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TANNIC ACID FERMENTATION. I
TANNIC ACID FERMENTATION. II
EFFECT OF NUTRITION ON THE PRODUCTION OF
THE ENZYME TANNASE

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TANNIC ACID FERMENTATION. I.

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I. PREFACE.

During 1907-1908, a preliminary investigation of the conditions of tannic fermentation was made by the writer with the purpose of improving the practical methods involved. In the course of this preliminary investigation a number of interesting observations were made, justifying a further study of the conditions of the fermentation and of the relation of various organisms to the process. With the progress of the investigation other phases suggested themselves, until ultimately four distinct but correlated parts of the subject were experimentally studied. Part I, here reported, includes chiefly: (1) the toxicity of tannic acid for various fungi; (2) a comparison of the organism *Aspergillus niger* and *Penicillium* sp. in the fermentation of tannic acid; (3) the conditions and influence of various factors on the fermentation process. Part II is concerned primarily with the influence of nutrition on the production of the enzyme tannase, and will be reported in a subsequent paper.

These investigations were begun at the suggestion of Prof. B. M. Duggar and prosecuted in his laboratory. It is a pleasure here to express my thanks for the advice, kindly criticism and assistance, which he has so generously given.

II. INTRODUCTION.

Chemical nature of tannic acid. Wagner¹ has grouped the tannins into a "physiological" and a "pathological" series, the latter including, as most important, the tannin of oak galls as

¹ R. Wagner: Beiträge zur Kenntnis und zur quantitativen Bestimmung der Gerbsäuren, *Zeitschr. f. anal. Chem.*, v, pp. 1-10, 1866.

well as the tannin of sumach and chestnut. The pathological tannins are hydrolyzed by boiling with acids or through the action of the enzyme tannase, gallic acid resulting. For other distinguishing characteristics of the tannins Trimble's² and Proctor's³ treatises may be consulted, and for a discussion of the diverse views held regarding their chemistry reference should be made to special papers on the subject.

This paper is concerned with the fermentation of the tannin from oak galls, which is frequently termed gallotannic acid.

History of tannic acid fermentation. Scheele⁴ found in 1786 that gallic acid was present in the gall nuts. Robiquet⁵ attributed the fermentation of the gall nuts to a ferment within the gall nut. Laroque⁶ considered the formation of gallic acid from tannic acid to be due either to a ferment or to oxidation. He further found that various toxic substances could inhibit the fermentation. Ed. Robiquet⁷ showed that the tannic acid was transformed during the fermentation and he believed the transformation to be due to the ferment pectase which he extracted from the gall nuts. Wittstein⁸ stated that beer yeast aided tannin fermentation by fermenting the sugars and other products present. Van Tieghem⁹ was the first, however, to demonstrate that the formation of gallic acid during fermentation is due to the action of fungus organisms, and not to enzymes pre-existing in the galls, nor to oxidation by the air. He stated further that the organisms were *Penicillium glaucum* and a new organism which he named *Aspergillus niger*. He found that if the growth was submersed, the tannic acid was converted into gallic acid and glucose, the glucose being gradually used up, the gallic acid remaining. He stated further that if the growth was on the surface, sporulation and greater growth occurred and that the tannic acid was destroyed directly, the slight hydrolysis being due to submerged mycelium, the resulting glucose and gallic acid being then assimilated.

² H. Trimble: *The Tannins*, Part I, 168 pp.; Part II, 172 pp., 1892.

³ H. K. Proctor: *Leather Industry. Laboratory Book*, 2nd edition, 450 pp., 1908.

⁴ Quoted from H. Trimble: *loc. cit.*

⁵ M. Robiquet: Faits pour servir à de l'acide gallique, *Ann. de chim. et de phys.*, 2^e serie, lxiv, pp. 385-409, 1837.

⁶ A. Laroque: Neue Untersuchungen über Gallussäure, *Ann. d. Chem. u. Pharm.*, xxxix, pp. 37-100, 1841.

⁷ Ed. Robiquet: Recherches sur la fermentation gallique, *Ann. de chim. et de phys.*, 3^e serie, xxxix, pp. 453-460, 1853.

⁸ Wittstein: *Jahresber. über die Fortschritte der Chemie*, 1853, p. 435.

⁹ Ph. VanTieghem: Sur la fermentation gallique, *Compt. rend. de l'Acad. des Sci.*, lxxv, pp. 1091-95, 1867.

Müntz¹⁰ found that fermentation occurred through the action of *Penicillium glaucum*. Fernbach¹¹ grew *Aspergillus niger* in Raulin's solution with the sugar replaced by tannic acid, and then extracted from the organism the enzyme tannase. Pottevin,¹² in a similar manner and at the same time, extracted the enzyme tannase from the same fungus. He noted also that the enzyme was developed when *Aspergillus niger* was grown on Raulin's solution with the sugar replaced by gallic acid. He stated that the tannase acted on tannate of gelatin and also hydrolyzed methyl salicylate and ethyl salicylate.

Manea¹³ showed that synthetically prepared digallic acid was not split up by *Aspergillus niger* and *Penicillium glaucum* into gallic acid, and therefore concluded that the hydrolyzable tannin of the gall nut could not be a digallic acid. The latter in high concentrations was toxic to the organism. Further, Manea estimated quantitatively the digallic and tannic acid used by each organism. In a study of the fermentation process he employed pure cultures, adding a previously sterilized Raulin's solution rendered strongly acid. The quicker the fermentation, the richer was the yield of gallic acid obtained.

Kunz-Krause¹⁴ found an octyl gallotannoid, $C_{54}H_{50}O_{33}$, which through the action of a mould was transformed to gallic acid.

III. METHODS.

Culture solution. Throughout all the work the culture solutions used were a slight modification of Richards'¹⁵ solution or of Czapek's solution.¹⁶ These solutions are designated respectively A and B and are as follows:

¹⁰ Müntz: *Ber. d. deutsch. chem. Gesellsch.*, 1877, p. 1773.

¹¹ A. Fernbach: *Sur la tannase*, *Compt. rend. de l'Acad. des Sci.*, cxxxi, pp. 1214-15, 1901.

¹² H. Pottevin: *La tannase. Diatase dedoublant l'acide gallotannique*, *Compt. rend. de l'Acad. des Sci.*, cxxxi, pp. 1215-17, 1901.

¹³ A. Manea: *Sur les acides gallotanniques et digallickes*. These, Geneva, 1904. (Cited from Lafar: *Handb. d. technische Mykologie*, I, p. 663.)

¹⁴ Kunz-Krause: *Fragmente zu einer Monographie d. Tannoide*, *Pharm. Centralbl.*, Halle, 1898. (Cited from Lafar: *Handb. d. technische Mykologie*, I, p. 662.)

¹⁵ H. M. Richards: *Die Beeinflussung des Wachstums einiger Pilze durch chemische Reize*, *Jahrb. f. wiss. Bot.*, xxx, pp. 665-688, 1897.

¹⁶ Quoted from A. W. Dox: *Intracellular Enzymes of Penicillium and Aspergillus*, U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120, 70 pp., 1910.

SOLUTION A.		SOLUTION B.	
KNO ₃	1.0 gram.	MgSO ₄	0.5 gram.
KH ₂ PO ₄	0.5 gram.	K ₂ HPO ₄	0.1 gram.
MgSO ₄	0.25 gram.	KCl	0.5 gram.
Distilled H ₂ O	100 cc.	NaNO ₃	2.0 gram.
		Distilled H ₂ O	1000 cc.

The source of carbon was cane sugar, tannic acid or gallic acid, either alone or supplementing each other, depending upon the experiment. A 10 per cent concentration of sugar was employed, as experience has shown that the better growth is secured with this concentration than with the lower concentration. This fact is developed in a subsequent table.

Methods of inoculation. In all of the fermentation experiments the method of inoculation employed was that proposed by Hasselbring.¹⁷

Methods of analysis. The volumetric method of Dreaper¹⁸ was used for some experiments, but for most of the work the volumetric method proposed by Jean¹⁹ was used. Both methods have imperfections, but they are approximately accurate. In all cases analyses were checked by duplicate determinations and usually by more.

Method of washing and weighing the fungus felt. For the experiments, the results of which are included in tables I and II, the method used in washing the felt free from gallic and tannic acid was as follows:

The felt was removed by means of a bent needle and floated on distilled water, the water being renewed until it gave no further coloration. In order to secure the submersed growth, the solution was poured into a cylinder and the submersed growth, which now usually floated on the surface, was then removed by needles. All of the mycelium was then placed in a crucible, which had been brought to constant weight at 105°, heated for five hours at the same temperature and then weighed. This method, as well as the use of filter paper, possesses obvious disadvantages as well as being inaccurate. For most of the work, therefore, the following method was used: The Gooch

¹⁷ H. H. Hasselbring: Carbon Assimilation of *Penicillium*, *Bot. Gazette*, xlv, pp. 176-193, 1908.

¹⁸ W. P. Dreaper: Estimation of Tannic and Gallic Acid, *Chem. News*, xc, pp. 111-112, 1904.

¹⁹ F. Jean: Die Bestimmung des Tannins und der Gallussäure, *Chem. Centralbl.*, 1900, pp. 1107-08.

filter was prepared in the usual manner, as employed in quantitative chemical analysis, and filtration was made by means of the Gooch funnel with suction. The original solution was first decanted into the Gooch crucible. The felt was then washed in the flask four or five times with distilled water at room temperature; or, if gallic acid had been precipitated, warm water was used. The washing of the felt continued until the wash water was perfectly clear. The felt was then placed in the Gooch crucible, the flask again washed and the wash water poured into the Gooch crucible. The advantages of the method consist in the rapidity of the filtration and the accuracy which results from the thorough washing, which latter is important when the culture solution is to be analyzed and absolute weight of mycelium is to be obtained. It is an especially accurate method of securing all the fungous mycelium, and by exercising a little care there is no noticeable loss of spores.

IV. TOXICITY OF TANNIC ACID FOR CERTAIN FUNGI.

In the literature of tannic acid fermentation only two organisms are mentioned as possessing the property of effecting this fermentation; these are *Aspergillus niger* and *Penicillium glaucum*. In order to determine whether other organisms are capable of effecting the transformation, a considerable number of filamentous fungi were carefully tested with respect to their ability to grow in tannic acid solutions.

As a nutrient medium a bean decoction was made by boiling 1 liter of laboratory preserved beans with a liter of tap water. The juice was then filtered off and diluted to 2 liters. With this decoction as a solvent, four concentrations of tannic acid were made; namely, 0.25 per cent, 2 per cent, 5 per cent and 10 per cent. Test tubes were employed as culture vessels, to each of which were added 10 cc. of the solution. Small wads of filter paper were added to afford a solid substratum. The tubes were prepared in duplicate, sterilized, inoculated and kept at room temperature. They were examined at intervals and the final observations made at the end of two weeks are recorded in table A.

It is especially noteworthy that the 5 per cent permitted the growth of only one-third of the organisms, while in the 10 per cent solution only *Aspergillus flavus*; *Aspergillus niger* and *Penicillium sp.* are able to grow. A separate experiment indicated that *Aspergillus oryzae* could withstand 10 per cent tannic acid.

An experiment was also made to determine if any of these organisms could utilize tannic acid as a source of carbon. Solution B with 11.6 grams of tannic acid per 100 cc. of solution was used.

TABLE A.

ORGANISM	CHARACTER OF GROWTH			
	0.25 per cent	2 per cent	5 per cent	10 per cent
1. <i>Penicillium brevicaulis</i>	good	good	good	
2. <i>Penicillium camemberti</i>	good	good	very slight	
3. <i>Penicillium claviforme</i>	good	good	good	
4. <i>Penicillium duclauxii</i>	good	good	none	
5. <i>Penicillium granulatum</i>	good	good	good	
6. <i>Penicillium italicum</i>	good	slight	slight	
7. <i>Penicillium lilacinum</i>	good	slight	none	
8. <i>Penicillium purpurogenum</i>	good	none	none	
9. <i>Penicillium sp.</i>	good	good	good	good
10. <i>Aspergillus flavus</i>	good	good	good	good
11. <i>Aspergillus niger</i>	good	good	good	good
12. <i>Trichoderma lignorum</i>	good	none	none	
13. <i>Mucor circinelloides</i>	good	slight	none	
14. <i>Mucor rouxii</i>	good	slight	none	
15. <i>Mucor spinosus</i>	good	none	none	
16. <i>Polyporus sulphureus</i>	good	none	none	
17. <i>Polyporus resinosus</i>	good	none	none	
18. <i>Fomes megaloma</i>	no growth	none	none	
19. <i>Chaetomium sp.</i>	good growth	none	none	
20. <i>Chaetostylon sp.</i>	good growth	none	none	
21. <i>Stysanus sp.</i>	good growth	none	none	
22. <i>Cephalothecium roseum</i>	good	none	none	
23. <i>Circinella umbellata</i>		good	none	

Erlenmeyer flasks of 150 cc. capacity were employed, and in each were placed 50 cc. of the culture solution. After sterilization these flasks were inoculated and maintained at room temperature. In addition to the above list of organisms the following were tested: *Aspergillus oryzae*, *Nectria ipomoeae*, *Fusarium oxysporum*, *Phycomyces nitens* and *Stilbella sp.* Of all organisms tested only *Aspergillus niger* and *Penicillium sp.*²⁰ developed. Duplicate cultures of all these organisms on Chinese galls gave similar results, except in this case *Aspergillus flavus* produced a very slight growth.

VanTieghem²¹ found that both *Aspergillus niger* and *Penicillium glaucum* could withstand a saturated solution of tannic acid, and

²⁰ One other species of *Penicillium*, as indicated in the appendix, is able to develop upon a 10 per cent tannic acid solution.

²¹ *Loc. cit.*

the fact that both develop in moistened gall nuts, which contain per dry weight 60 per cent of tannic acid, is evidence that for these two organisms the tannic acid is not toxic.

Toxicity of tannic acid for Aspergillus flavus and Aspergillus oryzae. An experiment was conducted to determine the growth at various concentrations of tannic acid with and without 10 per cent cane sugar. In one case tannic acid (Merck's tested reagent) was added to solution B; in the other, tannic acid + 10 per cent sugar was used. Test-tube cultures with 15 cc. of the solutions were employed. The results in general showed that these two fungi develop normally in the presence of 2.5 per cent tannic acid, but greater concentrations decrease the rate of germination and inhibit the growth. In the 15 per cent concentrations of tannic acid, after nine days, only one-third of the surface was felted. Up to 7.5 per cent concentration the entire surface was felted.

Conclusion and discussion. The experiments on the toxicity of tannic acid indicate that of all the organisms tested, *Aspergillus niger* and *Penicillium sp.*²² are best adapted for the tannic acid fermentation. These two organisms were, therefore, selected for more detailed investigation, though the other two organisms previously mentioned were also reserved for further study.

Since the above experiments were made, a bulletin has appeared on the toxicity of tannin by Cook and Taubenhaus.²³ The majority of a large number of parasitic organisms tested by them with respect to the toxicity of tannin show retardation of growth at from 0.1 per cent to 0.8 per cent of tannin. The few saprophytic forms tested exhibit a more marked resistance. My own experiments indicate also that the saprophytic forms can withstand relatively higher concentrations of tannic acid than the parasitic forms.

²² See appendix for description of this organism.

²³ M. T. Cook and J. J. Taubenhaus: The Relation of Parasitic Fungi to the Contents of the Cells of the Host Plant, Delaware Agric. Exp. Station, Bulletin 91, 77 pp., 1911.

V. COMPARISON OF ORGANISMS AND INFLUENCE OF TIME AND GROWTH ON FERMENTATION.

Comparison of organisms. Employing an infusion of gall nuts containing 10.5 per cent tannic acid, VanTieghem²⁴ found that the fermentation was completed by *Aspergillus niger* in six days and by *Penicillium glaucum* in eight days, when the temperature of incubation was 35° and the supply of oxygen was limited by keeping the flask stoppered. In the weaker concentrations, *Penicillium glaucum* fermented the tannic acid more vigorously. Since in VanTieghem's experiments the temperature was rather high and the conditions of growth approached anaerobic conditions, it was believed that a better comparison could be made by supplying the optimum conditions for the growth of each. In all the natural fermentations (in unsterilized solution) of the tannic acid, *Aspergillus niger* always developed first and then *Penicillium sp.* So marked is this succession that by exposing a nutrient solution of 10 per cent tannic acid to the air a pure culture of *Aspergillus niger* may usually be obtained. This suggests that *Aspergillus niger* may possess the greater fermentative capacity, but an experiment was required.

In the first experiment solution A and tannic acid were used, 50 cc. of the solution being placed in Erlenmeyer flasks of 150 cc. capacity. The flasks were sterilized for forty-five minutes at 115°, inoculated, and kept for two weeks at a temperature of from 18°-28°, the average being close to 24°. The felts were then removed, thoroughly washed, and the wash water added to the culture solution, the solution being then brought up to 500 cc. volume and analyzed according to Dreaper's²⁵ method. The figures below are for *Aspergillus niger*, the averages of six cultures, while for *Penicillium sp.* the averages of five cultures are given.

TABLE I.

ORGANISM	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	grams	grams	grams	grams	gram
<i>Aspergillus niger</i>	0.228	1.244	0.200	2.752	0.848
<i>Penicillium sp.</i>	0.332	1.140	1.780	1.172	0.537
Check.....	1.472		2.952		

²⁴ *Loc. cit.*²⁵ *Loc. cit.*

The loss in gallic acid indicates that this substance is used by the organisms as a source of carbon, which fact agrees with the observations of VanTieghem²⁶ and Pottevin.²⁷ According to VanTieghem, when growth occurs on the surface the tannic acid is utilized directly without previous conversion into gallic acid. There is no evidence for this assumption. If the tannic acid is not utilized directly, and it probably is not, then *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium sp.* for in the *Penicillium* culture more tannic acid remained and the decrease in gallic acid was only 39 per cent. The larger gallic acid content of the *Penicillium* culture is related to the smaller amount of growth and not to the greater practical efficiency as a fermentative organism, and this point is more apparent from later work.

In table II there are given, separately, data for four of the *Penicillium* cultures. This table emphasizes a general relation between the amount of growth and the extent of fermentation; furthermore, the disappearance of gallic acid is correlated with increased growth.

TABLE II.

CULTURE NO.	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS IN GALLIC ACID	WEIGHT OF FUNGUS
	grams	grams	grams	grams	gram
Check	1.472		2.952		
1	1.216	0.156	2.396	0.556	0.295
2	0.140	1.332	2.240	0.700	0.450
3	0.152	1.320	1.760	1.192	0.550
4	0.100	1.372	1.308	1.644	0.700

Culture No. 1 seems to indicate that the tannic acid transformation is dependent upon the amount of growth, for only a small amount of tannic acid was transformed, but a greater growth during the same period in the other cultures resulted also in almost complete transformation.

Since the most economical production of gallic acid is dependent upon the amount of growth, and growth amount is a function of time, temperature, aeration, and nutrition, then these factors should be important. Growth is emphasized because with it

²⁶ *Loc. cit.*²⁷ *Loc. cit.*

the amount of the enzyme is probably correlated, at least with respect to *Penicillium sp.*

Culture No. 1 shows a small decrease in tannic acid and a high decrease in gallic acid. In culture No. 2 the loss of tannic acid is greater than loss of gallic acid. The probable explanation is that *Penicillium sp.* utilizes first the gallic acid and then transformation of the tannic acid occurs. In culture No. 1 the smaller growth has been at the expense of tannic acid. It does not seem possible to demonstrate that tannic acid is not directly utilized but it seems probable that it must be converted into gallic acid.

Influence of duration and extent of growth and comparison of organisms. In order to determine the yield of gallic acid at different intervals, and so to note the influence of growth and duration on the fermentation, an experiment was made as follows: Solution B was used and to it were added 7.5 grams of Merck's purified tannic acid per 100 cc. of solution. The concentration of the tannic acid was such that it came within the limits, as found by Van Tieghem,²⁸ most favorable for *Penicillium glaucum*. For the investigation 50 cc. of the culture solution were placed in Erlenmeyer flasks of 150 cc. capacity. The cultures were sterilized for fifteen minutes at 5 pounds' pressure and then inoculated according to the method described. The cultures were kept in an incubator at 31° and, at intervals, as indicated, duplicate cultures were taken for analyses. The mycelium was removed by filtering with suction through the Gooch crucible, and it was then washed with warm water to dissolve all adhering gallic acid. The solution was then brought up to 500 cc. and analyzed according to the method of Jean.²⁹ The results of the experiment are given in Table III.

In culture No. 8 the amount of gallic acid decrease was 0.126 gram and the tannic acid decrease only 0.069 gram, recalling the case of No. 1, table II. *Penicillium sp.*, it seems, therefore utilizes the gallic acid first, and then the secretion of enzyme involves the transformation of the tannic acid. In all of the succeeding cultures the tannic acid decrease is greater than the increase of gallic acid, and this is to be accounted for, again, not by a direct utilization of tannic acid but by the fact that the tannic acid is first converted into gallic acid; and the constant increase of the

²⁸ *Loc. cit.*

²⁹ *Loc. cit.*

TABLE III.

Organism, Aspergillus niger.

CULTURE NO.	DURATION	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OR GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	days	grams	grams	grams	grams	gram
1	4	0.750	0.853	2.584	+0.484	0.0314
2	6	1.057	0.546	2.191	+0.091	0.1568
3	8	0.252	1.351	1.814	-0.286	0.1946
4	10	0.306	1.297	1.001	-0.499	0.4688
5	12	0.160	1.443	1.460	-0.640	0.4933
6	16	0.159	1.444	1.406	-0.694	0.5137
7	28	0	1.603	0.898	-1.202	0.6345
Check		1.603		2.100		

Organism, Penicillium sp.

8	4	1.534	0.069	1.974	-0.126	0.0157
9	6	1.330	0.273	2.191	+0.091	0.0800
10	8	1.296	0.305	1.769	-0.331	0.1458
11	10	0.750	0.853	1.669	-0.431	0.3031
12	12	0.427	1.176	1.333	-0.767	0.3880
13	16	0.597	1.006	1.530	-0.670	0.4096
14	28	0.106	1.497	1.277	-0.823	0.4284

gallic acid prevents the utilization of the gallic acid from being made manifest.

If the *Aspergillus niger* and *Penicillium sp.* cultures are compared, one finds that the tannic acid in the *Aspergillus* culture is transformed to the extent of 81 per cent by the tenth day; whereas in the *Penicillium* cultures this transformation, for the corresponding time, is only 53.3 per cent of the tannic acid. Moreover, by the fourth day the gallic acid had increased in culture No. 2 by nearly 0.5 gram, while in culture No. 8, for a corresponding time, there was a decrease of gallic acid. With this concentration and temperature, therefore, the *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium sp.*

If now the time factor and amount of growth be examined in their relation to the gallic acid, it is found that with the above solutions and under the specified conditions, the gallic acid decreases after the sixth day and is utilized in the further metabolism of the organism.

Comparison of fermentative capacity in an infusion of gall nuts. For the investigation an extraction of the gall nuts was made as follows: 1800 grams of the Aleppo gall nuts were placed in one jar and the same quantity of Chinese nuts in another. To each were added 3 liters of tap water, and the extraction was allowed to continue for five days, when the extract was filtered. To each jar were added again 2 liters of water, and after one day the extracts were filtered and combined with the previous filtrates. Then the two extracts from the Chinese and Aleppo galls were mixed. After a second filtration the solution was ready for the culture vessels. For this purpose cultures were made in the usual way, using Erlenmeyer flasks of 150 cc., each, with 50 cc. of the infusion.

TABLE IV.

CULTURE NO.	DURATION days	TANNIC ACID IN CULTURE SOLUTION grams	LOSS OF TANNIC ACID grams	GALLIC ACID IN CULTURE SOLUTION grams	GAIN OF GALLIC ACID grams	DRY WEIGHT OF FUNGUS gram
Check		1.637		1.797		
<i>Aspergillus niger.</i>						
1	4	0.545	1.092	2.752	0.955	
2	6	0.218	1.419	3.218	1.421	0.197
3	8	0	1.637	2.500	0.703	0.262
4	12	0	1.637	2.527	0.730	0.171
5	20	0.206	1.431	2.527	0.730	0.205
6	56	0	1.637	2.527	0.730	0.148
<i>Penicillium sp.</i>						
7	4	1.330	0.307	2.050	0.253	0.043
8	6	0.545	1.092	2.627	0.730	0.157
9	8	0.409	1.128	2.387	0.590	0.110
10	12	0.034	1.603	2.471	0.674	0.171
11	20	0.085	1.552	2.457	0.660	0.117
12	56	0.152	1.415	2.247	0.450	0.151

A comparison of the two organisms, as regards their fermentative capacity, shows again that *Aspergillus niger* is a more efficient organism. Note especially that in four days the *Aspergillus* cultures exhibit an increase of gallic acid nearly four times as great as that in the *Penicillium* cultures, and all of the tannic acid had been converted in the former by the eighth day, while in the *Peni-*

cillium cultures only 70 per cent was converted in the same time. Not only does *Aspergillus niger* produce a more rapid fermentation, but also a greater production of gallic acid is effected; for, as indicated, the maximum yield of gallic acid in the *Aspergillus* cultures is twice that in the *Penicillium* cultures, and this in spite of the fact that a greater weight of *Aspergillus niger* is produced. Furthermore, in the *Penicillium* cultures there occurs a decrease of the gallic acid even when considerable tannic acid is still present in the culture solution.

In order to understand these differences between *Aspergillus niger* and *Penicillium sp.*, the composition of the infusion must first be considered and an idea of this may be obtained from constituents of the gall nuts. In addition to tannic acid, the gall nuts contain (Guibert³⁰) gallic acid, chlorophyll, starch, gums, sugar, proteins and various inorganic salts and other compounds. A water extract of the gall nuts would contain in solution and suspension a certain amount of most of these substances, and the subsequent sterilization would probably result in transformations which would make certain of the organic compounds more available.

Aspergillus niger is an omnivorous organism in its relation to the utilization of carbon compounds. The growth of this organism for the first few days is probably at the expense of the other organic substances present, and the gallic acid, in this case, accumulates. All the facts indicate that while *Aspergillus niger* is utilizing the organic compounds other than tannic acid, it secretes the enzyme tannase (this point will be further developed in a separate paper), and consequently the transformation of the tannic acid goes on, and gallic acid accumulates. When the other organic compounds are exhausted, the gallic acid is utilized, and then the decrease begins. As shown previously, *Penicillium sp.* tends to utilize the gallic acid before it transforms the tannic acid, for gallic acid is a favorable nutrient carbon compound for this organism. Furthermore, as I will show in a later paper, the presence of the other organic compounds may decrease the secretion of the enzyme tannase by *Penicillium sp.*; and since the utilization of the gallic acid exceeds the formation of this substance, there results a decrease

³⁰ Quoted from H. Trimble: *loc. cit.*

of the gallic acid despite the fact that tannic acid is present in the solution.

A comparison of tables III and IV is of interest. The one experiment with a synthetic solution and the other with the infusion were conducted at the same time and under identical conditions as regards sterilization, inoculation and incubation. Moreover, the tannic acid and the gallic acid content of the culture solutions are nearly the same.

In table V, only the duration of experiment, decrease of tannic acid, the loss or gain of gallic acid and the dry weights of the fungus produced, are included.

TABLE V.

DURATION days	SOLUTION B + 1.603 GRAMS TANNIC ACID + 2.100 GRAMS GALLIC ACID			INFUSION OF GALL NUTS: TANNIC ACID = 1.627 GRAMS GALLIC ACID = 1.797 GRAMS		
	LOSS OF TANNIC ACID	LOSS OR GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS	LOSS OF TANNIC ACID	GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	grams	gram	gram	grams	grams	gram
<i>Aspergillus niger.</i>						
4	0.753	+0.484	0.031	1.092	+0.955	
6	0.546	+0.091	0.0156	1.419	+1.421	0.197
8	1.351	-0.286	0.194	1.637	+0.703	0.262
12	1.443	-0.640	0.493	1.637	+0.730	0.171
16	1.444	-0.694	0.513			
20				1.637	+0.730	0.148
<i>Penicillium sp.</i>						
4	0.069	-0.126	0.015	0.307	+0.253	0.043
6	0.273	+0.091	0.080	1.092	+0.760	0.157
8	0.305	-0.331	0.145	1.128	+0.590	0.110
12	1.176	-0.767	0.188	1.603	+0.674	0.171
16	1.016	-0.670	0.4096			
20				1.415	+0.450	0.151

It is interesting to note that in the synthetic solution the gallic acid decreased after the eighth day, while in the gall nut infusion it showed at that time a marked increase, and this increase was maintained thereafter. In the synthetic solution on the eighth day there was a loss of 0.286 gram of gallic acid, while in the gall nut infusion there was a gain of 0.703 gram of gallic acid, and in

the latter the tannic acid was completely transformed. The difference in the amount of tannic acid transformed was not sufficient, however, to account for the loss of gallic acid in the synthetic solution and the marked increase in the infusion culture. Despite the greater weight of the fungus this increase of gallic acid was more in the infusion culture. A similar condition existed at the end of six days. The explanation of this point seems to be found in the fact that the infusion cultures contain various organic compounds which are utilized in place of the gallic acid; that is, elected in preference. At the end of four days probably none of the gallic acid has been assimilated. The subsequent decrease in gallic acid was due to its use after the exhaustion of more favorable organic nutrients. After the sixth day in the infusion culture there was no further growth of the organisms and no decrease in the gallic acid. The growth was less than one-half of that in the synthetic cultures, probably due to the lack of inorganic nutrients, although the presence of injurious metabolic products might also have been a factor, as the organic food supply was by no means exhausted. The conditions which obtained for the *Aspergillus niger* cultures apply also to those of *Penicillium* sp.

Another point of interest brought out by the comparison is the more rapid fermentation in the infusion than in the synthetic solution. In the *Aspergillus* cultures the tannic acid was completely transformed in the gall nut infusion by the eighth day, when the weight of the fungus was 0.262 gram. In the synthetic cultures transformation was not complete by the sixteenth day, when the weight of dry mycelium was 0.513 gram. In the *Penicillium* cultures, also, the results are comparable.

In another experiment in which a gall nut infusion was used containing 2.04 grams of tannic acid and 0.56 gram of gallic acid per 50 cc., at room temperature, there was maintained for thirty days an increase of gallic acid. Practically all of the fermentation occurred before the eleventh day, and the gallic acid was protected by the other organic substances.

Since the experiments with the infusion cultures indicated that the gallic acid was protected to a certain extent by the election of other organic substances, it was determined to try the addition of sugar to the culture solution with respect to its effect on the election of foods and hence on the yield of gallic acid.

VI. INFLUENCE OF THE ADDITION OF SUGAR.

Effect of 5 per cent sugar. For these cultures solution A was used to which was added in the one series tannic acid alone, and to the other, tannic acid and cane sugar. Sugar was added at the rate of 5 grams per 100 cc. of solution, and the amount of tannic acid is indicated by the check. The cultures were made in liter flasks, and 500 cc. of the solution were used for each. The flask and contents were sterilized, inoculated and kept in the incubator at a temperature which varied from 27°–30°C. The results of the experiment given in table VI are in all cases obtained from duplicate cultures.

TABLE VI.

CULTURE NO.	DURATION	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	GAIN OR LOSS OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	days	grams	grams	grams	grams	grams
SERIES I. <i>Solution A + tannic acid.</i>						
1	10	4.568	8.193	11.095	+6.867	1.42
2	20	0.203	12.558	2.190	–2.038	3.66
3	30	0.050	12.711	0.142	–4.086	4.00
Check		12.761		4.228		
SERIES II. <i>Solution A + tannic acid + cane sugar.</i>						
4	10	0.913	11.848	8.761	+4.533	6.008
5	20	0.812	11.949	3.904	–0.324	10.35
6	30	0.101	12.660	0.143	–4.085	13.10
Check		12.761		4.228		

The results of the experiment seemed at first surprising. Instead of getting a protective action of the sugar with respect to the gallic acid, and thereby an increased yield of gallic acid, the opposite condition seemed to result. The yield of gallic acid was actually less at the end of ten days in the solution containing sugar than in the solution which lacked sugar, even though more of the tannic acid was transformed in the former. At the end of twenty days more gallic acid was left in the cultures of series II than in the corresponding culture solutions of series I, and so a certain protective action of the sugar is evident. At the end of thirty days practically all of the tannic acid and gallic acid had disappeared from

the culture solutions. In explanation of the seeming failure of the sugar to protect the gallic acid, the dry weights of felts produced in the corresponding cultures must be compared. The weight of fungus produced in each culture of series II was, at the end of each period, at least three times as great as the weight of the corresponding cultures of series I. This increased growth and the accompanying increased respiration were sufficient to utilize practically all of the organic nutrients supplied, usually all of the sugar and some of the gallic acid.

Effect of 10 per cent sugar. Since negative results were obtained as regards the protection of the gallic acid by 5 per cent sugar, a new series of cultures was made with 10 per cent sugar in solution B to which was added the tannic acid required. On analysis the solution showed after sterilization 4.171 grams of tannic acid and 2.198 grams of gallic acid per 50 cc. The cultures were incubated at a temperature of 28°, though it dropped occasionally, owing to an imperfect thermostat, to 25° and rose likewise to 32°. The cultures were taken down at definite intervals, the weight of the felts determined, and the analyses of the culture solutions made according to the methods previously described. The results follow in table VII.

The protective action of the sugar is at once evident. Since the concentration of tannic acid here is double that in the experiments which are included in table III, and therefore a comparison of the yields of gallic acid is not possible, yet the great increase of gallic acid and the maintenance of this increase prove that the sugar has been utilized in place of the gallic acid. Here it is obviously not true that the greater the weight the less the gallic acid. Cultures Nos. 4, 5, 6 and 7 all vary in weight, yet the amount of gallic acid in each is approximately the same, and any difference may be due to the imperfect method of analysis. Even at the expiration of thirty-five days no decrease of the gallic acid was evident. It may be concluded, therefore, and further experiments prove, that the 10 per cent sugar protects the gallic acid.

While an increase of gallic acid is maintained in the *Penicillium* cultures, the increase is relatively small and is due to a slower transformation of the tannic acid; practically no transformation of tannic acid occurred until after the fourteenth day. Previous to this time the growth was entirely submersed, but afterwards fructi-

TABLE VII.

CULTURE NO.	DURATION days	GALLIC ACID IN CULTURE SOLUTIONS grams	INCREASE OF GALLIC ACID grams	DRY WEIGHT OF FUNGUS gram
Check		2.198		
<i>Aspergillus niger.</i>				
1	3	4.929	2.731	0.1579
2	6	5.028	2.830	0.4093
3	8	5.533	3.335	0.4135
4	14	*6.320	4.122	0.5148
5	20	*6.320	4.122	0.4470
6	25	*6.292	4.094	0.4820
7	35	*6.348	4.150	0.4986
<i>Penicillium sp.</i>				
8	6	2.190	0	0.0076
9	14	2.261	0.063	0.332
10	20	3.764	1.574	0.265
11	25	3.764	1.574	lost
12	35	†3.820	1.630	0.4515
13	56	†4.101	1.911	0.5570

* Transformation of tannic acid complete.

† Considerable tannic acid still left in culture solution.

fication occurred. According to Malfitano³¹ the secretion of the enzymes from *Aspergillus* occurs just after fructification, and it is possible to conclude that the lack of transformation is explainable by non-secretion of the enzyme by the submersed growth. VanTieghem,³² however, found that transformation was effected when the growth was submersed; and some experiments of the writer subsequently confirm the fermentation by submersed growth. The non-transformation is probably due to the inhibiting action of the sugar on the secretion of the enzyme tannase, but not on the formation of the enzyme, for subsequent experiments have shown that with 2 per cent tannic acid and 10 per cent sugar the enzyme is formed. The inhibition of enzyme secretion by the presence of organic nutrients has been observed by Puriewitsch.³³

³¹ G. Malfitano: La proteolyse chez l'*Aspergillus niger*, *Ann. d. l'Inst. Pasteur*, xiv, pp. 60-81, 1900.

³² *Loc. cit.*

³³ K. Puriewitsch: Ueber die Spaltung der Glycoside durch die Schimmelpilze, *Ber. d. deutsch. bot. Gesellsch.*, xvi, pp. 368-377, 1898.

The transformation of salicin or arbutin was inhibited in the presence of certain amounts of glucose, cane sugar or starch. Likewise Katz³⁴ found that *Penicillium glaucum* did not secrete the enzyme diastase when, along with 0.25 per cent soluble starch, either 2 per cent glucose or 15 per cent cane sugar was offered. *Aspergillus niger* was not influenced noticeably in the secretion of the tannase by the presence of the 10 per cent cane sugar, while the *Penicillium* sp. was markedly influenced.

Another noteworthy fact in regard to the *Penicillium* sp. is the small increase of gallic acid between the twentieth and thirty-fifth day. It seems that the enzyme secreted was so very little, or of such feeble activity or destroyed, that a very limited transformation only was effected. Even at the end of fifty-six days the tannic acid was not entirely converted, and the increase of gallic acid after the end of thirty-five days was relatively small.

Influence of concentration of sugar on growth. For an explanation of the protective action of 10 per cent sugar the following table is suggestive. Each result is the average of sixteen cultures of *Aspergillus niger*.

TABLE VIII.

CULTURE NO.	COMPOSITION OF NUTRIENT SOLUTION	SUGAR IN CULTURE SOLUTION	DRY WEIGHT OF FUNGUS
		per cent	grams
1	Check: 50 cc. sol. A + trace Fe_2Cl_3 + 2.5 gms. sugar.....	5	0.779
2	50 cc. sol. A + trace Fe_2Cl_3 + 8.55 gms. sugar.....	17.1	1.239
3	50 cc. sol. A + trace Fe_2Cl_3 + 17.1 gms. sugar.....	34.2	1.513
4	50 cc. sol. A + trace Fe_2Cl_3 + 25.65 gms. sugar.....	51.3	1.590
5	50 cc. sol. A + trace Fe_2Cl_3 + 37.00 gms. sugar.....	74.0	1.308
6	50 cc. sol. A + trace Fe_2Cl_3 + 42.50 gms. sugar.....	85.0	1.230

The table shows that the optimum sugar content is above 5 per cent. It is possible that the increased growth in the culture solution of over 10 per cent sugar is produced not by an assimilation

³⁴ J. Katz: Die regulatorische Bildung von Diastase durch Pilze, *Jahrb. f. wiss. Bot.*, xxxi, pp. 599-618, 1898.

lation of more sugar but by a stimulus, the result of high concentration. The table is offered here, however, only to indicate that with the nutrient solution used 5 per cent sugar is not sufficient for greatest growth. It throws light also upon the failure of the 5 per cent sugar to protect the gallic acid in the previous experiment.

VII. ELECTION OF ORGANIC SUBSTANCES.

Historical. VanTieghem³⁵ stated that the glucose formed as a result of tannic acid fermentation was utilized and the gallic acid left behind. Pasteur demonstrated that *Penicillium glaucum* exhibited an election of the dextro-tartaric acid when both the dextro- and laevo-tartaric acids were present. Duclaux's³⁶ observations revealed the fact that when *Aspergillus niger* was offered salts of butyric and acetic acid in a mixture, it first used the latter and then the butyric acid. Furthermore, he proved that this was not due to the better nutrient value of acetic acid, for when the acetate was offered with the tartrate (an especially good nutrient) the acetate was utilized more rapidly. The election then was not merely a matter of relative food value.

Pfeffer³⁷ found that under certain conditions the use of glycerin by fungi may be protected by dextrose and even better by peptone, and he showed also that the relative concentration of each had an effect upon the election.

Puriewitsch³⁸ found with two organisms an election with respect to the products of amygdalin. With this substance as the source of carbon it was first transformed; then the dextrose and lastly the benzaldehyde was used. He found further that salicin was not transformed in the presence of six times its quantity of dextrose, twelve times its quantity of saccharose or fourteen to sixteen times its quantity of starch.

Election of cane sugar. In order to determine definitely whether or not *Aspergillus niger* and *Penicillium sp.* elect cane sugar, when it is offered together with gallic acid, two series of cultures were made. Solution B was used, to which was added in the one case the gallic acid and 10 per cent of cane sugar, the cultures being made in Erlenmeyer flasks as before. In a similar manner cultures were made in which the gallic acid alone was offered as the source of carbon. Sterilization and inoculation were made by the usual

³⁵ *Loc. cit.*

³⁶ E. DuClaux: Sur la nutrition intracellulaire, *Ann. de l'Inst. Pasteur*, iii, pp. 97-112, 1889.

³⁷ W. Pfeffer: Ueber Election organischer Nährstoffe, *Jahrb. f. wiss. Bot.*, xxviii, pp. 205-268, 1895.

³⁸ *Loc. cit.*

methods, and the cultures incubated at 28° C. The results obtained are included in table IX.

TABLE IX.

ORGANISM	DURATION <i>days</i>	GALLIC ACID IN CULTURE SOLUTION <i>grams</i>	GALLIC ACID USED <i>grams</i>	DRY WEIGHT OF FUNGUS <i>grams</i>
SERIES I. <i>Solution B + 10 per cent sugar + gallic acid.</i>				
Check.....		2.837		
<i>Aspergillus niger</i>	7	2.837	none	0.3491
<i>Penicillium sp.</i>	7	2.837	none	0.1109
<i>Aspergillus niger</i>	10	2.837	none	0.4589
<i>Penicillium sp.</i>	10	2.837	none	0.3676
SERIES II. <i>Solution B + gallic acid.</i>				
Check.....		2.837		
<i>Aspergillus niger</i>	7	2.429	0.337	0.1000
<i>Penicillium sp.</i>	7	2.837	not detected	0.010
<i>Aspergillus niger</i>	10	1.474	1.363	0.3434
<i>Penicillium sp.</i>	10	2.557	0.280	0.108

A glance at the table reveals the fact that both *Penicillium sp.* and *Aspergillus niger* elect cane sugar and leave behind in the culture solution the gallic acid. Cane sugar is therefore proven conclusively to protect the gallic acid. It is of interest to note that the addition of the sugar permits of a more rapid and more extensive growth during the ten-day period.

VIII. INFLUENCE OF AERATION.

Limiting supply of oxygen. VanTieghem³⁹ stated that under aerobic conditions the tannic acid was utilized directly, and that the small amount of gallic acid formed was also assimilated. The preceding experiments and others of the writer, not here mentioned, show that transformation of the tannic acid occurs even when all the growth is on the surface. However, if sugar is not offered with the tannic acid, the increased growth may, as shown, be at the expense of the gallic acid formed. If the tannic acid is

³⁹ *Loc. cit.*

offered as the only source of carbon, the yield of gallic acid is obviously dependent upon the amount of growth. Van Tieghem⁴⁰ found that 0.022 gram's weight of mycelium transformed 48.3 grams of tannic acid in ten days at 35°. The growth is greatly diminished by the absence of oxygen, consequently the oxygen supply is a factor influencing the yield of gallic acid. In order to compare the yield under aerobic and anaerobic conditions, another experiment was performed. Solution B was used, to which was added tannic acid. Into each of six Erlenmeyers of 150 cc. capacity were placed 50 cc. of the solution. Four of the flasks were plugged with cotton, while the other two were fitted with perforated rubber stoppers. After sterilization the perforations were plugged with pieces of glass rod and these, together with two of the flasks plugged with cotton, were inoculated with *Aspergillus niger*. The flasks fitted with the rubber stoppers contained approximately 100 cc. of air and therefore about 20 cc. of oxygen. The cultures were incubated at 31° and at the end of twenty-eight days and forty days analyses were made. The results follow in table X.

TABLE X.

CULTURE SOLUTION	DURATION	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OR GAIN IN GALLIC ACID	DRY WEIGHT OF FUNGUS
	days		grams	grams	grams	gram
Anaerobic (limited).....	40	0	1.603	3.286	+1.186	0.158
Aerobic.....	28	0	1.603	0.898	-1.202	0.634
Check.....		1.603		2.100		

It is at once evident that the inhibition of growth due to deficiency of oxygen is favorable to a good yield of gallic acid. No doubt if the culture in limited oxygen supply had been analyzed sooner a greater yield of gallic acid could have been obtained. With respect to the condition in aerobic cultures it may be stated that cultures, identical with those above, showed on the fourth day a gain of 0.484 gram of gallic acid, though only half of the tannic acid had been transformed, and the weight of the mycelium produced was 0.0314 gram.

Comparison of methods. In order to determine more definitely the yield of the gallic acid under conditions in which the supply of

⁴⁰ *Loc. cit.*

oxygen varied, as well as to compare the yields under these conditions with that obtained when the most favorable conditions were offered, as by the addition of sugar, the following experiment was made:

SERIES I. Solution B + tannic acid, flask plugged with cotton and aerobic conditions maintained.

SERIES II. Solution B + tannic acid + 10 per cent cane sugar, otherwise like the above.

SERIES III. Solution B + tannic acid, flasks stoppered with rubber stoppers and containing therefore only 75 cc. of air or approximately 15 cc. of oxygen.

SERIES IV. Solution B. + tannic acid, flasks stoppered with perforated rubber stoppers, fitted with glass and rubber tubing and clamps. The air was replaced by passing a stream of nitrogen (oxygen-free air) through the flasks for a period of five minutes after inoculation had been made.

All the inoculations were made with spores of *Aspergillus niger*, according to the method described by Hasselbring.⁴¹ The temperature of incubation varied from 30°-35°. Erlenmeyer flasks of 125 cc. capacity were employed. The results obtained are included in table XI.

TABLE XI.

Aspergillus niger. Duration, ten days.

SERIES	ATMOSPHERE OF GROWTH CHAMBER	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	GAIN IN GALLIC ACID	DRY WEIGHT OF FUNGUS
		grams	grams	grams	grams	gram
Check		4.433		3.089		
I	Unlimited air supply	0.409	4.024	5.618	2.529	0.3166
II	Unlimited air supply	0.683	3.760	6.515	3.426	0.3341
III	75 cc. air	1.023	3.410	6.151	3.062	0.0114
IV	Nitrogen	1.707	2.726	5.420	2.331	0.0013

In comparing the rate of tannic acid transformation under the different conditions it is found that in the order of rapidity of transformation they are series I to series IV. The yield of gallic acid was greatest in series II, the addition of cane sugar in this case protecting the gallic acid; somewhat less in series III; still less in series I, where the mycelium was abundant, despite the fact that it led in the amount of tannic acid transformed; and last in series IV. It is noteworthy that the amount of mycelium pro-

⁴¹ *Loc. cit.*

duced in this last was only 1.3 mgm., yet sufficient of the enzyme was liberated to transform a quantity of tannic acid more than 2000 times the weight of the mycelium produced.

From an economic standpoint the method of series I is wasteful of gallic acid, as the organism utilizes much of this substance in its metabolism. Series II has an advantage over series III, and series III over series IV, only in the rapidity of the transformation. The small amounts of growth in series III and IV require such a slight amount of gallic acid in their metabolism that the yield of gallic acid in those series would finally be practically equal to that obtained in series II, to the cultures of which sugar had been added. This fact is borne out by the larger amounts of tannic acid left in the culture solutions of series II and series III.

IX. SUMMARY.

1. Tannic acid is toxic to a large number of fungi at relatively low concentrations.

2. *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium* sp.

3. The fermentation was found to be more rapid in the gall nut infusion than in the synthetic solution in which tannic acid was the only source of carbon. The presence of other organic compounds in the gall nut infusion protected to a certain extent the gallic acid.

4. The addition of 5 per cent sugar did not protect the gallic acid but simply increased the growth. The addition of 10 per cent sugar protected the gallic acid entirely.

5. When gallic acid and cane sugar to the extent of 5.5 per cent and 10 per cent, respectively, were offered together, the cane sugar was elected and the gallic acid left in the culture solution.

6. Fermentation can take place under anaerobic conditions, and 1 mgm. of mycelium is sufficient to effect the transformation of 2.706 grams of tannic acid in ten days.

7. In an approximately 15 per cent solution of tannic acid, fermentation was most rapid when the tannic acid alone served as the source of carbon, and when aerobic conditions were maintained, yet the method of fermentation is wasteful from the standpoint of an economical yield of gallic acid.

8. The economical methods are (a) those in which growth occurs under aerobic conditions and the tannic acid is supplemented by cane sugar; or (b) those in which, with tannic acid alone, the supply of oxygen is limited to a small amount.

9. The presence of 10 per cent cane sugar does not inhibit the secretion of the enzyme tannase by *Aspergillus niger*, but it does seem to inhibit to some extent the secretion of the tannase by *Penicillium sp.*

10. The enzyme is secreted into the culture solution by submerged mycelium as well as by surface growth. There is no evidence that tannic acid is used directly; but the evidence seems to indicate that tannic acid is first transformed into gallic acid and the gallic acid then utilized.

APPENDIX.

Investigators previously occupied with tannic acid fermentation usually employed *Aspergillus niger* together with a species of *Penicillium* which they have designated *Penicillium glaucum*. As has been pointed out by Thom⁴² the name *Penicillium glaucum* has in the past been applied to so many different species that the only idea conveyed by its use is a general concept of the genus. VanTieghem⁴³ applied the name to denote the species of *Penicillium* which he isolated from gall nuts, and it is probable that other students of tannic acid fermentation used the same organism.

In the work of the writer a trial of a number of organisms was made and the *Penicillium* employed was secured from a culture labeled *Penicillium olivaceum*. That identification was incorrect, and then it was believed that the organism might be the one which develops on the gall nuts, but this also proved erroneous, as is indicated subsequently.

In attempting to determine the *Penicillium sp.* used in these experiments it was found that the organism did not correspond to any of the species described by Thom.⁴⁴ Instead, however, of withholding publication of these investigations until the organism should be definitely determined, it was thought best to present here a brief description and certain cultural characteristics of the organism. It is, furthermore, the intention of the writer to make a study of the relation of the various species of *Penicillium* to tannic acid fermentation, and it is hoped by that time to have determined definitely the two species of *Penicillium* which are now known to develop in a 10 per cent solution of tannic acid.

The *Penicillium sp.* used in these experiments possesses only a single whorl of unbranched conidia-bearing cells (sterigmata), and might therefore

⁴² Charles Thom: Cultural Studies of *Penicillium*, Bureau of Animal Industry, Bulletin 118, pp. 107, 1910.

⁴³ *Loc. cit.*

⁴⁴ *Loc. cit.*

be grouped with the genus *Citromyces* as founded by Wehmer; but Thom⁴⁵ does not consider this a valid basis for differentiation of genera, and prefers to include this form of conidiophore under the genus *Penicillium*. This latter concept is here followed.

Description of organism. Colonies on 15 per cent gelatin are, when young, of a faint green color which changes to an otter brown. On 15 per cent gelatin + 3 per cent sugar the olive green changes to ashy gray and then to greenish black. On bean agar the color is at first bluish green, and changes to dark olive green, and finally to a grayish color. The surface is velvety. The conidiophores arise vertically from the substratum and in length vary from 100μ to 700μ . The fructification averages 90μ in length (it may be 200μ), and its width is approximately 15μ . The conidiiferous cells average 10μ in length, the conidia are spherical, and 3μ in diameter. A single whorl of simple conidia-bearing cells only is present as is represented by figures 1 and 2.

FIG 1.

FIG 2.

Conidial fructification showing simple conidiiferous cells ($\times 600$).

Cultural character. At room temperature 15 per cent gelatin is liquified in six days with the production of a strong ammoniacal odor; in the presence of 3 per cent sugar the total liquefaction is retarded a day or more. The presence of bean juice still longer delays the liquefaction of the gelatin. On 15 per cent gelatin + 3 per cent sugar, the lower surface is colored yellowish to reddish brown, when grown in solution B + 10 per cent cane sugar the lower surface of the mycelial felt may be of a salmon color. Fruiting on such a solution at room temperature usually requires eight days. This organism possesses the ability to ferment tannic acid and with 10 per cent tannic acid in solution B at a temperature of 30°C ., gallic acid may be precipitated in about seven days.

Gall nut Penicillium. Only one other species of *Penicillium* has so far been found to grow on 10 per cent tannic acid, and this is the one isolated from the gall nuts. It grows more slowly in 10 per cent tannic acid. In the presence of sugar it produces an intensely red color in the substratum and is a slow liquefier of gelatin. It differs from the other also in possessing more than one whorl of conidiiferous cells, and has other distinguishing features. It appears to be *Penicillium rugulosum*.

⁴⁵ *Loc. cit.*

TANNIC ACID FERMENTATION. II.

EFFECT OF NUTRITION ON THE PRODUCTION OF THE ENZYME TANNASE.

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I. PREFACE.

In an investigation upon tannic acid fermentation reported in the previous paper, it was found that when cane sugar and tannic acid are offered simultaneously to either *Aspergillus niger* or *Penicillium sp.*, the sugar is utilized as the source of carbon while the tannic acid is fermented, gallic acid resulting. Some of the results indicated that the rate of fermentation was influenced by the concentration of the sugar. It was deemed important, therefore, to determine if varying the relative amounts of tannic acid and sugar in the nutrient solution has an influence upon the amount of the enzyme tannase produced in the fungus thus grown. Since tannic acid is probably not commonly utilized in nature by *Aspergillus niger* and *Penicillium sp.* as a source of carbon, experiments were also made to determine if the enzyme is produced when the fungus is cultivated on nutrient solution lacking tannic acid.

The writer wishes to acknowledge his indebtedness to Prof. B. M. Duggar for assistance received during the course of the investigation.

II. INTRODUCTION.

Regulatory production of enzymes. Historical. A number of investigations have been made on the regulatory formation of the enzymes, but for the most part conclusive investigations are lacking. Experimenting with two species of bacillus, Brunton and MacFayden¹ found that when culti-

¹ T. Lauder Brunton and A. MacFayden: The Ferment Action of Bacteria, *Proc. Roy. Soc.*, xlv, B, pp. 543-553, 1899.

vated on starch paste these developed the enzyme diastase; but if the starch were replaced by meat extract no diastase was formed. Fermi² found that of ten bacterial organisms, which developed the tryptic ferment in the presence of peptones or albumen, none developed the enzyme on a sugar-containing nutrient solution. Wortman's³ observations established the fact that the addition of tartaric acid prevented the formation of diastase in bacteria which inhabited decaying potatoes. Fermi and Montesano's⁴ investigations indicated that the presence of sugar is not absolutely necessary for the formation of the enzyme invertase. Various other investigators have studied the influence of nutrition on the formation of enzymes in bacteria.

Dubourg⁵ stated that a yeast which did not normally possess the inverting enzyme was capable of developing it by proper cultivation. As a culture solution in the latter case, yeast water was used, to which was added 5 per cent cane sugar and 5 per cent grape sugar. The yeast, after cultivation, was thoroughly washed and then transferred to a cane sugar solution. In the latter inversion occurred. The form of yeast was not an identified strain. He reported that he was also able to develop the enzyme which fermented galactose by similar methods.

Klöcker,⁶ employing the methods of Dubourg, was unable to develop invertase in *Saccharomyces apiculatus* or maltase in *Saccharomyces marxianus* which organisms do not normally possess these enzymes. The ability to form specific enzymes, according to Klöcker, is therefore a constant character of the yeast organism.

Recently Harden and Norris⁷ "have trained" the yeast *Saccharomyces Carlsberg I* to ferment galactose by cultivating the yeast on hydrolyzed lactose in yeast water to which was added 0.15 per cent of monobasic potassium phosphate. Normally galactose is not fermentable by the above mentioned yeast. According to Kohl⁸ the chemical nature of the solution and the aeration of the culture influence the amount or activity of the enzyme formed in the yeast organism, while the temperature of storage of the yeast also markedly affects the enzyme content.

² Claudio Fermi: Weitere Untersuchungen über tryptischen Enzyme der Mikroorganismen, *Centralbl. f. Bact.*, x, pp. 401-408, 1891.

³ J. Wortman: Untersuchungen über das diastatische Ferment der Bacterien, *Zeitschr. f. physiol. Chem.*, vi, p. 287, 1882.

⁴ C. Fermi and C. Montesano: Die von den Mikroben bedingte Inversion des Rohrzuckers, *Centralbl. f. Bact.*, i, Abt. II, pp. 482-87, 542-56.

⁵ E. Dubourg: De la fermentation des saccharides, *Compt. rend. de l'Acad. des Sci.*, cxxviii, pp. 440-42.

⁶ Alb. Klöcker: Ist die Enzymebildung bei der Alcoholgärungspilzen ein verwerthbares Artmerkmal?, *Centralbl. f. Bact.*, vi, Abt. II, pp. 241-45, 1900.

⁷ A. Harden and R. V. Norris: The Fermentation of Galactose by Yeast and Yeast Juice, *Proc. Roy. Soc.*, lxxxii, B, pp. 645-49, 1910.

⁸ F. G. Kohl: *Die Hefpilze*, 1908, pp. 79-81.

Büsgen⁹ showed that *Aspergillus oryzae* on bouillon, as well as in a sugar-containing solution, formed the enzyme diastase. According to Pfeffer,¹⁰ *Penicillium glaucum* did not secrete diastase in the presence of 10 per cent sugar and, even when only 1.5 per cent sugar was present, the starch was only slightly attacked. *Aspergillus niger* behaved differently, producing diastase even in the presence of 30 per cent cane sugar. Employing a nutrient solution containing 0.25 per cent of soluble starch Katz¹¹ found that starch was saccharified by *Penicillium glaucum*. The addition of 2 per cent grape sugar or 1.5 per cent cane sugar prevented the formation of the diastase. An addition of 1.5 per cent cane sugar depressed the formation of the diastase, while an addition of 0.05 per cent had no effect. Lactose and maltose in a 3 per cent concentration decreased the rate of starch transformation, while a 10 per cent concentration still further depressed the formation of diastase. A 4 per cent addition of erythrodextrin had no effect whatsoever in protecting the starch. Neither did a 10 per cent addition of quinic acid, 4 per cent glycerin or 2 per cent potassium tartrate have any effect upon the secretion of the diastase. The addition of peptone to the solution increased the secretion of the diastase. With *Aspergillus niger* the growth on starch nutrient solution was slow, and five days were required for the transformation of the starch. The addition of 1.5 per cent cane sugar decreased the time to two days, 15 per cent sugar increased the time of transformation by one day and 30 per cent sugar increased the time by two days. *Bacterium megatherium* behaved much the same as *Penicillium glaucum*.

Dox¹² has shown that the carbohydrate-splitting enzymes, amylase, inulase, raffinase, sucrase, maltase and lactase are formed in *Penicillium camemberti*, regardless of the carbohydrate which has served as the source of carbon in the nutrient solution. The amount of the particular enzyme could be increased, however, by cultivating the organism on the corresponding carbohydrate. Likewise, other enzymes are formed independently of the presence in the nutrient solution of the corresponding substance on which the enzyme acts.

According to Went,¹³ the ten enzymes which he investigated in *Monillia sitophila* could be divided into three groups according to the influence of nutrition on their formation. The first group includes those which are formed in slight amounts regardless of the nutrition, the second group in-

⁹ M. Büsgen: *Aspergillus oryzae*, *Ber. d. deutsch. bot. Gesellsch.*, iii, pp. 66-77, 1885.

¹⁰ Quoted from R. Green: *The Soluble Ferments and Fermentation*, p. 32.

¹¹ J. Katz: Die regulatorische Bildung von Diastase durch Pilze, *Jahrb. f. wiss. Bot.*, xxxi, pp. 599-618, 1898.

¹² A. W. Dox: The intracellular Enzymes of *Penicillium* and *Aspergillus*, U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120, 70 pp., 1910.

¹³ F. C. Went: Ueber den Einfluss der Nahrung auf die Enzyymbildung durch *Monillia Sitophila* (Mont.), *Sacc.*, *Jahrb. f. wiss. Bot.*, xxxvi, pp. 611-664, 1901.

cludes the enzymes which are formed only under several different forms of nutrition, while the third group includes such enzymes as are formed only when the substance on which the enzyme acts is present in the culture solution.

Butkewitsch¹⁴ also has shown that nutrition has an influence upon the secretion of the gelatin-dissolving enzyme by *Aspergillus* and *Penicillium*.

III. METHODS OF EXPERIMENTATION.

In all of the experiments upon the influence of nutrition on the production of the enzyme the organisms were cultivated in a synthetic nutrient solution, the inorganic composition being that of Czapek's¹⁵ formula. Throughout this paper the solution has been designated for convenience solution B and is as follows:

Magnesium sulphate (cryst.).....	0.5 gram
Dibasic potassium phosphate.....	1.0 gram
Potassium chloride.....	0.5 gram
Sodium nitrate.....	2.0 grams
Distilled water.....	1000 cc.

All cultures were grown at a temperature of 28° or 30°. The fungus felt, when formed and before spore production, was removed from the nutrient solution, as it has been shown by Malfitano¹⁶ that the most abundant secretion of enzymes into the culture solution takes place just after spore formation. The felt after removal was treated according to Albert and Buchner's method for the preparation of "Acetondauerhefe," as described by Dox,¹⁷ though in the method here employed the felt was not run through a hashing machine. After the mycelium was dry, it was pulverized in a mortar and then placed in a vial until its enzymatic activity was to be determined.

For determining the presence of the enzyme or the relative

¹⁴ W. Butkewitsch: Umwandlung der Eiweisstoffe durch die niederen Pilze im Zusammenhange mit einer Bedingungen ihrer Entwicklung, *Jahrb. f. wiss. Bot.*, xxxviii, pp. 147-240, 1903.

¹⁵ Quoted from A. W. Dox: The Intracellular Enzymes of *Penicillium* and *Aspergillus*, U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120, 70 pp., 1910.

¹⁶ G. Malfitano: La proteolyse chez l'*Aspergillus niger*, *Ann. de l'Inst. Pasteur*, xiv, pp. 60-81, 1900.

¹⁷ A. W. Dox: The Intracellular Enzymes of *Penicillium* and *Aspergillus*, *loc. cit.*

amount of it present, the procedure was as follows: Either a 0.5 per cent, a 0.75 per cent or a 1.0 per cent solution of tannic acid was employed. To the flasks containing the solution were then added equal weights of the pulverized mycelium, the enzymatic activity of which was to be determined. There was also added, as an antiseptic, 2 per cent toluene. The flasks containing the solution and mycelium powder, being tightly stoppered, were then incubated for a definite period and then the solution analyzed for gallic acid according to Jean's¹⁸ method. The relative increase in the gallic acid is taken as a measure of the amount of tannase present.

IV. INFLUENCE OF CONCENTRATION OF SUGAR AND TANNIC ACID ON PRODUCTION OF TANNASE.

Influence of concentration of tannic acid on the amount of enzyme produced. It was found in certain experiments,¹⁹ in which 10 per cent sugar plus tannic acid had been added to the nutrient solution, that the addition of sugar could not prevent the secretion of the enzyme tannase by *Aspergillus niger*, but the secretion by *Penicillium sp.* was markedly decreased. In order to determine the minimum concentration of tannic acid which would stimulate the formation of the enzyme tannase and also what influence the concentration of tannic acid would have upon the amount of tannase produced within the organism, the following experiment was made: Solution B was used, to which was added 10 per cent cane sugar. Liter flasks were employed, and to each were added 500 cc. of the solution. The flasks were plugged and sterilized, and when cool the tannic acid was added to each in varying amounts, as is indicated in the table. For inoculation the method described by Hasselbring²⁰ was employed, though 1 cc. of the spore-containing water was used instead of a single drop. These cultures were incubated at 28°C., and the felt was then removed and treated according to the method described. In determining the enzymatic activity of the mycelium powder 0.3

¹⁸ F. Jean: Die Bestimmung des Tannins und der Gallussäure, *Chem. Centralbl.*, 1900, pp. 1107-08.

¹⁹ L. Knudson: Tannic acid Fermentation I, this *Journal*, xiv, p. 159, 1913.

²⁰ H. H. Hasselbring: Carbon Assimilation of *Penicillium*, *Bot. Gazette*, xlv, pp. 176-193, 1908.

gram of the dried powder was added to a flask containing the tannic acid solution. Each flask contained 75 cc. of an approximately 0.9 per cent solution to which was added, as an antiseptic, 1 cc. of toluene. The results after one week's incubation at 34° are given in table I. The solution contained at the beginning of incubation 0.555 gram tannic acid and 0.327 gram gallic acid.

TABLE I.

*Effect of concentration of tannic acid on production of enzyme tannase; using nutrient solution B + 10 per cent sugar + tannic acid. Period of incubation, 7 days.**

	AMOUNT OF TANNIC ACID ADDED	GAIN IN GALLIC ACID		AMOUNT OF TANNIC ACID ADDED	GAIN IN GALLIC ACID
	per cent	gram		per cent	gram
<i>Aspergillus niger</i>	0.00	0	<i>Penicillium sp.</i>	0.01	0
	0.01	0		0.10	0.058
	0.10	0.059		0.40	0.065
	0.80	0.223		0.80	0.065
	1.00	0.262		1.00	0.108
	2.00	0.388		2.00	0.159
	4.00	0.515		4.00	0.293
	10.00	0.515			
	10.00	0.525			
	10.00	No sugar			

* The actual concentration of each culture was approximately only two-thirds of the figures given, the other one-third consisting of gallic acid.

It is evident from the preceding table that there is a regulatory formation of the enzyme. There is a progressive increase in the amount of tannase with increase of tannic acid in the culture solution. It is noteworthy that no tannase was produced when growth took place in a nutrient solution which lacked tannic acid. It is somewhat remarkable that the formation of enzyme could be stimulated by 0.1 per cent of tannic acid (actually about 0.066 per cent) when there was present at the same time cane sugar in an amount more than one hundred times as great as the tannic acid. The stimulation by this small amount of tannic acid is even more surprising when previous experiments²¹ are recalled in which the gallic acid formed from the tannic acid was protected by the cane sugar, no determinable amount of the gallic acid being assimilated.

²¹ L. Knudson: *loc. cit.*

Why the increase in concentration of tannic acid should increase the amount of tannase is difficult to explain. The amount removed by the organism from the solution is undeterminable with the present method of analysis.

Is the stimulation produced within the cell, or is it caused by contact of the tannic acid with the plasma membrane? Tannic acid precipitates albuminous substances, and it might be possible that it reacts in this manner with the plasma membrane and this precipitation might be the stimulus for the production of the enzyme. This explanation would not, however, include the stimulation to production of the tannase by gallic acid.

A suggestive explanation for the increase of the tannase with increased concentration of the tannic acid is afforded by the work of Katz.²² In his experiments Katz found that diastase could be precipitated by tannic acid and rendered inactive, though when freed from the tannic acid by washing with alcohol it becomes active. Bearing in mind this precipitation by tannic acid and working on the hypothesis that if the diastase formed and secreted into the culture solution were removed from solution more diastase would be formed, he added tannic acid to the culture solution, assuming that in this way there should be an increase in the quantity of the diastase formed. In his experiment Katz actually found that the total quantity of enzyme formed (that secreted into the nutrient solution and that present in the fungus) was greater in the culture which contained 0.5 per cent tannic acid than in the control, the ratio of the diastatic activity being 143 to 100. The results seem to confirm his hypothesis, but there are a number of factors which suggest another explanation of the results obtained. In some previous experiments²³ of the writer it is noted that in the presence of 10 per cent sugar the tannic acid was fermented, and in table I, here reported, it is clear that even in the presence of only 0.1 per cent tannic acid the enzyme tannase is formed and in all probability secreted. The tannic acid of the culture solution would, therefore, be fermented and rendered inactive as regards its capacity to precipitate the diastase liberated, and this condition doubtless occurred in the experiment of Katz.

²² J. Katz: Die regulatorische Bildung von Diastase durch Pilze, *Jahrb. f. wiss. Bot.*, xxxi, pp. 599-618, 1898.

²³ *Loc. cit.*

While a temporary precipitate of the diastase and tannic acid may exist, it is more reasonable to assume that the increase of diastase must be ascribed to some other cause. Increased growth may possibly occur, as a result of the addition of tannic acid, and with this may be correlated an increase of diastase.

A comparison of *Aspergillus niger* and *Penicillium sp.* in the formation of the enzyme tannase is of interest because it affords a partial explanation of the relatively slower transformation of the tannic acid by *Penicillium sp.*²⁴ In both cases the amount of tannase produced in a 0.1 per cent concentration of tannic acid is the same. At 2 per cent concentration the amount of gallic acid, resulting from the action of the *Aspergillus* powder, was 3.88 times the transformation effected by the *Penicillium* powder.

In the preceding experiment no attempt was made to determine the amount of enzyme secreted into the nutrient solution, and an experiment was required to determine this point as well as to verify the preceding results. The methods of experimentation were essentially the same as in the previous experiment except that 250 cc. Erlenmeyer flasks were employed with 100 cc. of the nutri-

TABLE II.

Aspergillus niger.

Effect of concentration of tannic acid on the production of tannase, using solution B + 10 per cent cane sugar + tannic acid. Average period of incubation for mycelial powder, sixty-four hours; for enzyme excreted, ninety hours.

AMOUNT TANNIC ACID ADDED	WEIGHT OF FUNGUS: AVERAGE OF 2 CULTURES	ENZYME OF POW- DERED MYCELIUM: AVERAGE OF 2. INCREASE OF GAL- LIC ACID	ENZYME EXCRETED: AVERAGE OF 2. INCREASE OF GAL- LIC ACID	TOTAL INCREASE OF GALLIC ACID
<i>per cent</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>
0	0.083	0	0	0
0.5	0.102	0.013	0	0.013
0.10	0.111	0.011	0	0.011
0.50	0.149	0.064	0.008	0.072
1.0	0.290	0.084	0.033	0.117
2.0	0.323	0.090	0.041	0.131
4.0	0.207	0.103	0.033	0.136
8.0	0.034	0.157	0	0.157

²⁴ *Ibid.*

ent solution. The tannic acid in all cases was added after the culture solutions had been sterilized. The fungus mat was removed and treated as previously described, and for testing its tannase content 0.05 gram of the mycelium powder was added to 50 cc. of 1 per cent tannic acid solution, containing as an anti-septic 1 per cent toluene. The experiments were all made in duplicate and one series of the solutions was incubated for forty-eight hours and the other for eighty hours.

After the fungus felts had been removed from the culture solution and the liquid filtered, alcohol was added to precipitate any enzymes present, the precipitate being collected on filter paper. The filter paper with whatever precipitates accompanying were then added to flasks containing the tannic acid solution. After ninety-six hours' incubation these solutions were analyzed for gallic acid. The results obtained are given in table II.

The results obtained verify the figures of the preceding table. With an increase in concentration of tannic acid there is a corresponding increase in the amount of enzyme produced. The excretion of enzyme into the culture solutions was evident only in the cultures having a relatively high percentage of tannic acid and at most the excretion was small.

Influence of concentration of sugar. Since in the preceding experiments the amount of tannase produced per unit weight of the fungus varies with the concentration of the tannic acid, it seemed desirable to determine the effect of maintaining constant the tannic acid concentration while varying the concentration of the cane sugar. In the first experiment duplicate cultures of *Aspergillus niger* were made in liter flasks containing 300 cc. of solution B + 2 per cent tannic acid + varying amounts of sugar. The mycelial mats formed were removed, treated as before described and then tested for tannase activity. In measuring the activity of the tannase of each culture 100 cc. of a 0.5 per cent tannic acid solution were employed to which was added 1 per cent toluene as an anti-septic. To each flask was added 0.2 gram of the powdered mycelium. Incubation was given at 34°, and at the end of ninety hours the solutions were analyzed for gallic acid. In table III are given the results of the experiment. The results are the averages of the two tests.

It is here clearly shown that with increased concentration of

TABLE III.

Aspergillus niger.

Effect of concentration of sugar on production of tannase; using solution B + 2 per cent tannic acid + cane sugar. Period of incubation, ninety hours.

AMOUNT OF CANE SUGAR ADDED	INCREASE OF GALLIC ACID	AMOUNT OF CANE SUGAR ADDED	INCREASE OF GALLIC ACID
per cent	gram	per cent	gram
0	0.224	+12	0.166
+1	0.206	+16	0.134
+2	0.206	+24	0.130
+8	0.179		

sugar, the tannic acid being maintained at a constant figure, there is a decrease in the amount of the enzyme produced.

In order to verify the above results another experiment was made along substantially the same lines with *Aspergillus niger*. To test the tannase activity of the dry mycelium 100 cc. of a 0.5 per cent tannic acid solution were used to which was added 1 per cent toluene. In each case 0.05 gram of the powdered mycelium was employed. The cultures were made in duplicate and the determinations likewise. One set was incubated at 24°C. for four days and the second set at the same temperature for six days before analyses were made for gallic acid. In the following table there is given the composition of the nutrient solutions used and the increase in the gallic acid resulting, which increase of course is a measure of the tannase activity of the various cultures.

TABLE IV.

Aspergillus niger.

Effect of concentration of sugar on production of tannase; using solution B + 2 per cent tannic acid + cane sugar.

AMOUNT OF SUGAR ADDED	DRY WEIGHT OF FUNGUS AVERAGE OF 2	INCREASE IN GALLIC ACID; 4 DAYS INCUBATION	INCREASE IN GALLIC ACID; 6 DAYS INCUBATION	AVERAGE INCREASE
per cent	gram	gram	gram	gram
32	0.158	0.179	0.179	0.179
16	0.083	0.270	0.278	0.274
12	0.074	0.233	0.270	0.251
8	0.101	0.270	0.278	0.274
4	0.093	0.270		0.270
2	0.065	0.270	0.296	0.283
0.5	0.074	0.303	0.332	0.317
0	0.035	0.303	0.368	0.335

The results confirm the evidence of the preceding experiment, though the differences due to sugar concentration are not so marked. This may have been due to the longer period of incubation.

V. INFLUENCE OF NUTRITION.

Since Dox has found in *Penicillium camemberti* that the various enzymes are produced irrespective of the nutrition of the fungus, an experiment was made to determine definitely if the enzyme tannase could be produced in two *Penicillium* species when tannic acid is withheld from the culture solution. *Penicillium* sp., which I have previously described,²⁵ and *Penicillium rugulosum* were employed in the experiment. They were cultivated in 100 cc. of a nutrient solution which was composed on the one hand of solution B + 5 per cent sugar and, on the other, solution B + 5 per cent sugar + 2 per cent tannic acid. The mycelial felts, as before, were removed just before spore formation, treated as previously described and pulverized. For determining the presence of the enzyme tannase 100 cc. of a 1 per cent tannic acid solution were used to which was added 2 per cent toluene as an antiseptic and 0.1 gram of the pulverized mycelium. The solutions were incubated for twenty-eight days at a temperature of 33°. They were then analyzed for increase in gallic acid. All cultures and determinations were made in triplicate. As is evident from the table tannase was formed only in the presence of tannic acid.

TABLE V.

ORGANISM	COMPOSITION OF SOLUTION	GALLIC ACID PRESENT	GAIN
<i>Penicillium</i> sp.....	Solution B + 5 per cent sugar	0.287	0
<i>Penicillium rugulosum</i> .	Solution B + 5 per cent sugar	0.287	0
<i>Penicillium</i> sp.....	Solution B + 5 per cent sugar + 2 per cent tannic acid	0.822	0.535
<i>Penicillium rugulosum</i> .	Solution B + 5 per cent sugar + 2 per cent tannic acid	0.822	0.535
Check.....		0.287	0

Experiments were next made to determine the influence of replacing the sugar by other compounds and of adding to the nutrient solution various reagents. For this experiment solution

²⁵ *Loc. cit.*

A²⁶ was used, and as culture vessels liter flasks were employed. Into each flask were placed 365 cc. of the solution, and to it were added the sugar and other reagent, or the sugar was omitted and some other carbon compound substituted for it. The solutions were sterilized, inoculated and incubated at a temperature of 28°C. The felt was removed just before spore production and treated in the manner previously described.

To determine the presence of tannase, 0.3 gram of the powdered, dried mycelium was introduced into 100 cc. of a 0.75 per cent solution of tannic acid, to which had been added as an antiseptic 2 cc. of chloroform. After incubation at 31° for one week, the solutions were analyzed for gallic acid. The results obtained are given in table VI.

TABLE VI.
Penicillium sp.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution A + 25 grams cane sugar.....	0.202	0
Solution A + 25 grams cane sugar 5 cc. $\frac{N}{10}$ HCl..	0.202	0
Solution A + 15 grams corn starch.....	0.208	0
Solution A + 25 grams glycerin.....	0.202	0
Solution A + 25 grams gallic acid.....	0.370	0.128
Solution A + 25 grams tannic acid.....	0.713	0.511

The control contained 0.202 gram gallic acid at the beginning and at the end of the incubation.

The gallic acid stimulated the formation of the enzyme only one-fourth as much as did the tannic acid. Slight acidity had no effect in stimulating the production of the enzyme. Glycerin and starch, both of which are relatively poor food compounds, were supplied, and if enzymes are stimulated to formation by conditions approaching starvation, as suggested by Wortman,²⁷ then the tannase should have developed; but the results were negative.

An experiment similar to that above was made with *Aspergillus niger*, 400 cc. of solution being used and the methods of experimentation the same as before. The results obtained are as follows:

²⁶ KH₂PO₄, 0.5 gram; KNO₃, 1 gram; MgSO₄, 0.25 gram; Distilled water, 100 cc.

²⁷ *Loc. cit.*

TABLE VII.
Aspergillus niger.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution A + 25 grams sugar.....	0.209	0
Solution A + 25 grams sugar + 5 cc. $\frac{N}{10}$ HCl.....	0.202	0
Solution A + 25 grams sugar + 5 grams gallic acid.....	0.202	0
Solution A + 25 grams sugar + 2 grams resorcin.	0.190	0
Solution A + 25 grams sugar + 1 gram hydro- quinone.....	0.202	0
Solution A + 25 grams sugar + 5 grams peptone.	0.202	0
Solution A + 25 grams sugar tannic acid.....	0.691	0.489

From the above table, it is evident that in *Aspergillus niger* there is a very marked regulatory formation of the enzyme. Peptone, which stimulates the secretion of diastase, according to Katz²⁸ has no influence in stimulating the formation of tannase. Gallic acid of the strength used had no effect, which result is surprising in view of the results indicated in table VI and of the further fact that Pottevin²⁹ found the enzyme tannase to be formed in *Aspergillus niger* when the organism is cultivated in Raulin's solution with the sugar replaced by gallic acid. Since pyrogallol is a decomposition product of tannic acid, it was thought that perhaps it might stimulate the formation of the enzyme, but the amounts used proved toxic in this particular experiment. Resorcin and hydroquinone were both employed because of their constitutional similarity to pyrogallol, both being dihydroxybenzenes, while the pyrogallol is a trihydroxybenzene. Negative results, however, were obtained.

In a similar manner experiments were made in which 10 per cent cane sugar plus various other substances were added to solution A. For the experiment 500 cc. of the solution were used. In determining the presence of tannase, 0.2 gram of the powdered mycelium was added to 75 cc. of a 0.9 per cent tannic acid solution to which had been added 2 per cent toluene as an antiseptic. The results are given in table VIII.

²⁸ *Loc. cit.*

²⁹ H. Pottevin: La tannase. Diastase dédoublant l'acide gallotanique, *Compt. rend. de l'Acad. des Sci.*, cxxxi, pp. 1215-17, 1901.

TABLE VIII.
Aspergillus niger.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID INCREASE
Solution B + 10 per cent sugar + 35 mgm ZnSO ₄ . H ₂ O..	None
Solution B + 10 per cent sugar + 20 mgm salicylic acid.	None
Solution B + 10 per cent sugar + 1 gram gallic acid...	None
Solution B + 10 per cent sugar + 200 mgm. methyl salicylate.....	None
Solution B + 10 per cent sugar + 200 mgm. ethyl salicylate.....	None
Solution B + 10 per cent sugar + 50 mgm. methyl salicylate.....	None
Solution B + 10 per cent sugar + 50 mgm. ethyl salicylate.....	None

Pottevin³⁰ states that tannase has the property of hydrolyzing methyl and ethyl salicylate. In my cultures the methyl or ethyl salicylate did not incite the development of the enzyme. The amount of salicylate present, however, was small. These concentrations may have been too weak to stimulate the production of the enzyme but probably the hydrolysis of these two substances is due to another enzyme. Salicylic acid also was used because of its relation to gallic acid, salicylic acid being a monohydroxybenzoic acid, while gallic acid is a trihydroxybenzoic acid. The former was necessarily used at a very low concentration, and was without effect. The zinc sulphate was used at a concentration which is stimulating to the fungus growth, as shown by Richards,³¹ but no formation of the tannase resulted.

Since in some of the experiments tannase was not produced when the organism was grown in a solution with the carbon supplied as cane sugar and gallic acid, an experiment was made with the carbon supplied only as gallic acid. At the same time experiments were made to determine the influence of certain glucosides. Solution B was used, and 500 cc. of it placed in each of three liter flasks. These were plugged and sterilization made at 115° for ten minutes. To each of the three flasks was added gallic acid, amygdalin and salicin, respectively, and the culture solutions

³⁰ *Loc. cit.*

³¹ H. M. Richards: Die Beeinflussung des Wachstums einiger Pilze durch chemische Reize, *Jahrb. f. wiss. Bot.*, xxx, pp. 665-88.

then inoculated. The growth in gallic acid was rapid, but not heavy. In the solutions in which the glucosides were present, growth was at first very slow, but after the transformation of the glucosides had begun, growth was rapid. While it required eight days to develop the first felts in the two glucoside solutions, the second felts (after the removal of the first) developed in two days. The felts first formed were removed and treated according to the methods previously described, and the powdered mycelium then added to 100 cc. of a 0.90 per cent solution of tannic acid which contained also 2 per cent of toluene. After incubating two days at 40°C., the solutions were analyzed for gallic acid. The composition of the nutrient solutions and the gallic acid formed by the powdered mycelium are given in table IX.

TABLE IX.
Aspergillus niger.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution B 500 cc. + 10 grams gallic acid.....	0.546	0.219
Solution B 500 cc. + 10 grams salicin.....	0.327	0
Solution B 500 cc. + 2 grams amygdalin.....	0.327	0

The control contained 0.327 gram gallic acid and 0.555 gram tannic acid.

In preceding tables it has been seen that gallic acid at certain concentration in the presence of 10 per cent sugar does not stimulate the formation of the enzyme tannase. When gallic acid is present alone as the source of carbon, the enzyme is produced. If tannic acid is a glucoside, it might be expected that the presence of another glucoside would stimulate the production of some tannase, but such was not the case. Nevertheless, glucosides were transformed by the organism. While the gallic acid stimulates the formation of the enzyme tannase, it does not do so as effectively as the tannic acid.

VI. EFFECT OF CONCENTRATION OF GALLIC ACID ON PRODUCTION OF TANNASE.

Since the amount of tannase produced is regulated by the relative concentrations of sugar and tannic acid it was deemed important to determine if the amount of the tannase produced could be in-

creased by increasing the concentration of the gallic acid. In the experiment, the results of which are given in table X, the usual methods were followed. Erlenmeyer flasks of 250 cc. capacity were used and 100 cc. of the nutrient solution. For determining the tannase activity of the various cultures 0.05 gram of the dried mycelium was added to 50 cc. of 1 per cent tannic acid solution and the solutions incubated for forty-eight and eighty hours respectively at a temperature of 30°. The culture solutions were tested for their tannase content by precipitating any enzyme present with alcohol and collecting on a filter any precipitate. The test for the enzyme was made as before. Incubation was made at a temperature of 30° for ninety hours. The results in table X are the averages of two cultures.

TABLE X.

Aspergillus niger.

Effect of concentration of gallic acid on the production of tannase. Average period of incubation for mycelial powder 64 hours and for enzyme excreted 90 hours.

COMPOSITION OF NUTRIENT SOLUTION	WEIGHT OF FUNGUS. AV. OF 2 CULTURES	INCREASE IN GALLIC ACID		
		Mycelium Av. of 2	Enzyme excreted. Av. of 2	Total increase in gallic acid
	gram	gram		gram
Solution B + 10 per cent sugar + 0.1 per cent gallic acid.....	0.240	0	0	0
Solution B + 10 per cent sugar + 0.5 per cent gallic acid.....	0.442	0.024	0	0.024
Solution B + 10 per cent sugar + 1.0 per cent gallic acid.....	0.324	0.045	0	0.045
Solution B + 10 per cent sugar + 1.5 per cent gallic acid.....	0.280	0.046	0	0.046

An examination of the above table reveals the fact that the enzyme tannase is produced when the concentration of gallic acid is 0.5 per cent in the presence of 10 per cent sugar. At a concentration of 1.0 per cent gallic acid in the presence of 10 per cent sugar the amount of tannase produced is double that produced when only 0.5 per cent gallic acid is present. In the presence of

10 per cent sugar the gallic acid, so far as my previous results³² show, is not at all absorbed from the nutrient solution. Why the increase in gallic acid should increase the amount of tannase produced is therefore difficult to explain. The writer is not prepared at present to offer an explanation.

VII. SUMMARY.

1. There is a progressive increase of tannase in the two organisms *Aspergillus niger* and *Penicillium sp.* with increased concentration of tannic acid in Czapek's solution containing 10 per cent sugar.

2. In a full nutrient solution containing, as a source of carbon, 2 per cent tannic acid, the addition of cane sugar decreases the quantity of the tannase produced by the organism. The higher the concentration of sugar the lower the quantity of tannase produced.

3. *Aspergillus niger* produces more tannase or a more active tannase per unit weight than *Penicillium sp.*

4. The production of tannase in *Aspergillus niger*, *Penicillium sp.* and *Penicillium rugulosum* is stimulated by tannic acid and gallic acid, only, and the former is more effective than the latter.

5. There is a progressive increase of tannase in *Aspergillus niger* with increased concentration of gallic acid in a nutrient solution containing 10 per cent sugar.

VIII. DISCUSSION.

Dox³³ in his excellent paper makes the following statement: "There is no evidence that enzymes not normally formed by the organism in demonstrable quantities can be developed by special methods in nutrition. The influence of adding a particular substance to the medium is, therefore, not to develop an entirely new enzyme, but to stimulate the production of an existing enzyme, which is normally formed under all conditions." In the light of Dox's results it seems somewhat surprising that the enzyme tannase should be formed only under special nutrition. It may be as Dox³⁴

³² *Loc. cit.*

³³ A. W. Dox: The Intracellular Enzymes of *Penicillium* and *Aspergillus*, *loc. cit.*

³⁴ A. W. Dox: Enzyme Studies of Lower Fungi, *Plant World*, xv, pp. 40-43, 1912.

has stated concerning my work that the formation of the enzyme tannase is an exception to the general rule. The work of other investigators previously mentioned has indicated, however, that the production of certain enzymes in other organisms is governed entirely by the character of the nutrition.

Dox³⁵ has considered only the influence of external environment upon the formation of the enzymes. It is to be expected that protease, lipase, nuclease, inulase and perhaps some of the other enzymes would be produced because the substances which they transform are present in the mycelium. If the action of an enzyme is reversible and they are synthesizing agents, then the question arises: "can the products of the decomposition induce the formation of enzyme?" In my experiments the only substance besides tannic acid capable of inducing the formation of the tannase is gallic acid, which is a decomposition product of tannic acid.

Might it be possible that all of the enzymes are produced only in response to the influence of the zymolyte or to the products of its decomposition present either in the nutrient solution or in the mycelium? There is a considerable amount of evidence indicating that one or the other is always present, but there is also evidence that certain enzymes are seemingly produced in the entire absence of the zymolyte or the products of its decomposition. The whole problem is a complex one and requires investigation.

³⁵ Enzyme Studies of Lower Fungi, *loc. cit.*

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