A BIO-CHEMICAL STUDY OF SOME SOIL FUNGI WITH SPECIAL REFERENCE TO AMMONIA PRODUCTION.

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The important role played in the soil by micro-organisms has long been recognised. Owing to the predominance of bacteria, attention was at first almost entirely restricted to the study of this group, but the later work of Russell and Hutchinson demonstrated that protozoa had important functions. Later still attention has been directed to the study of fungi, and the opinion now held by many workers in this field is that the importance of this group in the soil economy has been much under-estimated.

Enumeration of the fungi in a soil is difficult and no satisfactory method is at present available. The universal occurrence of certain species in soils indicates that these species are true soil inhabitants, and a further knowledge of their activity under varying conditions is much to be desired.

That fungi play an active part in the breaking down of organic matter under certain conditions can no longer be doubted, but the mechanism of this process is imperfectly understood. In the present paper an account is given of an investigation of the fungi of a typical soil from S. India. A large number of species have been isolated and the behaviour of some among them investigated with particular reference to (1) the power of such species to fix atmospheric nitrogen, (2) their ability to break down carbohydrate and protein matter and (3) the mechanism of the changes by which ammonia is produced from nitrogenous material.

Isolation and Identification of Predominant Species.

The extensive data presented by Waksman (*Soil Science*, 1917, **3**, 565) warrant the conclusion that fungi are true soil organisms and not mere invaders. The fact that such species as *Rhizopus nigricans*, *Mucor racemosus*, *Zygorhynchus vuilleminii*, *Aspergillus niger*, *Trichoderma koningi*, *Cladosporium herbarum*, different *Penicillia*, *Aspergilli*, *Fusaria* and others have been repeatedly isolated from different soils all over the world, tends to show that these organisms are normal inhabitants of the soil. The variations in fungal types found in soils are probably more quantitative than qualitative. The differences in the

climatic and soil conditions may modify the numbers of the particular groups of organisms and their activities without determining their presence or absence.

Only one soil was used throughout this investigation, a garden soil from the Institute grounds. It is a clay loam, neutral in reaction, neither manured nor irrigated. It appeared advisable to study a soil not previously manured or irrigated as this treatment may encourage one set of organisms at the expense of others. All samples were taken from the same soil through different seasons of the year to a depth of six inches, and were collected under sterile conditions, brought at once to the laboratory and immediately plated out on the four media used.

Direct Method of Isolation.—This method has been suggested and elaborated by Waksman (*Soil Science*, 1922, **14**, 153). A pea-sized portion is carefully transferred from the soil sample, with as little contamination from the air as possible, to the centre of a sterile plate into which 10 c.c. of a sterile medium, favourable for the growth of the fungi only, has been placed. The soil is lightly pressed down so as to be surrounded by the nutrient medium and the plates incubated at 35°. Fungus hyphæ are found to radiate from the soil into the surrounding medium after 24 hours' incubation. The method is based upon the fact that the fungi present in the soil in the form of mycelium will grow at once into the medium, before the spores can germinate and develop hyphæ. When a tip of a growing hypha, as far from the soil as possible, is transferred to a sterile medium, a pure culture of the particular fungus is obtained.

Dilution Method of Isolation.—A small quantity of the soil is weighed in a sterile flask and diluted 1,000 or 10,000 times with sterile water. Plates with various media are then prepared from the different dilutions and kept at room temperature, 23–25°, the fungi being separated after six days and isolated by a series of sub-cultures.

The first method was used to demonstrate that certain fungi actually live and produce mycelia in the soil. A number of species were isolated, the majority belonging to *Mucorales* and being included in the genera *Mucor*, *Rhyzopus* and *Zygorhynchus*; curiously enough, the organisms generally found in the largest numbers in the soil such as *Aspergillus* and *Penicillium*, were demonstrated in the soil by this method only in a very few cases. The method did not give satisfactory results in regard to the isolation of the less dominant species. When, on the other hand, the dilution method was used for the same soil sample the opposite was found to hold true; the *Mucorales* did not then develop as freely as the *Penicillia* or *Aspergilli*.

The dilution method, however, used with such convenience for the isolation of soil bacteria, is entirely inadequate for an investigation of soil fungi; since neither the numbers nor the species of fungi developing on the plates give any true indication of the relative abundance and possible importance of the fungi in the soil. This is due to the fact that the mycelium in the soil, however abundant, may be entirely absent when the soil is diluted 1,000 or 10,000 times, and the fungi developing on the plate may indicate only the spores originally present in the soil. These are so unevenly distributed that the results obtained in different determinations made with the same soil sample exhibit very wide variations.

In addition to these, a microscopic method has been suggested by Conn (*N.Y. Gen. Agric. Expt. St. Tech. Bull.*, 64, 1919). This consists in taking a very small fragment of soil, suspending this in the medium after dilution with sterile water and examining it under the microscope in the form of a hanging-drop-culture; the procedure, however, has not given satisfactory results in our hands.

Media used for Isolation.—To compare our results with those of other workers we have employed media in common use. Preference has been given to synthetic media of definite chemical composition since these can be reproduced with greater certitude, four varieties having been used.

1. *Modified Albumen Agar*, composed of distilled water, 1,000 c.c., dextrose, 10 gms., potassium hydrogen phosphate, 0·5 gm., magnesium sulphate, 0·2 gm., egg albumen, 0·15 gm. and agar, 20 gms. with a trace of ferric sulphate. The egg albumen was first dissolved in 50 c.c. of cold water to which a few drops of dilute caustic soda were added, and this solution then mixed with the hot medium already prepared and well shaken. In this way coagulation was completely avoided.

This medium was used for several reasons:—(*a*) It allowed the greatest development of both fungi and bacteria under the given condition and thus afforded the best means of comparing the relative numbers of the most important groups. (*b*) It is rather poor in plant food, thus allowing only a very slow growth of all the organisms; isolation of the more slowly growing varieties before the plates are overrun with the more vigorous species thus becomes possible. (*c*) It afforded satisfactory conditions under which bacteria and fungi, both slow and rapid growing forms, could be studied on the same plate.

2. *Cook's No. 2 Medium* (*Del. Agr. Exp. Sta. Bull.*, 91, 1911) has been used by several investigators. Though many organisms grew only slowly on it, the final growths are characteristic and

assist in isolation and identification. Other media were used for supplementary purposes; this one was used as a standard with which all the results could be compared.

3. *Raisin Agar*, prepared by heating 60 gms. of raisins in 1,000 c.c. of tap water for one hour and dissolving in this 35 gms. of agar. The reaction was adjusted to a P_H of 4·0 and the medium then sterilised.

4. *Modified Czapek's Synthetic Agar*, composed of distilled water, 1,000 c.c., magnesium sulphate, 0·5 gm., potassium hydrogen phosphate, 1·0 gm., potassium chloride, 0·5 gm., ferrous sulphate, 0·01 gm., sodium nitrate, 2·0 gms., cane sugar, 30·0 gms. and agar 15·0 gms.

Normal phosphoric acid (6 c.c. per. litre) is added to make the reaction equivalent to P_H 3·8, and 25 gms. more of agar are dissolved by boiling. The reaction of the medium after sterilisation is equal to P_H 4.

Media Nos. 3 and 4 are acid and keep down the bacterial numbers yielding cultures of fungi free from bacteria; they also allow slow development of the organisms. We have found that when the numbers of fungi were determined on the plates, the variability was so great, due to the high dilution of the soil, and therefore, to the small numbers of fungi obtained therein, that the probable error in as many as 20 plates inoculated from one soil sample is so large that the results have little quantitative value. Where low dilutions of the soil were employed so many bacteria would sometimes develop on the plate as to crowd out many of the fungi and make the counting entirely unreliable. Morever, supposing that forty colonies developed on any particular plate, this cannot be taken as proof that so many pieces of mycelium were present in that quantity of inoculum, but may be due merely to the presence of a clump of spores. This uncertainty will always exist because of the fact that any particular sample taken from a spot where sporulation has occurred would give a very high count though the species in question may not really play a predominant role. For example, such organisms as *Aspergillus* or *Penicillium* which produce a large number of spores separating with relative ease, are often found in predominant numbers, while at a different period of the year these species may be entirely absent. In view of the above considerations the actual plate counts are of little significance. On the other hand the constant presence of certain species in nearly all the samples examined is strong evidence that such species are normal soil inhabitants, playing an important part in the soil economy. Again there is no doubt that during the monsoon period, when the soil may be heavily charged with moisture for a considerable period, the fungal numbers are much increased.

Fifty distinct species of fungi were isolated. Not all of these have yet been identified but all of the following are common and occur in large numbers:—*Aspergillus flavus : A. candidus : A. niger : A. fumigatus : Rhizopus nigricans : Citromyces glaber : Penicillium glaucum : Aspergillus nidulans : A. fuscus : A. repens : Penicillium oxalicum : P. glaber : P. digitatum : Botrytis cinerea : Sporotrichum roseum : Oidium lactis : Mucor racemosus : M. glomerula : M. plumbeus : Cephalosporium acremonium : Trichoderma album : Verticillium glaucum :* Common soil *Fusaria, Verticillia, Sporotricha, Armillarii, Acrostalagma.* Most of these organisms have been isolated from soils by other investigators also.

Nitrogen-fixing Power of the Fungi.

During the last two decades many investigators have examined this question and in some thirty researches published during this period, opinion is found to be equally divided. Goddard (*Bot. Gaz.*, 1903, **56**, 249) who has studied the problem in detail reports negative results. The amount of free nitrogen taken up from the air by his cultures did not seem sufficient to make any appreciable differences in their nitrogen-content, alike whether the media contained nitrogen or not.

In our work on this subject the medium used was Lipman's solution (*N. J. Agr. Exp. Sta. Ann. Rept.*, 1904). This was distributed in 100 c.c. portions in 250 c.c. flasks and twenty different species were inoculated after sterilisation of the medium; growth was very small during four weeks. The above medium, which is commonly used to study the nitrogen-fixing power of bacteria, is distinctly alkaline; it was thought advisable to use a more acid medium and therefore potassium phosphate was replaced by potassium hydrogen phosphate. On repeating the above experiment only three organisms showed appreciable growth after four weeks; the nitrogen figures for these cultures are shewn in Table I.

TABLE I.

Organism	Total Nitrogen, mgms.		Remarks
	Blank	Culture	
Aspergillus flavus ...	3·36	3·51	Slight Growth
Penicillium glaucum ...	3·39	3·75	,,
Armillarius ...	3·41	3·39	,,

As seen in the above two experiments, there was practically no growth of the organisms and the amount of nitrogen fixed is negligible. It was considered advisable to add 0·02 per cent. of ammonium nitrate in order to induce better initial growth, the medium being kept acidic. The above three species were inoculated after sterilisation of the medium and allowed to grow for four weeks, other conditions being the same. The results are given in Table II.

TABLE II.

Organism	Total Nitrogen, mgms.		Remarks
	Blank	Culture	
Aspergillus ...	8·0	8·82	Good Growth
Penicillium ...	8·55	8·68	,,
Armillarius ...	8·1	8·9	,,

The amount of nitrogen fixed lies within experimental error and is negligible. The results confirm the investigations of Goddard and others, who could not prove any nitrogen-fixation by fungi. It should be mentioned here that growth of the organisms was found to stop when the quantity of added ammonium nitrate was exhausted.

Decomposition of Organic Matter by the Fungi.

1. *Cellulose Decomposition.*—Fungi seem to play an important part in the decomposition of cellulose. Among the species isolated from the soil twenty of very common occurrence were selected for testing this function. The precipitated cellulose agar was prepared in three stages :—

(*a*) One litre of ammonium hydroxide (sp. gr. 0·90) in a glass stoppered bottle was diluted with 250 c.c. of distilled water and shaken vigorously with 75 gms. of pure copper carbonate until all had dissolved. With this solution 15 gms. of high grade sheet filter paper was shaken vigorously at intervals of 10 minutes for one hour, or until the liquid was perfectly clear.

(*b*) The ammonium-copper-cellulose solution (250 c.c.) was diluted with 10 litres of tap water and shaken with slowly added portions of hydrochloric acid prepared by mixing 500 c.c. of concentrated acid with 10 litres of tap water; on disappearance of the blue colour a slight

excess of acid was added and the thoroughly shaken liquid allowed to stand a few minutes. The finely precipitated cellulose rose with the gas liberated, and when this had escaped the cellulose rapidly settled; it was then washed with repeated changes of water until free from copper and chlorine, allowed to settle and drained by siphon.

(*c*) The precipitated cellulose with 2 gms. of agar was added to 100 c.c. of water containing 0·21 gm. of potassium hydrogen phosphate, 0·2 gm. of crystallised magnesium sulphate, 0·02 gm. of calcium chloride, 0·02 gm. of ammonium nitrate and 0·02 gm. of sodium chloride. The solution was boiled to dissolve the agar and sterilised in the usual way. The organisms were inoculated into sterile Petri dishes containing this precipitated cellulose medium. The plates were examined under the microscope, and where cellulose decomposition had taken place the threads could be found broken down and destroyed. The results are given in Table III.

TABLE III.

Decomposition of Cellulose by Soil Fungi.

Organism	Cellulose decomposition in 10 days
Aspergillus niger ...	+ +
,, flavus ...	−
,, fumigatus ...	−
Penicillium glaucum ...	−
Mucor racemosus ...	+
Citromyces sp. ...	+ + +
,, glaber ...	+ +
Armillarius ...	+ + +
Rhyzopus nigricans ...	−
Fusarium sp. ...	+ +
Verticillium sp. ...	+ + +
Aspergillus sp. ...	+ +

\+ indicates slow decomposition.
\+ + ,, fairly strong decomposition.
\+ + + ,, very strong decomposition.
− ,, no decomposition.

Although this experiment is qualitative it has value in showing which species are capable of decomposing cellulose. It will be noticed in the above table that *Penicillium* gave negative results while *Aspergillus niger* and *Citromyces* are fairly strong cellulose decomposers. In this respect these results agree with those of other investigators such as Waksman, Goddard and others.

2. *Protein Decomposition with Production of Ammonia.*—If we consider the disintegration of nitrogenous organic matter in the soil, particularly during the first stage of decay leading to ammonia production, we have sufficient evidence to show that the soil fungi play a very important part. The precise mechanism of the changes involved is not however fully understood nor has it been clearly ascertained what part the ammonia plays in the metabolism of the organisms concerned.

3. *Experimental Details.*—In view of the difficulty of working with soil samples and the fact that when such samples are sterilised they no longer bear any relation to natural soils, we have throughout utilised an artificial medium with gelatin as the source of protein nitrogen, and prepared from water, 1,000 c.c., gelatin, 10 gms., glucose, 15 gms., potassium acid phosphate, 0·25 gm., zinc sulphate, 0·1 gm., magnesium sulphate, 0·25 gm. and ferric chloride, one drop.

Cultures were made in a litre of the above medium at room-temperature (28–30°). From the time of inoculation 25 c.c. was removed from each culture every third day by a sterilised pipette and immediately filtered twice to remove mycelium; it was then diluted to 100 c.c. with sterile water, 50 c.c. being used for determining ammonia which was estimated according to Folin's aeration method and 10 c.c. for the estimation of total nitrogen (micro-Kjeldahl's method). Amino-nitrogen in another 10 c.c. was determined by Van Slyke's micro-method. P_H determinations were made colorimetrically. The values obtained in the above determinations were then calculated back to the original 25 c.c. of sample. These details have been followed in all the experiments. The organisms isolated from the soil have been studied under these conditions for their capacity to produce ammonia in the culture medium.

In one series with *Aspergillus flavus* (Table V) sugar was excluded from the medium to note the influence of the absence of carbohydrates on the process of ammonification. In the case of *Humicola sp.* (Traaen) which was grown in sugar-free medium, amino-nitrogen was also determined in all the filtered samples (Table VI) along with the other nitrogen estimations.

TABLE IV.

Decomposition of Organic Matter by Aspergillus flavus (*Sugar present*).

Days	Total *N*, mgms.	Available *N*, mgms.	Ammoniacal *N*, mgms.	P_H
1	26·66	26·46	0·2	...
4	24·00	23·80	0·2	4·4
6	22·8	21·54	0·26	4·3
8	18·46	17·26	1·2	5·2
10	17·2	15·2	2·0	6·2
12	16·1	13·1	3·0	6·5
16	17·14	12·34	4·8	6·6
18	17·1	12·84	4·26	6·6
20	16·3	10·7	5·6	6·6
22	18·45	12·23	6·22	6·7
24	18·5	11·07	7·43	6·6
26	18·47	10·47	8·0	6·7
28	17·14	12·34	4·8	6·7
30	15·1	9·45	5·65	6·8
32	17·13	10·73	6·4	6·8
36	15·9	8·52	7·38	6·9
40	18·4	9·07	9·33	8·2

TABLE V.

Decomposition of Organic Matter by Aspergillus flavus (*Sugar absent*).

Days	Total *N*, mgms.	Available *N*, mgms.	Ammoniacal *N*, mgms.	P_H
1	32·0	31·8	0·2	5·9
6	27·0	26·52	0·48	5·6
9	24·2	23·67	0·53	6·4
12	21·6	19·8	1·8	7·0
15	27·0	21·6	5·4	7·2
18	30·1	24·6	5·4	7·8
22	30·2	24·2	6·0	8·0
25	31·1	21·1	10·0	8·2
29	30·9	17·7	13·2	8·2
32	31·3	21·55	9·75	8·4
35	31·2	21·2	10·00	8·4

TABLE VI.

Decomposition of Organic Matter by Hunicola (*Sugar absent*).

Days.	Total *N*, mgms.	Available *N*, mgms.	Ammoniacal *N*, mgms.	P_H	Amino *N*, mgms.
1	26·6	26·13	0·47	6·4	1·541
7	24·9	24·42	0·48	6·0	1·6
10	24·2	23·9	0·3	6·0	...
12	24·1	23·63	0·47	6·1	3·05
14	24·2	23·7	0·5	6·2	1·7
17	24·12	23·52	0·6	6·2	2·36
21	24·2	23·6	0·6	6·4	2·46
24	24·5	23·01	0·49	6·6	4·51
27	20·9	20·1	0·8	6·7	5·14
31	20·01	18·81	1·2	6·7	5·01
35	23·9	22·78	1·12	6·7	5·12
38	24·0	22·8	1·2	6·8	5·03
41	23·9	21·82	2·08	7·0	6·16
45	20·8	18·24	2·56	7·2	6·3
47	18·46	15·66	2·8	7·2	5·12
50	19·5	17·2	2·3	7·5	4·2
53	25·5	22·3	3·2	7·8	4·62
56	26·92	22·87	4·1	7·9	5·2
59	24·6	18·57	6·03	8·0	7·01
62	26·0	19·0	7·0	8·2	7·2

Conclusions.—These results (Tables IV and V), correlated with the observations noted on the growth of the organism, brought clearly to notice the important fact that accumulation of ammonia as a result of mould action seems to take place in different stages during the life-cycle of the organism; in other words there appears to be a definite relationship between the growth period of the organism and ammonia accumulation. Waksman and Cook (*Soil Sci.*, 1916, **1**, 375), in confirming the observations of McLean and Wilson (*N. J. Agri. Exp. Sta. Bull.*, **270**, 39, 1914) as regards ammonia production by fungi have called attention to the same fact.

The first stage consists in the organism attacking the protein molecule and thus deriving from it the nitrogen required for its vegetative growth. During this stage, although the protein and available nitrogen diminish, little ammonia accumulates in the solution, the nitrogen being retained by the organism. In Table IV it will be noticed that the nitrogen in the medium reaches a low minimum, this being attained at a time between the first and second week. A fall in the quantity of total nitrogen in the filtrate extending for a period of ten to twelve days indicates the first stage in the life-cycle of the organism, during which the organism assimilates most of the nitrogen. The second stage begins when the vegetative growth is completed and spore-formation takes place. During this stage there is a rapid accumulation of ammonia in the filtered culture medium. A third stage is reached when the content of ammonia remains more or less constant and an equilibrium is established after which the total nitrogen does not appreciably vary, the nitrogen required for new growth being supplied probably by the autolysis of the older cells. It will be seen from Tables IV and V that the organism during autolysis has restored much of its nitrogen to the medium in the form of ammonia.

The second experiment (Table V) was carried out using the same medium but without sugar, for the purpose of studying the influence of absence of carbohydrates on ammonia production, and it will be seen by comparing the results in Tables IV and V that *Aspergillus flavus* produced more ammonia in the medium wherein sugar was absent. This may be explained by the fact that where sugar or other available carbohydrates are present, the organism will utilise these as a source of energy, and split off from the protein only as much nitrogen as is required for its metabolism and consequently the quantity of ammonia accumulating in the medium will be small. But when available carbohydrates are absent the organisms will attack the protein molecule not only for nitrogen but also for carbon, and as the carbon requirement of the organism is high more protein will be decomposed in this case. Only a portion of the nitrogen will be used up by the organism and the balance will accumulate in the medium as a waste product in the form of ammonia.

The third experiment using *Humicola* (Traaen) was for the purpose of noting the change in amino-nitrogen during the process of ammonia-production. The data from this experiment and shewn in Table VI point to a distinct difference in the accumulation of amino and ammonia nitrogen from gelatin. The production of amino-nitrogen is comparatively slow and small in quantity. The fluctuation in the content of amino-nitrogen shows that the splitting of gelatin may proceed concurrently with utilisation of the amino-nitrogen formed and its further conversion into ammonia and other products. Other

species of fungi have been studied and they have shown the same general characteristics although they differed in their power of ammonia-production.

Production of Ammonia from Amino-acids and Amides.

In the course of their investigations on the proteolytic enzymes of fungi, Shibata (*Beitr. Chem. Physiol.*, 1904, **5**, 384), Pringsheim (*Biochem. Z.*, 1908, **12**, 15) and Waksman (*J. Bact.*, 1918, **3**, 529) observed very often that small quantities of ammonia, amounting to a few milligrams of nitrogen per 50 c.c. solution were produced in the protein-containing solutions by the action of their enzymes. The last worker also suggested the probable presence of deamidases among the enzymes produced. It has been definitely established by Waksman (*loc. cit.*) that the first stage in the protein decomposition, into amino-acids and amides, is accomplished solely by the activities of the proteolytic enzymes of micro-organisms. Little work has however been done on the further conversion of these substances to ammonia and the experiments now to be described were conducted in the hope of throwing some light on the mode of action of the enzymes responsible. As source of enzyme *Aspergillus flavus*, a very strong ammonifier, has been used.

Method of Extraction.—Among the various methods suggested for the extraction of enzymes from moulds, we have followed one devised by Duclaux which consists in growing the organism on one per cent. gelatin medium for about twelve days, i.e., up to the time when spore-formation is complete. Special care was taken to stop the growth of the organism before autolysis and disintegration of the cells began and so to prevent liberation of intracellular enzymes into the medium, which was composed of gelatin, 10 gms., potassium acid phosphate, 0·5 gm., magnesium sulphate, 0·5 gm., zinc sulphate, 0·1 gm., sugar, 5·0 gms., ferric chloride, a trace and distilled water, 1000 c.c.

The mycelial growth was washed on a Buchner funnel with repeated changes of distilled water and then pounded in sand to a pulpy mass in presence of toluene. It was afterwards allowed to remain in contact with distilled water for 24 hours. This procedure assists in the further disintegration of the ruptured cells and is therefore accompanied by liberation of the enzyme. The liquid containing the pulped mycelium was filtered and used directly as the source of enzyme in all the following experiments; it is faintly alkaline owing to small quantities of ammonia. When kept standing for a week the alkalinity increases and the activity is finally destroyed. The solution contains a large amount of protein matter. Purification was attempted by precipitation with alcohol and acetone, but this procedure leads to a

loss of activity. The solution has the power of liberating ammonia from asparagine, alanine, propionamide, acetamide and glycine; it does not act on urea, biuret, tyrosine, hippuric acid and allantoin. In order to eliminate ammonia in the enzyme extract the latter was dialysed in parchment thimbles for 24 hours in distilled water. It will be seen from experiment I that it becomes more active after dialysis. In all the experiments about to be described, unless otherwise indicated, this fresh solution was used as the source of enzyme. The only disadvantage of this procedure is that preparations of uniform activity could not be used in all the experiments. The dialysed solution when kept in the dark and in well stoppered bottles with toluene retains its activity during two weeks.

Experiment I. (Influence of dialysis on enzyme activity).—The following solutions were prepared, (*a*) 20 c.c. of 0·5 per cent. asparagine with 5 c.c. of boiled enzyme solution, (*b*) 20 c.c. of water with 5 c.c. of enzyme solution, (*c*) 20 c.c. of 0·5 per cent. asparagine with 5 c.c. of enzyme solution. Hydrolysis was allowed to continue at room temperature (25–28°) in presence of toluene for 24 hours after which it was checked by the addition of 10 c.c. of 0·125*N* sulphuric acid. The ammonia produced was determined by Nesslerisation, the results being shewn in Table VII.

TABLE VII.

Influence of Dialysis.

	Ammoniacal nitrogen, mgms.		
	A	B	C
Undialysed	0·04	0·06	0·32
Dialysed	nil	nil	3·25

These results indicate that the dialysed enzyme was much more active. It will be seen further that in the case of the undialysed enzyme on standing there was a slight accumulation of ammonia doubtless due to the action of the enzyme on some of the impurities. The enzyme solution after dialysis is faintly yellow, slightly turbid and opalescent. The action of the enzyme on asparagine is very easily detected by the naked eye by the gradual appearance of a fine white precipitate suspended in the solution. Further data in regard to the behaviour of this enzyme under various conditions are given in the following experiments.

Experiment II. (Relation between enzyme-concentration and ammonia-production).—Solutions were prepared consisting of (*a*) 15 c.c. of 0·5 per cent. asparagine and (*b*) the enzyme in varying proportions. The total volume of the reacting mixture in each case was 30 c.c. and enzyme-action was checked after 48 hours.

Other details were the same as in Experiment I. The ammonia formed is shewn in Table VIII.

TABLE VIII.

Enzyme, c.c.	0·1	0·2	0·5	1·0	2·0	5·0	8·0	10·0
Ammoniacal *N*, mgms. ...	1·2	1·6	1·8	2·0	5·0	9·6	9·6	9·6

The figures indicate as one would expect that an increase in enzyme concentration results in increase of ammonia production though this is not proportional to the enzyme concentration. The quantity of ammonia formed is constant in the case of the three highest concentrations, the hydrolysis being complete. Theoretically 15 c.c. of 0·5 per cent. asparagine on complete hydrolysis (if the amido and amino-groups were both attacked) would give 19·5 mgms. of ammoniacal nitrogen, but as it actually happens that half this quantity of ammonia, namely, 9·6 mgms. is produced, it is clear that only one group is hydrolysed by the enzymes.

Experiment III. (General course of the hydrolysis).—A solution containing 0·5 gm. of asparagine dissolved in 50 c.c. of freshly prepared enzyme solution was made up to 100 c.c. and the hydrolysis conducted at room temperature in presence of toluene. At regular intervals 5 c.c. samples were withdrawn and immediately analysed for ammonia by Nesslerisation, the action being stopped by standard sulphuric acid. The values thus found were calculated back to the total volume of the mixture and are shewn in Table IX, the velocity curve being plotted in Fig. I.

TABLE IX.

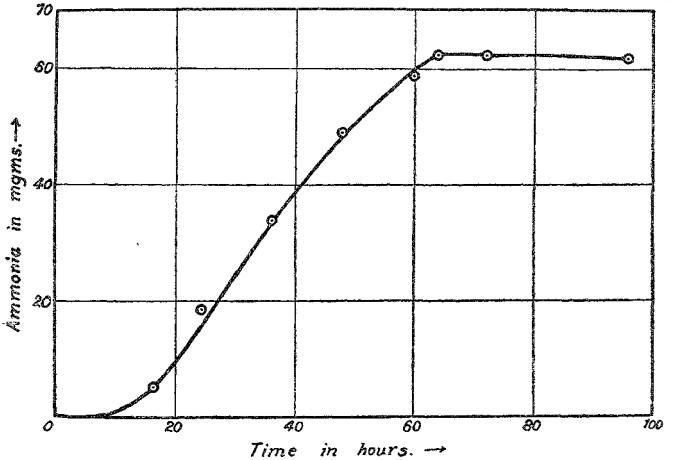
Hydrolysis of Asparagine.

Time in hours	4	8	16	24	36	48	60	72	96
Ammoniacal *N*, mgms. ...	0·1	0·6	5·0	18·2	33·5	48	58	62	62

This experiment was undertaken in order to study the normal course of change that would take place with a high concentration of the enzyme in 0·5 per cent. asparagine solution. It will be noted that the action is very slow and requires from 60–70 hours for completion. It is also clear that although the change taking place in the intermediate stages was approximately proportional to the time, there was a very marked lag at the beginning of the experiment, the amount of hydrolysis in the first twelve hours being very small. The reaction in the earlier stages is acid, the P_H being about 4·6; as hydrolysis proceeds the reaction becomes more alkaline, finally reaching 8·6 owing to

Fig. I.

Hydrolysis of asparagine Velocity Curve



the production of ammonia. This change in reaction is at first accompanied by an increase in the velocity of the hydrolysis when the quantity of ammonia produced after 72 hours becomes constant at 62 mgms., or half the amount producible if both the groups (amino and amido) in 0·5 gm. of asparagine were attacked by the enzyme. This observation justified the conclusion that in this reaction only one group in the asparagine molecule is attacked by the enzyme.

Experiment IV. (Influence of alkali on the enzyme).—The solution contained 10 c.c. of one per cent. asparagine, 5 c.c. of freshly prepared enzyme solution, 0·125*N* sodium hydroxide in varying quantities and was diluted in each case to 20 c.c. Hydrolysis was allowed to proceed at room temperature in presence of toluene for 24 hours when the ammonia was estimated. The results are shewn in Table X.

TABLE X.

Influence of Alkali on the Hydrolysis.

0·125 *N* NaOH, c.c.	Initial P_H	Amm. *N*, mgms.
0	4·6	1
0·1	6·6	1·2
0·5	7·6	1·2
1	8·4	2·4
2	8·8	3·2
3	9·2	2
5	9·8	0·5

The results indicate that hydrolysis is favourably affected by an increase in the hydroxyl-ion concentration within certain limits, namely, up to a P_H of about 8·8, but a more alkaline reaction leads to destruction of the enzyme. This confirms our previous observation that the enzyme works most efficiently when the P_H lies between 6·6 and 8·6. Similar experiments were carried out using very dilute hydrochloric, nitric and sulphuric acids instead of alkali, but it was found that very small quantities of these acids destroy the enzyme. Phosphoric acid however within reasonable limits did not exert any injurious influence on the enzyme, which can actually be extracted from the mycelium with an extremely dilute solution of phosphoric acid without impairing its activity.

Experiment V. (Influence of phosphoric acid on the enzyme).—The solution contained 25 c.c. of 0·5 per cent. asparagine, 15 c.c. fresh enzyme solution, 0·044 *N* phosphoric acid in varying proportions and was diluted in each case to 50 c.c., the action of the enzyme being stopped after 72 hours.

TABLE XI.

Influence of Phosphoric Acid on the Hydrolysis.

No.	0·044 *N*, acid, c c.	Initial P_H	Final P_H	Amm. *N*, mgms.
1	0	5	8·4	3·5
2	0·1	4·8	8·4	10
3	0·2	4·8	8·6	15
4	0·5	4·6	8·6	16
5	1	4·4	8·6	16
6	2	4·2	8·6	16
7	5	3·6	8·6	16
8	10	3	3	0·25

It will be seen from the figures that there is a decided increase in ammonia production as a result of adding phosphoric acid. The latter in small concentrations seems to exert a specific and favourable influence on this reaction. It is surprising to find that hydrolysis has taken place in experiments 6 and 7 wherein the initial P_H values were 4·2 and 3·6 respectively. From our previous experience it was to be anticipated that the enzyme would be inactivated in such a highly acid solution.

Experiment VI. (Influence of hydrogen-ion concentration).—A study of the influence of hydrogen-ion concentration on the rate of hydrolysis is complicated in the present case by the fact that the product is ammonia; there is thus a continual change in the reaction as hydrolysis proceeds and the solution becomes progressively more alkaline. As it was desired to study more closely the influence of reaction and in particular the effect such changes might have on the period of lag already mentioned, a series of solutions were buffered with phosphate mixtures of different reactions. Each solution contained 25 c.c. of 1 per cent. asparagine, 5 c.c. of enzyme solution and 20 c.c. of phosphate mixture the composition of which varied in each case to give a series of initial P_H values ranging from 4·7 to 8·8. The solutions were kept at room-temperature in presence of toluene, and the action was stopped after 48 hours.

TABLE XII.

Influence of Reaction on Rate of Hydrolysis.

No.	M/15 primary phosphate	M/15 secondary phosphate	Initial P_H	Final P_H	Amm. *N*, mgms.
1	...	...	4·6	4·7	1
2	19	1	5·5	5·5	...
3	18	2	6	7·3	12
4	16	4	6·3	7·3	18
5	12	8	6·7	8·3	18
6	8	12	6·9	8·3	18
7	6	14	7·1	8·3	18
8	2	18	7·8	8·5	18
9	1	19	8	8·5	18

The results indicate that the amount of buffer solution was not sufficient to keep the reaction constant in those cases where the hydrolysis was most active. It is clear however that the reaction proceeds most rapidly in neutral or alkaline solution the rate falling off when the acidity exceeds 6·3.

Experiment VII. (Specific influence of phosphate mixture on the velocity curve).—Three solutions were prepared : (i) Control, containing 0·5 gm. of asparagine and 25 c.c. of fresh enzyme solution diluted to 100 c.c. and having P_H 6·4. (ii) The same solution with 20 c.c. of phosphate buffer mixture having P_H 6·4. (iii) The same solution with 40 c.c. of phosphate buffer mixture having P_H 6·4.

The solutions were kept at room-temperature in presence of toluene, and 5 c.c. samples were removed at regular intervals for ammonia determinations.

TABLE XIII.

Influence of Phosphate.

Time in hours	Ammoniacal nitrogen, mgms.		
	I	II	III
4	0	0·6	0·3
8	0·12	1·2	0·72
12	0·3	2	4·8
24	3	7·2	36
36	5·9	25·9	60
48	10·5	60·1	62
60	20·3	62·07	62
72	33·2	62	62
96	36·5	62	62

The figures in the above are plotted in the form of velocity curves in Fig. II, and indicate very clearly the great increase in the rate of hydrolysis brought about by the addition of phosphates. The difference cannot be ascribed to any difference in the reaction of the solutions. In the first place this was initially the same in all three solutions and secondly the increase in alkalinity of the control solution without phosphate is quite insufficient to check the rate. The influence of the phosphate would therefore appear to be specific. It will be noted, however, that the phosphate-containing solutions still exhibit the initial period of lag though this is somewhat reduced in time.

Experiment VIII. (Influence of ammonia on the velocity of reaction).—Two solutions were prepared: (i) Control, containing 0·5 gm. of asparagine, 25 c.c. of fresh enzyme solution and 20 c.c. of phosphate mixture. (ii) The same solution with phosphate replaced by 10 c.c. of 0·095 *N* ammonia.

The total volume in each case was made up to 100 c.c., and the P_H was 8. The solutions were kept at room-temperature in presence of toluene and 5 c.c. samples were taken out at regular intervals for ammonia determination. Ammonia equivalent to that produced by a 20 per cent. hydrolysis of 0·5 gm. of asparagine, namely, 10 c.c. of 0·095 *N*, was added at the outset to II for the purpose of studying its effect on the velocity of hydrolysis.

TABLE XIV.

Influence of Ammonia on the Rate of Hydrolysis.

Time in hours	Ammonia, mgms.	
	I	II
6	2·9	0·2
12	6	1·2
24	12	9
36	20·1	24
48	36	62
60	50	62·3
72	62	62·5
96	62·5	62·5
	Final P_H was 8·6	Final P_H was 8·6

Fig. II.

Influence of phosphate mixture on the reaction

A = Blank

B = 20 c.c. Buffer mixture

C = 40 c.c. " "

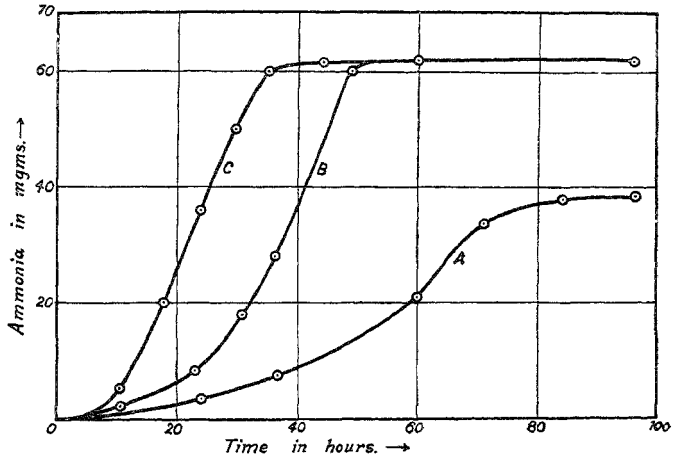
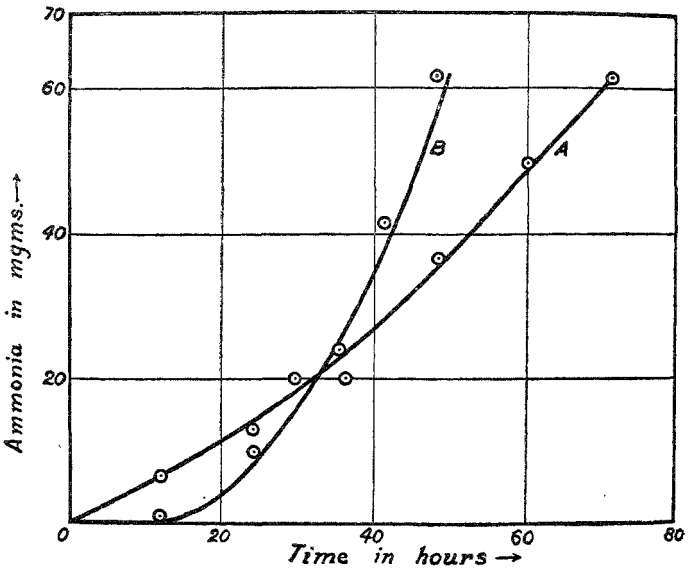


Fig. III.

Influence of ammonia on the rate of reaction

A : Ammonia
B : Phosphates } $P_H = 8$



The above results are plotted in Fig. III, and it is quite evident from the difference in the curves that the rate of hydrolysis is initially faster in the solution containing ammonia. In other words the addition of ammonia has resulted in the disappearance of the period of lag. The phosphate-containing solution, on the other hand, still exhibits this lag; but when once this is over, presumably owing to sufficient ammonia being formed, it overtakes the first solution on account of acceleration by the phosphate.

Experiment IX. (Effect of phosphates and ammonia together on the rate of reaction).—Four solutions were prepared: (i) Control, containing 0·5 gm. of asparagine and 25 c.c. of fresh nzyme solution made up to 100 c.c. (ii) The same solution with 10 c.c. of 0·095 *N* ammonia solution. (iii) The same as the control with 20 c.c. of phosphate mixture. (iv) The same as ii with 20 c.c. of phosphate mixture. Other conditions were the same as in the previous experiment.

TABLE XV.

Influence of Phosphate and Ammonia.

Time in hours	Ammonia, mgms.			
	I Initial P_H 4·6	II Initial P_H8	III Initial P_H8	IV Initial P_H8
12	0·36	3	1·2	6
24	2	9·5	20·9	18
36	3	20·6	38	44·5
48	5·2	35·2	64·2	64·5
60	13·1	54	64·2	64·3
72	20·5	64·5	64·2	64·3
84	36·4	64·5	64·2	64·3
96	64·5	64·5	64·2	64·3

Final P_H was 8·6.

The results are also plotted in Fig. IV and confirm the previous observations that ammonia and phosphates exert a favourable influence on the rate of hydrolysis. The initial presence of ammonia has again abolished the period of lag. The phosphate cannot do this, but when once the reaction has begun it accelerates the rate of hydrolysis. The reaction would appear to be a typical example of autocatalysis, the rate of hydrolysis being increased, in the initial stages at any rate, by one of the products.

Experiment X. (Purification of the enzyme).—It has already been mentioned that the enzyme solution, even after dialysis, loses its activity when kept for more then two weeks. In order to avoid this inconvenience the following adsorption method, based on Willstätter's process, has been employed for the separation of the enzyme. The method of extraction of the enzyme from the fungus mycelium was the same as previously described, but was conducted on a large scale. Purified kaolin (100 gms.) was added to the enzyme solution (one litre, undialysed), the mixture vigorously shaken for 30 minutes and the kaolin then allowed to settle. The supernatant liquid was drawn off by means of a siphon and the kaolin transferred to a Buchner's filter and washed five times with distilled water. By this procedure the enzyme remains absorbed on the kaolin, while the impurities are removed in the filtrate and washings. The kaolin on the filter was then transferred to a flask containing 200 c.c. of M/15 sodium hydrogen phosphate and vigorously shaken for 2 hours, the enzyme being thus again brought into solution. From the clear solution obtained after removing the kaolin, the phosphate can be removed by dialysis.

Fig. V shows a typical velocity curve obtained with enzyme thus purified. It exhibits the same characteristics, including the period of lag, as the previous examples.

SUMMARY.

1. A typical local soil has been examined for its fungus content and some fifty species isolated.

2. Three of these species were examined for their power to fix atmospheric nitrogen with negative results.

3. The ability of various species to break down cellulose and to liberate ammonia from protein material has been investigated.

4. The mechanism of ammonia-production by fungi has been examined and in particular the properties of an amidase isolated from *Aspergillus flavus*. The reaction is of an autocatalytic nature, the velocity curve first exhibiting a period of lag and then a period of acceleration as ammonia is formed.

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Fig. IV.

Influence of phosphate and ammonia together on the rate of reaction

A = Blank

B = Ammonia

C = Phosphate mixture

D = " & ammonia

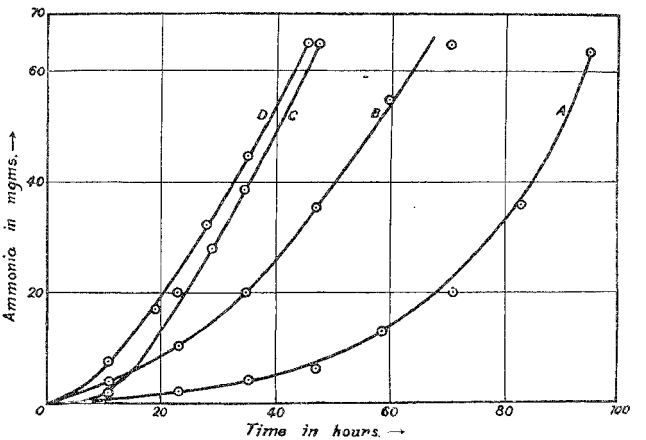

Fig. V.

Hydrolysis Curve with purified enzyme