Studies in the Physiology of Parasitism

XV. Effect of the Nutrient Medium upon the Secretion and Properties of Pectinase

BY

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A. INTRODUCTION

THE work to be described in this paper is a continuation along certain lines of the earlier enzymological studies of this series. In comparing the pectinase enzymes of a number of parasitic fungi Chona (1932) demonstrated that they reacted differently to a number of physicochemical factors. Thus the enzymic preparations obtained from some potato-attacking fungi, *Phytophthora erythroseptica*, *Pythium* sp. and *Fusarium caeruleum* were characterized by relative insensitiveness to potato extracts, i.e. the addition of the latter to the enzymic solution had a relatively slight retarding effect on the rate of tissue hydrolysis. On the other hand, these preparations were relatively sensitive to the presence of apple juice, which strongly depressed their activity. Enzymic solutions prepared from the apple-attacking fungi *Botrytis cinerea* and *Fusarium fructigenum* showed the converse relationship. In this connexion the active principle of potato juice was shown to be certain polyvalent salts, that of apple juice to be hydrogen-ion concentration. The enzymes of the apple-attacking and potato-attacking fungi had their optima in acid and alkaline solution respectively.

Chona's results were amplified by the later work of Menon (1934), who determined the relation between pH and enzymic activity for extracts

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prepared from a number of parasitic fungi. As a rule the curves obtained for any particular organism showed the same general trend whatever the medium used or whether the enzymic solution was derived from the hyphae (endo-enzyme) or from the culture medium (exo-enzyme). Nevertheless, in one case at least the shape of the curve was markedly affected by the nature of the nutrient used.

Menon also compared the specific retarding effects of certain plant extracts on a variety of enzymic preparations. While confirming the particular results which had been obtained in the earlier work, he pointed out that the nutrient used had an important modifying effect. The experimental data were hardly sufficient to justify the framing of a rule, but they strongly suggested as a working hypothesis that the pectinase solution prepared from a culture on a particular medium tends to be relatively insensitive to constituents of that medium. The suggestion was that the enzyme adsorbed various substances from the medium and was therefore less affected by any further additions of these.

The object of the present investigation was to examine in some detail the effect of nutrient composition upon the excretion of pectinase enzyme and upon the properties of the enzymic preparations obtained. Chief attention was paid to the pH factor. While the two lines of work were carried on more or less simultaneously it will be convenient to deal with each separately and to describe first of all the effect of pH upon enzymic activity.

B. Materials and Methods

The organisms used were the following:

1. *Bacillus carotovorus* Jones, isolated from tomato.
2. *B. subtilis* (Ehrenberg) Cohn.
3. *Botrytis cinerea* Pers., isolated from lettuce.
4. *Pythium de Baryanum* Hesse, isolated from cress.

Stock cultures were maintained in tubes of potato or malt extract agar.

Enzymic solutions were obtained from *Botrytis cinerea* according to the method developed by Brown (1915), i.e. dense spore sowings were made in various media on horizontal glass plates kept in a moist atmosphere. After two days' growth at laboratory temperature, the hyphal mass was removed by centrifugalizing and the culture solution which contained the excreted enzyme was used as such in the tests.

The above method being unsatisfactory for the preparation of Pythium enzyme, the latter was prepared by squeezing out the juice from autoclaved blocks of potato tuber which had been inoculated twelve days previously with the fungus. This extract was subjected to one purification by precipitation with alcohol.

Preparations of the bacterial enzymes were obtained by adding a definite number of drops of a bacterial suspension in water to each of a series of flasks containing 20 c.c. of a liquid nutrient medium. The aim was to present

the latter as a shallow layer so that good aeration would be ensured throughout the medium. From time to time a sample flask was withdrawn from the incubator and the enzymic activity of the culture fluid determined. The enzyme was used in the unpurified form and with no addition of antiseptics except where the action was prolonged on account of the low activity shown. In that case a few drops of toluene were added. After some experience it was fairly well known whether it was desirable or not to add toluene at the beginning of the tests, but for safety some experiments were run in duplicate, one set with toluene and the other without. Whenever the solution under test was so weak that the reaction was incomplete after seven hours, toluene was then added. From the purely enzymological point of view the addition of toluene is allowable, but is open to objection in so far as the idea is to study the effect of the enzyme as an agent of parasitism. In carrying out this work, therefore, the general plan was to avoid the use of antiseptics except where they were absolutely necessary.

The standard method of testing the various enzyme solutions was to determine the time required, at known temperature, for the decomposition of discs of potato tuber of standard thickness (0·5 mm. × 1·8 cm.). In each test three such discs were used, and the average taken. In any one experiment, all the test discs were cut at the same time from the medullary tissue of the same tuber, this precaution being necessary on account of the marked variation from one tuber to another. The time required for complete loss of coherence, as tested mechanically, gives an inverse measure of the enzymic activity of the solution tested.

Throughout the work parallel determinations were made of the drift of the reaction in the various cultures and of the rate of growth of the organisms.

Estimations of pH values were made with the Universal Buffer Solution prepared by British Drug Houses according to the formula prescribed by Prideaux and Ward (1924). By suitable additions of $\frac{1}{2}$-normal soda, the range pH 3·1 to 11·5 can be accurately covered. The usual indicators were used.

The rate of multiplication of *B. carotovorus* was determined by the standard dilution method. This method is generally inapplicable to cultures of *B. subtilis* on account of the tendency to aggregation and scum formation. For this organism a rough estimate of amount of growth was obtained by centrifugalizing and measuring the volume of precipitate. By this means all but the finest aggregates were carried down.

Determinations of the amount of fungal growth were similarly made with the centrifuge, the wet volume of the precipitate being noted.

C. Experimental Results

1. *The pH value as affecting enzymic activity.*

A representative set of curves illustrating the behaviour of the enzyme of *B. carotovorus* in relation to pH value is given in Fig. 1. Here pH is plotted against reaction time (R.T.), i.e. the time required for complete loss

in coherence of the standard test discs of potato tissue. The preparations were derived from cultures on a variety of media and after different periods of incubation so that they showed a considerable range of activity. The optimum pH in all cases is in the neighbourhood of 8. A point of interest

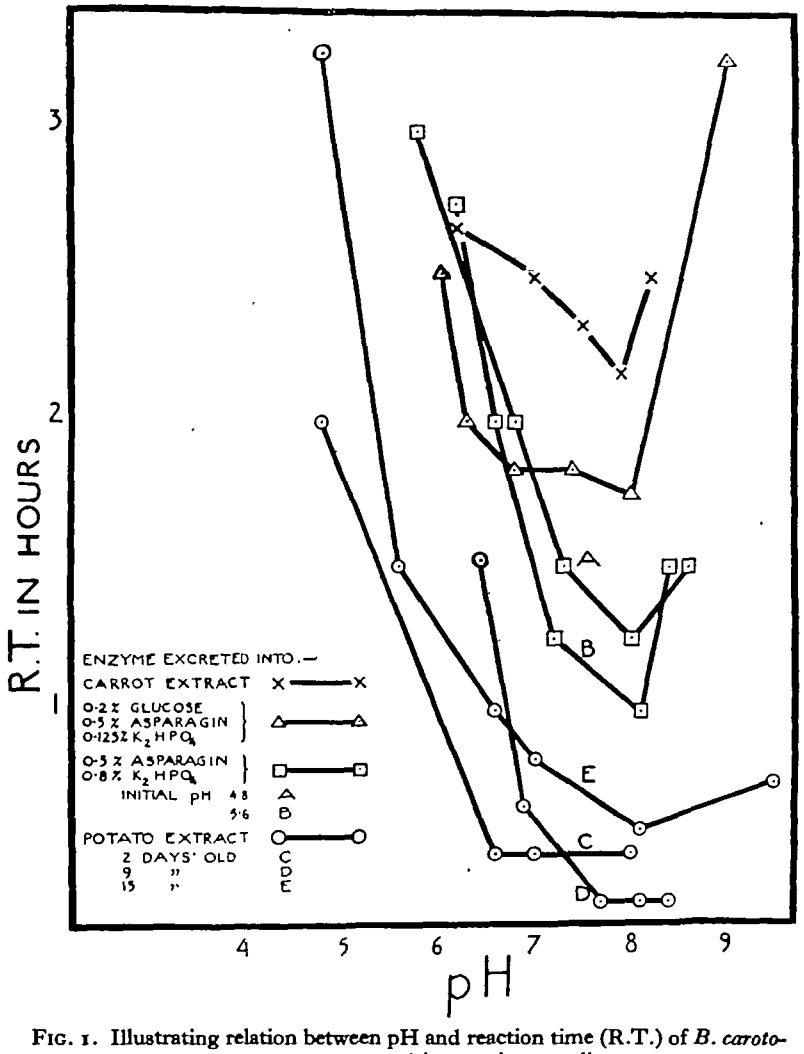

FIG. 1. Illustrating relation between pH and reaction time (R.T.) of *B. carotovorus* enzyme excreted into various media.

is that, although in the more dilute solutions the optimum is sharply defined, at the higher concentrations the curves exhibit a flat-topped optimum. This difference could be explained on the assumption that the rate of diffusion of the enzyme into the test discs of tissue is the slowest process, and as such assumes the function of a master reaction limiting the rate of maceration. The effect described has been repeatedly met with in the course of this work,

whence it follows that solutions of high enzymic activity should be avoided if the position of the optimum is to be accurately determined.

While the position of optimum activity in terms of pH value is more or less sharply defined and lies in the neighbourhood of pH 8, there is evidence that it tends to shift during the course of bacterial growth if the latter leads to the development of an acid reaction. Furthermore, exposure of the enzyme to acid conditions alters it in such a way that it exhibits an optimum activity at a lower pH value. These statements will now be illustrated.

In one experiment, *B. carotovorus* was grown on an apple extract (100 gm. per litre) to which the following constituents were added: 0·5 per cent. asparagin, 0·8 per cent. K_2HPO_4, and 0·075 per cent. $MgSO_4$, $7H_2O$.

The pH value moved from 7·2 to 5·5 by the third day and remained steadily at that low level for the rest of the experiment (18 days altogether). The determinations of the effect of the pH value at various intervals are reproduced in Fig. 2, in which the reciprocal of the reaction time in hours multiplied by 100 is taken as a measure of the activity of the enzyme.

From the figure it is seen that the enzyme of a 3-days-old culture had its optimum at about pH 8·5. After six days this had drifted to the region pH 6·8–7·6. In the 10-days-old culture its position was not accurately determined (6·9–8·0), but at the end of the experiment an optimum in the neighbourhood of pH 7·0 was indicated. Though more detailed examination would be required for the exact location of the various optima, the curves of Fig. 2 indicate clearly that the pH for optimal activity of the bacterial enzyme has moved in the acid direction as the cultures developed.

The same feature is brought out in a somewhat different way in the following experiment. To a basal medium consisting of 0·15 per cent. glucose, 0·5 per cent. asparagin, 0·075 per cent. magnesium sulphate was added Clark and Lubs' (1928) KH_2PO_4–NaOH buffer mixture in such proportions that the medium had initial pH values of 8·0 and 6·2. These two media were inoculated with *B. carotovorus*. Over the first five days, the pH of the former fluctuated between the limits 7·4 and 8·1; that of the latter within the limits 5·7–6·7, i.e. the one medium remained definitely alkaline and the other definitely acid throughout. Examination of the culture media after five days' incubation showed that the region of optimal activity was pH 7·7–8·3 and pH 6·8–7·8 for the enzyme secreted in alkaline and acid solution respectively. In other words, as the pH conditions prevailing in the culture solution are altered, the pH which is optimal for enzymic activity tends to move in the same direction.

The results just described could be interpreted to mean that the organism secretes an enzyme of somewhat different properties in accordance with the reaction of the medium into which the enzyme is excreted; or that the enzyme is fundamentally the same when excreted, but that its properties are modified on exposure for some time to acid solutions. The latter view appears to fit the observed results best, as the following experiments show.

In a number of experiments *B. carotovorus* was grown on the same medium which was adjusted to different initial pH values over the range 4·8–8·1. On the second or third day of incubation, the culture media were tested as to the optimal pH range of the enzyme present, and this was found

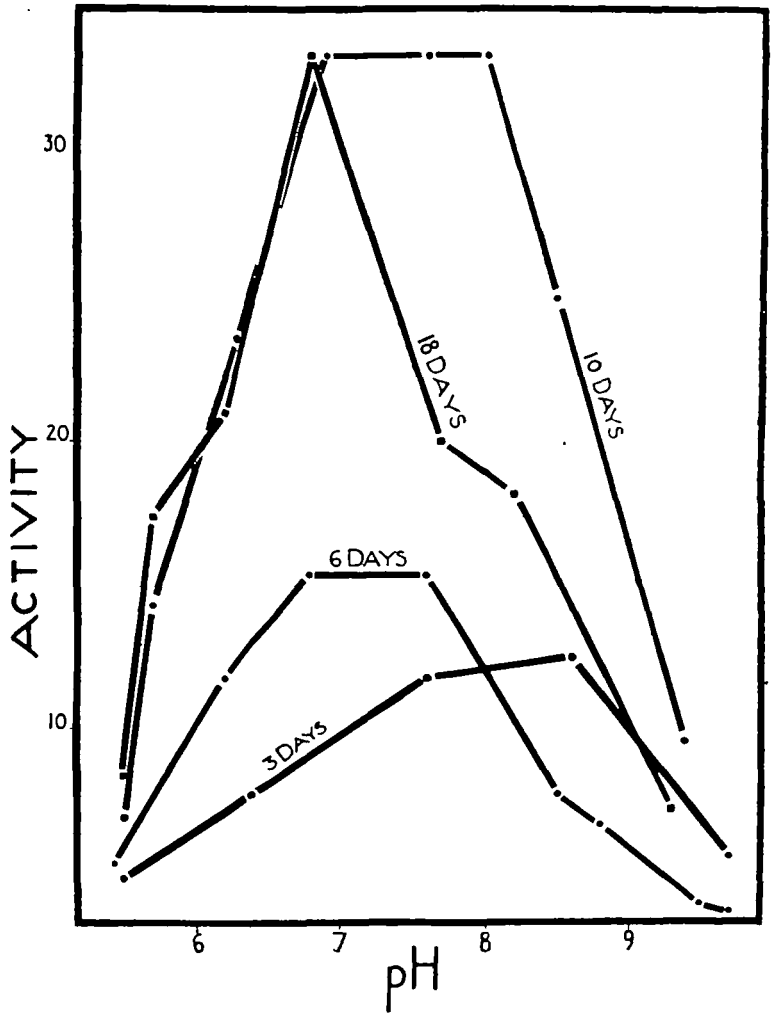

FIG. 2. Illustrating effect of pH on activity of *B. carotovorus* enzyme of various ages.

to be in the neighbourhood of pH 8·0 for all the preparations. There was no indication that, in these young cultures, there was any difference in the pH response of the enzyme. The suggestion, therefore, is that the enzyme, when excreted, is fundamentally the same, and that it requires exposure for some length of time to an acid solution before its properties are definitely altered.

The effect of exposure of the enzyme to acid solutions was clearly shown in a series of experiments in which the enzyme was kept for varying lengths of time, in the presence of toluene, in slightly alkaline (pH 8·0) or in acid (pH 3·0) solution. The presence of toluene, in addition to preventing contamination, ensured that there was no continued secretion of enzyme throughout

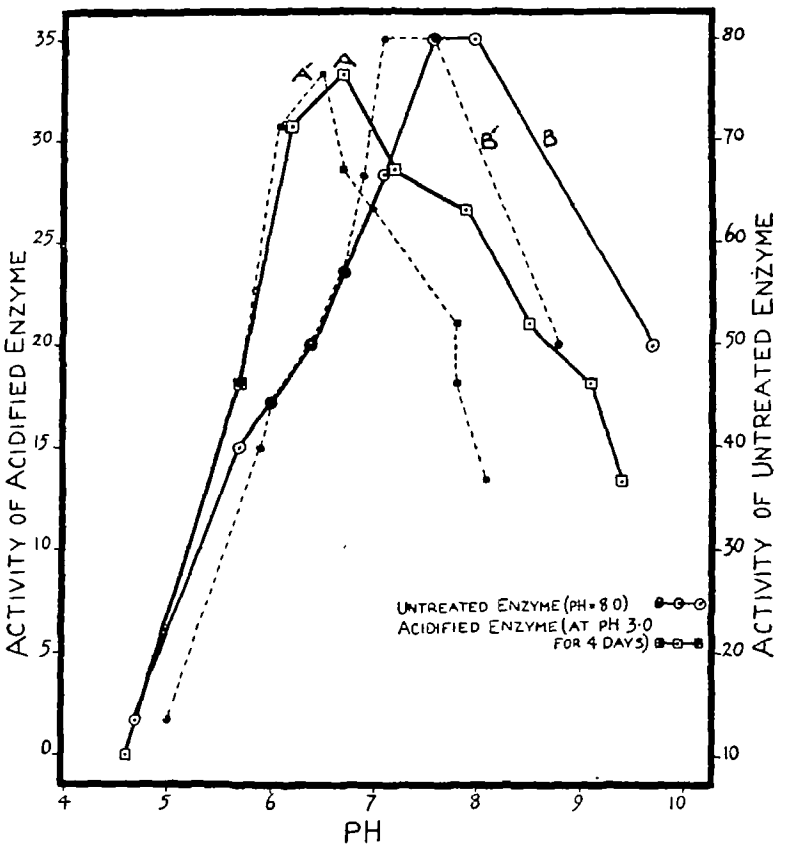

FIG. 3. Illustrating effect of exposure of *B. carotovorus* enzyme to acid conditions.

the period of the experiment. Fig. 3 illustrates the distinct shift of the optimal point arising from storage of the enzyme in acid solution.

It will be observed that in the figure each curve is plotted in duplicate (*A* and A^1, *B* and B^1). The continuous curves give the activity of the enzyme against the pH of the latter before the test discs of tissue are added. Through the liberation of juice from the latter, the alkaline enzymic solutions become less alkaline and the distinctly acid solutions become less acid. The dotted curves give the enzymic activity plotted against the pH of the solutions after maceration of the discs is completed. The true curve would obviously be somewhere between the continuous and the dotted curves. This effect

mentioned, which has been noted throughout the whole work, is not very great and it could be reduced by buffering the enzymic solutions or by using a larger quantity of the latter for each maceration test. The dotted curves have been omitted from Figs. 1 and 2 in order to avoid confusion.

The shift of the pH optimum, which is brought about by four days' exposure to a pH of 3·0 is from pH 7·6–8·0 to pH 6·7 or, if the dotted curves be considered, from pH 7·1–7·6 to pH 6·4.

Incidentally it will be noted that exposure to acid conditions increases the rate of inactivation of the enzyme in watery solution. This is clear on comparison of the scales used in plotting the two pairs of curves, that of the enzyme exposed to acid (*B* and B^1) being shown on the left, that of the enzyme kept at pH 8·0 (*A* and A^1) being on the right. After eight days' exposure to an acid concentration of pH 3, the activity of the enzymic solution was almost zero.

Further experiments showed that the change in position of the optimum could be demonstrated after two days' exposure to acid conditions corresponding to pH 2·0.

The most suitable hypothesis therefore appears to be that the enzyme of *B. carotovorus* has a characteristic optimum at about pH 8, but that exposure to acid conditions causes this to shift towards an acid point.

The effects of the pH value on the activity of the enzymes of *Bacillus carotovorus*, *Bacillus subtilis*, *Botrytis cinerea*, and *Pythium de Baryanum* are shown in Fig. 4. The enzymes of all the organisms except Pythium were excreted into potato extract. It has not been found possible to secure an enzyme of sufficient strength from Pythium by using potato extract. The Pythium enzyme employed was extracted from rotted autoclaved potato tissue and prepared according to the method previously described.

The curves for the activity of the enzymes of *B. carotovorus*, *B. subtilis*, and *P. de Baryanum* are very much alike, with optimal pH values distinctly on the alkaline side. The behaviour of the Botrytis enzyme is, however, different, the position of the optimum pH value being slightly on the acid side. The activity declines gradually towards the higher H-ion concentrations, but has a much steeper downward gradient on the alkaline side. The reverse holds for the enzymes of the other three organisms. Here the downward slope on the acid side is sharper than on the alkaline. In the region extending from about pH 6·7 to pH 7·5, the curve for the Botrytis enzyme shows a rapid descent, while the activities of the other enzymes exhibit a rise. At a pH value of about 9·5, at which the other enzymes have lost at most a small fraction of their activity, the activity of the Botrytis enzyme is almost nil. Again, whereas the activities of the enzymes of *B. carotovorus* and *P. de Baryanum* have fallen nearly to zero long before a pH value of 3 is reached, the Botrytis enzyme still retains at pH 3 a considerable proportion of its activity.

Brown (1915) studied the effect of the H-ion concentration on the activity

of the enzyme of *B. cinerea*, but he represented his results in terms of titrable acidity and not of pH values. He found that the optimum reaction for the activity of the Botrytis enzyme was a little on the acid side of neutrality, with

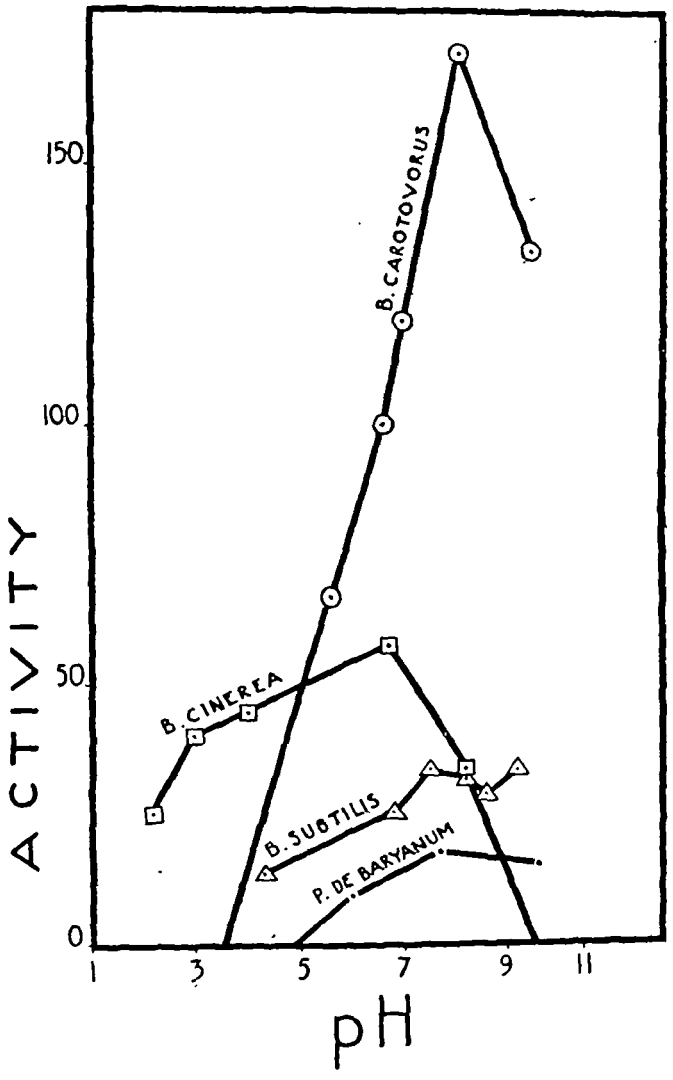

FIG. 4. Illustrating effect of pH on activity of enzyme of *B. carotovorus*, *B. subtilis*, *Botrytis cinerea*, and *Pythium de Baryanum*.

a sharp gradient towards the alkaline side. The curve for Botrytis in Fig. 4 resembles the curve that Brown would have obtained if he had expressed his results in terms of pH values. Menon (l.c.), on the other hand, observed that the curve for the activity of the Botrytis enzyme rose continuously till a pH value below 3 was reached. Thus while there may be some disagreement

as to the exact trend of the curve for the Botrytis enzyme in acid solution, and while in fact the details may vary according to the strain of the fungus used or to the method of preparing the enzyme, all workers agree that the enzyme of Botrytis maintains a high degree of activity up to quite low pH values in the solution. Menon's results for Pythium agree closely with those obtained by the writer.

2. *Factors affecting enzyme production in cultural media.*

(*a*) *Effect of pH value and C/N ratio of synthetic cultural media.* The subject has been studied in fullest detail for *B. carotovorus*. For the sake of clearness the main conclusions will be stated first, and the illustrative detail given later.

Granted suitable conditions of aeration and temperature, the factor which is most important in determining whether a culture of *B. carotovorus* does or does not produce the enzyme in quantity is the pH value of the medium. Speaking generally, one may say that, whatever the initial pH of the medium, if the growth of the organism causes the pH value to move into the region pH 5·5–8·5 or thereby, good secretion of enzyme takes place. Active secretion is correlated with good growth of the organism, though from time to time enzymic solutions are obtained which show a greater activity than would correspond with the bacterial numbers present, and vice versa. The carbon/nitrogen ratio (C/N) of the initial medium is of considerable importance in connexion with growth and secretion of enzyme, but with slight qualification this factor appears to operate by determining the course of pH reaction.

In the experiments to be described, cultural media of various composition (more particularly with regard to the C/N ratio) were set up at different initial pH values, inoculated in a standard way with *B. carotovorus*, and from time to time examined as to

(*a*) drift of pH reaction,
(*b*) bacterial numbers present,
(*c*) enzymic activity of culture solution.

With regard to (*a*), the solutions were either unbuffered, with the result that the pH value would in some experiments move so rapidly to an extreme point that the multiplication of the organism was strongly checked; or they were buffered by the appropriate additions of phosphate mixtures, in order that the pH value should remain reasonably steady throughout the growth period. This was successfully accomplished in those experiments in which the C/N ratio of the medium was properly arranged.

The methods of determining (*b*) and (*c*) have already been described.

In the ultimate analysis one would presume that the enzymic activity of a culture solution depends on the three factors:

(1) Amount of bacterial growth.
(2) The rate of excretion per bacterium.
(3) The pH value of the solution at the time of testing.

The influence of the last factor can be determined by adjusting the solution tested to the pH value which was found in the preceding section to be optimal for enzymic activity; and such a method of standardizing was adopted. On the other hand, the separation of factors (1) and (2) was rarely possible. The method of testing enzymic activity which is employed is of little value when the solutions concerned are very weak, i.e. when the reaction time is much greater than twenty-four hours, and suitably active solutions are only obtained when there is vigorous bacterial growth. It is quite possible that the individual organism is actively secreting enzyme under conditions where the bacterial growth is severely checked, but the concentration of enzyme so produced may be too small to be determinable by the method available. It is only in the relatively rare cases where the curve of enzymic activity runs counter to the curve of bacterial numbers that one can suggest greater individual secretion in one set of conditions than in another.

After preliminary experiments the optimum pH value for the growth of *B. carotovorus* was determined by the type of experiment illustrated in Table I.

TABLE I

	After 1 day.		After 2 days.		After 4 days.	
Initial pH.	pH.	Bacterial nos. per c.c.	pH.	R.T. in hrs.	pH.	R.T. in hrs.
8·0	8·0	10×10^6	7·4	3	8·1	2·5
7·6	7·6	20×10^6	7·2	3	7·8	2·25
7·0	7·0	23×10^6	6·9	2·25	7·4	2
6·8	6·8	24×10^6	6·8	2·25	7·0	2
6·2	6·2	19×10^6	6·2	3·5	6·7	2

The medium used had the following composition: glucose 0·15 per cent., asparagin 0·5 per cent., $MgSO_4.7H_2O$ 0·075 per cent., KH_2PO_4 0·68 per cent. The different initial pH values were set up by the appropriate additions of N/5 NaOH.

Throughout the experiment the pH value did not change appreciably in each culture medium, i.e. the buffering was successful. After one day, at which time bacterial counts were made, no drift was apparent. The more alkaline media by and by became somewhat less alkaline, but by the fourth day had returned to approximately the initial pH values. This effect, which appears to be inevitable even in the best buffered solutions and which will be illustrated more strikingly in later tables, is probably due to the preferential attack by the organism of the glucose in the early stages. This would lead to formation of organic acids. As the glucose became depleted the organism would attack the organic acids and the carbon part of the asparagin, so that a drift to the alkaline side would set in. The same drift towards greater alkalinity, but without a preliminary drift to greater acidity, is shown in the media of lower initial pH (pH 6·2 and 6·8). This is what would be expected if the above explanation is a true one.

The optimum pH value for the growth of *B. carotovorus* is shown in Table I to lie within the range 6·2–7·6, and the best value suggested is pH 6·8.

In marked contrast to the results of Table I are those shown in Tables II and III. In both the latter the basal medium contained 0·075 per cent. $MgSO_4.7H_2O$ and 0·125 per cent. K_2HPO_4, i.e. the phosphate concentration was much less than that of the media in Table I. The buffering effect was thus much reduced. The concentrations of glucose and asparagin used were as follows:

	Glucose.	Asparagin.
Table II	0·1%	0·5%
Table III	0·2%	0·2%

TABLE II

	2 days.		4 days.		6 days.		10 days.	
Initial pH.	pH.	R.T. hrs.	pH.	R.T. hrs.	pH.	R.T. hrs.	pH.	R.T. hrs.
7·1	8·2	8	8·4	2·25	8·5	1·5	8·8	1·25
6·4	7·9	8	8·3	3	8·4	1·5	8·6	1·75
5·8	5·4	37	7·0	3·25	8·3	1·75	8·6	1·75
5·0	4·4	50	4·4	37	4·4	33	4·4	29

TABLE III

	4 days.		9 days.	
Initial pH.	pH.	R.T. in hrs.	pH.	R.T. in hrs.
8·3	7·9	9	8·5	1·75
7·6	6·4	10	8·4	2
7·1	5·3	>24	5·4	18
6·1	5·1	>24	5·2	>24
5·8	5·0	>24	5·2	>24
4·2	4·2	>24	4·2	>24

As a result of the low C/N ratio in the media of Table II a steady alkaline drift was shown in the culture solutions with initial pH 7·1 and 6·4. The solution with initial pH 5·8 first became more acid, then drifted to a pronounced alkaline point. On the other hand, the medium of initial pH 5·0 became too acid for active growth of the organism, an effect which was obvious from the solution remaining clear, whereas the others became distinctly turbid. High enzymic activity was shown in the first three but not in the last.

The relatively high C/N ratio of the media of Table III produced a tendency to development of acidity, so that it was only in the media of initial pH 8·3 and 7·6 that a final drift to an alkaline point occurred. All the remainder became acid and remained so till the end of the experiment. In all the media of Table III except the first two, bacterial growth was severely checked and enzymic activity was low.

Summarizing Tables I–III one can say that, independently of the initial pH value, good growth and enzyme production take place provided the medium is such that the reaction does not drift to an acid point and remain

there. An acidity corresponding to about pH 5·4 represents the limit at which a reversal of drift can occur. At pH values less than this, growth is severely checked and little enzymic activity is developed; above this point the alkaline drift is able to set in, and when this is established there is a rapid development of enzymic activity (cf., in this connexion, the two days' and four days' old cultures of initial pH 5·8 in Table II).

The effect of the C/N ratio in a relatively unbuffered medium (K_2HPO_4 = 0·125 per cent.) is shown in detail in Table IV. All the media (*A*–*N*) had

TABLE IV

Media.	% glucose/ % asparagin.		2 days.	4 days.	6 days.	10 days.	16 days.
A	0/0·5	pH	8·3	8·6	8·7	8·7	8·9
		R.T.	15·5	3·5	3·25	2·5	2·25
B	0·02/0·5	pH	8·3	8·5	8·6	8·5	8·5
		R.T.	9·5	2·75	2·75	2	1·5
C	0·05/0·5	pH	8·3	8·4	8·5	8·5	8·7
		R.T.	9·5	2·5	2	2	1·5
D	0·02/0·2	pH	8·0	8·3	8·6	8·6	8·6
		R.T.	17	13·5	4·75	4·25	17
E	0·004/0·04	pH	8·3	8·3	8·2	8·2	8·2
		R.T.	33	20	18	21	22
F	0·1/0·5	pH	7·9	8·4	8·4	8·5	8·7
		R.T.	8·5	3	2	1·5	1
G	0·02/0·1	pH	7·9	8·4	8·4	8·4	8·4
		R.T.	18	17	16	7·75	20
H	0·004/0·02	pH	7·9	7·9	7·9	7·9	7·9
		R.T.	31	31	18	24	24
J	0·2/0·5	pH	5·3	5·5	5·6	8·2	8·4
		R.T.	45	37	19	2·25	1·25
K	0·02/0·05	pH	7·9	8·2	8·3	8·3	8·3
		R.T.	18	18	17	10	19
L	0·008/0·02	pH	7·9	7·9	7·9	7·9	7·9
		R.T.	33	30	18	24	26
M	0·5/0·5	pH	5·1	5·2	5·2	5·5	7·4
		R.T.	48	48	42	25	12
N	0·5/0·1	pH	4·8	4·8	4·8	4·8	5·5
		R.T.	48	48	48	48	46

an initial pH of 7·0–7·3. In the table the upper row corresponding to each medium gives the pH drift, the lower row gives the reaction time of the enzyme.

The main features shown in Table IV are as follows:

(1) With media of the same high content of asparagin (*A*, *B*, *C*, *F*, *J*, and *M*), the pH drift is steadily to the alkaline side when the glucose concentration is low (*A*, *B*, *C*, and *F*). With higher glucose concentrations (*J* and *M*), the cultures first become acid, but later a reversal sets in, by the tenth day for *J* and by the sixteenth day for *M*. Good enzymic activity is shown in media *A*, *B*, *C*, and *F* from the fourth day onwards, whereas the same does not occur in *J* until the tenth day, and *M* is still weak even on the sixteenth day.

(2) With media of the same C/N ratio, but of different concentrations, e.g.

C, *D*, and *E* (ratio 1 : 10), the pH drift is the same in all, but enzymic activity progressively diminishes as total concentration decreases. The same holds for media *F*, *G*, and *H* (ratio 1 : 5). With *J*, *K*, and *L* (ratio 2 : 5), the activity of *J* is very low until the tenth day on account of a preliminary acid drift. When this drift is reversed, and conditions become alkaline, there is a relatively sudden increase in enzymic activity. With media *K* and *L* there is no such preliminary acid drift, whence it follows that, for the same ratio of carbon to nitrogen, increase of concentration accentuates the tendency to an acid drift.

(3) With media of the same glucose concentration (*B*, *D*, *G*, and *K*) the drift is much the same in all, but enzyme activity progressively diminishes with the fall in asparagin concentration.

A noteworthy feature of media with very low C/N ratio, e.g. *A*, is the deposition, after a few days, of crystals of ammonium magnesium phosphate.

Table V records the behaviour of media *A*, *F*, *J*, *M*, and *N* of Table IV, but with bacterial counts added for the sixth day.

TABLE V

Medium.	C/N ratio (%glucose/% asparagin).	After 6 days pH.	After 6 days bacterial no. per c.c.	After 6 days R.T. hrs.
A	0/0·5	8·7	$2{\cdot}8 \times 10^6$	3·25
F	0·1/0·5	8·4	800×10^6	2
J	0·2/0·5	5·6	1700×10^6	19
M	0·5/0·5	5·2	$0{\cdot}003 \times 10^6$	42
N	0·5/0·1	4·8	$<10^3$	>48

The series of media *A*–*N* is one of increasing C/N ratio. Within this series there is a pronounced optimum for bacterial growth, viz. in medium *J*. Much greater enzymic activity, however, is shown in media *A* and *F*, which is the more remarkable in medium *A* when the relatively weak bacterial growth is borne in mind. Such a comparison is unfair in that the pH reaction of medium *J* is unfavourable to enzymic activity. When the comparison was made after adjustment of medium *J* to pH 8, which is optimal for enzyme activity, it was found that medium *A* was nearly twice as active as medium *J*. The obvious interpretation of this result is that much greater excretion of enzyme per individual bacterium took place under the conditions prevailing in *A* than in *J*.

The marked effect of the C/N ratio upon enzyme production illustrated in the foregoing, especially in Table IV, is not due in the main to carbon and nitrogen concentrations as such, but to the effect of the C/N ratio upon the pH value. If the media are sufficiently buffered to prevent extreme drifting of the pH value, very much the same enzymic concentrations are obtained for a wide range of C/N ratios in the culture medium. This point is illustrated in Table VI. It is only when the C/N ratio becomes too high, as in the last medium of the table, that the buffering has proved incompletely effective, with the result that a relatively weak enzymic solution is obtained.

Subsidiary experiments showed that, provided the pH value does not run to the acid side, a phosphate concentration of about 0·1–0·2 per cent. is optimal for enzyme production. The active secretion of enzyme which occurs

TABLE VI

(*Solutions buffered with* 0·8 per cent. K_2HPO_4)

C/N ratio (% glucose/ % asparagin).	Initial pH.	After 3 days. pH.	After 3 days. R.T. in hrs.	After 6 days. pH.	After 6 days. R.T. in hrs.
0·02/0·5	7·2	8·4	1·16	8·6	<0·5
0·1/0·5	7·0	8·2	2	8·6	<0·5
0·25/0·25	7·1	7·8	1	8·0	<0·5
0·5/0·5	7·0	7·0	1·25	8·0	<0·5
0·5/0·1	7·1	6·2	3·75	5·8	1

in well buffered media, such as those of Table VI, is therefore not a direct effect of the high concentration of phosphate which is present.

The general conclusion from the experiments of Tables I–VI is that, apart from a stimulating effect of the nitrogenous constituent on enzymic production which may be noted in some cases, the controlling factor in enzymic production is the pH value arising in the medium. The C/N ratio of the culture solution is important in so far as it determines the drift of the reaction.

Table VII records an experiment in which *Botrytis cinerea* was grown on a basal medium consisting of 0·125 per cent. KH_2PO_4 and 0·075 per cent. $MgSO_4$, with different proportions of glucose and asparagin. A comparison

TABLE VII

C/N ratio (% glucose/ % asparagin).	After 2 days. Vol. of mycelium in c.c.	Final pH.	R.T. in hrs.
0/0·5	0·4	7·3	1·75
0·1/0·5	0·5	7·5	<0·5
0·2/0·5	0·6	7·7	<0·5
0·5/0·5	1·8	7·9	<0·5
1·0/0·5	6·2	7·7	<0·5

of this Table with Table IV shows how differently the two organisms behave in respect of the pH drifts produced. On the two last media of Table VII, *B. carotovorus* would quickly develop a highly acid reaction which would inhibit growth and therefore produce very weak enzymic preparations.

It is to be noted, however, that media of formula 0·1 per cent. glucose/0·5 per cent. asparagin and 0·2 per cent. glucose/0·5 per cent. asparagin give strong enzymic preparations with both organisms, so that the possibility of obtaining vigorous extracts from both on the same medium exists.

(*b*) *Effect of the nitrogenous constituent of synthetic media.* The availability of simple inorganic salts for enzyme production by *B. carotovorus* was also investigated. In salts, according as the nitrogen is present in the kation (e.g. NH_4Cl) or the anion (e.g. KNO_3), utilization of the nitrogen results in

an accumulation of the anion or the kation with consequent development of an acid or an alkaline reaction. In view of the large extent to which the enzymic activity of *B. carotovorus* has been shown to depend upon the pH value, it was interesting to determine how far different inorganic sources of nitrogen had a direct effect on enzyme production, or alternatively an indirect one, through their influence on the pH drift.

Illustrative contrasting data are set out in Tables VIII and IX, in each of which the various nitrogenous compounds were used in equivalent proportions.

TABLE VIII

Basal medium: glucose, 0·2 per cent.; K_2HPO_4, 0·125 per cent.; $MgSO_4$, 0·075 per cent.
Conc. of N source: asparagin 0·2 per cent.; KNO_3, 0·307 per cent.; NH_4Cl, 0·162 per cent.

		After 4 days		After 9 days.	
Nitrogen source.	Initial pH.	pH.	R.T. hrs.	pH.	R.T. hrs.
Asparagin	7·1	5·3	>24	5·4	18
Potassium nitrate	6·9	6·4	14	5·5	12
Ammonium chloride	6·9	4·4	>24	4·2	>24

TABLE IX

Basal medium: glucose, 0·1 per cent.; K_2HPO_4, 0·8 per cent.; $MgSO_4$, 0·075 per cent.
Conc. of N source: asparagin 0·47 per cent.; KNO_3, 0·72 per cent.; NH_4Cl, 0·38 per cent.

		After 2 days.	After 3 days.	
Nitrogen source.	Initial pH.	pH.	pH.	R.T. hrs.
Asparagin	7·0	7·2	7·8	0·5
Potassium nitrate	7·3	7·2	7·7	0·75
Ammonium chloride	7·0	6·4	6·9	1

In the experiment of Table VIII, the high C/N ratio combined with the low degree of buffering resulted in an acid pH drift in all three media used, but this was greatest in the medium with ammonium chloride. All the media gave weak enzymic solutions, and this was especially so with the ammonium salt. On the other hand, the high degree of buffering and the low C/N ratio of the media of Table IX had the effect of maintaining a fairly steady pH value in all the cultures (though the acid drift was not suppressed in the medium with ammonium chloride), and good enzymic activity was shown in all. The effect of the nitrogenous constituent thus resolves itself, in the main, if not entirely, into a pH effect.

Parallel experiments with *B. subtilis* gave very similar results, except that the enzymic preparations obtainable from this organism are considerably weaker.

The effect of the nitrogenous compound on growth and enzyme secretion of *Botrytis cinerea* was studied by adding to a basal medium (glucose, 0·4 per cent.; KH_2PO_4, 0·125 per cent.; $MgSO_4$, 0·075 per cent.) equivalent

concentrations of asparagin (0·047 per cent.), Witte's peptone, urea, potassium nitrate, sodium nitrate, and the following salts of ammonium: chloride, sulphate, phosphate (HPO_4), nitrate, and tartrate. The cultures were grown on horizontal glass plates, according to Brown's method, and at the end of two days enzymic activity, pH drift and amount of mycelium formed were determined. The main results may be summarized as follows:

1. Asparagin, peptone, and ammonium tartrate cause the pH value to drift to the region 6–7, and all produce very active enzyme solutions.

2. The ammonium salts of strong acids: chloride, sulphate, and nitrate, while giving growth comparable to that on asparagin, cause the pH value to move to the vicinity of pH 3 and enzymic activity is very low in all. With ammonium phosphate the effect is intermediate, both as regards pH and enzymic activity.

3. With urea and both nitrates growth was rather weak, and so also was enzyme production.

The data obtained indicated that when the pH value sank to the neighbourhood of 5·5 (as with ammonium phosphate) or lower (as with ammonium chloride), enzyme secretion was reduced. Thus the same rule appears to apply to *Botrytis cinerea* as to *B. carotovorus* (see p. 740). This result was rather unexpected for a fungus like *B. cinerea*, the optimum for growth of which is about pH 6.

(*c*) *Enzyme production on certain natural decoctions.* In the light of the preceding work with synthetic solutions of various compositions it was interesting to examine the course of the reaction produced by *B. carotovorus* and *B. subtilis* on natural plant extracts. These were prepared from potato, turnip, carrot, and apple in the usual way. Data illustrative of the results are included in Tables X and XI.

On potato extract a definitely alkaline reaction is produced which is quite favourable for the activity of the pectinase enzyme. Potato extract thus behaves like a synthetic medium with rather low C/N ratio. On this medium, *B. subtilis* reacts similarly, except that the enzymic preparations show less than one quarter the activity of those of *B. carotovorus* under the same conditions.

On the other hand, in the turnip extracts an acid reaction is developed. With *B. carotovorus* this reaches a pH value of about 5, which approaches the limit which this organism is able to tolerate. *B. subtilis* has a less pronounced acid-producing tendency, but here again the enzymic solutions obtained are weaker. Carrot extract shows the same features as turnip extract. In contrast to the preceding the apple extracts used were entirely unsuitable for the growth of *B. carotovorus*. In the first place the initial pH value was near 3·5, at which the organism would not grow; when the initial pH value was adjusted to neutrality, growth took place but the reaction rapidly moved to a low pH value (4·6), which stopped further growth. Even when the medium was buffered so that this extreme acid drift was

TABLE X

B. carotovorus on Potato Extract

	2 days.				7 days.			
		R.T. in hrs.				R.T. in hrs.		
Initial pH.	pH.	at prevailing pH.	at optimum pH.	*Growth.	pH.	at prevailing pH.	at optimum pH.	Growth.
5·2	5·3	40	15	+	8·4	<0·5	<0·5	++
5·6	6·6	2	1·5	++	8·4	<0·5	<0·5	++
6·9	6·4	1·5	0·75	++	8·4	<0·5	<0·5	++
8·1	8·0	0·5	0·5	++	8·5	<0·5	<0·5	++

* As determined by turbidity records; — indicates apparent absence of growth, + visible though poor growth, and + + profuse growth.

TABLE XI

B. carotovorus on Turnip Extract

	2 days.				7 days.			
		R.T. in hrs.				R.T. in hrs.		
Initial pH.	pH.	at prevailing pH.	at pH 7·0.	*Growth.	pH.	at prevailing pH.	at pH 7·0.	Growth.
4·3	4·3	>48	33	—	4·3	>24	>24	—
5·5	5·1	10	5	++	4·9	>24	6·25	++
6·1	5·2	6·5	3·25	++	5·0	11	3·5	++
7·3	5·2	10	3·5	++	5·2	5·25	1·25	++

* Notation as in Table X.

prevented, enzymic production was poor and was made appreciable only by the addition of an extra supply of nitrogenous substance. The composition of apple extract, and presumably, therefore, of apple tissue also, is of a kind that inhibits the growth and the parasitic mechanism of *B. carotovorus*.

SUMMARY

1. The optimum pH value for the pectinase enzyme of *Bacillus carotovorus* depends to some extent on the nature of the medium into which the enzyme is excreted. In young cultures the optimum pH of this enzyme is near to pH 8. If the culture medium is such that bacterial growth causes a pH drift to an acid point, or if the reaction is so maintained by appropriate buffering, the pH which is optimal for enzymic action likewise moves in the acid direction.

2. Subjection of the enzyme for some time to acid conditions displaces the optimum to a lower pH value.

3. The effects of the pH value on the activity of the enzymes of *B. carotovorus*, *B. subtilis*, *Botrytis cinerea*, and *Pythium de Baryanum* are compared and contrasted.

4. The production of active enzymic solutions by *B. carotovorus* is largely controlled by conditions which govern the rapid multiplication of the organism. On numerous media there is a tendency for an acid drift of the reaction, to be followed later by a reversed drift to alkalinity. Good growth and active enzyme solutions are obtained only when the pH drift towards acidity does not exceed pH 5·0.

5. On the basis of equal growth, cultures of *B. carotovorus* excrete pectinase enzymes more freely under alkaline than under acid conditions.

6. The chief effect of the carbon-nitrogen ratio of the cultural medium upon enzyme production is that it determines the direction and intensity of the pH drift. When this ratio is low, the reaction becomes alkaline and vigorous enzyme production occurs. With high C/N ratio, a high H-ion concentration which inhibits growth and enzyme production develops. Over a narrow range of media an inverse correlation between growth and enzyme production has been observed.

7. The effect of the nitrogenous constituent is also to a large extent an indirect one, though its influence on the reaction developed. With *Botrytis cinerea* nitrate appears to be a poor source of nitrogen from the point of view of enzyme production.

8. The behaviour of *B. carotovorus* and *B. subtilis* on vegetable decoctions has been studied and correlated with that on synthetic media.

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