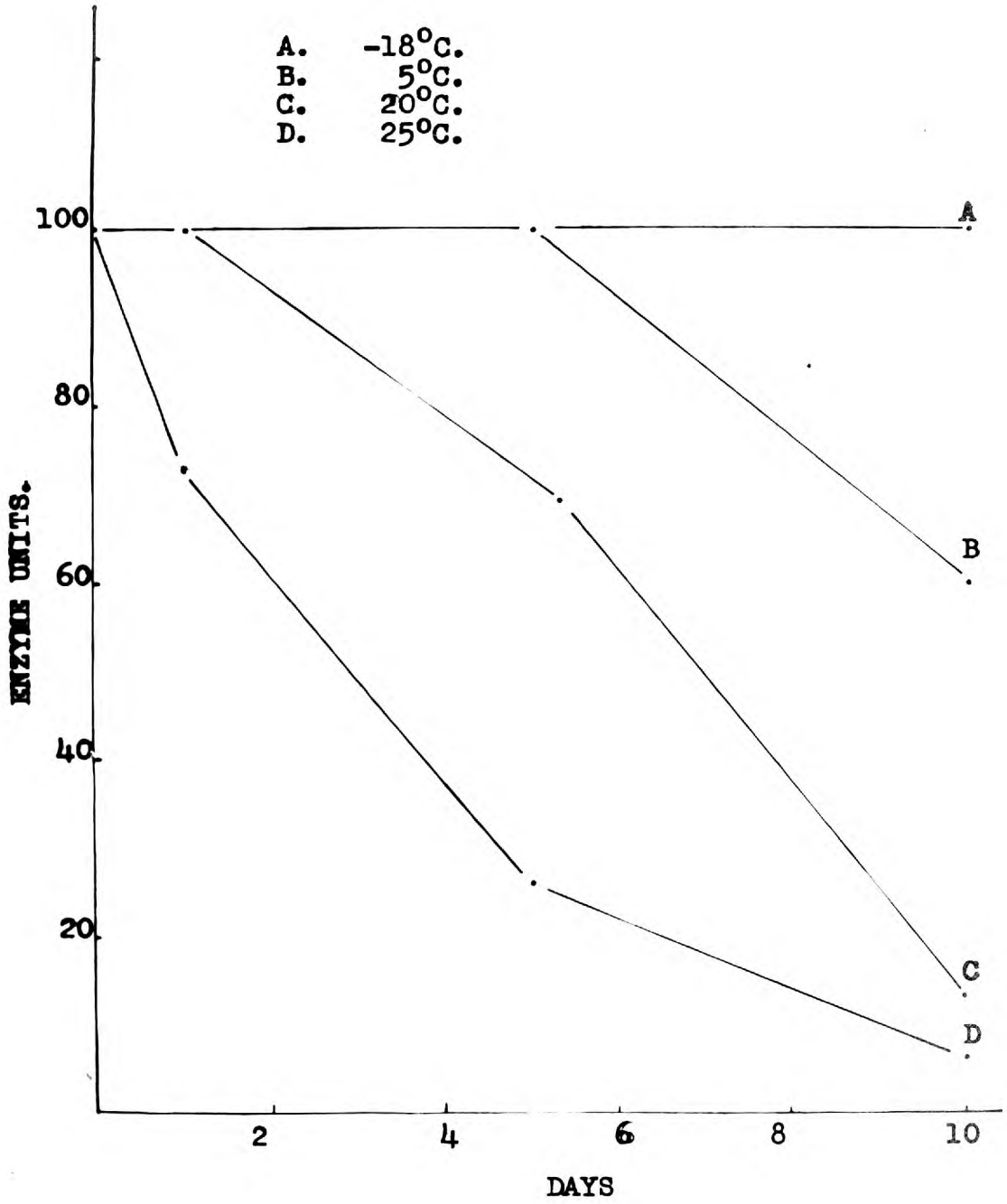


Fig. 6. Effect of storage on the activity of F.moniliforme enzyme solution.

volumes of acetone or alcohol were added to these samples which were then kept at 5°C for 5-10 minutes. The pH of the solutions was checked and adjusted to the original, where necessary. They were kept for 2 hours at 5°C and the precipitate which formed in each case was collected by centrifuging. They were then kept in the vacuum desiccator for 20-30 minutes to remove most of the acetone or alcohol. The precipitate was dissolved in 2.5 ml. of distilled water and activity testedat the pH 8.0. Fig. 7 illustrates the effect of pH on the precipitation.

The optimum pH for precipitation with acetone was 6.0 with reasonable recovery over the range 5.3-8.0. With alcohol, the optimum was pH 8.0 with a range of 5.3-8.0 for good recovery.

(ii) Effect of concentrations of acetone or alcohol.

Various concentrations of acetone or alcohol were used for the precipitation at pH's 6.0 and 8.0 respectively. Fig. 8 illustrates the results obtained.

Maximum recovery was obtained with concentrations of 60% or above.

Precipitation with ammonium sulphate at various concentrations up to 70% saturation and at pH's 6.0 and 8.0 did not give active precipitates.

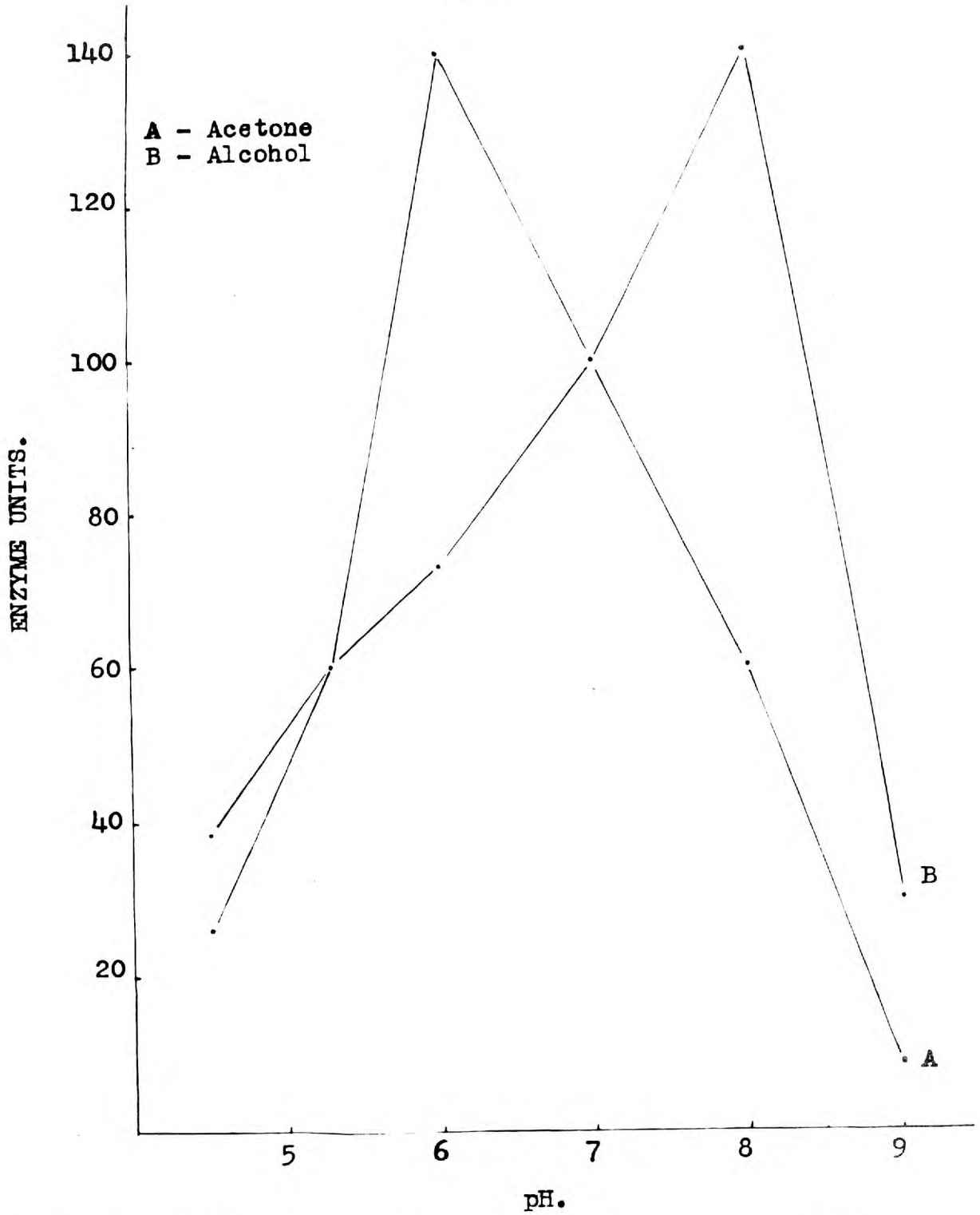


Fig. 7. Effect of pH on the precipitation of F.moniliforme Enzyme.

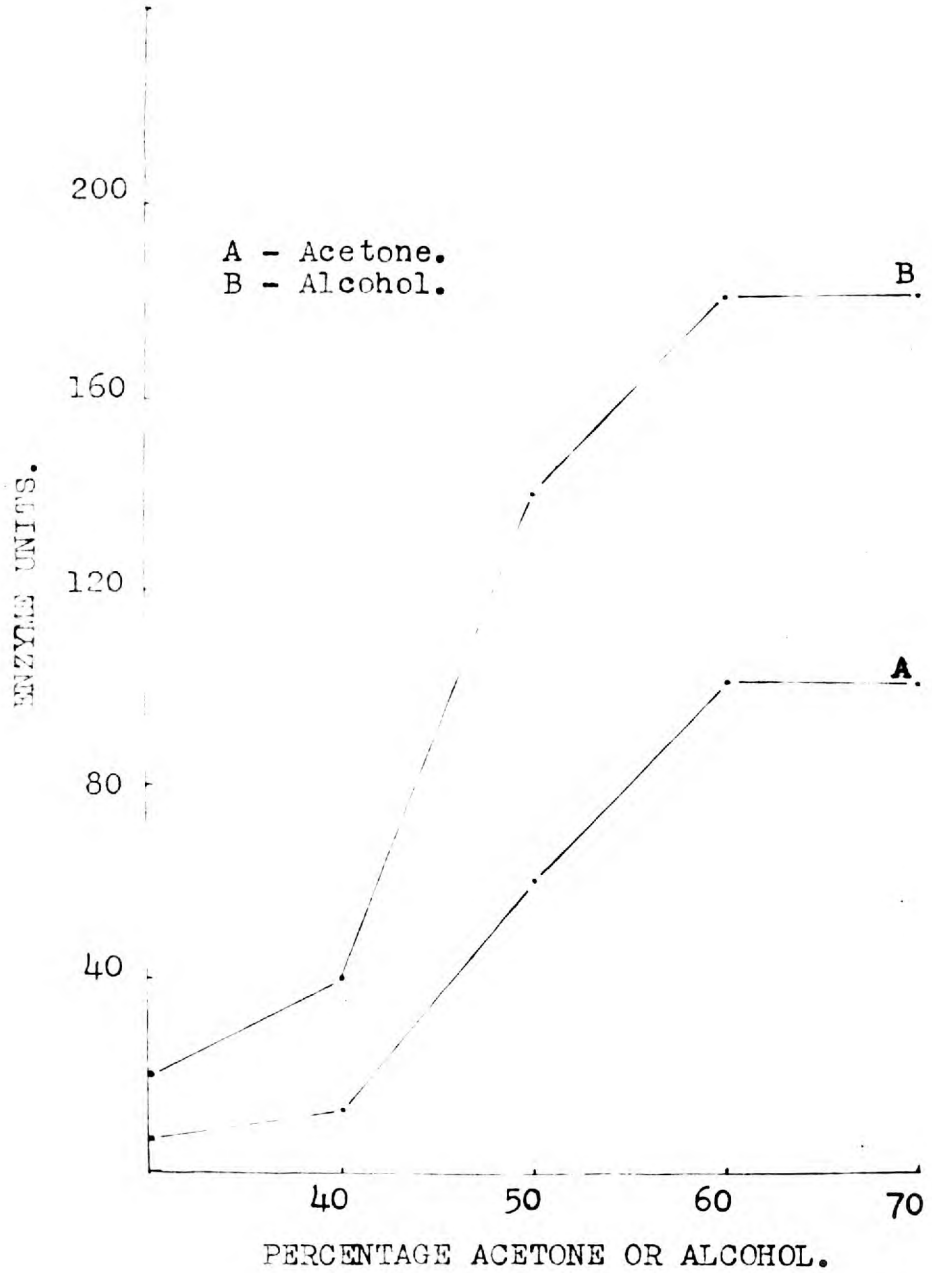


Fig. 8. Effect of concentration of acetone or alcohol on the precipitation of F.moniliforme enzyme.

B. By Viscometric Methods.

Many pectic materials which have not been significantly degraded during preparation give colloidal and highly viscous solutions at low concentrations. One of the most striking effects of enzymes of the pectinase group is to cause a rapid drop in viscosity of such solutions under suitable conditions and since such changes in viscosity can be rapidly and accurately determined with relatively simple apparatus, viscometric methods have been much used for measuring the activity of preparations of these enzymes under prescribed conditions (Wood and Tribe, 1953).

Pectin is a very suitable material to use for such determinations but in view of the influence of methoxyl groups on the activity of certain polygalacturonases if difficulties in the interpretation of results are to be avoided, it can only be used in the absence of pectinesterase. Active preparations from F.moniliforme cultures were therefore first assayed for pectinesterase activity. At the optimum pH for pectinase action, little activity was detected over the relatively short period (25 minutes) used in the viscometric tests. In routine determinations a pectin solution was therefore used.

During the course of preliminary experiments, it was found that phosphate inhibited enzyme activity. It was therefore thought worthwhile to run two series of experiments

using undialysed and dialysed enzyme solutions.

The general composition of the substrate used in these viscosity tests was as follows:

1% pectin	-	5.0 ml.
N/10 NaOH	-	0.8 ml.
Veronal/acetate buffer at pH 7.9	-	2.0 ml.
Distilled water	-	0.2 ml.

The above was placed in a water-bath at 25°C. An Ostwald viscometer, thoroughly cleaned and dried was also placed in the water-bath and both were left for a few minutes to acquire the temperature of the bath. Then 2.0 ml. of an enzyme solution was added to the substrate and a stop-watch was started at the same time. The substrate was stirred quickly and then 5.0 ml. was transferred to the viscometer. Viscosity readings were taken at intervals of 1, 3, 6, 10, 15, 20 and 25 mins. after adding the enzyme. In the case of the sodium polypectate and sodium pectate solutions which were used in some experiments, 0.1 and 0.2 ml. of N/10 NaOH were used respectively. The amount of water in the substrate was adjusted accordingly. The following precautions were taken while conducting the viscosity tests.

(a) When pipetting pectin or pectate solutions, the pipette was hung in the vertical position, and the solution was allowed to drain for 5 mins. A bulb pipette with wide aperture was used for this purpose.

(b) The viscometer was washed each time thrice with distilled water and finally rinsed with 90% alcohol, then air was drawn through till completely dry.

(c) Pipettes used for transferring the enzyme solution and the substrate were washed thoroughly with distilled water each time.

(d) When transferring the substrate to the viscometer, the pipette was placed on the viscometer, not touching its sides and was allowed to drain for 10 minutes.

Smooth curves were obtained when viscosity, represented by the time of flow of the test solution through the capillary of the viscometer, was plotted against time. From such viscosity/time curves the activity of unknown solutions was estimated in the way now to be described.

(a) Effect of dilution on enzyme activity.

Untreated and dialysed enzyme solutions were diluted with corresponding autoclaved preparations to give a series of solutions having varying active enzyme concentrations in the same concentration of thermostable substances. The effect of these on 0.5% pectin solutions was studied and a series of viscosity/time curves drawn for the dialysed and untreated preparations.

Similar series with other organisms have been used as follows. With Botrytis cinerea, Bacterium aroideae (Wood and Tribe, 1953) and Pythium debaryanum, a linear relation

is obtainedwhen the enzyme concentration is plotted against activity represented as the inverse of the time taken by a solution to show a given loss of viscosity. This being so the activity of an unknown solution may be estimated directly from the time taken for it to produce a given viscosity change under standard conditions.

Other workers (Roboz et al. 1952) have shown that with preparations from Neurospora crassa a linear relation is obtained when the logarithm of the enzyme concentration is plotted against activity represented by the percentage of viscosity of the test solutions in a given time.

Neither of these methods gave linear relations with the data referred to above. An arbitrary standard curve was therefore drawn showing the relation between enzyme concentration and percentage drop in viscosity after 25 minutes.

The percentage loss of viscosity was calculated as follows:

Vo = Viscosity at time 0.

Vf = " " " = water.

Vt = " " " t.

$$\% \text{ loss of viscosity} = \frac{Vo - Vt}{Vo - Vf} \times 100$$

These curves (Fig. 9.) were drawn for both dialysed and untreated enzyme solutions and subsequently used for estimating the activity of solutions of unknown enzyme concentrations.

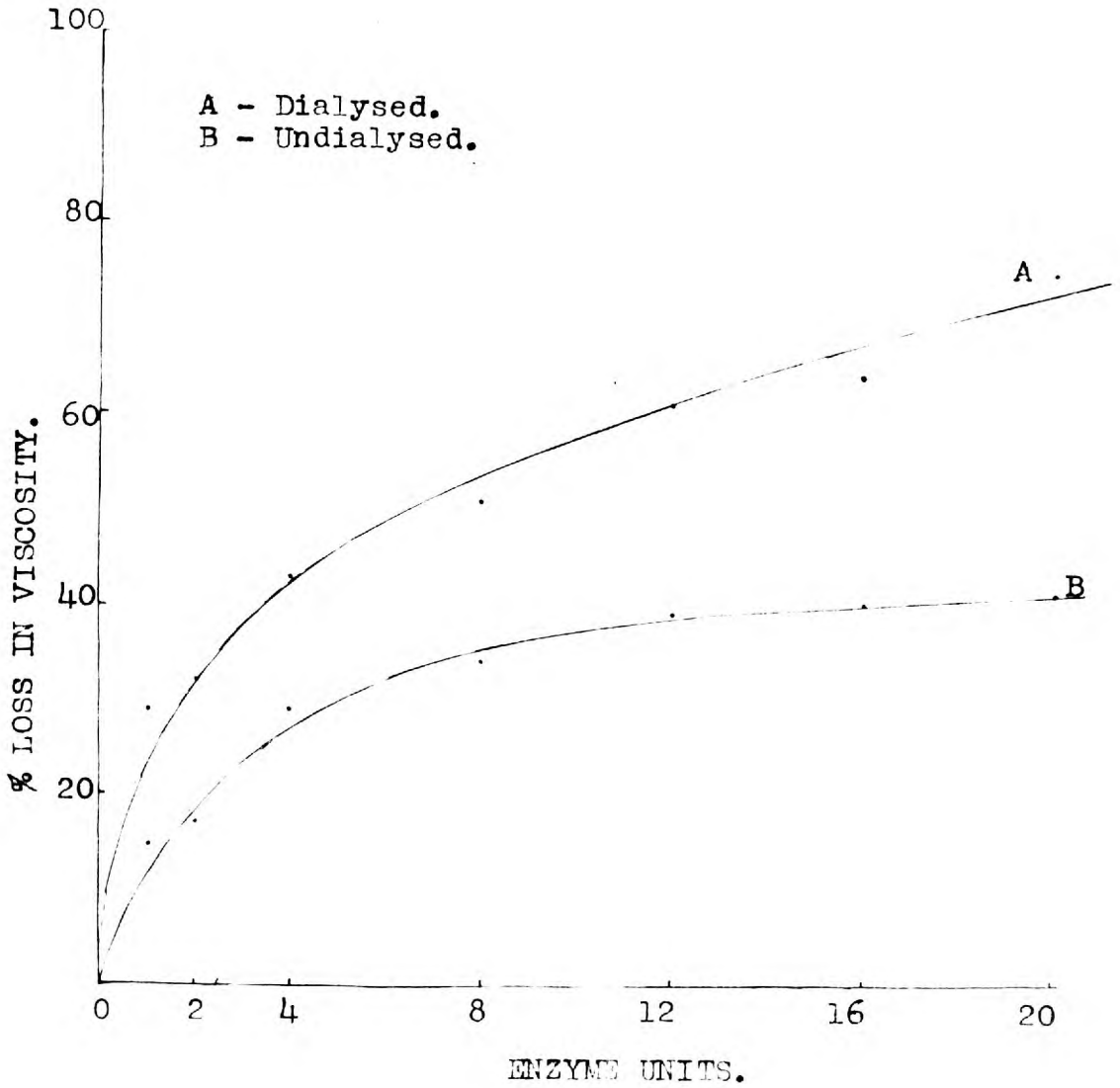


Fig. 9. Effect of dilution on the activity of F.moniliforme enzyme (viscometric method).

(b) Thermal inactivation.

5.0 ml. samples of dialysed and undialysed enzyme solutions were heated at various temperatures for 5 minutes (for details see page 41). The temperature inactivation curves are shown in Fig. 10. The position of the first point of curve B cannot be given exactly owing to the fact that the enzyme preparation used was slightly more active than the one used for the calibration curve.

The activity of the undialysed enzyme solution was rapidly reduced by exposure over the range 40-50°C and almost completely lost after 5 minutes at 70°C. Some recovery of activity was obtained after heating at 80°C, there was a further decrease at 90 and 100°C. The dialysed enzyme preparation is considerably less affected by high temperatures. Again, some increase in activity occurs over the range 70-80°C. In contrast with the undialysed solution, some 20% of the original activity remained after 5 minutes at 100°C. Autoclaving at 120°C for 15 minutes led to complete loss of activity in both cases.

(c) Effect of pH.

The effect of pH on the activity of dialysed and undialysed enzyme solutions has been illustrated in Fig. 11.

The substrates, before the addition of 2.0 ml. of dialysed or undialysed enzyme solutions, were adjusted to

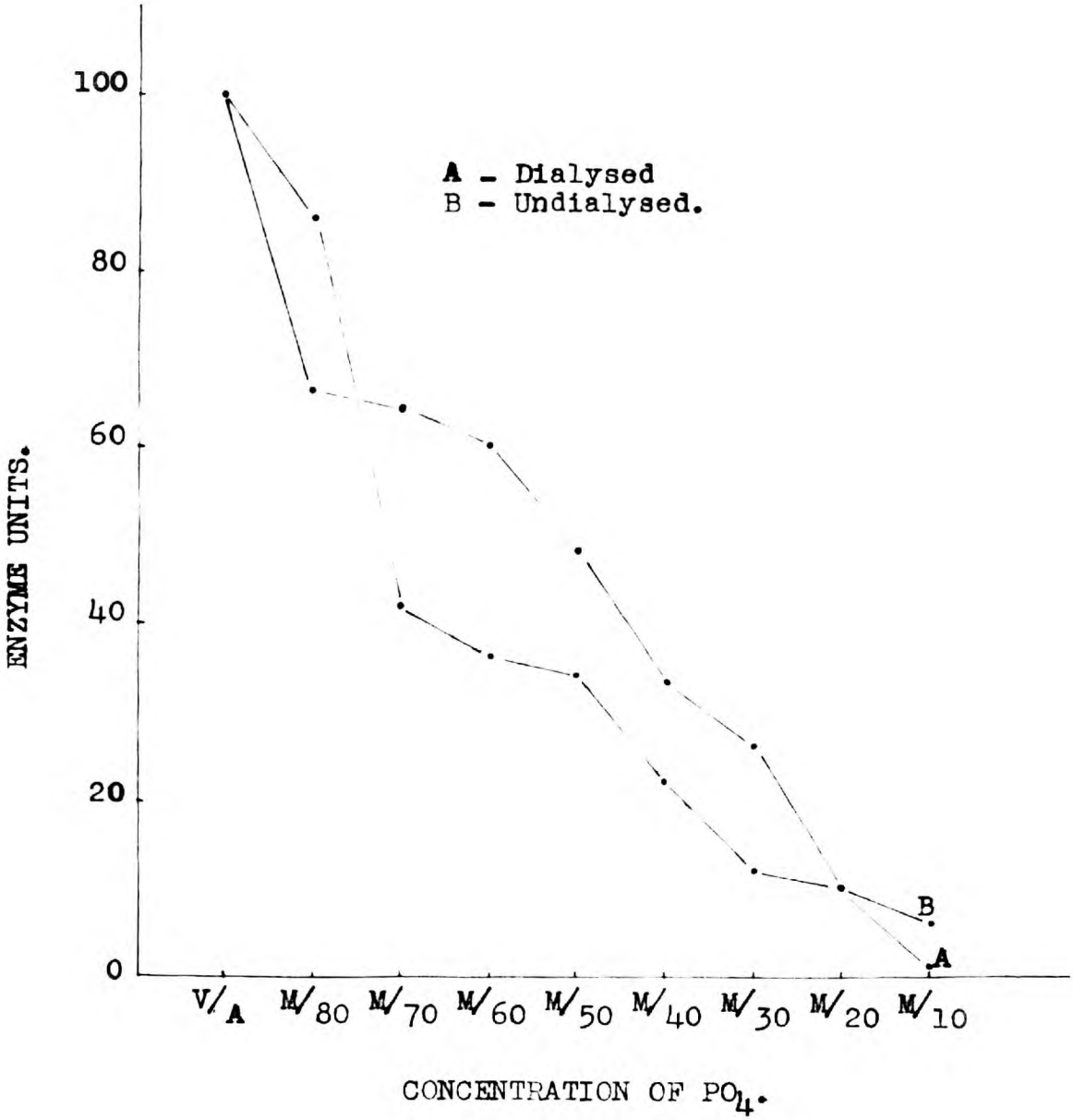


Fig. 10. Effect of heating on the activity of F.moniliforme enzyme (viscometric method).

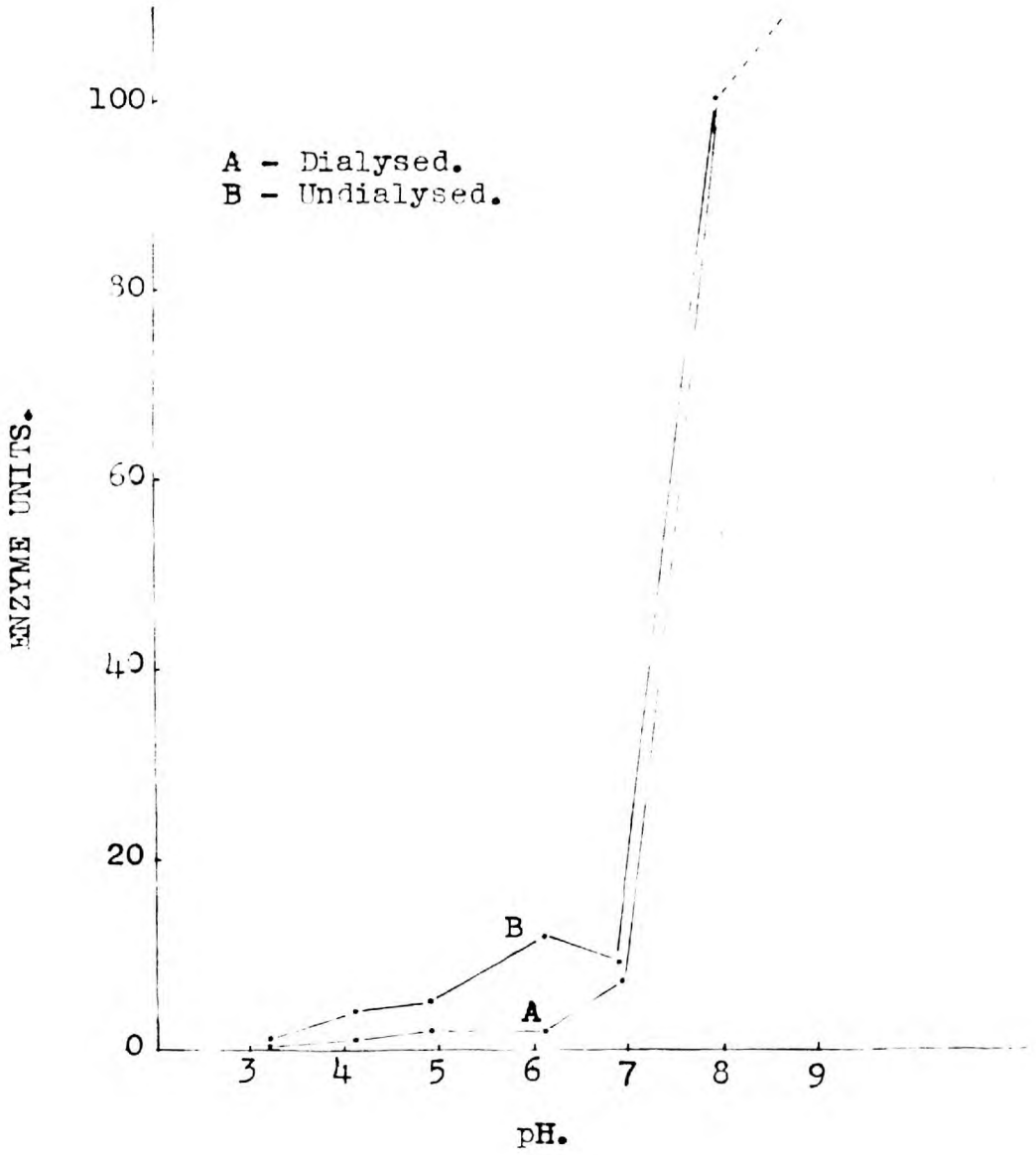


Fig. 11. Effect of pH on the activity of F.moniliforme enzyme (viscometric method).

various pH values with 0.1 N NaOH and veronal/acetate buffers at the same pH's. There was no significant change of pH during the viscosity determinations. Both preparations behaved similarly. There was negligible activity at pH 3.2 and only a slight increase up to pH 6.9. A tenfold increase in activity occurred between pH 6.9 and 7.9 and further increases above pH 7.9. The latter are not included in Fig. 11 as, owing to the effect of these high pH values in reducing the viscosity of the controls, precise interpretation of the data is difficult. It was apparent that the optimum was at pH 7.9 or above. Routine determinations of activity were therefore made with solutions buffered at pH 7.9.

(d) Effect of phosphate.

Samples of a 1.0% pectin solution were first adjusted to pH 7.9 with 0.1 N NaOH and to them were them added 2.0 ml. of various concentrations of Na_2HPO_4-KH_2PO_4 buffer at 8.0. The procedure thereafter was as already described. The results are given in Fig. 12. Similar curves were obtained with both preparations.

Some inhibition of activity was observed at a concentration of M/80. The inhibition increased steadily with concentration until at M/10 only 1 to 6% of the original activity was obtained. It is to be noted however that

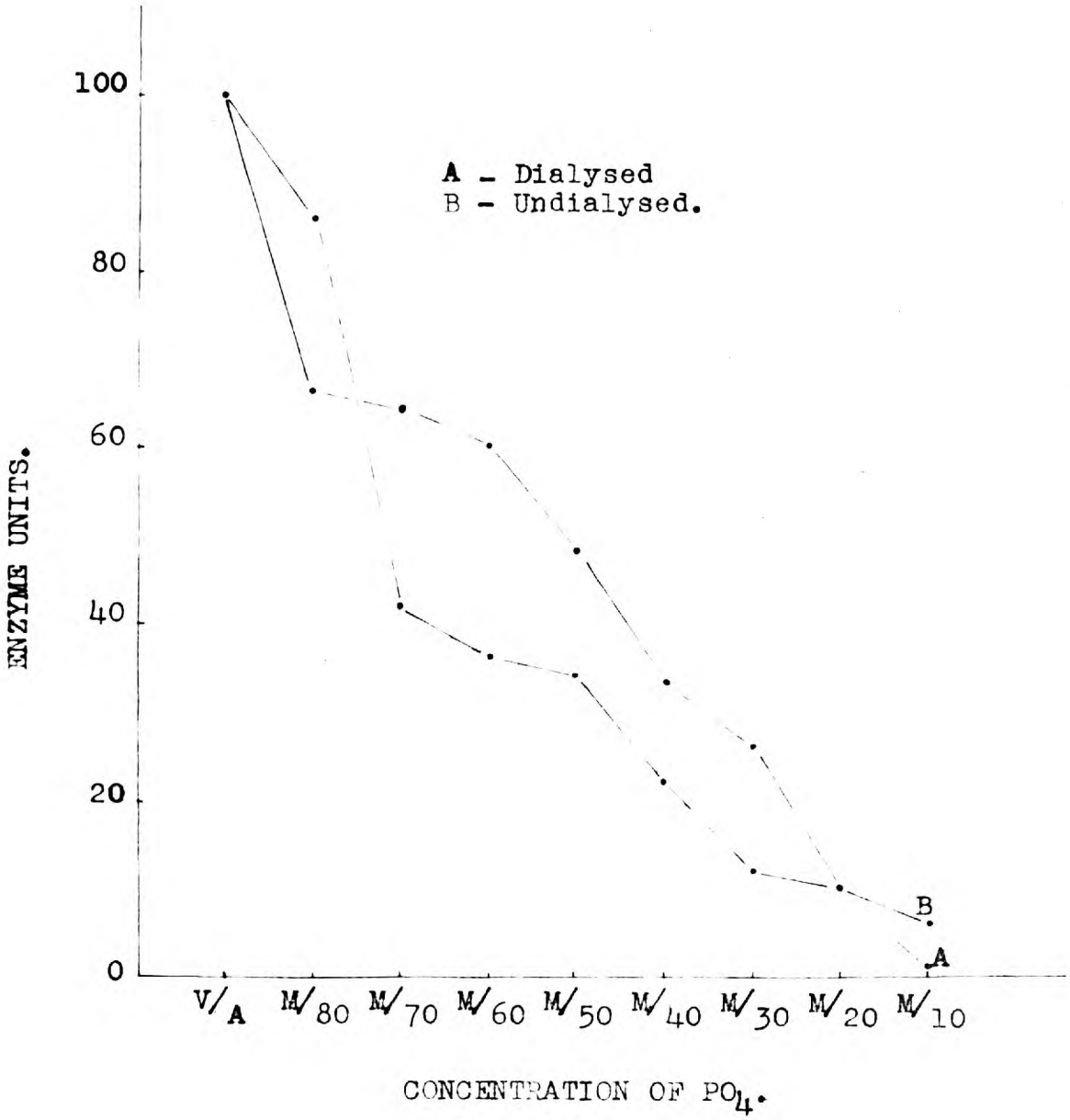


Fig. 12. Effect of various concentrations of phosphate (KH_2PO_4-Na_2HPO_4) on the activity of F.moniliforme enzyme by viscometric method.

relatively high concentrations were required for any significant reduction in activity. As the concentration of phosphate in the crude undialysed enzyme solution is only M/140, the substantial increase in activity after dialysis cannot therefore be attributed to removal of phosphate.

(e) Effect on pectin, Na- polypectate and Na- pectate solutions.

The effect of F.moniliforme enzyme solutions both dialysed and undialysed on the viscosity of 0.5% solutions of pectin, Na- polypectate and the Na- pectate was compared in view of recent work (Seegmiller and Jansen, 1952) which showed that certain pectic enzymes have pectin but not sodium pectate as a substrate. Three different preparations of Na- pectate were used. The details of their preparation are given below.

(a) From pectic acid (C.F.G.) - see page 8.

(b) By de-esterifying a pectin solution with NaOH. 50.0 ml. of a 2.0% pectin solution was titrated with 0.5 N NaOH solution to pH 10, with constant stirring. The pH was maintained in the neighbourhood of 10 by adding NaOH from time to time. This was done over a period of two days after which there was no further change in pH on standing, indicating that the de-esterification was complete. The mixture was then made up to 100 ml. to give a 1% solution of sodium-pectate.

(c) By de-esterifying a pectin solution with pectin-esterase.

To 50.0 ml. of a 2.0% pectin solution was added sufficient 0.5 N NaOH to give a pH between 7.0-8.0. 2.0 ml. of tobacco-leaf* pectin-esterase (prepared in this laboratory) in 0.2 M NaCl was added to it. The pH was maintained between 7.0-8.0 by adding 0.5 N NaOH from time to time and cheking the pH. This was continued till no further change in pH took place. The amount of NaOH added was recorded and the mixture made up to 100 ml. to give a 1.0% sodium pectate solution.

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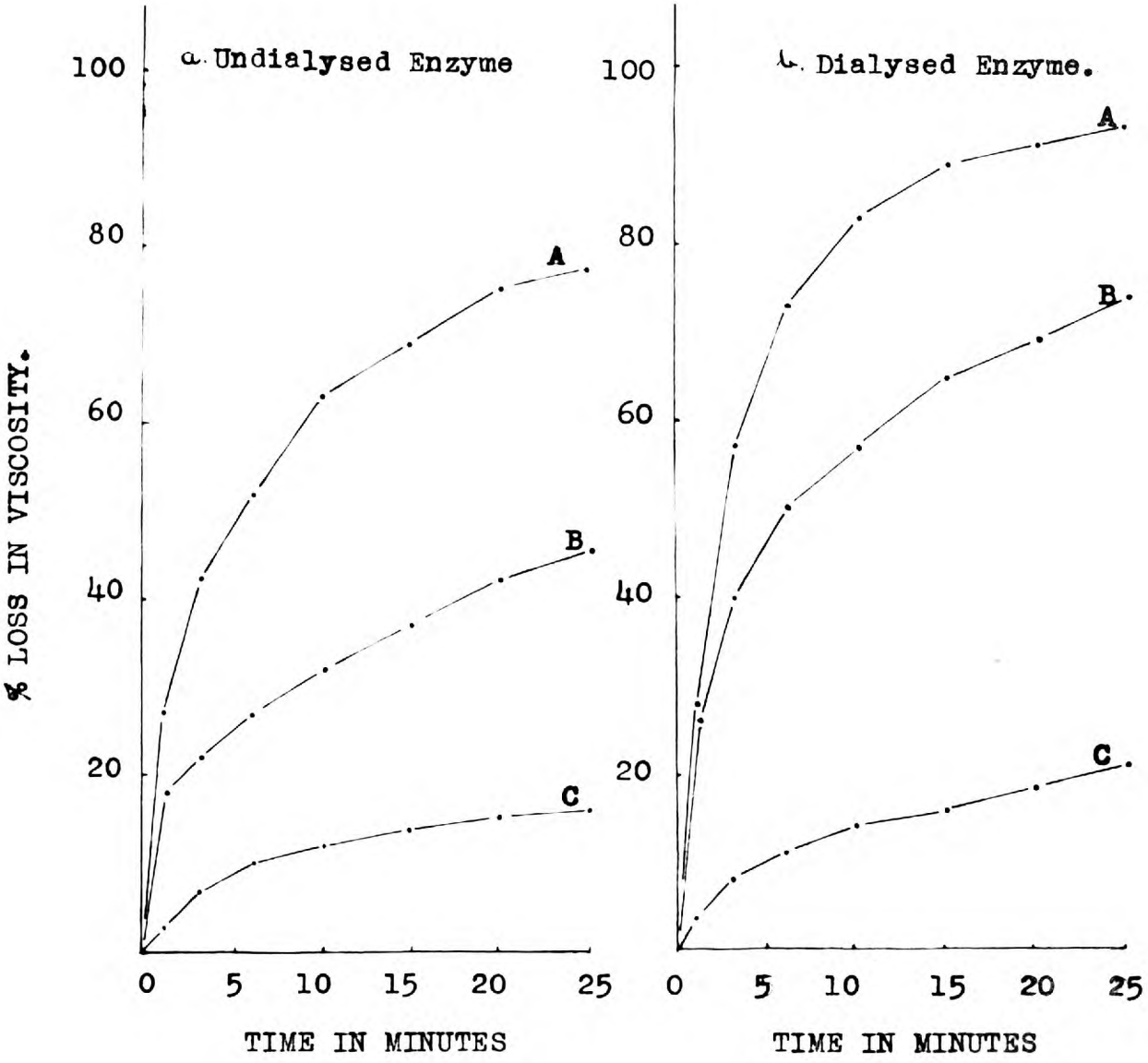
* 160 gm. of fresh tobacco leaves, free from midrib, were homogenised in a Waring blendor using a small quantity of distilled water. It was collected on a Buchner funnel over 3 layers of Whatman No. 1 filter paper and the liquid discarded. The tissue was now suspended in 100 ml. of 0.2 M Na_2HPO_4 solution and left overnight at 5°C, after which the liquid was pressed out and to it ammonium sulphate was added to give 30% saturation at room temperature. The solution was left for 1 hour at 5°C after which it was cleared by centrifuging, the precipitate being discarded. More ammonium sulphate was added to give 65% saturation. The precipitate, which formed within 1 hour was collected on a thin cake of Hyflosupercel on a Buchner funnel. The cake with precipitate was then suspended in 20 ml. of 0.2 M NaCl and wascentrifuged to remove the supernatant which was then used as a source of pectinesterase (PE).

Figs. 13 a and b. illustrate the reduction of viscosities of pectin, Na- polypectate and Na- pectate (prepared from pectic acid) solutions by dialysed and undialysed solutions. Both preparations gave similar curves, the dialysed solutions being more active in each case. Na- pectate solutions showed the lowest, Na- polypectate solutions the highest rate of viscosity loss, pectin solutions being intermediate.

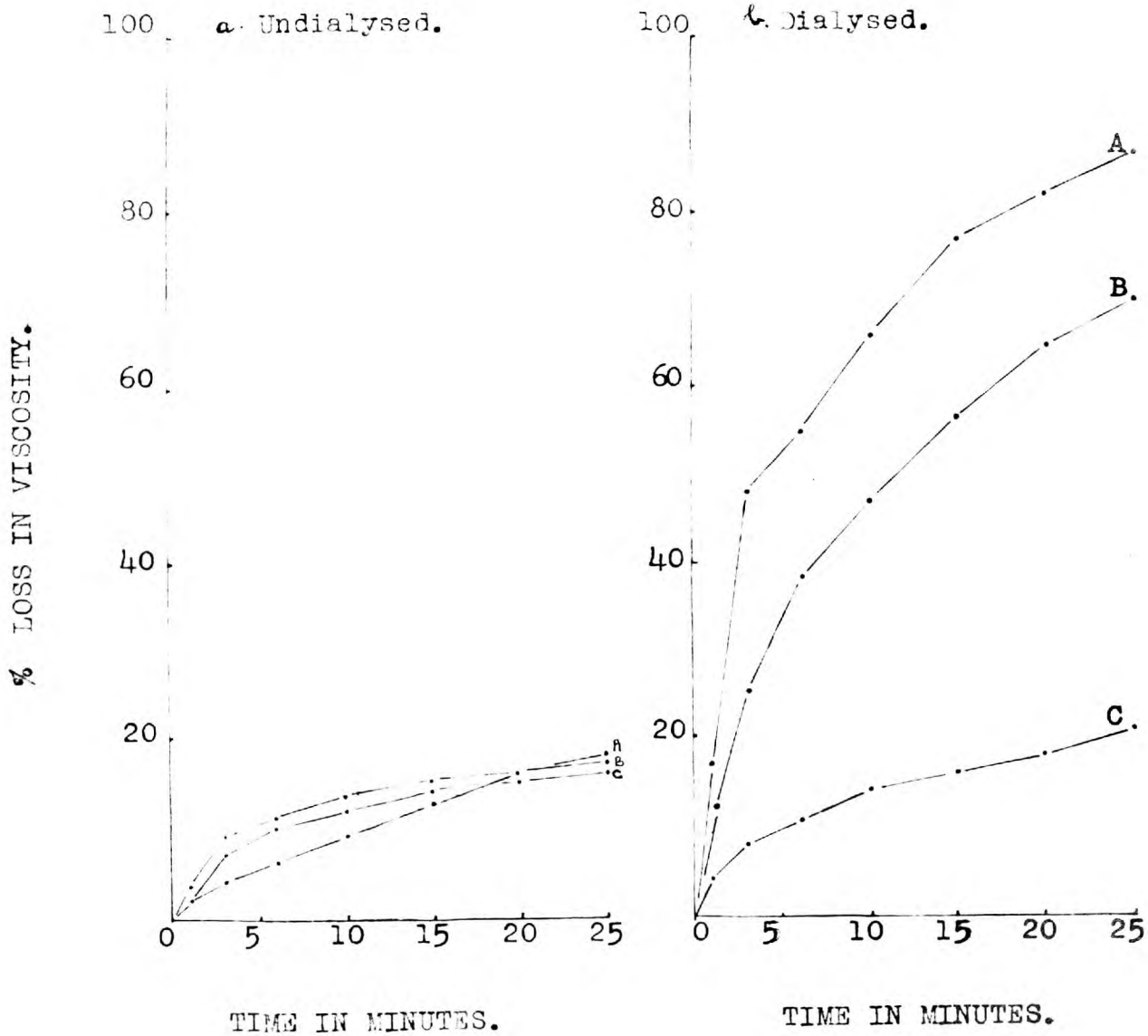
The effect of the enzyme preparations on solutions of Na- pectate prepared in different ways is illustrated in Figs. 14 a and b. Rather surprising results were obtained in that the undialysed and dialysed preparations behaved differently with certain Na- pectate solutions. Their behaviour with Na- pectate produced by neutralising pectic acid was similar. With Na- pectate solutions directly derived from pectin however, while the undialysed preparation behaved as with neutralised pectic acid, the dialysed preparation showed much greater activity. This anomalous behaviour requires further investigation.

C. Measurement of Activity by Reducing Group Determinations.

Fission of the 1, 4 glycosidic linkages of the polygalacturonic chain liberates reducing groups and their estimation has formed a basis for the determination of activity of polygalacturonase preparations (Jansen and McDonnell, 1945).



Figs. 13 a & b. Effect of F.moniliforme enzyme on solutions of pectin, Na- pectate and Na- polypectate.
A. Na- polypectate solution.
B. Pectin solution. C. Na- pectate solution.



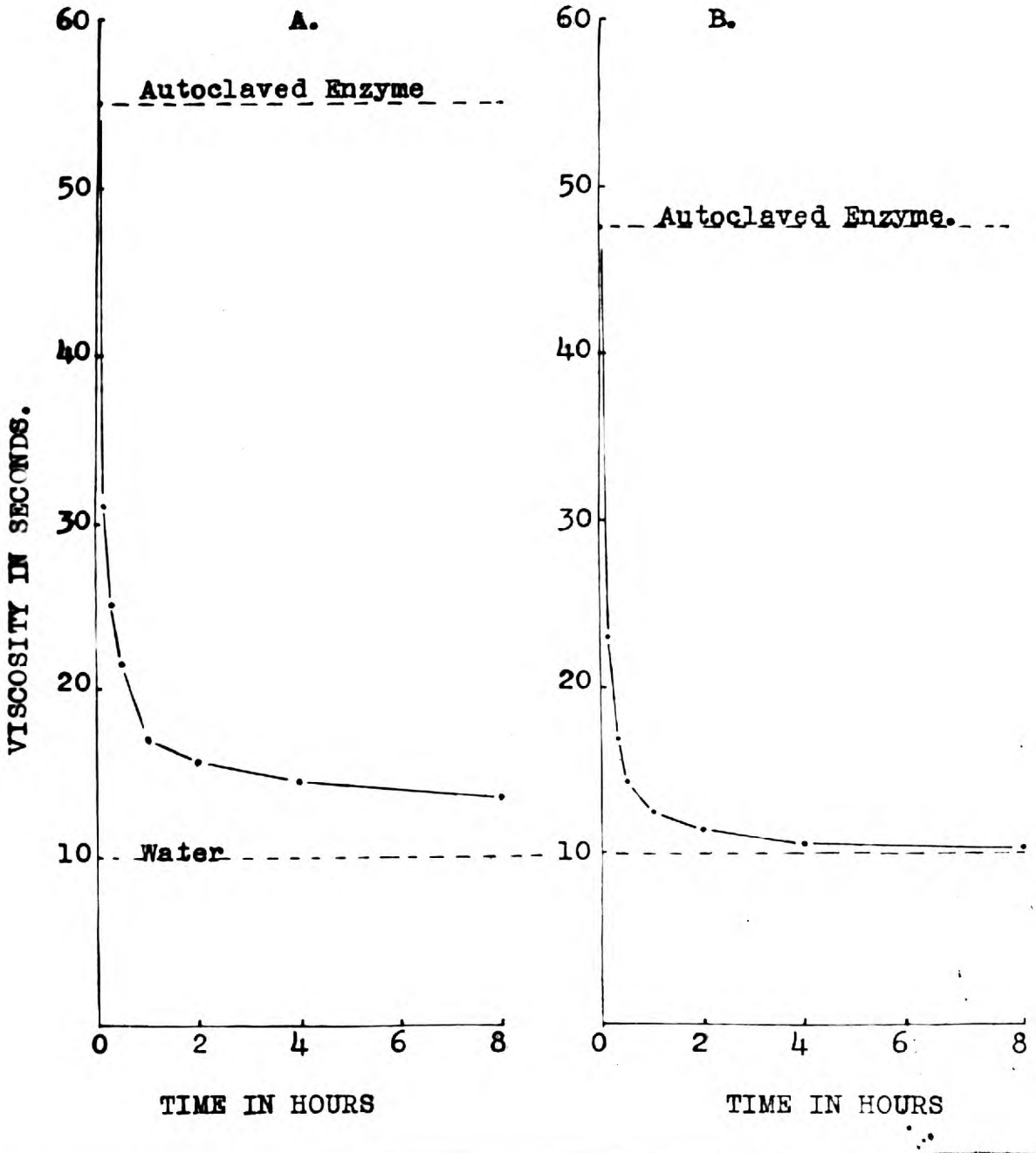
Figs. 14 a & b. Effect of F.moniliforme enzyme on various preparations of Na- pectate solutions.
A. PE de-esterified pectin solution.
B. De-esterified pectin solution.
C. Neutralised pectic acid.

The method used by these workers has been followed in a study of the effect of F.moniliforme preparations on solutions of pectin and Na- pectate derived directly from the pectin by enzyme de-esterification (for details of latter see p.65). The following mixtures were used:-

A		B	
1% Pectin soln.	- 20.0 ml.	1% Na- pectate soln.	- 20.0 ml.
0.1 N NaOH	- 3.2 ml.	0.1 N NaOH	- 0.8 Ml.
Veronal/acetate buffer at pH 7.9	- 4.0 ml.	Veronal/acetate buffer at pH 7.9	- 4.0 ml.
Dialysed enzyme soln.	- 12.0 ml.	Dialysed enzyme soln.	- 12.0 ml.
Water	- 0.8 ml.	Water	- 3.2 ml.
	40.0 ml.		40.0 ml.

The above was kept at 25°C and in both cases after adding the enzyme, 5.0 ml. samples were removed for viscosity determinations in the usual way. Viscosity/time curves are given in Figs. 15 A & B. Further samples of 3.0 ml. were removed at the time intervals given below -

Sample No.	Time taken.
1	½ hour.
2	1 hours.
3	2 "
4	4 "
5	8 "
6	24 "



Figs. 15 A & B. Effect of dialysed F.moniliforme enzyme solutions on the viscosity of pectin and Na- pectate solutions.
A. Pectin Solution. B. Na- pectate solution.

Sample No. (contd.)	Time taken (Contd.)
7	48 hours
8	96 "
9	24 + 24 hours. (after 24 hours 1 ml. of more enzyme soln. added).
10	48 + 24 " (after 48 hours 1 ml. of more enzyme soln. added).
11	96 + 24 " (after 96 hours 1 ml. of more enzyme soln. added).

They were placed in a boiling water bath for 10 minutes, and then stored at -18°C until the following analyses were made:

Reducing groups. 2.0 ml. from the above samples were placed in glass stoppered flasks and to it added 0.4 ml. of 1M Na_2CO_3 and 2.0 ml. of 0.1N I_2. This was left for 20 minutes at 20°C; 1.0 ml. of 2 M H_2SO_4 then added and residual iodine titrated with 0.01N $Na_2S_2O_3$. The increase in reducing power of the solution following enzyme action is expressed in the Table XXX below as equivalent mg. galacturonic acid which, theoretically, is the breakdown product of complete hydrolysis.

The experiment was conducted on two occasions with different enzyme preparations and the results are given in ~~below:-~~ Table XXX.

Table XXX.

Measurement of Reducing Groups in Pectin and Na-pectate Solutions.

Sample No.	Hours.	Pectin		Na- Pectate	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
1	0.5	0	0	0	0
2	1	0.1	-	0.19	-
3	2	0.1	-	2.03	-
4	4	0.1	0	2.13	2.23
5	8	0.1	0.1	2.71	2.91
6	24	0.97	1.26	4.07	4.56
7	48	1.94	2.43	4.37	4.95
8	96	3.29	3.59	5.24	6.11
9	24 + 24	2.32	-	5.12	-
10	48 + 24	2.67	-	5.24	-
11	96 + 24	3.14	3.72	5.82	6.17

The results from the two experiments agree quite closely and indicate the following.

(a) The enzyme acts much more rapidly on Na- pectate than on pectin solutions particularly in the earlier stages of the reaction. Thus at the end of 8 hours the solutions of Na- pectate show almost thirty times the reducing power of the pectin solutions. At the end of 24 hours however it is

reduced to four times the value and at the end of 96 hours is less than twice the value. The different action on the two substrates is illustrated in Figs. 16 a and b.

(b) Some inactivation of the enzyme seems to occur in the solutions under these conditions since in all but one there is an increase in reducing power on the addition of further enzyme.

(c) After 96 hours the addition of more enzyme, the solutions of pectin and Na- pectate yield reducing groups corresponding to approximately 30 and 60% respectively of the theoretical figure if complete hydrolysis to mono- D- galacturonic acid had occurred.

D. Analysis of Breakdown Products by Chromatography.

The samples described in section C above were also used for preliminary analysis by paper chromatography. The following were placed on spots at 2 cm. intervals on Whatman No. 1 filter paper measuring 58 x 44 cm.

1. 0.006 ml. 1% galacturonic acid.

2-12. 0.006 ml. x 4 of samples 1-11 Pectin soln. Expt. 1 of Table XXX.

13. 0.006 ml. 1% galacturonic acid.

14. 0.006 ml. 1% glucose.

15-25. 0.006 ml. x 4 of samples 1-11 Na- pectate soln. Expt. 1 of Table XXX.

26. 0.006 ml. 1% galacturonic acid.

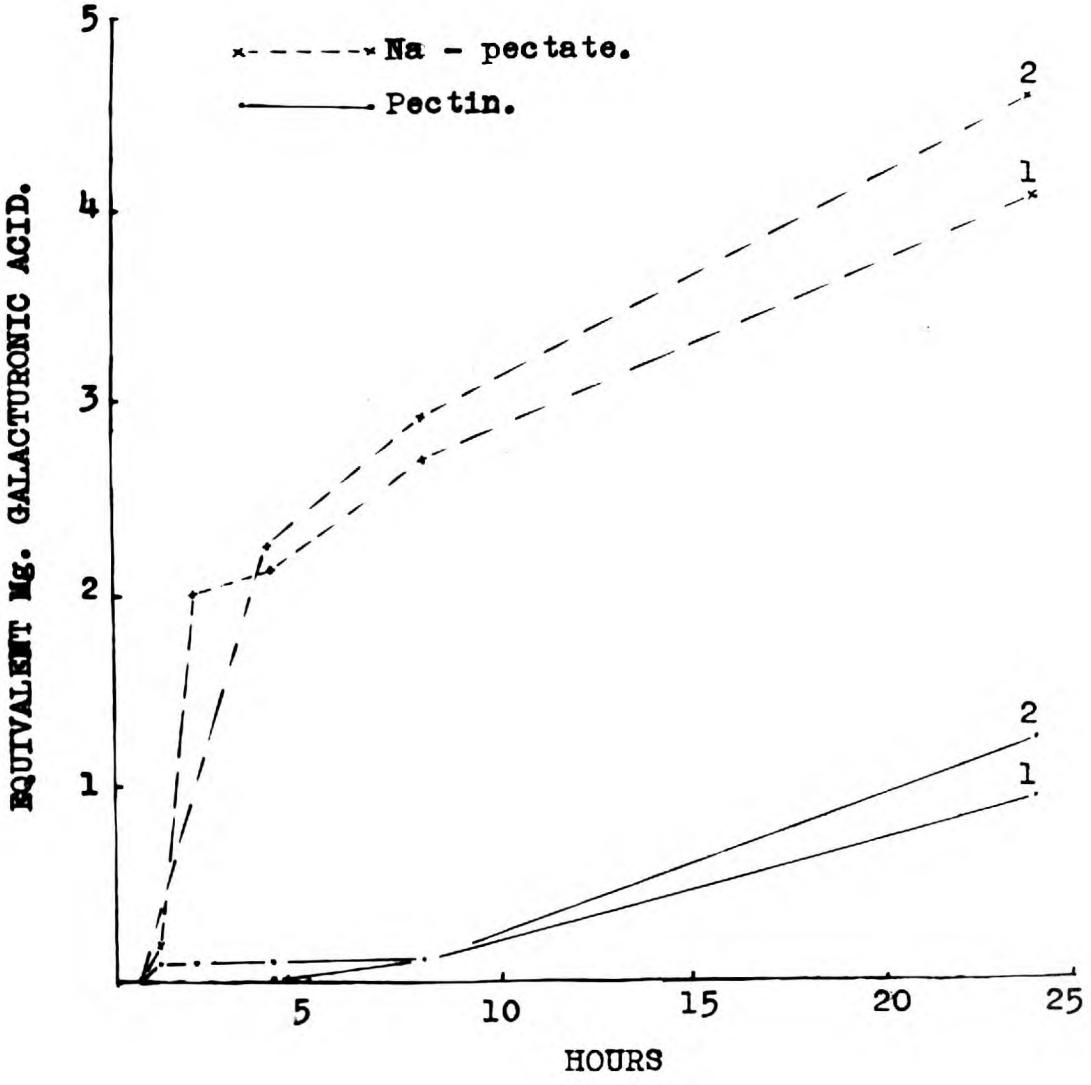


Fig. 16 a. Effect of F.moniliforme enzyme on the reducing power of pectin and Na- pectate solutions in 24 hours.
1. Expt. 1 of Table XXX. 2. Expt. 2 of Table XXX.

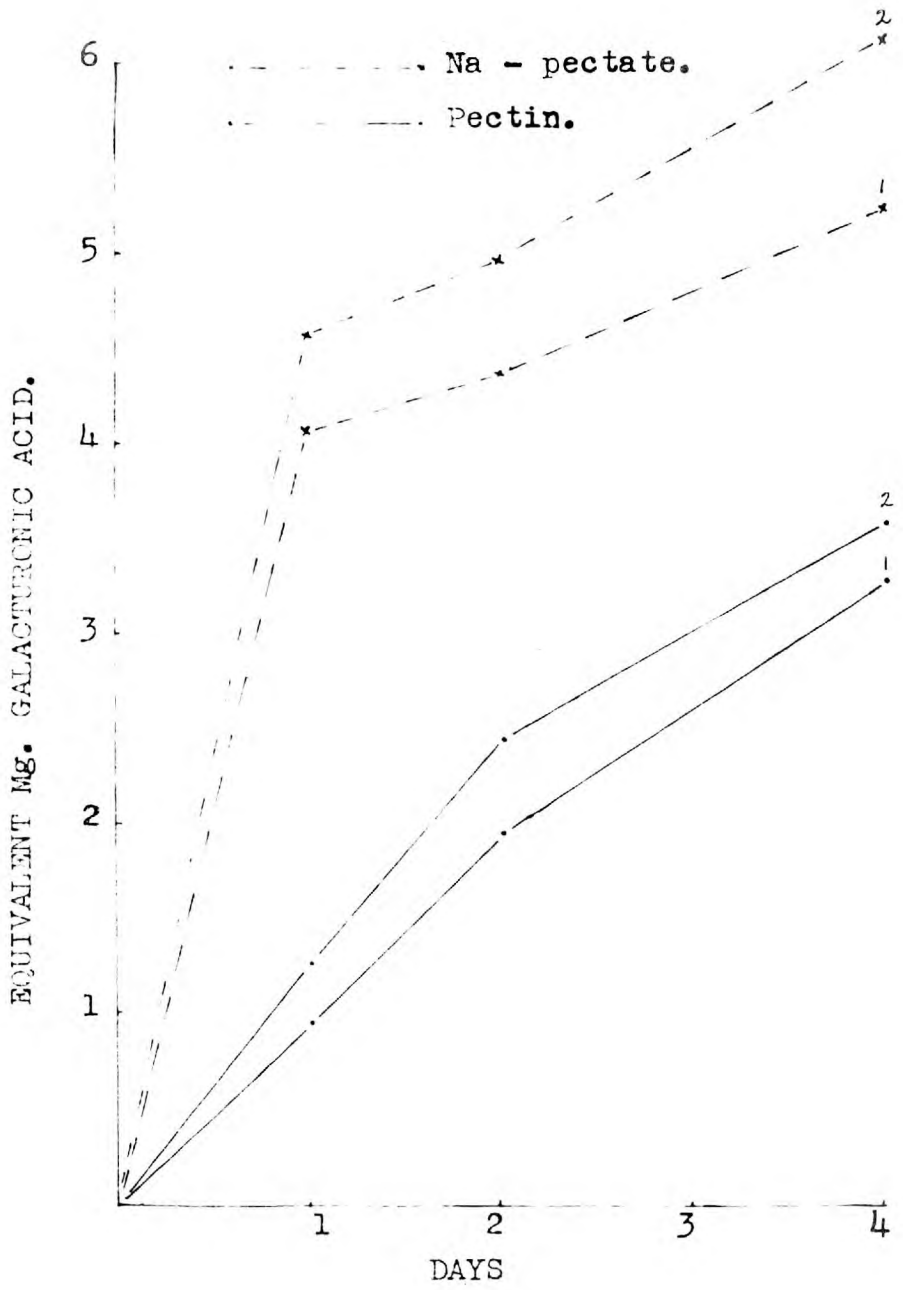


Fig. 16 b. Effect of F.moniliforme enzyme on the reducing power of pectin and Na- pectate solutions in 4 days.
1. Expt. 1 of Table XXX. 2. Expt. 2 of Table XXX.

The following mixture of solvents was used: Butanol (redistilled) 4 parts, glacial acetic acid 1 part and water 5 parts. The lower layer was placed in the base of the chromatography cabinet and the paper suspended in the cabinet overnight to equilibrate. The upper layer was then added to the trough and the chromatogram developed for 48 hours at room temperature. The solvent front had almost reached the bottom of the paper after 24 hours. The paper was dried in an air stream at 50°C, and then sprayed with the following reagent - Benzidine, 0.5 gm; acetic acid, 10.0 ml.; 45% trichloro-acetic-acid, 10 ml. and 95% ethanol, 100 ml. It was then heated at 100°C for 5 minutes to give the results shown in Figs. 17 a and b which are photostat copies of the chromatograms after shading detectable spots with pencil to an intensity corresponding to the depth of colour. Substantially the same results were obtained with the samples of Expt. 2. given in Table XXX.

The results clearly indicate that the action of the enzyme on pectin and Na- pectate solutions is quite different even although the latter were derived directly from the former with little degradation. In the case of pectin a trace of galacturonic acid (or methyl galacturonate?) appears after 8 hours and the intensity of the spot thereafter gradually increases. No intermediate compounds were detectable on the chromatogram.

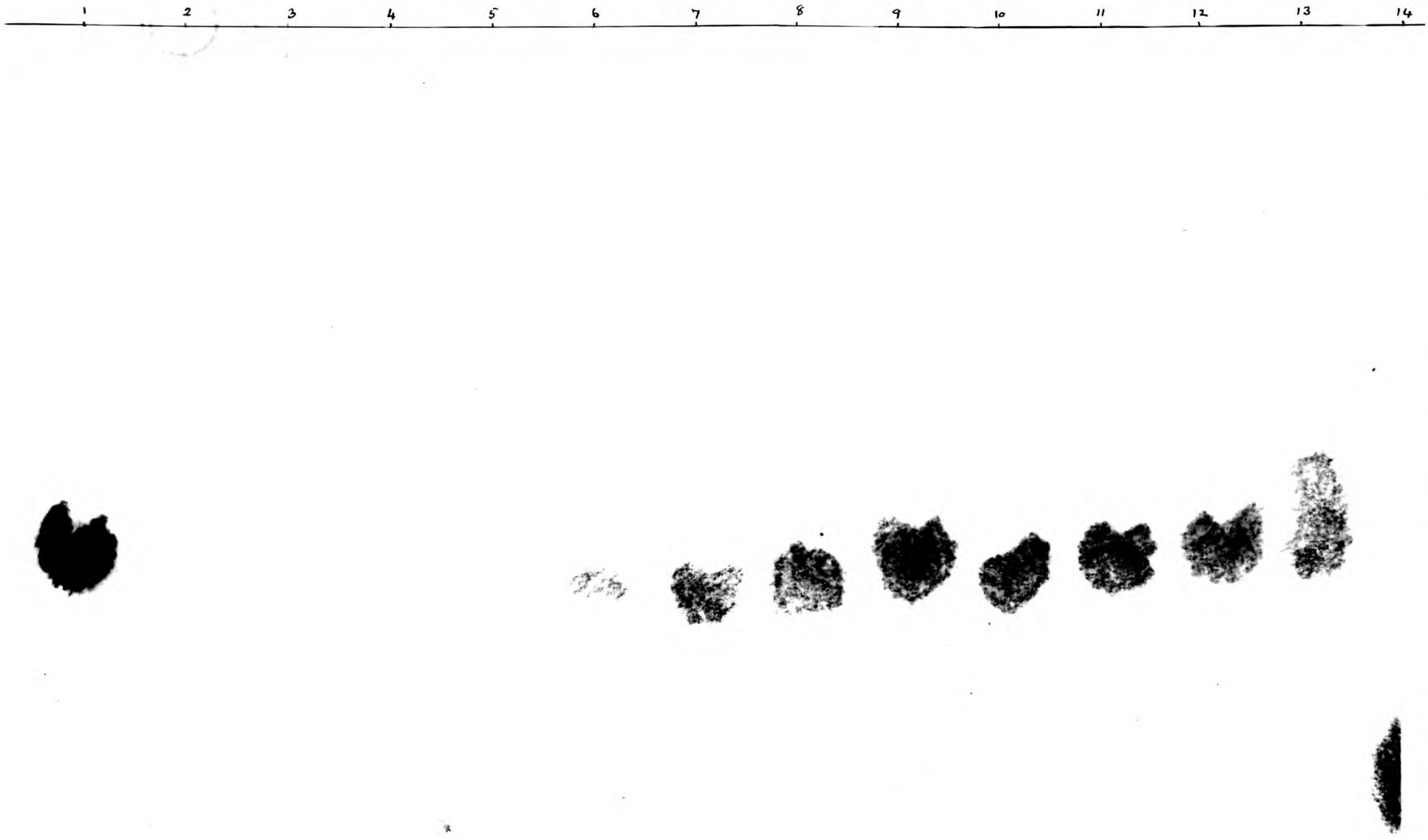


Fig. 17 a Analysis of breakdown products of pectin solution by F.moniliforme enzyme.

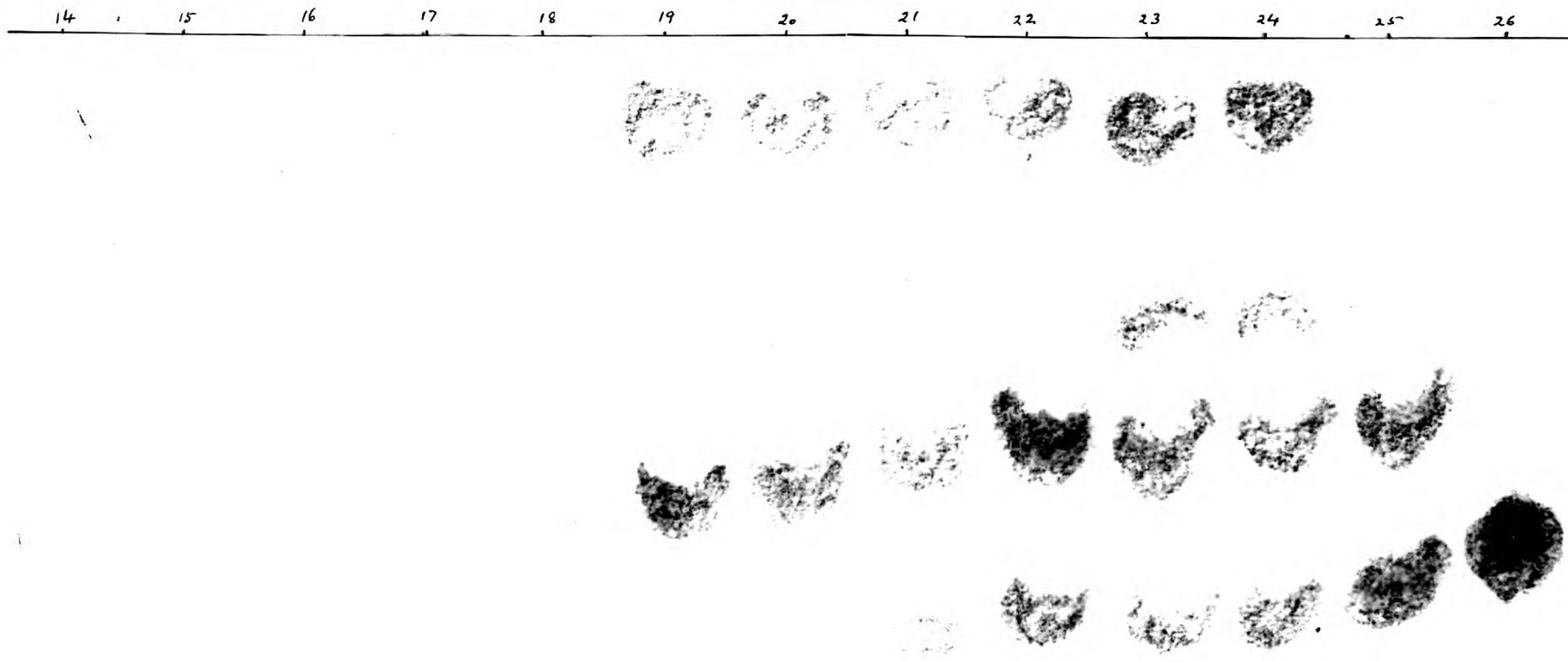


Fig. 17 b Analysis of breakdown products of Na- pectate solution by F.moniliforme enzyme.

With Na- pectate solutions traces of a component of higher molecular weight than galacturonic acid appear after 4 hours. Thereafter a series of components appear, galacturonic acid showing only after 48 hours. At least three components appeared between galacturonoc acid and the base line. Only one of these persisted with the most extensive enzyme treatment.

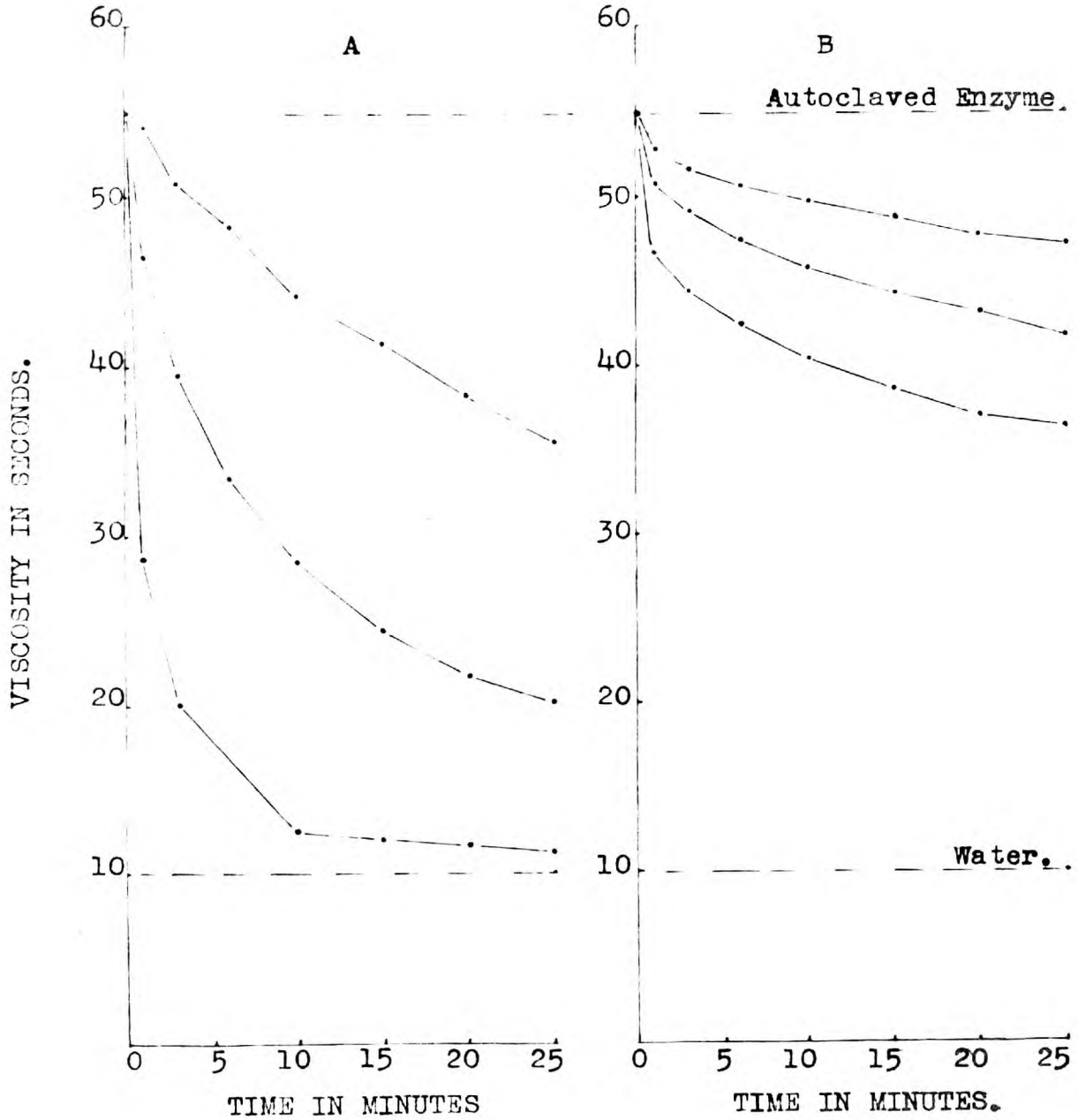
The results, which at present can only be regarded as preliminary, suggest that the enzymes secreted by F.moniliforme act upon esterified and de-esterified pectin in two different ways. The pectin chain is broken down by the splitting off of relatively small units and possibly galacturonic acid itself to cause a gradual shortening of the chain and a steady increase in the concentration of galacturonic acid with no or only a transient formation of higher molecular weight compounds. With Na- pectate however, much larger units are split off and galacturonic acid is formed at a relatively later stage in the reaction. A series of compounds of high molecular weight are therefore formed as intermediate products. It may well be that different enzymes are involved in the two cases.

E. Comparison of F.moniliforme and B.aroideae Enzyme. Solutions.

The Bacterium aroideae preparation was obtained from

Mr. Jarvis. A comparative study of the two enzymeswas made on the viscosity of 0.5% pectin solutions at pH 7.9. In both cases undialysed enzyme preparations were used. Three different samples of equal activity measured by the maceration method were tested. Their R.T. values with potato discs at pH 7.9 were: A, 10-15 mins; B, 30-35 mins and C, 55-60 mins.

Figs. 18A and B give viscosity/time curves for each of the above preparations. It is apparent that the B.aroideae preparation have a far greater effect on the viscosity than have those of F.moniliforme and the results indicate that the enzymes produced by these organisms have a different mode of action.



Figs. 18 A & B. Comparison of F.moniliforme and B.aroideae enzyme solutions.
A. B.aroideae enzyme. B. F.moniliforme enzyme.
1. R.T. 10-15 enzyme. 2. R.T. 30-35 mins.
3. R.T. 55-60 mins.

VI. EFFECT OF AGE OF CULTURE ON ENZYME PRODUCTION.

In this experiment flats containing 20 ml. of standard medium were inoculated with 1.0 ml. of a dense spore suspension of F.moniliforme and incubated at 25°C. After 3 days' growth, samples of three flats were removed daily, the mycelia removed, washed dried and weighed separately and the supernatant culture fluid assayed for enzyme activity by the maceration and viscometric methods already described. All tests were made in solution buffered at pH 7.9. The results are summarised in Table XXXI.

Table XXXI.

Effect of Age of Culture on Enzyme Production.

Age of culture (days)	Final pH of medium	Dry wt. of mycl. (mg.)	Enzyme activity			
			Maceration		Viscosity	
			R.T.(min.)	E.U.	% Loss	E.U.
3	4.8	100	110-120	3	28	17
4	7.8	119	10-15	100	43	100
5	8.3	125	"	"	43	100
6	8.4	131	"	"	39	60
7	8.5	137	"	"	38	50
8	8.6	137	"	"	37	42
9	8.7	131	"	"	36	37
10	8.8	125	"	"	33	27
11	8.9	119	"	"	27	15
12	"	113	"	"	26	14
13	"	100	"	"	23	11
14	"	"	"	"	22	10
15	"	"	"	"	20	9

It is evident from the above that when tested by the maceration method, solution reach maximum activity after four days growth. The activity does not diminish subsequently. With viscometric determinations however, although again the most active preparations are obtained from cultures 4-5 days old, the activity then drops rapidly as the age of the culture

increases. Such loss of activity was not associated with any major change in the pH of the media but was accompanied by a steady fall in the dry weight of the mycelium presumably owing to autolysis. The final result was that after 15 days growth, a preparations was produced which had little effect on the viscosity of a pectin solution but which rapidly macerated potato tissue.

VII PATHOGENICITY.

The following materials were used for pathogenicity tests: apple and tomato fruits, narcissus and onion bulbs, potato tubers, turnip and swede roots.

Method of inoculation.

The inoculations were performed by the plug method, the technique of inoculation differing slightly according to the type of material.

Apple, narcissus, onion, carrot, turnip and swede: These were surface sterilised by scrubbing with 90% alcohol which was then allowed to dry. A plug 3 mm. deep was taken out by means of a sterile no. 2 cork-borer (diam. 5 mm.). The inoculum was placed in the hole and the plug re-inserted and the broken surface sealed with a mixture of molten wax and vaseline to check contamination and desiccation of inoculum.

Potatoes were washed thoroughly and left overnight to dry. They were surface sterilised by means of 90% alcohol and the alcohol was allowed to dry. The inoculations were made by Isaac's method (Isaac, 1952). A plug 2 mm. deep was taken out, the inoculum was placed in the hole and sealed without re-inserting the plug.

Tomatoes were surface sterilised by 90% alcohol and left to dry. A slit 4 mm. long was made by means of a sterile scalpel. The inoculum was placed in the slit which was then sealed.

Agar discs cut from the edge of 7-8 day old plate cultures on potato extract agar with a no. 2 cork borer were used as inocula. The material after inoculation were kept in sterile tins at 25°C for 2 weeks.

In a preliminary experiment it was found that F.moniliforme produced no attack on carrot, turnip, swede, narcissus or onion; on potatoes (King Edward var.) there was slight attack without measurable rotting, the lesion being cut off by a cork layer. Apples (Bramley seedling) and tomatoes were attacked, giving respectively 6.7 and 13.8% of the tissue rotted.

The inoculation tests on apple and potato were repeated at the series of temperatures 5, 15, 20, 25 and 30°C. With apples there was no rotting at 5°C; from 15°C upwards rotting increased reaching 5-6% of the fruit weight at 25° or 30°C. With potatoes there was no attack at 5°C; at 15°-30°C it was slight, being cut off by a cork barrier.

Pretreatments for 1-2 weeks of apples and turnips at 5°C and of potatoes at 5°C and 35°C after the manner described by various workers (Vasudeva, 1930; Chona, 1932; Fernando, 1934; Chattopadhyay, 1947; and Isaac, 1952) had no noticeable effect on the amount of attack produced.

Similar negative results were obtained when the fungal inoculum was reinforced by the following nutrients; 1% glucose, 1% pectin and the standard medium.

Increase in the water content of potato cylinders after

the method adopted by earlier workers (Hall, 1932; Fernando, 1952; Stevenson, ; Chattopadhyay, 1947 and Isaac, 1952) likewise did not lead to fprogressive attack.

A comparison of the pathogenicity of Fusarium moniliforme, Fusarium avenaceum and Fusarium coeruleum on potato tubers is set out in Table XXXII. Ole tubers (Majestic var.) and young (Jersey) were used for this test.

Table XXXII.

Production of Rot on Old and New Potatoes.

Fungus	Type	Result of inoculation		Mean % rotted tissue
		No. inoculated	No. infected	
F.moniliforme	Old	5	5	Negligible
"	New	"	"	"
F.avenaceum	Old	"	"	6.3
"	New	"	"	20.5
F.coeruleum	Old	"	"	Negligible
"	New	"	"	"

Though initial attack was shown by all three isolates, the only one which gave a measurable amount of rotting was F.avenaceum. In contrast to this result, the enzymic behaviour of these fungi is set out in Table XXXIII. All tests were made at pH 8 with 0.5 mm. thick potato discs.

Table XXXIII.

Production of Enzyme on Potato Extract and Standard Medium.

Medium	Fungus	pH		R.T.(min.)	Dry wt. of mycl.(mg.)
		Initial	Final		
Potato Ext.	F.moniliforme	7.8	8.8	45-50	119
"	F.avenaceum	"	9.0	20 hours.	125
"	F.coeruleum	"	8.8	"	78
Standard Med.	F.moniliforme	3.6	8.1	10-15	122
"	F.avenaceum	"	8.2	24 hours.	128
"	F.coeruleum	"	3.6	80-90	36

It is evident from the foregoing results that there is no correlation between pathogenicity and activity of enzyme secretion. F.avenaceum in particular, the only isolate which was active on potatoes gave very weak enzyme preparations; F.moniliforme was the most active enzymically but had negligible pathogenicity.

VIII DISCUSSION.

The enzymological behaviour of Fusarium moniliforme has hitherto not been studied at all, so that all the findings of this paper are new: Several features of special interest have emerged and these will now be reviewed.

When the fungus is grown on a variety of natural decoctions, preparations which macerate plant tissue, i.e., which contain protopectinase enzyme, are readily obtained; when however a synthetic medium such as one based upon glucose-asparagine is used, growth is good but enzyme production is poor. The addition of pectin to such a medium greatly enhances enzyme production. This is an example therefore of an adaptive formation of enzyme. Several workers in the past, vis., Harter and Weimer (1923), Willaman and Kertesz (1931), Proskuriakov and Ossipov (1939), Phaff (1947) and Gaumann and Hohni (1947) have described this type of enzyme secretion for variousfungi. A variety of pectic compounds such as pectin, Na- pectate and Na- polypectate are effective, and strikingly also D- galacturonic acid which is the end point of the hydrolysis of pectic compounds stimulates enzyme production. A number of naturally occurring compounds such as galactose, alginic acid, mucic acid, gum arabic and gum tragacanth which do not possess the fundamental structure of pectic substances are ineffective.

Analysis of the pectic enzyme system of F.moniliforme has shown that pectinesterase is absent but polygalacturonase is present. The viscosity of solutions of pectin, Na- pectate and Na- polypectate is rapidly reduced and in the case of pectin and Na- pectate galacturonic acid with or without the formations of intermediate compounds is produced. The enzyme system of F.moniliforme thus compares with the corresponding systems of other parasites which have been worked out in this laboratory, as follows:

Table XXXIV.

Comparison of Enzyme Systems of Fusarium moniliforme, Bacterium aroideae, Botrytis cinerea and Pythium deBaryanum.

Fungus	PP.	PE.	PG.	Optimum pH	T.I.
F.moniliforme	+	-	+	8	>70°C.
B.aroideae	+	-	+	8	>70°C.
B.cinerea	+	+	+	5.5	50-60°C.
P.deBaryanum	+	-	-	8	50-60°C.

Abbreviations: Protopectinase (PP); Pectinesterase (PE); Polygalacturonase (PG); Temperature inactivation (T.I.); Plus (+), presence of enzyme and Minus (-), absence of enzyme.

It isevident from Table XXXIV that closest resemblance of F.moniliforme enzyme system is to that of B.aroideae but there are differences in detail. Thus when samples of F.moniliforme and B.aroideae enzyme solutions, having the same protopectinase activity, are used for viscosity tests on pectin solution, theB.aroideae enzyme reduces viscosity much more quickly than F.moniliforme (see p.). This indicates that although these enzyme samples have the same amount of protopectinase in them they differ considerably in their polygalacturonase content. B.aroideae enzyme solutions have more polygalacturonase than F. moniliforme enzyme solutions, protopectinase being the same in both. This result indicates that protopectinase is different from polygalacturonase as has been suggested by several workers in the past. It is possible that protopectinase is the same enzyme as the depolymerase known to occur in extracts of P.deBaryanum (Gupta, 1953) and the presence of which in F.moniliforme extracts is masked by the simultaneous presence of polygalacturonase.

That protopectinase and polygalacturonase are not the same enzymes is indicated by the effect of ageing on F.moniliforme cultures. After 4 days' growth maximum activity is reached with respect to macerating and viscosity reducing activities; after 15 days rate of macerating is still the same whereas viscosity reducing activity has dropped to 10% of its original value. In other words, whereas proto-

pectinase content has remained steady, polygalacturonase content has fallen to a low value.

It has been found that the fungus F.moniliforme does not produce pectinase ~~enzyme~~ on apple media, in spite of the fact that it attacked apples. The addition of citrus pectin or glucose or asparagine did not induce the production of any detectable enzyme nor was apple juice made suitable for enzyme production by subjecting it to dialysis before the fungus was grown in it. It was also found that the presence of one part of apple juice in three parts of the standard medium considerably reduced the activity of the enzyme produced although the growth of fungus was normal. These results clearly indicate the presence of some substance which retards or prevents the production of enzyme. It is also possible that the enzyme is produced but that its activity cannot be detected because of the presence of inhibitory substance or substances in apple media. Similar results have been found by Cole (1953) working with Monilia fructigena, Monilia laxa and Botrytis cinerea.

The results of the pathogenicity tests and of the studies of enzyme secretion on natural decoctions show that there is no correlation between pathogenicity and amount of enzyme produced under standard conditions. F.moniliforme rots apples but does not produce any detectable enzyme in

apple media. On the contrary, it produces the macerating enzyme on potato decoction but it does not produce rotting. Similar results have been indicated by earlier workers (Fernando, 1934 and Stevenson, 1938) with respect to Botrytis cinerea and Pythium deBaryanum on potato. F.moniliforme therefore presents a two-fold problem - why is it that it is able to attack and rot apple tissue without revealing the presence of protopectinase ~~enzyme~~ when grown on apple medium, and conversely why is it unable to attack and rot potato tissue seeing that it produces a potato macerating enzyme when grown on potato media.

IX SUMMARY

(1) Fusarium moniliforme produced pectolytic enzymes on a variety of natural media viz., extracts of tomato, potato and turnip but it did not produce these enzymes on apple media.

(2) Only negligible enzyme production was found on a number of synthetic media viz., glucose-asparagine, glucose-peptone, Ashour's medium, Czapek-Dox and various forms of synthetic potato extract though growth was good on all these media.

(3) The addition of pectin to any of the media listed under (2) gave active secretion of pectic enzymes, thus showing its adaptive nature. This effect was examined more fully with a glucose-asparagine medium, when it was shown that various pectic substances, including galacturonic acid itself stimulated enzyme production. On the other hand a number of naturally occurring substances which are not structurally related to pectin were ineffective.

(4) A synthetic medium (containing 1% pectin) was elaborated as being suitable for production of pectinase ~~enzyme~~ by F.moniliforme. The efficiency of various carbohydrates and nitrogenous sources for growth and enzyme production was determined.

(5) Decoctions of apple gave negligible enzyme production, even when, fortified by addtion of sugar or asparagine. Addition of a proportion of apple juice to media otherwise suitable renders the latter unsuitable for enzyme production. The nature of the substance (S) responsible for this effect had not been worked out. A small amount of enzyme was detectable in tissue of apple fruit rotted by the fungus.

(6) The macerating and viscosity reducing enzymes of F.moniliforme require fairly strong heating for complete inactivation, their optimum pH is 8-9, they are dialysable without loss of activity and are retarded by certain concentrations of phosphate, are readily precipitated by alcohol or acetone precipitation, but not by ammonium sulphate.

(7) Reduction of viscosity proceeds more rapidly on pectate than on pectin solutions. Galacturonic acid, with or without intermediate products is formed, i.e., the enzyme polygalacturonase is present.

(8) A comparison of F.moniliforme and B.aroideae enzyme preparations having equal macerating capacity showed that B.aroideae was much more active in reducing the viscosity of pectin solution than F.moniliforme enzyme.

(9) A comparison of F.moniliforme cultures of different ages showed that protopectinase did not lose its activity even after 15 days whereas polygalacturonase was almost completely destroyed in that time. In both cases, the maximum activity was attained in 4 days old cultures.

(10) F.moniliforme attacks apples, tomatoes and potatoes and produces rotting in measurable quantities in the two first mentioned; on potato it is checked by cork-formation. No correspondence was found between pathogenicity of the fungi tested (including two other species of Fusarium) on a number of hosts and their capacity to secrete pectinase ~~enzyme~~ in decoctions of these particular plants.

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