Studies in the Physiology of Parasitism.¹

IV. On the Distribution of Cytase in Cultures of *Botrytis cinerea*.

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IN the first of these studies a method was described by which a powerful enzymic solution could be obtained in reasonable quantities from fungal germ tubes of one day's age. It was claimed on *a priori* grounds that such an extract was more suitable for the study of the question of the nature of the active principle responsible for the breaking down of the host tissue in advance of the growth of the hyphae than the extracts which had been employed by previous investigators. The present paper relates to an investigation carried out with the object of determining in what way enzymic preparations obtained from the same strain of fungus may differ from each other. Though of a purely enzymological interest, these results have been incorporated in the present series, partly on account of the light which they throw on the nature of the extracts employed by previous investigators, and partly because of their bearing on the manner in which fungi secrete enzymes and on the technique of extraction.

It will add to clearness to tabulate first of all the factors which have been found to influence markedly the activity of the enzymic preparation obtainable from a fungal culture. These are :

1. The density of sowing of the spores.
2. The length of the period allowed for development.
3. The nature of the nutrient employed.

It must be emphasized at this point, in order to prevent misconception, that the first factor mentioned is not a factor *per se*. It will appear in the sequel that the essential factor concerned in the obtaining of a strong extract is the proportion of actively growing mycelium to that which has already ceased to grow. Variation of the density of spore-sowing, together

¹ For No. I of this series, see Ann. Bot., vol. xxix, 1915, p. 313; for No. II, see Ann. Bot., vol. xxx, 1916, p. 389; for No. III, see Ann. Bot., vol. xxx, 1916, p. 399.

with the variation of the length of the period allowed for development, is merely an experimental method by which the ratio of actively growing to non-growing mycelium can readily be altered while at the same time ensuring economy of space and labour. With this understanding, factors (1) and (2) will be considered as independent factors during the account of the experimental part of the work. Their exact bearing on the phenomena concerned will be set forth in the general discussion of results.

FACTORS INFLUENCING ACTIVITY OF A GIVEN ENZYMIC SOLUTION.

It was shown in the first paper of this series that the activity of an extract of germ tubes increased up to a certain limit with the amount of enzyme present and was reduced by the presence of certain other substances, e. g. salts, acids, &c. (except, in the case of acids, in the immediate neighbourhood of the neutral point). As therefore the enzymic activity is the resultant of two antagonistic factors, amount of enzyme and amount of retarding substances, it is possible for two extracts of equal activity to contain widely different amounts of enzyme. If we suppose, as seems legitimate, that deactivation of an extract by heating to 65° C. merely affects the enzyme (and it may be, some other colloidal substances which, however, are not likely to be of importance in this connexion), then it is possible to separate the two factors. The method of separation is as follows:

Suppose two extracts x and y are to be compared. Portions of x and y are heated to 65° ; such deactivated extracts may be termed x' and y' respectively. If now 1 c.c. of x be mixed with 1 c.c. of y' —which mixture may be represented by the symbol xy' —and if similarly we prepare the mixture $x'y$, it is plain that the retarding factors (salts, acids, &c.) in both mixtures are equal in amount. We can therefore judge from any differences in the activities of xy' and $x'y$ as to whether x or y is richer in enzyme.

The method of estimating the activity of the extracts has been described in the paper already referred to (No. 1, p. 324). The discs employed were of 0.5 mm. thickness, and were of potato, turnip, or swede.

Notation. In the following account, estimations are made of the activities of preparations obtained by extracting the washed and dried hyphal mass of the fungus, and of preparations consisting of the medium in which the fungus has been grown. For convenience in description these will be referred to as H (hypha) and M (medium) preparations respectively. Furthermore, as preparations of different ages will be compared with one another, a suffix will be added to indicate the age (in days) of the preparation under consideration. Thus by H_2 is meant an extract of hyphae of two

days' age; by M_2 , the medium in which the fungus has germinated and developed for two days, and so on.

EXPERIMENTAL METHOD.

In the case of thin sowings of spores, a quantity of the nutrient fluid (turnip extract) was infected in a flask with a small tuft of mycelium containing spores: for the thick sowings the method described in No. I of this series was adhered to. At the end of the period allowed for development, the nutrient medium was separated from the fungal mass by filtration through muslin. The fungal mass was thoroughly washed, dried, ground, and extracted in the manner already described.

Spores sown thinly. The general nature of the results obtained is indicated in the following experiment:

Three Erlenmeyer flasks, each containing 25 c.c. of turnip extract, were infected with a tuft of mycelium, and incubated at 20° C. Development in all three flasks appeared equal. Flask A was tested after 1 week, B after 2 weeks, and C after 3 weeks, the M and H preparations (0.1 gram. to 3 c.c. water in the case of the latter) being compared with standard extract. The activities of the various preparations are given in the following table, that of standard extract being taken as unity:

<i>A</i> (1 week).		<i>B</i> (2 weeks).		<i>C</i> (3 weeks).	
H_7	M_7	H_{14}	M_{14}	H_{21}	M_{21}
0.2–0.25	0.17	0.03–0.04	0.06	less than 0.02	trace (?)

In the case of both sets of preparations, the activity is seen to diminish continuously after one week, and at the end of the third week it is reduced to a negligible quantity in both cases. The further consideration of the H preparations will be taken up later. In the case of the M preparations, seeing that initially the enzymic activity is nil, it is plain that activity increases with time to a maximum and subsequently declines. The nature of this relationship is shown more clearly by the following more detailed figures. The dry weight of hyphal material derived from each culture is added:

Age in days	2	4	6	9	12
Activity of M prep.	<0.03	0.2	0.2	0.13	0.08
Dry wt. of mycelium	0.017 gram.	0.27	0.36	0.33	0.37

It will be observed that enzymic activity is slight on the second day; that the maximal activity is reached in the neighbourhood of the fourth to sixth days; also that about the latter time stagnation in the growth of the mycelium has been reached.

By applying the method described on p. 488, it is possible to determine the factor responsible for the reduced activities of the H preparations tabulated on p. 489. The table gives the enzymic activities of the various preparations indicated.

H₇, H₁₄, H₂₁ are, as before, the preparations obtained by extracting hyphal material of 7, 14, 21 days' age respectively; H₇', H₁₄', H₂₁' are the corresponding deactivated extracts.

S = Standard extract.

S = 1	H ₇ = 0.2	H ₁₄ = 0.03-0.04	H ₂₁ < 0.02
SS' = 0.8	H ₇ S' = 0.16	H ₁₄ S' = 0.04	H ₂₁ S' < 0.02
S'S = 0.8	H ₇ 'S = 0.8	H ₁₄ 'S = 0.8	H ₂₁ 'S = 0.8

The reduction of activity of S'S, H₇'S, H₁₄'S, and H₂₁'S, as compared with S, is about equal to that produced by dilution of S with an equal volume of distilled water. It is therefore plain that S, H₇, H₁₄, H₂₁ all contain amounts of retarding substances too small to be recognized by the method. The weakness of the preparation H₇S' as compared with H₇'S shows that the small activity of the H₇ preparation is due simply to the small concentration in it of the enzyme; and this is true *a fortiori* in the case of the H₁₄ and H₂₁ preparations.

Spores sown thickly. Spores were sown on circular glass plates in the ratio of 0.1 c.c. of spores to 10 c.c. turnip extract. The following table gives the data obtained as to yield of mycelium and activity of the corresponding H preparations:

Stage of Germination.	Yield per Plate (dry wt.).	Notation.	Activity.		
			0.1 gm. in 1.5 c.c.	0.1 gm. in 3 c.c.	0.1 gm. in 4.5 c.c.
Ungerminated	0.01 gm.	H ₀	0.1	—	—
8 hrs. germination	0.02 "	H.	0.19	—	0.12
1 day's "	0.14 "	H ₁	1	0.75	0.6
2 days' "	0.17 "	H ₂	1	0.75	0.6
4 " "	0.16 "	H ₄	0.75	0.5	0.38
6 " "	0.14 "	H ₆	0.25	—	0.17

The features brought out by the above table are:

1. The yield of dry material for a given quantity of spores sown reaches a maximum in the neighbourhood of the second day. Subsequently a slow decrease in dry weight takes place. This decrease is probably to be ascribed to respiration.

2. The activity of the extract obtainable from the dried mycelium reaches a maximum about the first or second day; on the fourth day it is distinctly, and on the sixth day very considerably, reduced.

In the above table, extraction was made on the basis of equal quantities of dry material. By combining the curves of activity and of yield, it is readily seen that when extraction is made on the basis of equal

quantities of spores originally sown, the curve of activity obtained is of the same kind, but grades off more rapidly on both sides of the maximum.

The material H_1 in these experiments is the same as the material employed for the preparation of standard extract (S). A comparison of H_0 , H_3 , and H_6 with H_1 according to the method which has already been described showed the reduced activity of the extracts derived from these sources to be due to lack of enzyme and not to the presence of large quantities of retarding substances.

Spores sown thickly—M preparations. The activities of the various M preparations corresponding to the H preparations just described are given in the following table, that of standard extract being taken as unity :

$M_3 = \text{trace}$	$M_1 = 1$	$M_2 = 1$	$M_4 = 0.87$	$M_6 = 0.25-0.3$
	$M_1S' = 1$	$M_2S' = 1$	$M_4S' = 0.87$	$M_6S' = 0.25-0.3$
	$M_1'S = 0.17$	$M_2'S = 0.2$	$M_4'S = 0.2$	$M_6'S = 0.15$

It is seen from the above table that the activity of the M preparations passes through a maximum which is reached on the first to second day. The activity is very considerably reduced by the sixth day.

A comparison of the figures in the second and third rows of the above table shows that the concentration of enzyme in M_1 , M_2 , and M_4 is much greater than in S; and that the same applies in a less degree to M_6 . Nevertheless the activity of M_6 is less than a third that of S. The preparation M_6 therefore illustrates the case of an enzymic solution in which the presence of a large quantity of enzyme is masked by the presence of retarding substances so that the full activity of standard extract is not reached.

That the concentration of enzyme in S is relatively so small involves no contradiction, as a number of factors, such as degree of dilution, adsorption of enzyme on the fungal debris, loss on desiccation, might be expected to play a part in producing this result.

The high concentration of enzyme in the M preparations is also shown in the following ways :

1. While the activity of standard extract diminishes with dilution, that of such a preparation as M_2 remains unaltered up to a considerable degree of dilution. Thus by dilution to one-fourth the activity of S diminishes to a half (No. 1 of this series, p. 342), whereas dilution to one-eighth produces no appreciable diminution in the activity of M_2 .

2. The M preparations are much less sensitive to the addition of salts and of plant extracts than is standard extract. The following table shows the retarding action of concentrations of KNO_3 upon M_2 and standard extract :

Concentrations of KNO₃.

	<i>n/∞.</i>	<i>n/128.</i>	<i>n/32.</i>	<i>n/8.</i>	<i>n/2.</i>
M ₂	1	1	0.87	0.5	0.33
S	1	1 (—)	0.37	0.08	0.015–0.025

A comparison of the M preparations in the case where the spores are thickly and where they are thinly sown is instructive :

*Manner of Sowing.**Time in Days.*

	0	1	2	4	6	9	12
Thick	0	1	1	0.87	0.25–0.3	—	—
Thin	0	—	< 0.03	0.2	0.2	0.13	0.08

The features of note are :

1. In both cases the activity of the M preparations passes through a maximum.

2. The effect of thick as compared with thin sowing is to increase the magnitude of the maximum, and to bring forward in time the date of its attainment.

Effect of medium. Various media may be expected to give different quantitative results in view on the one hand of the retarding factors which they introduce, and on the other of the vigour of the growth to which they give rise. Only a few experiments have been carried out in this connexion. The various media employed were dilutions of a strong turnip extract. The following table gives the activities of the M preparations in the various media after twenty-four hours' germination of the spores :

T = turnip extract.

m = a density of sowing equal to 0.1 c.c. spores in 10 c.c. nutrient.

*Density of Sowing.**Nutrient.*

	1	T/10	T/100	T/1000
<i>m</i>	1	0.2	0.1	trace
<i>m/10</i>	0.2	0.2	0.1	trace
<i>m/100</i>	trace	—	trace	trace
<i>m/1000</i>	trace	—	trace	trace?

The results of the above table can be summarized thus : The activity of the M preparation obtained is reduced both by diminishing the strength of the nutrient and the density of sowing of the spores.

FURTHER COMPARISONS BETWEEN M PREPARATIONS AND STANDARD EXTRACT.

1. *Amount of retarding substances present.* The amount of these substances present in standard extract (and extracts of mycelium generally) has been seen to be negligible (p. 490). The amount present in the M preparations, on the other hand, is considerable, being only somewhat

less than that present in the original turnip extract. This is shown by the following table of activities :

$M_2 = 1$	$M_2S' = 1$	$M_2M_2' = 0.75$	$M_2T = 0.58$
$S = 1$	$SS' = 0.8$	$SM_2' = 0.2$	$ST = 0.13.$

2. *Amount of crystalloids present.* This can be determined by estimation of the osmotic pressure. This was done by finding what concentration of a cane-sugar solution balanced the given solution on the opposite side of an osmometer. The osmometer consisted of a semi-permeable collodion thimble with capillary tube attached.¹

The figures were :

Turnip extract balances 0.43 molar cane-sugar.

M_2	„	„	0.29	„	„	„
S	„	„	0.03	„	„	„

GENERAL DISCUSSION OF ABOVE RESULTS.

Enzymic preparations of two types can be obtained from a fungal culture, viz. (1) watery extracts of washed and triturated mycelium, and (2) the fluid in which germination has taken place. The activity of the preparation obtained has been shown to be dependent upon (1) the density of sowing of the spores, (2) the time allowed for germination and growth, and (3) the nature of the nutrient medium.

Fluid in which germination has taken place. With any given concentration of spores, the enzymic activity increases to a maximum with lapse of time, and subsequently falls off. When the spores are sown thinly, the absolute value of the maximum reached is diminished, and its attainment takes place at a longer interval from the time of sowing.

These enzymic solutions are characterized by the high concentration of enzyme present. They also contain an amount of retarding substances comparable with, but somewhat less than, that of the original nutrient medium.

Mycelial extract. In the case of thick sowings of spores, the curve of activity was shown to rise during the first day's growth, and then to decline after about the second day. In the case of the thin sowings in Erlenmeyer flasks, only the latter part of the curve was obtained. It is obvious

¹ An air-dried collodion thimble is placed in 50 per cent. alcohol in water for 24 hours, then washed in water till the alcohol is removed. The open end of the thimble is then dried in the air and sealed by means of a solution of collodion to the rubber cork through which the capillary tube passes. The junction is then allowed to dry completely in the air, when the osmometer is ready for use. This osmometer is permeable to simple salts like chlorides and nitrates of the monovalent metals, but not to sulphates or salts of bivalent metals such as barium chloride, &c. For a full account of the technique of preparing membranes of graded permeability, see papers by the writer in *Biochemical Journal*, vol. ix, 1915, p. 591, and vol. xi, 1917, p. 40.

on general considerations that the ascending part of the curve would also be found on the first day, but no experimental verification was attempted on account of the laborious processes which the preparation of the necessary amount of material would entail.

Enzymic solutions of this type are characterized by the presence of a medium amount of enzyme. They contain a negligible amount of retarding substances and of crystalloids generally.

The following practical rules may therefore be framed as bearing on the technique of preparing solutions of the enzyme under consideration :

(a) In order to obtain a very active enzymic solution from the nutrient medium, it is essential that simultaneous development of a large number of spores take place throughout the whole nutrient medium. Where a small number of spores develop on the surface of a relatively large volume of nutrient, the enzymic activity of the latter never reaches a high value.

(b) In order to obtain a strong mycelial extract, the germinated spores must be extracted while still young (one to two days old). Conditions of yield and of economy of space and labour necessitate as thick sowing of the spores as is consistent with their vigorous germination.

In the case of both types of extract, the maximum activity is attained at a time roughly coincident with that at which vigorous growth of the hyphae ceases.

The above results throw light on the relative merits of the extract employed in these studies and those employed by certain other investigators in the examination of the nature of the active principle concerned in the process of parasitic attack. The extract employed by the present writer shows the maximal activity obtainable from mycelial extracts, and is at the same time comparatively free from crystalloidal contamination such as occurs in the equally active preparations derived from the nutrient in which the fungus has developed. In Marshall Ward's experiments,¹ extraction of the hyphae took place too late (after three weeks' growth) to allow of a strong extract being obtained. The extracts of de Bary and Behrens were obtained from the nutrient medium and contained therefore crystalloidal contamination. It seems certain that the lethal effects of boiled extract in Behrens's experiments were due to the large amount of crystalloidal substances present, while the great age (six months) of his cultures precluded the presence of anything but the smallest traces of enzyme in his unboiled extracts.

¹ References to the papers cited here are given in No. I of the series.

GENERAL REMARKS ON THE SECRETION OF CYTASE BY *Botrytis cinerea*.

In the case of the watery extracts of mycelium it has been shown that a weak enzymic preparation is obtainable from the mycelial film in the case where the spores were sown thinly, while in the case of the dense sowings on glass plates a much stronger extract is obtained. Now the outstanding difference between the two cases mentioned is that in the latter case the great proportion of the hyphal mass is in a state of vigorous growth, or, at any rate, has very recently passed through its growth period; whereas in the former, though the number of growing tips may be considerable, a large proportion of the fungal mass has ceased to grow. The small amount of enzyme obtainable in the latter case, as compared with the former, indicates that the source of enzyme is the growing region of the hypha. The older parts of the hypha do not contribute an appreciable amount of enzyme, but would probably tend to reduce by adsorption the amount going into solution during the process of extraction.

This view, that enzymic formation is confined to the growing apex, is further supported by the results obtained in extracting hyphae which have ceased growing. In the glass plate experiments it was found that there was a marked diminution in the amount of enzyme obtainable from plate sowings of four and six days' age as compared with that from plates one to two days old. As the plates were identical at the commencement, the older cultures must possess at least as many hyphal tips as the younger ones. The dry weight measurements show, however, that active growth ceases from about the second day. The reduced activity of the older cultures is thus to be set down in this case, not to a reduced number of hyphal tips in the extracted mass, but to reduction in the amount of enzyme in each hyphal tip, this reduction being correlated with the fact that these hyphal tips have ceased growth on account of the development of stale conditions in the culture.

The elaboration of cytase in the fungal hypha would thus appear to be a process bound up with the protoplasmic activity associated with growth in the hyphal tip. Whether this enzyme plays a direct part in the changes undergone by the cell wall during the period of growth it is as yet impossible to say. Again, the cause of the disappearance of this enzyme, both from parts of the hyphae which have ceased to grow and also from the medium in which growth has taken place, is not clear. It is known that its solution in water is not stable, and thus the disappearance of enzyme from cultures as time goes on may be due simply to this fact, though a process of actual resorption by the fungus is not impossible.

SUMMARY.

1. Two types of enzymic preparations derivable from cultures of *Botrytis cinerea* are described. These are:

(a) Watery extracts of the ground mycelium.

(b) The media in which germination and growth have taken place.

2. The amounts of (a) enzyme, (b) enzyme-retarding substances present in these under various experimental conditions (density of sowing, age of culture, nature of medium) were determined in each case.

3. A discussion is given of the bearing of these results on the technique of enzyme extraction on the one hand, and on the process of enzyme excretion by fungi on the other.

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