LXXXIII. THE ACTION OF TANNASE ON GALLOTANNIN.

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(Received March 31st, 1931.)

TANNASE hydrolyses gallotannin with production of gallic acid. The activity of tannase can thus be measured by estimating either the unchanged gallotannin or the gallic acid formed. Micro-methods were therefore elaborated in this laboratory for the estimation of gallotannin [Spiers, 1914; Rhind and Smith, 1922] and gallic acid [Nicholson and Rhind, 1924]. The application of these methods to the tannase problem, using gallotannin freed from glucose [Nierenstein, Spiers and Hadley, 1925], gave an unexpected result, namely, that more gallotannin was hydrolysed than corresponded with the gallic acid produced. It is now shown that this is due to the presence of an enzyme in tannase which destroys gallic acid. This enzyme, for which the name pyrogallase is suggested, is also produced by growing *Aspergillus niger* on media which contain pyrogallol or gallic acid. The presence of pyrogallase in tannase obviously forbids the use of tannase in connection with the chemistry of gallotannin, as was originally planned by us, and this also applies to other tannins which yield gallic acid on hydrolysis [*cf.* Fischer and Bergmann, 1918; Freudenberg, 1919, 1920; Freudenberg and Peters, 1920; Perkin and Uyeda, 1922; Freudenberg and Blümmel, 1924; Freudenberg and Frank, 1927]. The production of pyrogallase by moulds thus affords an explanation of the fact, well-known in industry, that the action of moulds produces appreciably less gallic acid from gallotannin than does acid hydrolysis, although Pottevin [1901] claims a yield of 98 % of gallic acid by the action of *A. niger* on gallotannin.

EXPERIMENTAL.

I. *Cultivation of A. niger.* Czapek's medium as modified by Knudsen [1913] was used; the concentrations of gallotannin, pyrogallol and gallic acid being 4, 2.5 and 0.8 % respectively. The crops were grown in Fernbach flasks or in ordinary large bottles placed on their sides. Each flask held about 350 cc. of medium and each bottle about 150 cc. when exposing a maximum surface. They were plugged tightly with cotton wool, covered with leadfoil and sterilised by intermittent steaming for an hour on three consecutive days. They were then inoculated from the required stock with a sterilised platinum needle. After being well shaken to induce the spores to spread over the surface

of the medium the vessels were placed in an incubator at 23°. After about 4 days the surface was covered generally fairly evenly by a thick white felt, and spores were beginning to form. At this stage the felt was removed, as during the spore formation the fungus excretes enzymes freely into the medium [Dox, 1910]. The felt was removed with a glass rod, washed with tap-water to remove both spores and medium, squeezed as dry as possible, put into a flask and covered with acetone, in which it was allowed to stand overnight. This killed the mycelium, dissolved out the colouring matter and removed the final traces of medium. The following day it was ground up in a mortar with fresh acetone and filtered by suction, the process being repeated until the acetone washings were practically colourless and the mycelium reduced to a fine powder. This was finally washed in a little ether, and after being again filtered by suction was left exposed in a cupboard in an evaporating dish until no smell of ether could be detected. The resulting mouse-grey powder is referred to as "mycelium-powder." It was put into a stoppered bottle and kept in the dark until required.

In a few cases one medium was used to grow more than one crop, but between each crop it was filtered through glass wool and re-sterilised. It was found, however, that although the felt was generally greater in amount in the second crop than in the first, it was less reactive, and in the majority of cases, therefore, only one crop was grown on each medium.

II. *Observations on the behaviour of A. niger during spore formation.* Reference has already been made to the observation recorded by Dox that the fungus excretes enzymes during spore formation. Our results using the methods described by Dox are tabulated below. They confirm Dox in every respect.

Table I.

Enzymes other than tannase and pyrogallase	Substrate used for detection	Mycelium- powder	Cultural solution before sporulation	Cultural solution after sporulation
Oxidase	Guaiacum	—	—	—
Peroxidase	Guaiacol	—	—	—
Laccase	Pyrogallol	—	—	—
Tyrosinase	Tyrosine	—	—	—
Protease	Fibrin	? +	—	—
	Gelatin	—	—	—
	Egg-white	—	—	—
Emulsin	Salicin	+	—	—
	Amygdalin	+	—	+
Diastase	Starch	+++	—	++
Invertase	Sucrose	+++	—	++
Maltase	Maltose	++	—	+
Lactase	Lactose	++	—	+
Raffinase	Raffinose	+	—	+
Amidase	Urea	+	—	+
Hippurase	Hippuric acid	—	—	—
Lipase	Ethyl acetate	++	—	+

III. *Hyper-cultivation.* The term hyper-cultivation is used here to designate the growing of the fungus for several successive asexual generations on gallotannin, a freshly prepared medium being used for each generation.

The results obtained show that it is possible to increase the yield of tannase by this method, and that a physiological change is apparently brought about, which persists even after the fungus is removed from contact with gallotannin. Thus in working with three cultures of *A. niger* and growing each for six asexual generations on gallotannin, the following values, definitely showing an increased tannase production, have been obtained. Taking the activity of the first generation of strain A to be 100 after 24 hours' hydrolysis:

Table II.

Strain A		Strain B		Strain C	
Generation	Activity	Generation	Activity	Generation	Activity
1	100	1	254	1	247
2	113	2	300	2	449
3	88	3	161	3	269
4	167	4	184	4	198
5	137	5	216	5	328
6	155	6	264	6	420
<hr/> α 136		<hr/> β 306		<hr/> γ 435	

In the case of all three cultures an inoculation was made from the fifth generation into a prune-agar medium (which was free from gallotannin). The new generation was again inoculated into a gallotannin-containing medium, and the crops thus produced have been labelled α, β and γ respectively. As will be seen, in each case they showed a greater activity than the first generation had done. This is of importance since it apparently indicates the persistence of the increased tannase production, probably due to a physiological change. As far as could be observed there was no morphological change.

IV. *Preparation of tannase and pyrogallase.* A suspension of 500 g. of mycelium-powder in 2 litres of distilled water containing a little chloroform is shaken for 200 hours and the solution filtered at the pump, the remaining solid being washed 4 times with 100 cc. of water containing a little chloroform. To the filtered solution are added 6 litres of absolute alcohol and the precipitate (40 g.) is collected after standing for 2-3 days. The solid, suspended in 150 cc. of distilled water containing a little chloroform, is shaken for 24 hours and filtered through thick filter-paper, and 2 litres of absolute alcohol are added to the filtrate. The precipitate formed (about 18 g. in the case of tannase and 15 g. in the case of pyrogallase) is collected and again purified by dissolving in 100 cc. of water, shaking for 24 hours, filtering and precipitating with 2 litres of absolute alcohol. In this manner the following average yields were obtained: 9 g. tannase, 6 g. pyrogallase and 2 g. pyrogallase by growing on media containing gallotannin, pyrogallol and gallic acid respectively.

V. *General properties of tannase and pyrogallase.* Both enzymes are completely soluble in cold water. Tested by Dox's methods they are found to give positive reactions for diastase, invertase, maltase, raffinase, amidase and lipase. The pyrogallol nucleus undergoes a profound change when acted on by pyrogallase, it loses its colour-producing properties with iron salts after

some time. The mechanism of this reaction is difficult to explain and all our attempts to isolate some definite disintegration product have led to disappointing results. Table III gives the values observed for the action of 0.1 g. of pyrogallase on 50 mg. of pyrogallol in 100 cc. of distilled water.

Table III.

Exp. 25.		Exp. 34.	
Time hrs.	Pyrogallol found mg.	Time hrs.	Pyrogallol found mg.
24	30.76	48	27.16
72	12.22	72	21.70
96	16.72	93	23.24
144	17.04	147	18.60
168	13.40	174	15.54
192	12.26	226	17.10
216	11.14	248	10.62
240	12.12	336	8.88
164	11.88	357	3.18

It must be noted that in these two experiments two different preparations of pyrogallase were used.

Table IV gives a typical experiment showing the amount of gallic acid produced by tannase from 100 mg. of anhydrous gallotannin. Column A records the amount of gallic acid found, column B the calculated amount of gallic acid from the estimated amount of the unchanged gallotannin.

Table IV.

Exp. 12.		
Time hrs.	A. Gallic acid found mg.	B. Gallic acid calculated mg.
0	1.59	—
24	13.93	19.10
48	19.74	24.80
72	26.16	31.60
144	37.46	40.70

SUMMARY.

It is shown that *Aspergillus niger* produces in addition to tannase an enzyme (pyrogallase) which destroys gallic acid.

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