NOTE TO USERS

THE DISSIMILATION OF GLUCOSE BY *CHAETOMIUM FUNICOLA* CKE.

by

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INTRODUCTION

Chaetomium funicola Cke, because of its marked ability to decompose cellulose and because it is commonly associated in nature with various cellulosic materials, seemed a promising fungous subject for an investigation of the manner of decomposition of plant materials. However, before such an investigation of a complex substratum as cellulose could be profitably undertaken, an investigation into its behavior in relation to a simpler substrate such as glucose alone was deemed adviseable. Hence in the present study the following lines were investigated: (1) to determine the manner of glucose dissimilation by Chaetomium funicola Cke., and (2) to throw some light on the inner mechanism of this process, especially the role of phosphorus.

PREVIOUS WORK

Cultural and biochemical characteristics of the genus Chaetomium are not known. Seven species have been found by Dickson (22) to exhibit considerable variation in the frequency of saltant formation induced by X-rays, but in all cases the saltants resembled the parents more than the other species. On different concentrations of glucose, starch and potassium phosphate, development was observed to be linear with the production of no staling products. Color changes in the media were small but could be markedly influenced by altering the glucose:asparagin ratio. Nine species of Chaetomium (C. funicola included) were found (114) to develop poorly in peptone or two percent alcohol. On glucose growth was found to occur between pH 4.2 to 11.0 with the best growth occurring on the alkaline side and no growth at pH 3.0.

On Czapek-Dox medium with glucose as the sole source of carbon 48 percent of the glucose was found (94) to be decomposed by a species of Chaetomium after an incubation period of 70 days under conditions of partial anaerobiosis. Of this carbon used as glucose 81.2 percent was converted into CO_2 and mycelium (53.5 percent as CO_2 and 27.7 percent as mycelium) 5.2 percent as non-volatile acids and 3.2 percent as synthetic compounds. Volatile neutral compounds were found

to be negligible in amount while volatile acids were absent. The medium was alkaline in reaction.

Cellulose has been found by Lovell (61) to be acted on by C. funicola in a very similar manner to glucose. He maintained cultures of this organism for periods up to 60 days in one-litre flasks containing 400 cc. of nutrient salts and 10 gms. of filter paper as the carbon source. In 20 days, the filter paper decreased in amount by 3 percent and in 60 days by 40 percent. Acetic acid was the acid produced. Its formation and accumulation occurred only after 20 days of incubation. However, at no time was the production large. Attempts to increase the production by aeration or altering the inorganic salt concentrations were unsuccessful. Acetic acid production was likewise obtained from glucose and sucrose.

MATERIALS AND METHODS

Methods in Part I.

Fungi

Chaetomium funicola Cke., culture No. 10, isolated from cornstalks (17) was used throughout in the present study. Other fungi used for comparative purposes were:

Fusarium bulbigenum (Cke.) Mass. v. *niveum* (E.F. Sm.) Wr. obtained as an isolate from wilt damaged watermelons at Muscatine, Iowa;

F. oxysporum Schl. v. *cubense* (E.F. Sm.) Wr. et Rg. obtained as an isolate by Mr. Clifford Meredith from infected banana trees in the West Indies;

F. Lini Bolley obtained as culture 1 W, from Dr. J.J. Christensen, University of Minnesota;

Aspergillus niger v. Tieghm. isolated at Ames, Iowa, from apples stored at high temperatures.

Spore suspension

For purposes of seeding the media, spore suspensions were used throughout in these studies. Such suspensions were usually obtained from two-week old cultures growing in 125 cc.

erlenmeyer flasks containing a layer of potato-dextrose agar approximately one-half centimeter in depth. For any particular experiment, the spores from several such cultures were combined by aseptically pipetting definite volumes into individual flasks.

Czapek-Dox liquid medium

Unless otherwise stated, Czapek-Dox liquid medium of the following composition was used: 1 litre of distilled water; 5.0 grams Pfanstiehl's technical dextrose; 2.0 grams $NaNO_3$; 1 gram KH_2PO_4; 0.5 grams KCl; 0.5 grams $MgSO_4 \cdot 7H_2O$ and 0.01 grams $FeSO_4 \cdot 7H_2O$. The salts were all of chemically pure grade.

Preparation of experimental cultures

Variations in the method used for each experiment in the preparation of media prior to seeding with spores will be indicated as the individual experiments are considered. For any particular experiment, however, the different media were treated in a uniform manner. Sterilization was effected in an autoclave at 15 pounds pressure for 15 minutes. To avoid any expected chemical change in the media, certain constituents were sterilized separately. In such cases the different portions of the media were prepared of such concentrations that

when combined, and after the addition of a definite volume of spore suspension, the desired concentrations in the final culture were obtained.

Pyrex erlenmeyer flasks cleaned with cleaning solution (potassium dichromate-sulphuric acid mixture) were used throughout as culture flasks. With the different experiments the quantities of media introduced into the flasks were not the same. In all cases, with the exception of one, the ratio of the depth of the liquid medium in the flasks to the surface area was approximately the same, the area being near its maximum. With the exception of two experiments, non-absorbent cotton plugs were used as stoppers for the culture flasks during the course of the experiments. Pipettes were wrapped either in paper or non-absorbent cotton and sterilized in an autoclave before use. Culture flasks were maintained upright on flat horizontal surfaces. The temperatures maintained were between 25° to 30°C.

Analysis of cultures

Cultures were decanted and filtered by suction through previously washed, dried, and weighed pieces of broad-cloth. Use of cloth as a filter proved advantageous over filter-paper in the matter of greater ease of filtration, easy removal of the mycelium from the filter and greater reliability because weighings could be made in weighing bottles. The my-

celium was usually washed several times with hot distilled water in the culture flask and on the filter. The drying of mycelium to constant weight was effected at approximately 70°C. followed by maintenance for several days in vacuo over fresh concentrated sulfuric acid. When absolute dry weights were necessary, additional drying was done at 100°C. for one day. The filtrates from cultures containing approximately 50 cc. of medium were made to 200 cc., while cultures containing 300 cc. of medium were made to 500 cc. Analyses of the filtrates were made immediately.

Residual glucose. Residual glucose in the filtrates was determined in most instances by the Bertrand modification of the Munson - Walker method (60). In certain experiments, glucose was determined by the Shaffer-Hartmann copper-iodate method as first modified and adapted to a semi-micro scale by Somogyi (103) and subsequently further modified by Shaffer and Somogyi (102), Harding and Downs (40) and Van der Plank (116). This method had the advantage over the former in being more rapid and less costly. The solutions were prepared as directed with the exception of the standard sodium thiosulphate solution which was used in the strength of 0.005 N instead of 0.01 - 0.02 N. Starch solution was prepared according to the method of Willard and Furman (124) and the suggested precautions observed. Sodium thiosulphate solution

was prepared according to the method of Stiles, Peterson and Fred (104) and their tables were used for direct readings of glucose equivalents.

Glucose determinations in all cases were made on the filtrate directly, without any previous clarification with lead or mercury salts. No significant differences were observed when clarification was omitted.

Hydrogen-ion concentration. The Coleman glass-electrode apparatus was used for determinations of the hydrogen-ion concentrations of the media as well as in the various hydrogen-ion adjustments made of the solutions used. In a few instances La Motte indicators were used. In all cases, hydrogen-ion concentration readings of the culture substrata were made on the culture filtrates after they were made to volume. Such dilutions introduced no appreciable errors in the values obtained.

Carbon distribution. To determine the distribution of carbon resulting from the development of a culture on glucose the following method was used. (Fig. 1) An erlenmeyer flask of two-litre capacity containing the culture was provided with an air-inlet and an air-outlet of glass tubing fixed in a rubber stopper of a size sufficient to fit tightly into the mouth of the flask. The inlet tube was of such

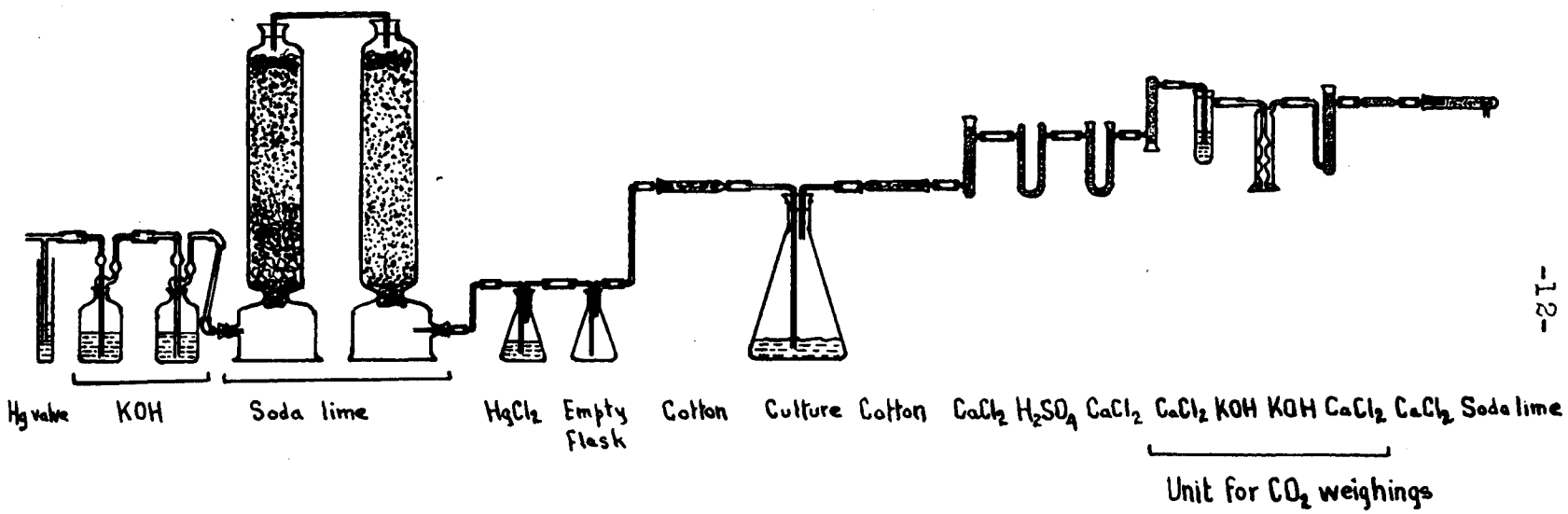


Fig. 1. Arrangement of apparatus for studies in glucose dissimilation

length that it projected below the surface of the medium while the outlet tube led off from just below the lower surface of the rubber stopper. Carbon-dioxide-free air was passed continuously through the culture at a rate of approximately one bubble of air per second. The source of air was that compressed and stored in gas mains from a central plant. Excessive pressure from the mains was counteracted by the insertion of a mercury safety-valve between the culture and the air tap. Removal of carbon dioxide from the air was effected by passage through several solutions of 40 percent potassium hydroxide and several towers containing soda-lime. The carbon dioxide evolved by the culture was determined gravimetrically and absorbed in 40 percent potassium hydroxide solution containing a few drops of phenolphthalein indicator. This solution was contained in a side-arm test tube and a Bowen potash bulb fastened together. It was protected on both sides by two calcium chloride tubes, the whole constituting a unit which was weighed to determine the carbon dioxide evolved. Between this unit and the culture were placed in succession a calcium chloride tube, a U-tube containing concentrated chemically pure sulphuric acid and glass beads, and another calcium chloride tube. Sulphuric acid was included to absorb any volatile carbon compounds that might be removed with the slow air stream. Adequate protection was given to maintain pure cultures by insertion of cotton part way into the inlet and

outlet glass tubing to which also were connected other tubes of cotton as indicated. In addition, a 1-1000 mercuric chloride solution was inserted in the portion indicated. All rubber connections were made of fresh black rubber tubing of three-sixteenths inches bore and one-sixteenth inches wall thickness.

To make serial analyses for carbon distribution, a number of cultures were necessary that were prepared simultaneously and given identical treatment but which were allowed to develop for different periods of time. A system was arranged for this purpose about an incubation chamber (Fig. 2) which could be adjusted to maintain relatively constant desired temperatures of 28°C. In this system, arrangement was made for six cultures which could all be placed in the incubator at the same time. Each culture had its own carbon dioxide-absorbing unit which was held outside the incubator. The single absorption system for insuring carbon dioxide-free air, likewise outside the incubator, served for all cultures. This was made possible by the insertion of a series of six connected T-tubes in the system (Fig. 1) between the empty flask and the sterilized cotton. Adjustments for equal air flow through each culture were made with screw clamps and water-air bubble counters placed between the T-tubes and the sterilized cotton.

To determine the distribution of carbon in the medium,

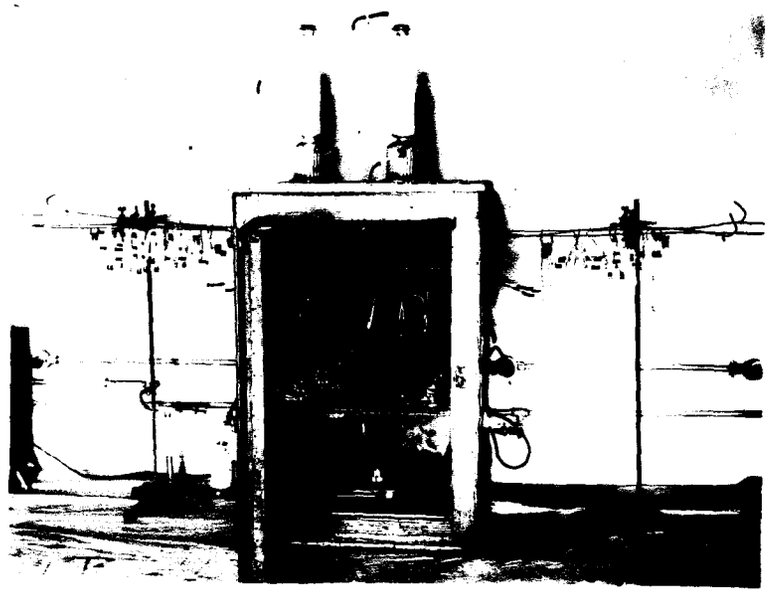


Fig. 2. Arrangement of apparatus about an incubation chamber for studies in glucose dissimilation.

the following fractionation method was used: a 300 cc. aliquot part of the filtrate was made acid to congo red with sulphuric acid and distilled through a long condenser into an ice-cooled container to obtain 250 cc. of distillate. The residual liquid was maintained above a volume of approximately 150 cc. during distillation by addition of water. The distillate was made alkaline to phenolphthalein with sodium hydroxide and further distilled to produce 100 cc. of distillate containing neutral volatile materials (fraction A). The residue of this distillation was combined with the residue of the first distillation, made acid to congo red and subjected to steam distillation to drive off the volatile acids (fraction B). Two litres was the amount distilled over. The residue of this distillation (fraction C) contained the non-volatile materials. Further subdivisions of fraction C were made by ether extraction in a continuous extracter for 48 hours. The fraction (approximately 300 to 400 cc. in volume), however, was first reduced in volume to approximately 100 cc. in a vacuum distillation apparatus. The procedure found most convenient to control the excessive frothing that occurred was to allow the fraction to enter drop-wise through a long capillary tube into a one-litre or two-litre distillation flask. Further provision for this difficulty was made by inserting a stop-cock between the flask and the water pump. By opening the stopcock momentarily the pressure within the flask could

be reduced and the frothing subdued. To facilitate distillation, bubbles of air were allowed to enter the flask through a capillary tube that projected into the liquid. The temperature for distillation was maintained by a water bath at approximately 30° to 35°C.

Subdivision of the concentrated fraction C was made by making the fraction alkaline to phenolphthalein with sodium hydroxide and then acid to congo red with sulphuric acid, and extracting the fraction both times. Such a procedure should result in an ether-soluble, non-volatile, neutral compound fraction, (D), ether-soluble, non-volatile acid compound fraction, (E), and an ether-insoluble, non-volatile fraction,(F).

Carbon analysis on aliquot parts of some these fractions were made by the method of Osburn and Werkman (90). The carbon dioxide formed was caught in ascarite and weighed. Precaution was exercised to use a good fresh supply of potassium persulphate as well as to maintain the rubber connections in good condition.

Methods in Part II.

Source of large amounts of mycelium

To obtain mycelia in amounts sufficient for the studies presented in Part II, cultures were grown in petri dishes and in two-litre erlenmeyer flasks containing 250 cc. of medium.

The former method was used in most of the present studies. For this purpose usually 180 cc. of Czapek-Dox medium were prepared in each of a number of 250 cc. erlenmeyer flasks, sterilized in the autoclave and sown with 20 cc. of a heavy suspension of spores. The preparations were then aseptically portioned out by direct pouring into petri dishes in approximately 20 cc. amounts (10 petri dishes for each flask), as determined by practice. These dishes, nine centimeters in diameter and one and one-half centimeters in depth, were then stacked, usually five deep, on a flat horizontal surface. Six days at 25° to 30°C. was the time usually allowed for the fungus to develop. In this time a thin surface mat usually had developed and sporulation had just begun.

Treatment of mycelium prior to use

Immediately before use in an experiment, the fungal mycelia were removed from the individual petri dishes and bulked on several layers of cheesecloth. After allowing approximately five to 10 minutes for drainage, the liquid substratum was squeezed out by hand. Subsequent treatment of the mycelium varied somewhat with the experiments. In general, however, the procedure was to wash the mycelium by repeated immersion in cold (10°C.) distilled water for several minutes with intervening drainage and squeezing by hand. From three to five times were the usual number of washings carried out. After

this treatment only a slight Fehling's test for glucose was obtained. Further treatment of the mycelium involved grinding it by hand in a mortar using quartz sand and pestle with additions of water to form a thick soup. This soup shall be referred to as "mycelial preparation." The time involved in the entire operation from washing the mycelium to the preparation of the soup was usually about one hour. Unless otherwise stated, the entire operation was carried out at room temperature of approximately 25°C. Standing of the mycelial preparations for longer than 15 minutes (time taken to grind all the mycelium) was avoided by immediately introducing calculated portions of the mycelium into flasks previously prepared to receive them. Precautions were taken against excessive contamination of the mycelial preparation with other organisms by using previously steam-sterilized utensils, vessels, distilled water and quartz sand.

Use of trichloracetic acid

An aqueous solution of trichloracetic acid in five percent final concentration when added to experimental samples of material was used in the present work. Two purposes were served by this acid: (1) to arrest further enzymatic action, (2) to separate the non-colloidal constituents of the mycelium from the colloidal. This acid was used in some of the trials for methylglyoxal, pyruvic acid and acetaldehyde isolation and

throughout in the analyses for the acid-soluble phosphorus constituents.

With analysis for the acid-soluble phosphorus in experimental mixtures of mycelial preparations, the usual practice was to remove 15 cc. of the mixture (or some small amount) and to add 5 cc. of 20 percent trichloracetic acid. In the experiments on autolysis, each sample was removed at different times during the experiment and placed at 0.5°C. after treatment with trichloracetic acid. The contact of the acid with the mycelial material was allowed to remain till the experiment was completed (usually 24 to 48 hours). From one to three days were usually allowed for the final sample to become extracted. These samples were then centrifuged and the centrifugate passed through filter paper. Analysis of this acid extract was made immediately or kept in the cold room till used. Any deviation from this method shall be indicated with the individual experiments.

Analysis for phosphorus

Inorganic orthophosphorus. Inorganic orthophosphorus was determined by three colorimetric methods, namely; Kuttner and Lichtenstein (46), Martland and Robison (72) and King (44). The latter method was used for the most part in the present

work. A Dubosq comparator was used to make phosphorus readings. The source of light was that reflected by white paper from an electric light bulb. The same source of light was used throughout.

Total phosphorus. Total phosphorus was determined by ashing the sample in pyrex test tubes 1.5 cm. in length and 2 cm. in diameter. Total phosphorus in the mycelium was determined on a small portion of the weighed mycelium. To this was added 1 cc. of concentrated sulphuric acid. After standing for several days at room temperature to allow for slow digestion, the ashing was carried out over a low flame and completed by small additions of 30 percent hydrogen peroxide. Care was exercised against over-heating the sample as judged by sputtering and fuming of the sulphuric acid. The final clear liquid was made to volume and the phosphorus determined by one of the above methods.

With total phosphorus determination in solution, a sample (usually 2.5 cc.) was used which could subsequently be diluted, if required, to a determined volume. Such dilutions were necessary in certain experiments of large amounts of phosphorus in order to obtain the necessary amount of phosphorus for colorimetric determinations. In other experimental samples, however, as in the studies for the nature of the acid-soluble phosphorus fractions present in the mycelium and in the autolysis studies to which no inorganic phosphorus was added, a

sample of from 1.0 to 2.5 cc. of the trichloracetic acid solution was usually sufficient to obtain an amount of phosphorus equal to 0.05 to 0.1 milligrams. In such cases ashing was carried out by the addition of 0.6 cc. of 50 percent sulphuric acid (this amount when diluted to 15 cc. approximates the concentration of sulphuric acid required for colorimetric determinations of phosphorus by the above methods). Considerable variations in concentration of sulphuric acid can be had without affecting the values for phosphorus.

To restore to the ortho form any pyrophosphate phosphorus formed in the process of ashing, water was added to make the volume 4.0 cc. and 1.0 cc. of 4 N HCl was added. This solution was then heated in a boiling-water bath for seven minutes, cooled and 1.0 cc. of 4 N NaOH was added. At the completion of this hydrolysis the sample was either diluted to a determined volume to obtain a required approximate amount of phosphorus for the determination or the phosphorus was determined directly in the pyrex test tube. In the latter case, additions of water and other ingredients were made (sulphuric acid being omitted) as called for by the above methods.

Other phosphorus fractions. In experiments on the nature of the acid-soluble phosphorus present in the mycelium and its behavior on autolysis, the analysis of the acid-soluble phosphorus fractions present in the trichloracetic acid extract

were determined by the method of Lohmann (55). For such determinations aliquots of the extract were pipetted out in amounts (usually between 1.0 and 2.5 cc.) depending on the concentration of phosphorus present, into soft glass test tubes of 15 cm. length and 1.6 cm. inside diameter. An equal amount of 2 N HCl was then pipetted into each and the test tubes were immersed immediately into boiling water. After definite intervals of time from seven to 180 minutes, tubes were removed from the bath and cooled immediately in cold running water. A number of drops of distilled water were usually added to each test tube to bring the contents to the original volume (as indicated by a mark on the test tube). In no case were more than five drops of water ever needed to restore the original volume.

With zero-minute hydrolysis samples, an equivalent amount of 2 N HCl - 2 N NaOH mixture was added to obtain comparable conditions (this addition was later found to have no effect). The inorganic orthophosphorus present and liberated by hydrolysis in the various samples was determined in the same test tubes. Test tube samples of the various hydrolysis treatments were held till all samples could be determined simultaneously for direct comparisons.

Phosphoglyceric acid isolation

Trials were made to isolate phosphoglyceric acid as its

barium salt. The method used was that of Neuberg and Kobel (79, 80) with some slight modifications introduced by Stone (105). The latter worker had observed that holding the flasks containing the experimental mixture for several days at 5°C. increased the yield of phosphoglyceric acid. Accordingly, the mycelium was removed by centrifugation and the centrifugate analyzed. The centrifugate was made ammoniacal and the inorganic phosphorus precipitated by additions of magnesium acetate. The precipitate was centrifuged off and the centrifugate made neutral with glacial acetic acid. An amount of glacial acetic acid was then added to equal 10 percent of the volume and followed by one-half of this quantity of 50 percent barium acetate to correspond to four percent of the final volume. The solution was then placed at 0.5°C. for several days and observed from time to time for the formation of phosphoglyceric acid crystals. Two volumes of 95 percent ethyl alcohol were added at the end of this time to facilitate crystallization.

Methylglyoxal, pyruvic acid and acetaldehyde

Simon and Neuberg (101) were followed on this phase of the investigation. By their method methylglyoxal, pyruvic acid and acetaldehyde can be simultaneously separated from solution as derivatives of 2:4 dinitrophenylhydrazine. After 48 hours of incubation to allow the formation of these compounds, the

mycelia were removed by centrifugation and trichloracetic acid added to the centrifugate to make a final concentration of five percent of the acid. Isolation of methylglyoxal, pyruvic acid and acetaldehyde from this solution was carried out by the addition of a saturated solution of 2:4 dinitrophenylhydrazine in 2 N HCl solution (0.5 grams in 60 cc. of hot 2 N HCl solution) in proportion of 10 cc. of the compound solution to 50 cc. of the centrifugate. Usually two to three hours were allowed for the formation of a precipitate but longer times were also allowed. These shall be indicated at the time of the consideration of the experiments. The formed precipitate was separated and fractionated according to the method of Simon and Neuberg.

RESULTS

Part I. Glucose Dissimilation by *Chaetomium funicola* Cke.

The partition of glucose carbon by *Chaetomium funicola*

Each of six cultures of *Chaetomium funicola* on 300 cc. Czapek-Dox medium in two-litre erlenmeyer flasks were analyzed for the partition of carbon after different periods of incubation. The results obtained are presented in table I.

The table shows that glucose dissimilation is a slow process which increases in velocity subsequent to the eighteenth day of incubation. However, even at 33 days the amount of glucose decomposed amounts only to approximately 60 percent of that added. This is considerably greater than the decomposition observed by Raistrick et al (94) for their *Chaetomium* sp. Accompanying the more rapid decomposition of sugar subsequent to the eighteenth day, there is also a more rapid evolution of carbon dioxide and mycelium formation. Likewise there occurs in this time a formation and accumulation of substances in the medium which are non-volatile. This production apparently reaches its maximum yield of approximately five percent of the glucose consumed at the twenty-

Table I. The partition of glucose carbon by Chaetomium funicola CKe.

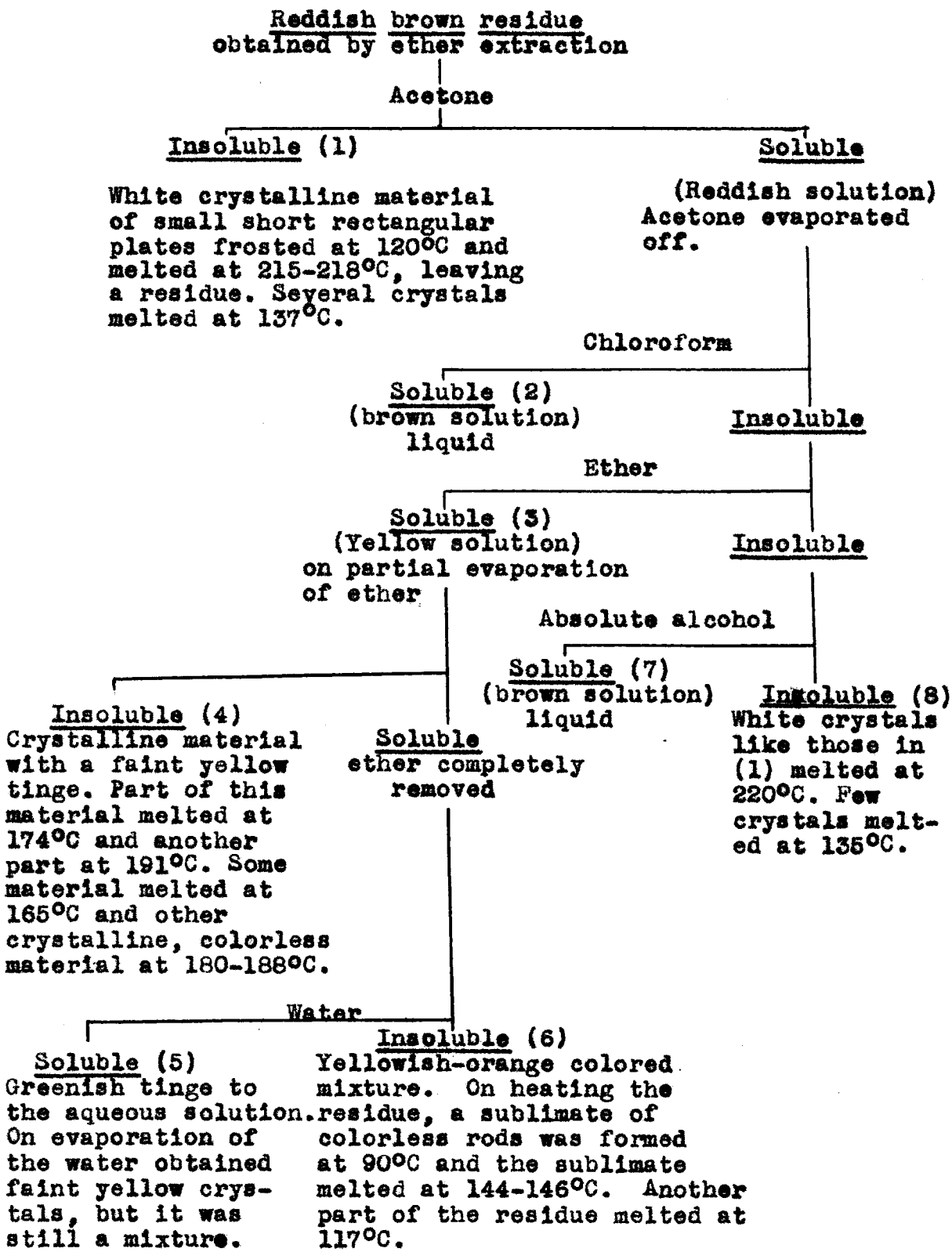
	Time of incubation in days					
	3	7	11	18	26	33
Initial glucose present in the medium as carbon, gms.	6.20	6.38	6.14	5.88	5.97	6.45
Final glucose present in the medium as carbon, gms.	5.33	5.01	4.52	4.16	2.88	2.58
Initial total carbon in the medium, gms.	6.48	6.55	6.30	6.40	6.32	6.47
Final total carbon in the medium, gms.	5.37	4.99	4.62	4.16	3.49	3.04
Carbon in the medium as glucose, gms.	5.33	5.01	4.52	4.16	2.88	2.58
Carbon in medium as products other than glucose, gms.	0.04	-0.02	0.10	0.00	0.61	0.46
Carbon as neutral volatile material, gms.	.0028	.0016	.0027	.0016	.0011	.0045
Carbon as volatile acids, gms.	.0101	.0243	.0194	.0102	.0056	.0027
Carbon as carbon dioxide evolved, gms.	.022	.159	.378	.421	.942	1.360
pH of the medium	6.2	6.4	6.8	7.1	7.7	7.1
Weight of mycelium, gms.	.204	.693	1.045	1.189	1.711	2.102

sixth day and then undergoes further conversion. The production of volatile neutral compounds is at no time very significant. The same can be said for the volatile acid fraction which did not consume more than 0.5 cc. of 0.02 N NaOH per 100 cc. of distillate on titration to phenolphthalein. It appears probable that the higher carbon values found for this fraction can be attributed to the presence of substances in the volatile acid fraction which are readily carried over by steam distillation. Such distillates in fact did exhibit very slight cloudiness as well as the formation of a faint paraffin-like scum on the surface. The progressive change of pH of the medium to the alkaline side speaks against any significant production of non-volatile acids. The production of only small amounts of lead precipitates during the clarification process for sugar analysis support this fact. Likewise the method of Raistrick et al (94) for estimating non-volatile acids by precipitation as calcium salts from 80 per-cent ethyl alcohol solution of the medium yielded only small amounts of amorphous precipitate.

The color of the liquid media of the cultures are a light yellow during the early stages of development of the culture and become brownish-orange at the later stages. Ether extraction of the alkali or acid non-volatile concentrated residue results in a yellowish or a brownish-orange ether solution. The color of the residue does not appear to

be appreciably removed by this means. Evaporation of the ether produces a residue that consists in part of a reddish-brown fatty liquid in which are contained other materials that are solids. Crystals of yellow flakes, and crystals that are short rods (m.p. 113.0° to 113.5°C.) and other solid material (m.p. 118°C.) appear. The presence of fatty substances in the medium is witnessed by the frothing of the medium on distillation. The fatty liquid obtained above was found to have a refractive index of 1.5107 at 24.5°C., and a specific gravity of 0.880 at 21.5°C. Distillation of this liquid at ordinary pressures and in air produced fumes and an odor of burned fat. A yellow liquid distillate was obtained with a refractive index of 1.5017 at 20°C.

The formation of various substances in the medium is indicated by the following: cultures of C. funicola were developed for 18 days on Czapek-Dox medium containing 10 percent sucrose. These consisted of five cultures on 250 cc. of medium in each of two-liter erlenmeyer flasks. After the mycelium was removed the medium was acidified to congo red and concentrated in vacuo. On extraction with ether there was obtained approximately 0.5 gram of a reddish-brown mass which was definitely a mixture. Fractionation of this mixture was effected by solvents as follows:


Reddish brown residue obtained by ether extraction
Acetone
Insoluble (1)
White crystalline material of small short rectangular plates frosted at 120°C and melted at 215-218°C, leaving a residue. Several crystals melted at 137°C.
Soluble
(Reddish solution) Acetone evaporated off.
Chloroform
Soluble (2) (brown solution) liquid
Insoluble
Ether
Soluble (3) (Yellow solution) on partial evaporation of ether
Insoluble
Absolute alcohol
Soluble (7) (brown solution) liquid
Insoluble (8) White crystals like those in (1) melted at 220°C. Few crystals melted at 135°C.
Insoluble (4) Crystalline material with a faint yellow tinge. Part of this material melted at 174°C and another part at 191°C. Some material melted at 165°C and other crystalline, colorless material at 180-188°C.
Soluble ether completely removed
Water
Soluble (5) Greenish tinge to the aqueous solution. On evaporation of the water obtained faint yellow crystals, but it was still a mixture.
Insoluble (6) Yellowish-orange colored mixture. On heating the residue, a sublimate of colorless rods was formed at 90°C and the sublimate melted at 144-146°C. Another part of the residue melted at 117°C.

In 47 days old cultures (Table II) initially containing 2.5 percent glucose, concentration of the medium in vacuo produced a separation from the medium of white, small rectangular plates similar to (1) in the foregoing. Similar aged cultures, but with higher initial glucose content, did not liberate crystals from solution.

Change of cultural conditions

Changing the cultural conditions is known to affect the products formed by fungi, qualitatively and quantitatively. Similar knowledge was sought for C. funicola.

Effect of varying the initial glucose concentration of the medium. Fifty cultures were prepared in 250 cc. erlenmeyer flasks. To each flask were added 35 cc. of Czapek-Dox medium and, after sterilization, 5.0 cc. of spore suspension. Analyses on duplicate cultures were made for glucose, dry weight of mycelium and pH of the medium after different periods of incubation. From the results obtained as shown in table II and in figures 3, 4, and 5, the following points are of interest:

1. Glucose disappearance occurs in an approximately linear manner (Fig. 3).
2. The rate of glucose disappearance is greater at the higher concentrations of glucose than at the

lower.

3. In 26 days of incubation the disappearance of glucose from the medium in a concentration of 2.5 percent glucose is nearly complete.
4. Mycelium formation (Fig. 4) on different concentrations of glucose is approximately the same for the first 17 days after which the increase in mycelial weight depends on the glucose concentration.
5. Autolysis of the mycelium occurs when the glucose content in the medium has been reduced to a low value.
6. The medium becomes progressively alkaline up to 17 days of incubation (Fig. 5) and then decreases. Subsequent to the twenty-sixth day of incubation, the medium again becomes more alkaline with cultures containing 2.5 and 5.0 percent of glucose, while with cultures containing higher concentrations of glucose, the change to the acid side is progressively maintained.

Effect of varying the initial hydrogen-ion concentration of the medium. A number of cultures were prepared in 250 cc. erlenmeyer flasks on Czapek-Dox medium with 10 percent glucose and with the initial hydrogen-ion concentration adjusted to various values. Calculated quantities of hydrochloric

Table II. Effect of glucose concentration in the medium on cultures of *Chaetomium funicola*

Days of incubation	Initial Concentration of Glucose in per cent				
	2.5	5.0	7.5	10.0	12.5
	pH of the medium				
0	4.72	4.95	4.58	4.77	4.61
7	6.29	6.20	6.10	6.22	6.35
17	7.87	7.52	7.63	7.71	7.84
26	7.62	7.15	7.10	7.46	7.35
47	8.70	8.04	6.46	6.25	6.28
	Mycelium formed in milligrams				
7	105.8	130.6	75.1	120.5	141.9
17	339.1	332.6	378.9	326.8	327.5
26	347.5	409.0	428.6	649.2	568.2
47	279.4	620.0	753.7	752.2	945.7
	Glucose not consumed in grams				
0	1.147	2.210	3.025	4.041	5.424
7	0.976	1.895	3.002	3.688	5.096
17	0.206	1.133	2.132	2.885	3.826
26	0.013	0.820	1.698	2.365	3.433
47	0	0	0.542	1.380	1.973

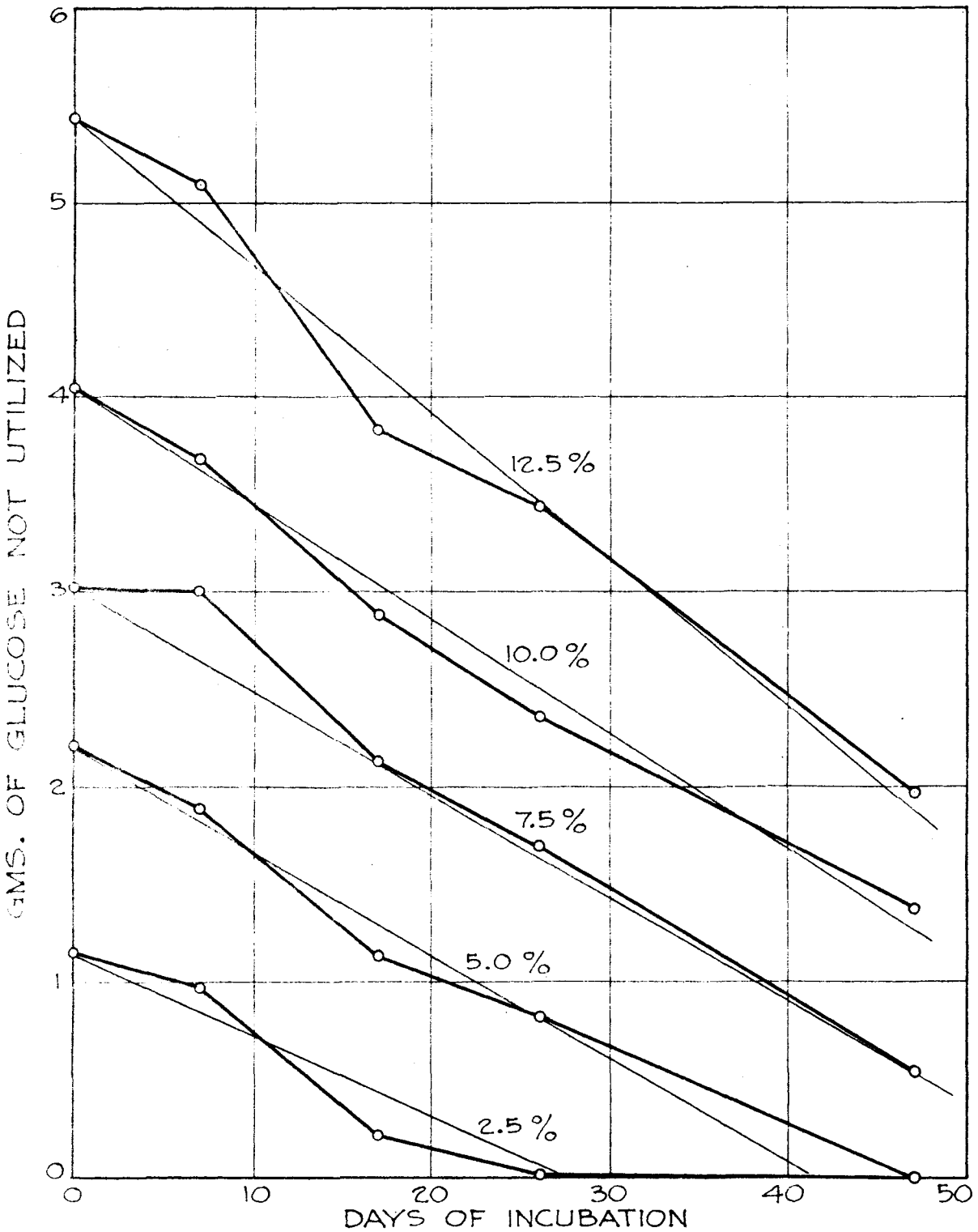


Fig. 3. Showing glucose remaining in the medium with cultures of Chaetomium funicola grown on different concentrations of glucose in the medium.

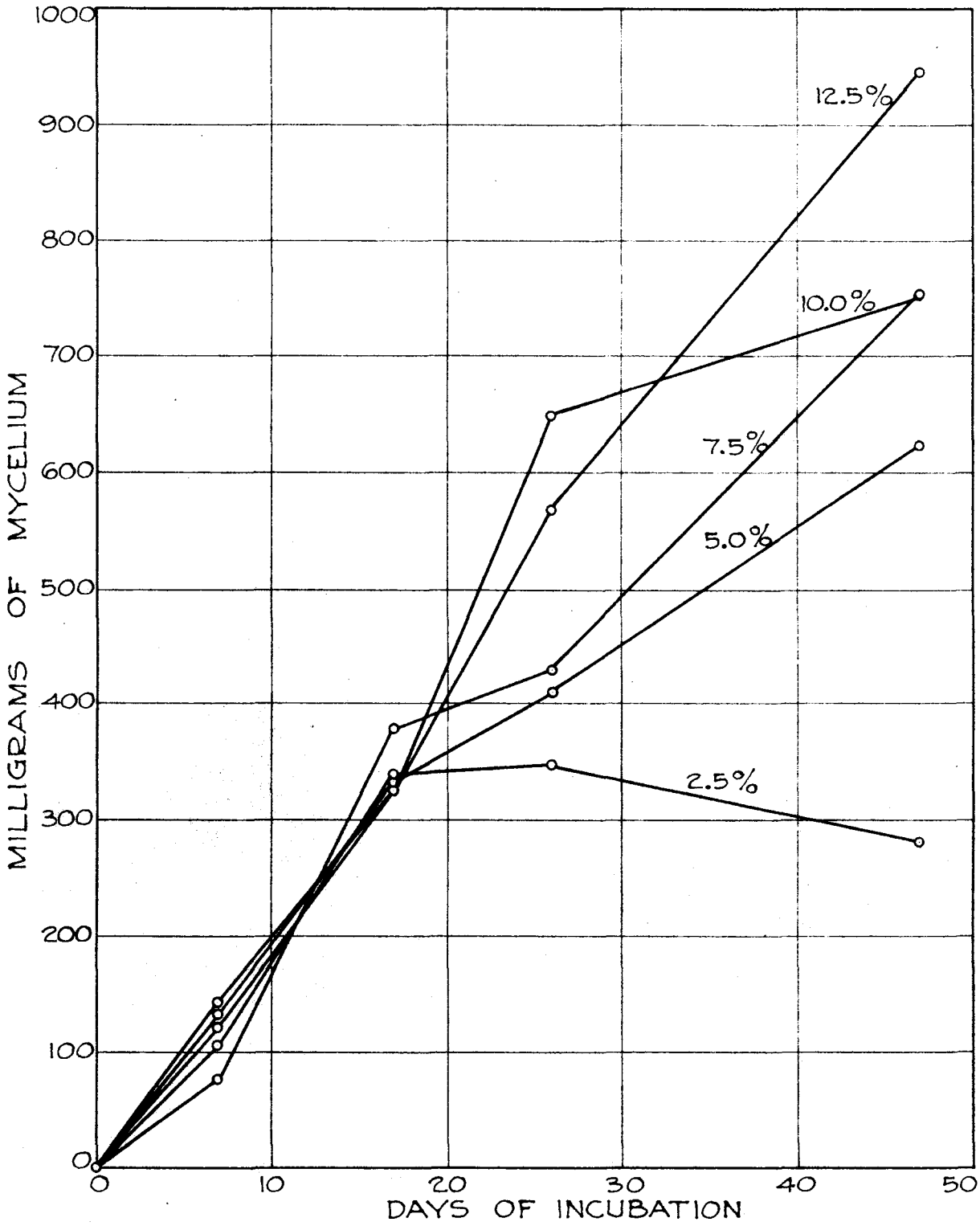


Fig. 4. Showing weight of mycelium produced in cultures of Chaetomium funicola grown on different concentrations of glucose in the medium.

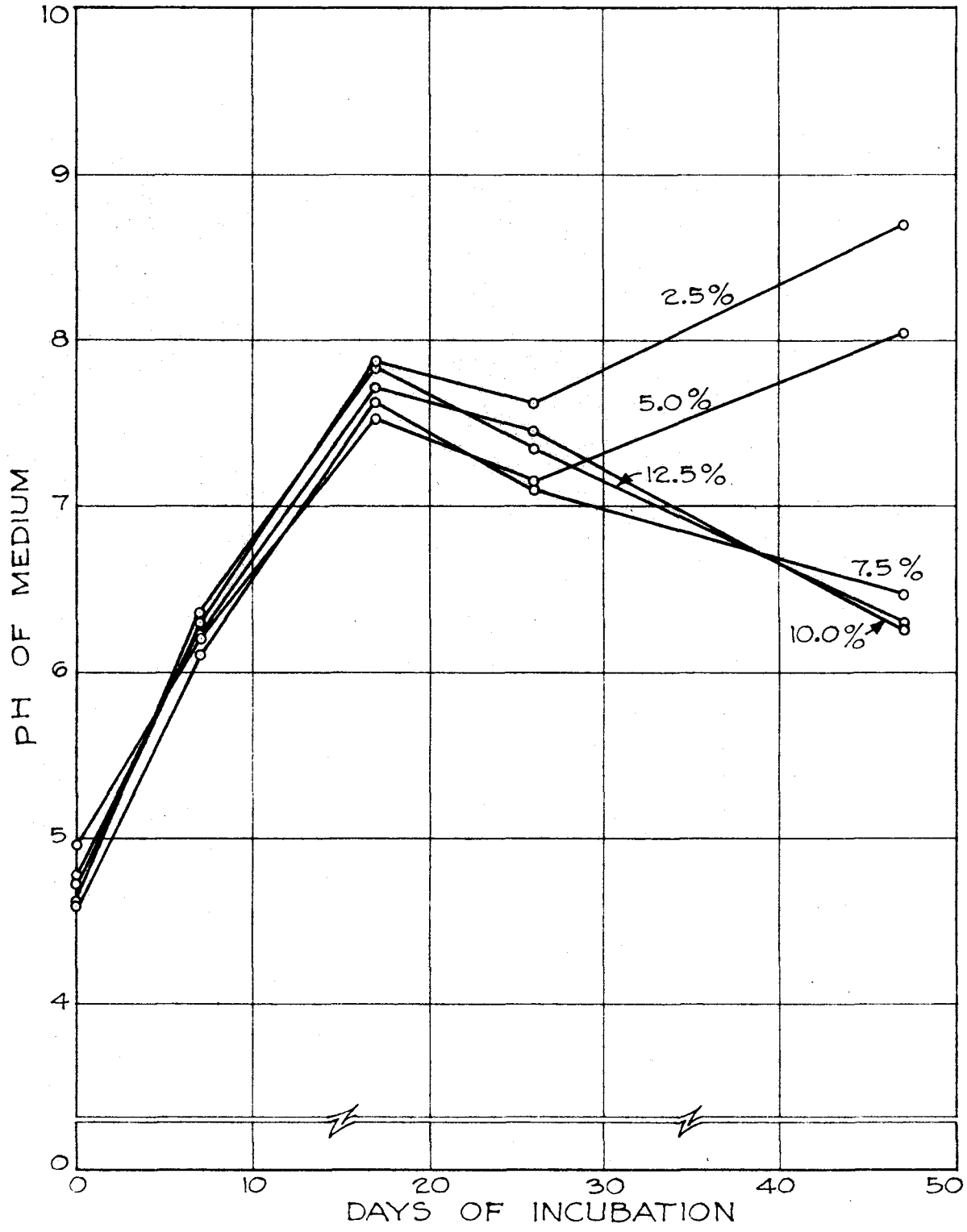


Fig. 5. Showing change in pH of the medium of cultures of Chaetomium funicola grown on different concentrations of glucose in the medium.

acid and sodium hydroxide solutions were added to the medium after sterilization, and after the addition of 5.0 cc. of spore suspension the volume was 40 cc. Analyses were made after different periods of incubation for glucose present, dry weight of mycelium and hydrogen-ion concentration of the medium. Five cc. samples were aseptically removed from each flask for zero time analyses of hydrogen-ion concentration and glucose present. The results obtained are shown in table III.

It will be observed from this table that the fungus failed to grow when the initial pH was 2.12 and that only very slight growth was obtained when the initial pH value was 2.90. At higher initial pH values the fungus grew quite readily but was considerably less when the initial pH value of 8.68 was reached. Tschudy (114) records the upper limit for Chaetomium spp. to be pH 11.0 with no growth at pH 3.0, the optimum being on the alkaline side. It appears that the optimum initial pH value is approximately 4.03 as obtained after 10 days indubation but after an incubation period of 23 days the cultures with an initial pH value of 7.20 produced the greatest yields of mycelium as well as greatest glucose decomposition. On the basis of analyses made in the latter period of incubation it might be concluded that there are three optima initial pH values, namely, 4.03, 7.20, and 8.15, the latter being the lowest of the three. In all cases the

Table III. Effect of initial hydrogen-ion concentration of the medium on culture of *Chaetomium funicola*.

Days of incubation	Initial pH of the medium								
	2.12	2.90	4.03	4.98	6.05	7.20	7.78	8.15	8.68
	pH of the medium								
10	2.50	3.25	7.01	6.92	6.89	6.98	7.02	7.41	7.47
23	2.55	4.45	7.28	6.86	7.34	6.78	7.97	8.05	8.15
	Mycelium formed in milligrams								
10	0.0	27.5	211.8	195.8	183.1	173.8	99.5	45.9	16.3
23	0.0	33.0	410.7	270.6	355.9	571.5	208.6	383.6	180.2
	Glucose not consumed in milligrams per 1 cc. of medium								
10	109.0	105.9	91.7	95.7	95.9	95.5	100.0	104.6	106.1
23	109.3	107.7	62.2	79.6	69.1	47.0	83.2	67.4	87.9

reaction of the medium shifted towards neutrality, both from the alkaline and acid sides. In no time, however, with the different initial pH values did the medium become markedly acid as a result of fungous growth.

Effect of varying the inorganic nitrogen sources of the medium. The observed decrease of acidity in the medium in the previous experiments might well be due either to the production of basic substances or merely to the uptake of nitrate ions from solution. In the absence of any appreciable formation of organic acids, the effect of sodium present in the medium would be expressed by a rise in hydrogen-ion concentration. To test this possible effect as well as to determine the relative availability of the different sources of inorganic nitrogen, cultures were prepared on different sources of inorganic nitrogen. To each 250 cc. erlenmeyer flask were added in succession 30 cc. of Czapek-Dox solution, 5.0 cc. of solutions containing 17.5 milligrams of elementary nitrogen (N) of the various inorganic nitrogen compounds. The nitrogen solutions were sterilized separately and the final concentration of the glucose was five percent. Adjustments in the initial hydrogen-ion concentration were made previous to sterilization. Analyses of duplicate cultures were made for pH of the medium, weight of mycelium and glucose present. The results obtained are presented in table IV.

Table IV. Effect of the inorganic nitrogen source in the medium on cultures of Chaetomium funicola.

Days of incubation	$(NH_4)_2CO_3 H_2O$	$(NH_4)_2SO_4$	$(NH_4)_2HPO_4$	$(NH_4)H_2PO_4$	NH_4NO_3	$Ca(NO_3)_2$	KNO_3	$NaNO_3$	$NaNO_2$	None
					pH of the medium					
0	6.33	4.80	6.95	4.65	4.65	4.48	4.65	4.78	5.35	5.11
16	6.52	3.75	5.02	3.48	4.63	4.15	6.98	7.01	----	5.69
35	3.95	3.67	3.67	3.47	3.64	6.38	7.23	7.23	----	4.57
					Mycelium formed in milligrams					
16	149.0	19.4	173.4	32.5	152.3	10.9	168.9	184.7	----	13.6
35	273.4	20.0	213.6	43.0	217.8	325.6	403.5	445.2	----	18.2
					Glucose not consumed in milligrams per 1 cc. of medium					
0	2.060	2.060	2.056	2.100	2.100	2.020	2.108	2.060	----	2.116
16	1.812	2.062	1.664	2.060	1.634	2.054	1.756	1.734	----	2.106
35	1.324	2.086	1.482	1.956	1.438	1.690	0.914	0.794	----	2.166

These data show that all inorganic nitrogen sources support growth except sodium nitrite which apparently is toxic since the spores failed to germinate. Subsequent addition of sodium nitrate did not induce growth. It is observed from the table that all ammonium salts rendered the medium more acid with time. On the other hand the medium became more alkaline with sodium or potassium nitrate. On the basis of change of pH of the medium with ammonium nitrate, it is possible to say that the ammonium ion is more available to the fungus than the nitrate ion. This relationship, however, is only evident in the early stages of development with cultures containing the other nitrogen compounds. With longer development on ammonium salt media, the relative accumulation of the acid radical effects a decrease in pH of the medium rendering it unfavorable for the further growth of the organism. The behavior with nitrate salts on the other hand is in the other direction which is favorable for the growth of the organism.

The amount of mycelial development and glucose decomposition in cultures containing the various ammonium salts can thus be explained on the basis of rapidity with which the medium is made unfavorable in pH to support fungal growth. On the other hand a similar explanation cannot be used for the differences between calcium, potassium and sodium nitrates. With calcium nitrate a certain stage of development by the fungus

was reached early and was maintained through 16 days of incubation and even up to approximately 28 days. Then rapid development occurred resulting in the formation of a heavy mycelial mat similar to that presented in cultures containing sodium and potassium nitrates. In the absence of supplied nitrogen very little development occurred and the medium became more acid indicating the possible formation of a small amount of acid under conditions of supplied nitrogen.

Effect of varying the concentration of phosphorus in the medium. Cultures were prepared to contain varying amounts of phosphorus. In 250 cc. erlenmeyer flasks were introduced 30 cc. of Czapek-Dox medium and 5.0 cc. of spore suspension. Glucose concentration was 10 percent and the initial pH was adjusted prior to sterilization. The data recorded in table V show that the media become progressively alkaline with incubation and that the change towards the alkaline side is less marked at the higher than at the lower concentrations of phosphorus. The stronger buffering action of the phosphate present in the higher concentrations would account for such a change. In the absence of phosphate additions only a slight development of the fungus occurred which is attributed to a certain amount of phosphorus included in the spores. Testing the spore suspension gave negative results for inorganic orthophosphate. The optimum concentration of phosphorus for mycelial development appears to be at the lower values of

Table V. Effect of Concentration of phosphorus in the medium on cultures of *Chaetomium funicola*

Time of incubation in days	Amount of phosphorus in milligrams per 1 cc. of medium						
	00	1.81	7.02	21.13	42.0	104.53	211.3
	pH of the medium						
0	4.4	4.4	4.4	4.6	4.7	4.9	4.9
6	6.4	7.4	6.4	6.0	5.6	5.5	5.3
13	6.8	8.0	7.8	6.7	6.4	5.8	5.6
23	6.2	7.4	7.5	6.9	6.5	6.0	5.8
	Mycelium formed in milligrams						
6	0.65	66.0	82.3	79.4	81.6	64.5	56.2
13	35.7	287.6	267.8	260.8	233.2	185.4	209.2
22	40.8	400.8	418.0	339.3	355.3	354.4	333.4
	Glucose not consumed in milligrams per 1 cc. of medium						
0	2.02	2.02	1.99	2.00	2.01	2.00	1.96
6	1.99	1.94	1.75	1.79	1.82	1.90	1.89
13	1.86	1.60	1.61	1.55	1.60	1.66	1.55
22	1.94	1.45	1.35	1.43	1.40	1.34	1.12

1.81 and 7.02 milligrams of phosphorus. Higher amounts of phosphorus resulted in lower weights of mycelium. However, sugar decomposition appears to be greater after 22 days of incubation with greater concentrations of phosphorus. The increase, however, is not very marked.

Effect of aeration and agitation of the medium. In the previous experiments the cultural media were allowed to remain undisturbed during development of the fungus. In the present experiment an attempt was made to induce the fungus to grow throughout the medium by vigorous passage of sterile air through the medium to result in combined aeration and agitation. For this purpose a two-litre flask was used in which was placed 1500 cc. of Czapek-Dox solution containing five percent glucose. To it was added aseptically an entire liquid culture of 150 cc. of similar composition contained in a two-litre flask in which the organism had developed for a period of three days. A vigorous stream of carbon dioxide-free air was passed through the medium by means ofa four-way outlet glass tubing set deep in the medium. The agitation induced by the air stream was sufficient to keep the medium in constant slow rotatory motion. In the presence of this agitation the fungus developed profusely throughout the medium as small individual colonies that increased in size with incubation. After approximately two weeks of agitation, the

colonies increased to a considerable size and made agitation difficult. At about this time the culture became contaminated with bacteria and the experiment was stopped. The results for sugar analysis and pH of the medium are presented in table VI.

Table VI. Effect of agitation and aeration on a culture of Chaetomium funicola.

Time of agitation and aeration (days)	pH of the medium	Milligrams of glucose present per 1.0 cc. of medium
0	5.01	41.75
1	5.75	40.20
5	6.55	38.30
17	6.30	34.10

The data show that even under this condition of vigorous aeration, agitation and growth of the fungus throughout the medium the pH of the medium again moves toward neutrality and at the same time glucose decomposition is still a slow process. Since air passed through the medium was in fairly large sized bubbles the aeration efficiency was probably not very high despite the rapid passage of air through the medium. Attempts to subdivide the air into fine bubbles by passage through small blocks of wood immersed in the medium presented the difficulty that the organism grew into the pores of the wood. Similar difficulty was observed in the above experiment in using the four-way outlet glass tubing.

Part II. The Intermediate Mechanism of Carbohydrate Dissimilation by Chaetomium funicola Cke., Especially the Role of Phosphorus

Introductory discussion

Considerations of the intermediate mechanism of the respiratory dissimilation of carbohydrates by fungi have been closely bound with the treatment of respiration of higher plants and yeasts. On the basis of carbon dioxide and ethyl alcohol production in a 1:1 ratio under anaerobic conditions and the formation of methylglyoxal, pyruvic acid and acetaldehyde, fungi are regarded as possessing a zymasic system. In consequence of this attribute the initial stages of the respiratory carbohydrate breakdown is taken to be identical with the Neuberg scheme of alcoholic yeast fermentation.

Chrzaszcz and Tiukow (19) and subsequently Bernhauer and Siebenäuger (11) accepted the Neuberg scheme for fungi to explain the formation of citric, oxalic, fumaric, succinic, and malic with the exclusion of gluconic and kojic acids.

Recent evidence (113) has cast doubt on the Chrzaszcz-Tiukow hypothesis (19) and the Pfefferian hypothesis as modified by Blackman (13) cannot be generally applied to all plant tissues (113). Lundsgaard (62, 63, 64) has effected a separation of fermentation from respiration by the use of

monoiodoacetic acid and has expressed the view earlier held by Boysen-Jensen that no connection exists between these two phenomena. Turner (115) has recently summarized this material. The separation is supported in part by the fact that tissues poisoned with the acid continue to take up oxygen and an enzyme glucose-oxidase (78) is present in Aspergillus niger which is not affected by this poison.

The existence of an initial fermentative phase in the carbohydrate dissimilation by strongly aerobic organisms like Chaetomium funicola is little known. Its investigation would prove of value in the further understanding of this phase of oxidative carbohydrate dissimilation.

Effect of iodoacetic acid and sodium fluoride on growing cultures

In recent studies in the intermediate mechanism of carbohydrate dissimilation, the use of differential poisons have assume an important place. Monoiodoacetic acid and sodium fluoride are the compounds now commonly used for this purpose. With the discovery by Lundsgaard (62, 63, 64) of the inhibitory effect of iodoacetic acid on anaerobic (fermentative) carbohydrate breakdown, the connection between the anaerobic phase and the aerobic phase of the respiratory dissimilation of carbohydrates had become considerably weakened

and even doubted. The lack of connection was particularly evident when, in the presence of air, tissues no longer formed lactic acid and ethyl alcohol, but continued to utilize oxygen. The action of this acid has been recently pointed out to be one of degree, affecting the fermentative phase more than the oxidative phase (115). The action of sodium fluoride is mainly on the phosphatase enzymes (52, 53, 89), preventing the conversion of hexosemonophosphate to hexose diphosphate (83) and of phosphoglyceric acid to phosphopyruvic acid (75). The effect, therefore, is mainly on the phosphorylating system. In the present study a test was made on C. funicola to determine (1) whether monoiodoacetic acid would inhibit the development of this fungus in culture, and (2) whether sodium fluoride would act likewise. Should both poisons stop the growth of the fungus, an initial anaerobic phase of carbohydrate dissimilation by C. funicola and a phosphorylating mechanism in such carbohydrate breakdown would be suggested.

Cultures were prepared to contain 50 cc. Czapek-Dox medium in 250 cc. erlenmeyer flasks. The glucose concentration was five percent and the poisons (pH 6.8) were added after separate sterilization. The results obtained after nine days of incubation in the presence of different concentrations of poison are presented in tables VII and VIII.

Table VII. Effect of concentrations of sodium fluoride on the development of C. funicola.

	Concentration of NaF in solution							
	0	M/500	M/200	M/100	M/75	M/50	M/25	M/10
Weight of mycelium in milligrams	260.3	67.2	19.7	19.7	11.9	8.6	6.15	0

Table VIII. Effect of different concentrations of monoiodoacetate on the development of C. funicola.

	Concentration of monoiodoacetate in solution							
	0	M/10,000	M/1,000	M/500	M/200	M/100	M/50	M/10
Mycelial growth in milligrams	260.3	149.6	--	--	--	--	--	--

The data show that iodoacetic acid in concentrations of M/1000 and greater, inhibited the development of C. funicola completely. Since a concentration of M/100 iodoacetate has usually been found to effect a separation of the anaerobic from the aerobic process, the results with C. funicola would point to a respiratory system very sensitive to iodoacetate. Likewise, the data for the sodium fluoride show the effect to be a very marked reduction in development of the fungus even in M/500 concentrations of sodium fluoride, a concentration which is 10 times less than the usual amount taken to interfere

with the phosphatase enzymes. From the results with these two poisons the anaerobic phase of carbohydrate breakdown in C. funicola is strongly suggested. The participation of phosphatase enzymes in the anaerobic phase appears quite probable.

Phosphorus in relation to the intermediate mechanism of glucose dissimilation.

Introduction. Recent work in the mechanism of yeast alcoholic fermentations and muscle lactic acid formation has encircled the Neuberg scheme with a considerable amount of doubt, especially in view of the fact that the scheme does not include phosphorus as an integral part of the mechanism. A new basis was laid for a reconsideration of the proposed intermediates by the isolation of phosphoglyceric acid as a product of yeast carbohydrate dissimilation (83). The inclusion of this acid in the scheme of muscle glycolysis proposed by Embden, Deuticke and Kraft (26) served as a beginning for the present schemes for alcoholic fermentations and lactic acid formation (74, 75, 76, 77, 91, 96). Methylglyoxal no longer assumed the central position given it by the Neuberg scheme but was replaced by phosphorylated products.

In the light of these findings on the role of phosphorus in carbohydrate dissimilation, application of the phosphory-

lating mechanism of carbohydrate dissimilation to other biological agents is being made (4, 6, 30, 31, 32, 105, 106, 109, 122, 123). In this respect fungi should form no exception. Should oxidative carbohydrate dissimilation with fungi undergo initially an anaerobic fermentation as is generally assumed, then the fermentative mechanism should conform to the new concepts of alcoholic fermentations and fungi should possess an enzymic system permitting phosphorylation to take place. This part of the dissertation is concerned with the possible extension of the hypothesis to the fungi.

Phosphoglyceric acid. The occurrence of phosphoglyceric acid as a product of carbohydrate dissimilation was first demonstrated for yeasts (83) and subsequently for muscle (26) and bacteria (123). Embden, Deuticke and Kraft (26) proposed a mechanism for its formation in muscle glucolysis while subsequent work with muscle and with yeasts have confirmed this mechanism in principle and have introduced modifications (74, 75, 76, 77, 91, 96).

Neuberg and Kobel (80) modified the procedure of Nilsson (83) to effect an accumulation of phosphoglyceric acid. Knowing that fresh yeast in the presence of toluene causes a phosphorylation of glucose to hexose diphosphate, these authors subjected fresh yeast to a period of two and one-half hours of incubation at 37°C. in the presence of glucose, tolu-

ene and inorganic phosphate; after which acetaldehyde and sodium fluoride were added and phosphoglyceric acid was allowed to form and accumulate in the next three and one-half hours. Free glucose was considered necessary for the formation of phosphoglyceric acid because in its absence very little of the acid was formed. Additions of acetaldehyde and sodium fluoride were necessary at the proper time. Vercellone and Neuberg (117) further simplified the method with fresh yeast by making all the additions at one time and stirring for a period of three and one-half hours.

Stone (105) showed that variations in time of incubation and concentration of acetaldehyde and sodium fluoride markedly influenced the formation of phosphoglyceric acid.

In the present work an attempt was made to determine the formation of phosphoglyceric acid from fungous tissue preparations, since the occurrence of phosphoglyceric acid in such preparations would indicate:

1. the definite occurrence of an initial anaerobic phase in carbohydrate dissimilation;
2. the similarity of (1) to the Embden-Meyerhof scheme of glucolysis;
3. the dissimilarity of (1) to the Neuberg scheme of alcoholic fermentation.

Failure to isolate phosphoglyceric acid in the same fungous tissue would suggest:

4. the absence of an initial anaerobic phase;
5. the presence of an initial anaerobic phase in which the mechanism of carbohydrate dissimilation is not similar to the Embden-Meyerhof scheme of glucolysis;
6. unsuitable methods and conditions used to effect its formation and accumulation.

The methods used were essentially those employed for yeast and bacterial studies. In the present case the mycelium was ground with sand and water to a thick soup with the object of effecting a distribution of the mycelial substance throughout the medium comparable to that attained by yeasts and bacteria. The grinding in no case caused complete destruction of the mycelium since on examination, intact mycelial hyphae were found present.

In no instance in the trials made was phosphoglyceric acid found. Even when the conditions, as to the preparation of the mycelium previous to grinding, time of incubation, temperature and the omission, concentration and type of the various ingredients pointed out by Stone (105) to be influential on phosphoglyceric acid formation were changed, no acid of this type could be isolated. The usual time of three and one-half hours allowed for the accumulation of this acid by yeasts and bacteria was lengthened in certain instances up to 22 hours. This change was considered necessary because fungi are much slower in their action in glucose decomposition

than yeasts and bacteria and a similar behavior might be in operation. However, despite such changes no formation of phosphoglyceric acid could be induced. The following experiments represent the trials performed:

Experiment 1.

Conditions varied: amount of acetaldehyde, amount of sodium fluoride, time of incubation, temperature.

Mycelium used: mycelium grown on 250 cc. Czapek-Dox medium in six two-litre erlenmeyer flasks for six days.

Treatment of mycelium: washed, pressed out to remove excess water and stored at 0.5°C. for 36 hours.

Experimental: mycelium divided into two equal parts. One part was used in the present experiment. Ground with sand and 20 cc. of water. 10 cc. of this mixture were removed to each of three 100 cc. erlenmeyer flasks. Further additions to the flasks were as follows:

	Flask number 1	2	3
2/3 M phosphate buffer[1] pH 6.85, cc.	3	3	3
20 percent dextrose, cc.	3	3	3

[1] K_2HPO_4 - KH_2PO_4 mixture

2.5 percent Na H.D.P., cc.[2]	2	2	2
2.0 percent acetaldehyde, cc.	5	3	5
0.2 M NaF, cc.	1.2	0.6	1.2
Toluene, cc.	1	1	1
Time, hours	9	6	6
Temperature, °C.	30	30	37

Experiment 2.

Conditions varied: dry mycelium used, time and temperature of incubation, amount of NaF, toluene and acetaldehyde.

Mycelium used: the second one-half of the mycelium used in Experiment 1.

Treatment of mycelium: dried eight hours over concentrated sulphuric acid in vacuo.

Experimental: the dry mycelium was portioned out equally amongst three 100 cc. erlenmeyer flasks. Further additions to the flasks were as follows:

	Flask number 1	2	3
Weight of dry mycelium, gms.	0.31	0.31	0.31
2/3 M phosphate buffer, pH 6.85, cc.	3	3	3
20 percent dextrose, cc.	3	3	3
2.5 percent Na H.D.P., cc.	2	2	2
2.0 percent acetaldehyde, cc.	4	2	4

[2]Na H.D.P. = sodium hexosediphosphate.

0.2 M NaF, cc.	1.0	0.5	1.0
Toluene, cc.	1	0	1
Water added, cc.	5	7.5	5
Time in hours	22	6	6
Temperature, °C.	30	30	37

Experiment 3.

Conditions varied: mycelium 14 days old, time of experiment lengthened, delayed addition of sodium fluoride and acetaldehyde, toluene omitted in one flask, magnesium chloride added.

Mycelium used: mycelium grown for 14 days on 250 cc. Czapek-Dox solution in eight two-litre erlenmeyer flasks.

Treatment of mycelium: washed, pressed out to remove excess water and stored at 0.5°C. for 36 hours.

Experimental: mycelium ground with sand and water and made to volume of 100 cc. This was divided between two 150 cc. erlenmeyer flasks of 50 cc. each. Further additions were as follows:

	Flask number 1	2
2/3 M phosphate buffer, pH 6.85, cc.	20	20
20 percent glucose, cc.	20	20

1.0 percent Mg. Cl_2 cc.	0.2	0.2
Toluene, cc.	3	0
0.2 M NaF, cc.	4 [1]	3 [2]
2 percent acetaldehyde, cc.	20 [1]	10 [2]
Total time, hours	21	20.75

Experiment 4.

Conditions varied: hydrogen acceptor, pH, time and amount of sodium fluoride

Mycelium used: mycelium grown for 14 days on 150 cc. Czapek-Dox solution in five two-litre erlenmeyer flasks and on 100 cc. Czapek-Dox solution in seven one-litre erlenmeyer flasks.

Treatment of mycelium: washed, pressed out to remove excess water and stored at 0.5°C. for 12 hours.

Experimental: mycelium ground with sand and water, made to 180 cc. and portioned out 20 cc. to each of eight 100 cc. erlenmeyer flasks. To each of the flasks were then added 7 cc. of phosphate buffer, 6 cc. of 20 percent dextrose, 3 cc. of 2.5 percent Na H.D.P. and 1 cc. of chloroform. Further additions to the flasks were as follows:

---

[1] Added after nine hours. [2] Added after four hours.

Flask number	pH of phosphate buffer	Hydrogen acceptor used	Hydrogen acceptor cc.	0.2 M NaF cc.	Time hours
1	6.7	2 percent acetaldehyde	8	1	6
2	6.7	4 percent acetylmethylcarbinol	8	2	6
3	6.7	2.7 percent pyruvic acid	12	2	6
4	6.7	2.7 percent pyruvic acid	12	3	10
5	7.1	2 percent acetaldehyde	8	1	6
6	7.1	4 percent acetylmethylcarbinol	8	2	6
7	7.1	2.7 percent pyruvic acid	12	2	6
8	7.1	2.7 percent pyruvic acid	12	3	10

Experiment 5

Conditions varied: agitation and aeration.

Mycelium used: mycelium grown for 11 days on 250 cc. Czapek-Dox medium in 10 two-litre erlenmeyer flasks.

Treatment of mycelium: washed at 0.5°C. and stored 36 hours (approx.) at 0.5°C.

Experimental: mycelium ground with sand and water and made to volume of 150 cc. Sixty cubic centimeters were placed in each of two 250 cc. erlenmeyer flasks to which were added further 35 cc. of 2/3 M phosphate buffer at pH 6.85, 30 cc. of 20 percent glucose, 125 cc. of Na H.D.P., 40 cc. of 2 percent acetaldehyde, 7 cc. of 0.2 M NaF and

3.5 cc. of toluene. The flasks were placed in a water bath at 37°C. and the contents of one flask was agitated by a vigorous passage of air. The time of the experiment was three and one-half hours.

In all the foregoing trials to isolate phosphoglyceric as the barium salt, small amounts of a light brown precipitate containing phosphorus were obtained. Similar precipitates were found by Stone (105).

Uptake of inorganic orthophosphorus. Inorganic phosphorus uptake is a fundamental phenomenon in the concept of phosphorylation. Stone (105) observed a significant correlation to exist between inorganic orthophosphorus uptake and phosphoglyceric acid formed with yeast and bacterial cell preparations. In view of the failure to detect phosphoglyceric acid formation in the present work, tests were made to determine inorganic orthophosphorus uptake in certain of the trials made for this acid. The following table IX contains the results obtained for inorganic orthophosphorus analysis made directly on the aqueous centrifugate. Kuttner and Lichtenstein's method (46) was used for phosphorus analyses.

Experiments 1 and 2 in the table show no phosphorus uptake but rather a liberation of phosphorus. In Experiment 3,

Table IX. Phosphorus analyses on aqueous solution of the mixture used in trials to isolate phosphoglyceric acid.

Flask number	Milligrams inorganic orthophosphorus per 1 cc. of centrifugate: Analyses: Initial	Final	Time of incubation in hours
	Experiment 1		
1	2.7	2.8	9
2	3.0	3.1	6
3	2.7	2.9	6
	Experiment 2		
1	3.4	3.4	22
2	3.4	3.4	6
3	3.4	3.5	6
	Experiment 3		
1	4.18	3.4	9
2	4.35	3.7	4

on the other hand, a definite phosphorus uptake is observed. The differences in these experiments might be attributed to the age of the mycelium; the mycelium used in Experiment 3 had developed on Czapek-Dox medium for 14 days as compared with the mycelium used in the other experiments that had developed for only six days. Nilsson (83) had observed dry yeast with greater carbohydrate reserve to phosphorylate more than dry yeast with lower carbohydrate reserve. A similar difference in reserve content might explain the differences observed in the present experiments.

Further experiments were planned to ascertain the effect the various ingredients used in the trials to determine phospholyceric acid might have on probable phosphorus uptake. For this purpose experimental mixtures were prepared to which mycelial preparations were added and from which samples were removed after different periods of incubation. The samples were centrifuged and the centrifugate analyzed immediately for inorganic orthophosphorus.

Experiment 6.

Mycelium used: mycelium grown for six days on Czapek-Dox solution containing five percent glucose in 80 petri dishes.

Treatment of mycelium: mycelium was washed, pressed out by hand and stored at 0.5° over night.

Experimental: 10 grams moist mycelium, ground in sand, were added to each of 10 125 cc. erlenmeyer flasks. To each of these was then added from one to all of the following ingredients: 10 cc. of 20 percent glucose; 8 cc. of 2.5 percent sodium hexosediphosphate; 10 cc. of acetate buffer (acetic acid-sodium acetate mixture), pH 6.8; 13.0 cc. of 2 percent acetaldehyde; 2.0 cc. of 0.2 M sodium fluoride; 1.0 cc. of toluene; 5.0 cc. of 2/3 M phosphate buffer (K_2HPO_4 + KH_2PO_4), pH 6.85; and water to make the total volume to 50 cc.

In table X are presented the data obtained. The data show that despite the presence of the various ingredients added to the mycelial preparations no significant phosphorus uptake occurred after nine hours of incubation. With 24 hours of incubation, however, flask No. 3. showed uptake. However, the uptake remains anomalous because no uptake was observed in the other flasks.

Experiment 7.

Mycelium used: mycelium grown for 18 days in two-litre erlenmeyer flasks on 250 cc. Czapek-Dox solution containing 15 percent sucrose solution.

Treatment of mycelium: the nutrient media from each

Table X. Effect of various additions on inorganic phosphorus uptake by mycelial preparations of Chaetomium funicola.

Flask No.	Contents of flasks Mycelial preparation - water Further additions	Milligrams inorganic orthophosphorus per 1 cc. of centrifugate			
		Time of incubation, hours			
		1/2	4	9	24
1	Toluene	-----	0.12	0.07	0.10
2	Toluene - NaF - acetaldehyde	-----	0.11	0.08	0.08
3	Toluene - NaF - acetaldehyde - phosphate buffer	2.28	2.48	2.54	1.69
4	Toluene - NaF - acetaldehyde - Na H.D.P.	-----	0.36	0.20	0.28
5	Toluene - NaF - acetaldehyde - Na H.D.P. - phosphate buffer	2.37	2.74	2.74	2.82
6	Toluene - NaF - acetaldehyde - Na H.D.P. - phosphate buffer - glucose	2.28	2.48	2.60	2.98
7	Toluene - NaF - acetaldehyde - glucose - phosphate buffer	2.38	2.48	2.45	2.40
8	Glucose - NaF	-----	0.10	0.07	0.07
9	Glucose - NaF - phosphate buffer	2.08	2.32	2.45	2.45
10	Glucose - NaF - phosphate buffer - acetaldehyde	2.12	2.42	2.54	2.40

flask were decanted and replaced by 100 cc. of Czapek-Dox inorganic salt solution containing no carbon. After six hours duration at room temperature, the mycelia were removed, washed several times and pressed by hand to remove excess water.

Experimental: 15 grams of moist mycelium ground with sand were added to each of seven 125 cc. erlenmeyer flasks. The various ingredients contained in the flasks were of the same concentration and amount as used in the foregoing experiment.

Table XI presents the results of the experiment. The data show a phosphorus uptake in flasks 2, 3, 4, 5, 6, and 7, and a liberation in flasks 1 and 5. The liberation of phosphorus in flask 1 can be attributed to autolysis having set in, which is less marked in flask 5.

Similar to the earlier experiments, these experiments show differences in the behavior towards phosphorus. The older mycelium exhibits phosphorus uptake while the younger mycelium does not.

Because of the lack of any significant decrease of inorganic orthophosphorus in the foregoing experiments, a similar experiment (Experiment 8) was tried to determine inorganic phosphorus uptake with mycelia grown under conditions of different amounts of phosphorus in the medium. The results obtained are presented in table XII.

Table XI. Influence of various additions on inorganic phosphorus uptake by mycelial preparations of Chaetomium funicola.

Flask No.	Contents of flasks: Mycelial preparation - toluene - water: Further additions	Milligrams inorganic orthophosphorus per 1 cc. of centrifugate: Time of incubation in hours: 1/6	1	9	29
1	No additions	0.25	0.37	0.31	0.60
2	Acetaldehyde - NaF	0.17	0.13	0.07	0.04
3	Acetaldehyde - NaF - Na H.D.P.	0.84	0.81	0.78	0.58
4	Acetaldehyde - NaF - Na H.D.P. - phosphate buffer	1.75	1.69	1.69	1.63
5	Acetaldehyde - NaF - Na H.D.P. - phosphate buffer - glucose	1.73	1.76	1.76	1.76
6	Acetaldehyde - NaF - phosphate buffer - glucose	1.76	1.80	1.67	1.60
7	Acetaldehyde - NaF - phosphate buffer	1.86	1.69	1.67	1.56

Table XII. Inorganic phosphorus uptake with mycelia grown on Czapek-Dox solution containing different concentrations of inorganic phosphorus.

Flask No.	Additions to mycelial preparations	Acid-soluble phosphorus fraction	Phosphorus per 2.5 cc. of extract: Time of incubation, hours 1/6	2 1/2	5	8	12
a) Mycelium grown on Czapek-Dox solution with normal amounts of phosphorus.							
1	NaF	Ortho-P	27.4	31.9	31.2	32.3	34.2
2	NaF + phosphate	Ortho-P	48.9	49.1	48.9	48.5	48.5
3	Glucose + NaF	Ortho-P	27.9	31.2	32.8	34.4	35.3
4	Glucose + NaF + phosphate	Ortho-P	50.3	50.0	49.9	49.3	50.4
b) Mycelium grown on Czapek-Dox solution with one-fourth the amount of phosphorus.							
5	NaF	Ortho-P	36.6	38.2	41.1	41.0	41.6
6	NaF + phosphate	Ortho-P	50.6	50.0	48.5	48.9	49.6
7	Glucose + NaF	Ortho-P	37.8	37.8	40.2	42.4	42.1
8	Glucose + NaF + phosphate	Ortho-P	51.1	49.9	50.0	39.2	51.9

Experiment 8.

Mycelium used: mycelia were grown for six days in 40 petri dishes each on two types of Czapek-Dox solution containing five percent glucose; one solution contained the normal amount (1 gm. KH_2PO_4 per litre) of phosphorus while the other contained one-fourth the normal amount (0.25 gms. KH_2PO_4 per litre).

Treatment of mycelia: mycelia were washed, ground with sand and each made to 220 cc. volume with water.

Experimental: 50 cc. of mycelial preparation were added to each flask containing one or more of the following: 4 cc. of 0.2 M sodium fluoride, 1 cc. of 2/3 M phosphate buffer, 20 cc. of 10 percent glucose solution and water to make the total volume of 80 cc.

The data in table XII show inorganic orthophosphorus to increase during incubation in the absence of added inorganic phosphorus with both types of mycelia. With phosphorus additions, however, an inorganic orthophosphorus uptake is evident but lasts for only a few hours. This uptake is small and is slightly greater with other mycelium grown on one-fourth the normal phosphorus concentration.

From the foregoing experiments to determine phosphorus uptake the results indicate that phosphorus uptake is not a significant phenomenon with mycelial preparations of C. funicola. In all cases the amount of phosphorus uptake is much less than that obtained by others for yeast and bacterial preparations. However, because of the fact that phosphorus is required for living organisms in general, the necessity of phosphorus for C. funicola and the role it assumes in the metabolism of the organism was put to question and investigated.

Phosphorus in relation to growing cultures of Chaetomium funicola. In order to ascertain the relationship of phosphorus to the development of C. funicola phosphorus in relation to growing cultures was investigated as to:

1. the necessity of phosphorus for mycelial development;
2. the extent of phosphorus uptake by the mycelium;
3. the formation of organic phosphorus in the medium.

Two experiments were performed in one of which the initial concentration of inorganic orthophosphorus was varied and in the other the initial pH was varied. It was considered that varying the initial inorganic phosphorus concentration might result in greater formation of organic phosphorus in the medium and possibly also in greater phosphorus uptake by the mycelium. On the other hand, the initial pH value of the

medium was varied because phosphatase enzymes are known (89) to possess different optima pH values for their activity and it was thought that such changes might result in different phosphorus uptake by the mycelia and organic phosphorus formation in the media.

The experimental cultures were prepared in a large number of 250 cc. erlenmeyer flasks with 35 cc. of Czapek-Dox solution containing 10 percent glucose in each flask. Duplicate cultures were removed after different periods of incubation and analyzed for pH of the medium, sugar remaining in the medium weight of mycelium, total phosphorus in the mycelium, total and inorganic orthphosphorus in the medium. The results obtained are presented in tables XIII and XIV.

The data in table XIII show that in the absence of added phosphate very little mycelial growth occurred, indicating the necessity of phosphorus for mycelial development. Similar observations have been made for Aspergillus niger by others (95). What little development occurred might be explained by the inclusion of a certain amount of phosphorus in the spores of the spore suspension used. Additions of inorganic phosphorus up to about 7.0 milligrams to the medium resulted in greater growth of mycelium than in cultures with no phosphorus additions. With progressively greater amounts of phosphorus additions there occurred progressively lower amounts of growth after 13 days of incubation. After 23 days

Table XIII. Effect of different initial concentration of phosphorus on living growing cultures of Chaetomium funicola.

Time of incubation in days	Milligrams of phosphorus added as KH_2PO_4 in 35 cc. of medium						
	0	1.75	7.0	21.0	42.0	105.0	210.0
Weight of mycelium in milligrams							
6	0.65	60.0	82.3	79.4	87.6	64.5	56.2
13	35.7	287.6	287.8	260.8	233.2	185.4	209.2
22	40.8	400.8	418.0	339.3	355.3	354.4	533.4
Glucose present in milligrams per 1 cc. of medium							
0	2.02	2.02	1.99	2.00	2.01	2.00	1.96
6	1.99	1.94	1.75	1.79	1.82	1.90	1.89
13	1.86	1.60	1.61	1.55	1.60	1.66	1.55
23	1.93	1.45	1.35	1.43	1.40	1.34	1.12
pH of the medium							
0	4.38	4.35	4.45	4.58	4.73	4.88	4.88
6	6.39	7.38	6.42	5.96	5.65	5.50	5.32
13	6.8	8.0	7.8	6.7	6.4	5.8	5.6
23	6.2	7.4	7.5	6.9	6.5	6.0	5.8
Inorganic orthophosphorus in the medium in milligrams							
0	0.0	1.81	7.02	21.13	42.60	104.5	211.3
6	0.0	1.85	6.47	19.55	41.15	107.0	208.4
13	0.0	Trace	4.06	15.33	36.70	99.7	196.1
23	Trace	0.20	3.89	13.83	33.60	97.8	187.2
Total phosphorus in the medium in milligrams							
0	Trace	Trace	7.97	21.90	43.0	106.6	210.0
6	1.01	1.39	5.94	19.77	40.9	106.6	210.6
13	0.68	0.80	5.26	18.05	38.7	103.9	205.5
23	0.70	1.03	5.72	16.44	33.7	91.0	181.4
Percent total phosphorus in the mycelium							
13	0.27	0.59	0.78	0.95	1.63	1.76	3.90
23	----	0.82	1.45	1.76	1.77	2.10	3.00

of incubation the same general relationship was obtained with, however, some intervening fluctuations of the mycelial weight with the amount of phosphorus supplied. These results are contrary to those obtained for Aspergillus niger by Braun and Frey (16) who observed a positive relationship between phosphorus concentration and the amount of mycelial growth in 27 days. On the other hand Vorbrodt (118) observed with A. niger that whereas dihydrogen potassium phosphate concentrations in the medium between 0.5 and 0.1 percent did not result in any change of the amount of mycelial growth, lower concentrations of 0.05 and 0.01 percent resulted in reduced growth. The relationship between the amount of phosphorus taken up and the amount of mycelial growth in the present experiment is close. On the basis of change in the phosphorus concentration in the medium with the development of Chaetomium funicola the data show less phosphorus to be taken up during the first six days of incubation than in the succeeding period of seven days. With further incubation the amount of phosphorus taken up depends on the concentration of the phosphorus in the medium; more phosphorus being taken up with greater concentrations of inorganic phosphorus. Uptake of phosphorus by C. funicola apparently continues for a long time. This phosphorus uptake agrees with a similar phosphorus uptake observed for Oidium lactis, Penicillium W 11 and Dematium pullulans by Schnücke (100) but differs in this respect from Aspergillus

niger observed by the same author. The latter fungus took up phosphorus from the medium for only a short while, the time being approximately six days subsequent to which a liberation of phosphorus from the mycelium occurred.

By subtracting the value for total phosphorus and inorganic orthophosphorus in the medium, small values are obtained for organic phosphorus, which are negative at times. Thes discrepencies are attributed to the errors obtained in the total phosphorus analyses. In this analysis for total phosphorus in the present experiment no precaution was exercised to hydrolyze the pyrophosphate formed after ashing the samples. Since pyrophosphate phosphorus is not determinable directly by the colorimetric methods employed, the values obtained are therefore slightly lower than the actual total amount of phosphorus present. Though precaution was exercised to effect uniform heating treatment in the process of ashing, there was no assurance that such was the case. Consequently the amount of pyrophosphate phosphorus formed differed and hence the values obtained are not uniformly alike. It is evident from the table, however, that the error is not very great. The increased inorganic phosphorus uptake from the medium is observed to be accompanied by a similar increase in the total phosphorus content of the mycelium. A similar increase in phosphorus content of the mycelium have been observed for A. niger (118).

The nature of the organic phosphorus fraction in the medium is not known. Schnücke (100) observed a similar small fraction in the media of A. niger cultures and considered it to be derived from the phosphorus liberated from the mycelium. A similar origin for this organic phosphorus was given by Braun and Frey (16) who considered that a balance existed between the phosphorus taken up from the medium by the mycelium and the organic phosphorus liberated. A greater organic phosphorus fraction was observed in the medium when nitrogen was supplied in organic form (asparagin and peptone) than when supplied in inorganic form (16).

The results for phosphorus changes as affected by the initial pH of the medium are presented in table XIV. With initial pH values of 4.03 and greater, the uptake of phosphorus from the medium after 10 days of incubation runs parallel to the weight of mycelium formed. After 23 days of incubation, the uptake is no longer proportional to the weight of the mycelium formed though the general trend is in that direction. The observed variations could be explained from the data on the total phosphorus percentage in the mycelium. According to these data changes occur in the mycelium between 10 and 23 days of incubation, probably in carbohydrate and other cell constituents, which are not accompanied by a proportional uptake of phosphorus from the medium. And further, the initial pH of the medium affects the extent of these

Table XIV. Effect of different initial pH of the medium on living growing cultures of Chaetomium funicola.

Time of incubation in days	Initial pH of the medium							
	2.90	4.03	4.96	6.05	7.20	7.78	8.15	8.68
Weight mycelium in milligrams								
10	27.5	211.8	195.8	183.1	173.8	99.5	45.9	16.3
23	33.0	410.7	270.6	355.9	571.5	208.6	383.6	180.2
pH of the medium								
0	2.90	4.03	4.98	6.05	7.20	7.78	8.15	8.68
10	3.25	7.01	6.92	6.89	6.98	7.02	7.41	7.47
23	4.45	7.28	6.86	7.34	6.78	7.97	8.05* 6.75*	8.05
Inorganic orthophosphorus in milligrams per 1 cc. of medium								
0	.29	.28	.31	.31	.30	.29	.31	.27
10	.26	.20	.18	.22	.23	.24	.25	.26
23	.21	.09	.09	.08	.11	.16	.14	.16
Total phosphorus in milligrams per 1 cc. of medium								
0	.31	.30	.31	.31	.30	.29	.29	.26
10	.30	.25	.25	.26	.29	.30	.36	.32
23	.23	.15	.15	.13	.14	.19	.20	.18
Organic phosphorus in milligrams in 1 cc. of medium								
0	.02	.02	.00	.00	.00	.00	-.02	-.01
10	.04	.05	.07	.04	.06	.06	.11	.06
23	.02	.06	.06	.05	.03	.03	.06	.02
Percent total phosphorus in the mycelium								
10		1.40	1.26	0.97	0.86	0.78	0.84	1.06
23	0.90	0.67	0.99	0.93	0.61	0.72	0.63	0.93

changes. With an initial pH value of 2.90, only slight mycelial growth was obtained accompanied by a definite phosphorus uptake.

In regard to the organic phosphorus in the medium the data in table XIV show that this phosphorus constitutes a definite fraction of the total phosphorus remaining in the medium. In no instance does this organic phosphorus fraction increase in amount with incubation, but rather a reduction in amount especially with alkaline and extremely acid initial pH values. In these same regions the organic phosphorus fraction constitutes a smaller part of the total phosphorus present in the medium than is the case with the intervening initial pH values.

From the foregoing considerations of the relation of phosphorus to living growing cultures of Chaetomium funicola, there is nothing that can be directly deduced that would indicate the functional role of phosphorus apart from its generally recognized necessity in the building up of nucleoproteins and other cell constituents. However, apart from this latter consideration it is evident that the site of action of phosphorus is within the mycelium since organic phosphorus occurs only in small amounts in the medium. An investigation of the phosphorus in the mycelium therefore would probably be more fruitful in the solution of the phosphorus problem in fungal metabolism.

The nature of the acid-soluble phosphorus in the mycelium of Chaetomium funicola and several other fungi. The nature of the phosphorus compounds in the mycelium of fungi has been the subject of very little study. Koch and Reed (45) observed that protein phosphorus (nuclein phosphorus) was the most important form of phosphorus in the mycelium of Aspergillus niger. Lecithin phosphorus came next in order while the extractive phosphorus (water soluble) held only an intermediary position between the inorganic phosphorus and the phosphorus in organic combination. It was considered that the extractive phosphorus gave rise to protein phosphorus and lecithin phosphorus but that lecithin phosphorus did not go directly to protein phosphorus. Goupil (36) considered lecithin phosphorus and nuclein phosphorus as the important phosphorus fractions in Amylomyces Rouxii. Contrary to Koch and Reed, this author considered lecithin phosphorus to give rise to nuclein phosphorus. The mineral phosphorus contained in the organism was considered the resultant of the degradation of organic phosphorus during the life of the Mucorineae.

Vorbrodt (118) studied the relative amounts of the different phosphorus fractions in the mycelium of Aspergillus niger when grown under conditions of different concentration of inorganic phosphorus in the medium. He observed that the mineral phosphorus (P_2O_5) in the mycelium grown on an inorganic salt-glucose medium containing 0.5 percent KH_2PO_4 con-

stituted the largest portion, being approximately 1.8 percent of the total dry matter. Acid-soluble organic phosphorus and albuminoid (protein) phosphorus made up the next largest portion (0.5 percent each), while lecithin phosphorus formed only a negligible fraction (0.01 - 0.04 percent of the total dry matter). Of the total phosphorus in the mycelium approximately 50 percent was mineral phosphorus, 15 percent acid-soluble organic phosphorus and the remainder protein phosphorus (lecithin phosphorus being neglected). Under conditions of different phosphorus concentrations (0.5-0.01 percent KH_2PO_4) in the medium the following observations on the phosphorus in the mycelium were made:

1. The percentage total phosphorus of the dry weight of the mycelium decreased with decreasing concentrations of phosphorus in the medium.
2. The percentage total phosphorus in the mycelium decreased with incubation.
3. The percentage protein phosphorus of the total phosphorus in the mycelium was approximately the same (30 to 40 percent) for concentrations of phosphorus in the medium between 0.5 to 0.05 percent KH_2PO_4. With lower concentratinns of phosphorus in the medium (0.025 and 0.01 percent KH_2PO_4) lower percentage protein phosphorus of the total phosphorus (20 to 40 percent) was obtained.

4. The percentage protein phosphorus in the mycelium decreased with incubation except at the extremely low phosphorus concentrations in the medium where mycelial development was slower.
5. The percentage acid-soluble organic phosphorus of the total phosphorus in the mycelium was lower, reaching a low value of zero, as the phosphorus concentration in the medium was lowered.
6. At low phosphorus values of the medium, the acid-soluble fraction of the mycelium decreased with increasing time of incubation of the cultures.
7. In all instances of different phosphorus concentrations in the medium and different periods of incubation, the mineral phosphorus content of the mycelium constituted the largest fraction of the total phosphorus.
8. The mineral phosphorus fraction in the mycelium increased with incubation.

From these observations the following important conclusions were made by Vorbrodt:

1. The acid-soluble organic phosphorus compounds in the mycelium have a transitory role.
2. Mineral phosphorus plays an essential role.
3. Part of the mineral phosphorus accumulated in the mycelium is converted into albuminoid substances.

The constant high content of mineral phosphorus in the mycelium lead Verbrodt to the conclusion that mineral phosphorus was the most important form of phosphorus. However, no indications were made as to its role. Two possible functions present themselves: (1) that phosphorus acts as a catalyst furthering chemical reactions; (2) that phosphorus participates in carbohydrate breakdown through the phenomenon of phosphorylation.

The catalytic activity of phosphorus has been observed in various biological oxidations (3, 68, 69, 70, 111 , 112). Lyon (70) has shown phosphorus to function through the phosphate-ion as a promoter catalyst on respiration by Elodea, while Nord (85) considers it to function as a protector catalyst. The inclusion of the second possibility derives from a consideration of the mechanisms of glycolysis by yeast, blood, muscle and other animal tissues, where phosphorus plays an important role. While the possible occurrence of phosphorylation in certain higher plants has been demonstrated by a number of workers (4, 7, 21, 41, 109), Nord et al (86, 88) have recently questioned the occurrence of phosphylation as a significant process in the breakdown of carbohydrates by Fusarium Lini and Fusarium oxysporum.

The presence of an acid-soluble phosphorus fraction has been observed for many tissues. Such fractions have been found to contain different phosphorus compounds. In blood,

for instance, the following acid-soluble phosphorus compounds have been found in the cells: inorganic orthophosphorus, pyrophosphorus, diphosphoglyceric acid and possibly one or more hexosephosphates (30, 37, 42, 121). Some of these fractions have been found in liver (35) and in muscle together with phosphagen-phosphorus (24). With yeasts, McFarlane (71) observed that 50 percent of the phosphorus contained in the cells was extractable with trichloracetic acid and consisted of 30 percent orthophosphorus, 50 percent labile phosphorus (pyrophosphorus) and 20 percent in organic combination. Lohmann (58) observed 57 percent of the acid-soluble phosphorus of yeast to be inorganic phosphorus and 16.2 percent pyrophosphorus.

The significance of these phosphorus fractions have been found in their intimate relationship with the dissimilation of carbohydrates as products of the dissimilation process and as co-enzymes, as is the case with adenylpyrophosphoric acid. A similar possibility arises for Chaetomium funicola.

To determine the nature of the phosphorus in the mycelium of C. funicola which might be related to a phosphorylation in carbohydrate breakdown, the acid-soluble phosphorus fraction was investigated. For this purpose several other fungi such as Aspergillus niger, Fusarium lini, F. oxysporum v. cubense, and F. bulbigenum v. niveum were included in the analysis to serve as a basis for comparison with the findings

and observations made by Vorbrodt and Nord et al. Mycelia of the different fungi were grown in 20 petri dishes each on Czapek-Dox solution containing five percent glucose. The time of growth allowed was six days for the reasons that:

1. Schnücke (100) had observed the major phosphorus constituent to be absorbed in the early developmental stages of Aspergillus niger.
2. Mycelium is still in an actively growing condition and therefore relatively high in protoplasmic content. Such a condition was desired because it was felt that a better picture would be obtained of the phosphorus compounds functioning in the active metabolism of the organisms.

The results of an analysis of the acid-soluble phosphorus are presented in table XV. These data were obtained from an analysis by the method of Lohmann (55) of the trichloracetic acid extracts of different mycelia. Inorganic orthophosphorus was analyzed by the method of King (44). The acid extracts were obtained by the following treatments of the mycelia at 0.5°C.: washing, flooding with trichloracetic acid, grinding with sand, and extracting for four hours; total volume of the mixture was made to 150 cc. by further addition of five percent trichloracetic acid. Analyses of the extracts were made immediately by removing the mycelia by centrifugation and filtration through filter paper.

Table XV. Inorganic orthophosphorus formation on hydrolysis of trichloracetic acid extract in 1 N HCl at 100°C.

Time of hydrolysis in minutes	Aspergillus niger	Chaetomium funicola	Fusarium lini	Fusarium cubense	Fusarium niveum
	Inorganic orthophosphorus in 5 cc. of extract				
0	6.0	20.2	46.1	37.3	61.4
7	12.8	69.5	113.0	192.2	219.5
30	14.0	69.5	111.7	208.2	227.5
180	15.4	71.5	118.5	208.2	236.0
Total	30.0	99.0	171.2	565.0	305.0
	Phosphorus fractions present in the extract				
Pyrophosphate phosphorus fraction $P_{7'-0'}$	6.8	49.2	66.9	154.9	158.1
Organic phosphorus fraction $P_{total-7'}$	17.2	29.5	58.2	72.8	86.0
Hydrolyzable phosphorus $P_{180'-7'}$	2.6	2.0	5.5	16.0	16.5
$\frac{P_{180-7}}{P_{total-7}} \times 100$	15.1	6.8	9.5	22.0	19.2

Total acid-soluble phosphorus. The data in table XV show that the total acid-soluble phosphorus fractions vary considerably, being greatest with Fusarium species, least with Aspergillus niger and mid-way for Chaetomium funicola. Likewise, similar differences are observed with the various fractions of the acid-soluble phosphorus. Of the Fusarium spp. the total acid soluble phosphorus is greatest with F. niveum, the least with F. lini while F. cubense compares closely with F. niveum.

Inorganic orthophosphorus. Inorganic orthophosphorus in all cases constituted the smallest part of the total acid-soluble phosphorus varying in amount with the different fungi from approximately 14 to 25 percent. It is quite probable that the absolute inorganic phosphorus content in the mycelium is slightly less than indicated because of incompleteness of washing the mycelium.

Pyrophosphate fraction. The table shows that the pyrophosphate fraction constitutes a considerable part of the total acid-soluble phosphorus, even with Aspergillus niger where its relative amount is lower than is the case with the other fungi. In all cases, however, it forms a definitely determinable fraction. When expressed as a percentage of the total acid-soluble phosphorus, the following values are obtained: A. niger

22.7 percent, Chaetomium funicola 49.7 percent, Fusarium lini 39.0 percent, F. cubense 58.4 percent and F. niveum 51.8 percent. A correction of the formula (pyrophosphate = $P_7 - O$) for the accompanying hydrolysis of other phosphorus compounds offers no particular advantage. Considering the pyrophosphate fraction and the inorganic orthophosphorus together it can be determined from the table that they form the following percentage values of the total acid-soluble phosphorus: Aspergillus niger 42.7 percent, Chaetomium funicola 70.2 percent, Fusarium lini 66.0 percent, F. cubense 72.6 percent and F. niveum 72.0 percent. The remainder constitutes the organic phosphorus fraction. In this connection it might be pointed out that Vorbrodt (118, 120) obtained mineral P_2O_5 values ranging from 75 to 90 percent or greater of the total acid-soluble phosphorus for Aspergillus niger when grown under conditions of different phosphorus concentrations in the medium. His method, however, involved a procedure that no doubt resulted in a certain amount of enzymatic and acid hydrolysis of the phosphorus compounds.

Organic phosphorus. Phosphorus in organic combination forms a smaller but considerable fraction of the total acid-soluble phosphorus being approximately 30 percent with the species of Fusarium and Chaetomium, while greater than 50 percent with Aspergillus niger.

The nature of the hydrolysis curve shows it to be of the difficultly hydrolyzable type, the hydrolysis in three hours expressed as a percentage ($P_{180-7}/P_{total-7} \times 100$) being in all cases lower than for the Embden or Robison esters (55). The possibility of the presence of several different compounds with different ease of hydrolysis is not excluded, particularly since the extent of hydrolysis of the organic phosphorus fractions during the three hours is so variable with the fungi. The fungi might well differ in the relative content of such esters.

Phosphagen phosphorus. Analysis for phosphagen phosphorus on the acid extract according to the colorimetric method of Eggleton and Eggleton (25) and observing the precautions of temperature pointed out by Fiske and Subbarrow (33) was done immediately following centrifugation. No phosphagen phosphorus was detected. The presence in any abundance of extremely labile phosphorus compounds in the mycelium is therefore excluded.

To test this point further, the phosphorus compounds in the extract were separated as the barium salts according to the method of Eggleton and Eggleton (25). Twenty cubic centimeters of the extract were treated with solid powdered barium hydroxide till just blue to thymol blue paper. The precipitate thus formed was centrifuged off and washed several times

with five percent trichloracetic solution treated with barium hydroxide till blue to thymol blue paper. With such treatment barium phosphagen phosphate would be stabilized and would remain in solution. Analysis of the solution after removal of the barium with sulphuric acid showed the presence of neither inorganic phosphorus nor extremely labile phosphorus.

In view of the presence of acid-soluble organic phosphate, pyrophosphate and inorganic orthophosphate phosphorus fractions in the young mycelium of Chaetomium funicola detaminations of their relative amounts were made with older mycelium. For this purpose, 80 petri dishes of C. funicola cultures were prepared and the mycelium analyzed for acid-soluble phosphorus after eight and 18 days development. At each of these times the mycelia from 40 petri dishes were removed, washed, treated with an equal weight of 10 percent trichloracetic acid without grinding, and the samples were made to 200 grams total weight by additions of five percent trichloracetic acid. Extraction was allowed to proceed for three days at 0.5°C. The results of analysis of the extracts according to Lohmann's method are shown in table XVI and figure 6.

Figure 6 shows that the forms of the hydrolysis curves are approximately the same at the two ages of the mycelium. Total acid-soluble phosphorus (Table XVI) is greater in the older mycelium than in the younger, a difference which is to

Table XVI. Effect of age of mycelium of Chaetomium funicola on the nature of the acid-soluble phosphorus components of the mycelium.

	Inorganic orthophosphorus per 2.5 cc. of extract	
	Age of mycelium	
	8 days	18 days
Time of hydrolysis in N/1 HCl in minutes		
0	57.2	54.3
1	72.3	103.3
7	111.0	320.0
30	152.0	409.0
60	150.0	416.0
90	153.5	423.0
180	164.0	422.5
Total	207.0	486.5
Acid-soluble phosphorus components of the mycelium		
P_o	57.2	54.3
$P_{7'-0'}$	53.8	265.7
$P_{15'-7'}$	96.0	166.5
$P_{180'-7'}$	53.0	102.5
$\frac{P_{180'-7'}}{P_{total-7'}} \times 100$	55.3	61.6

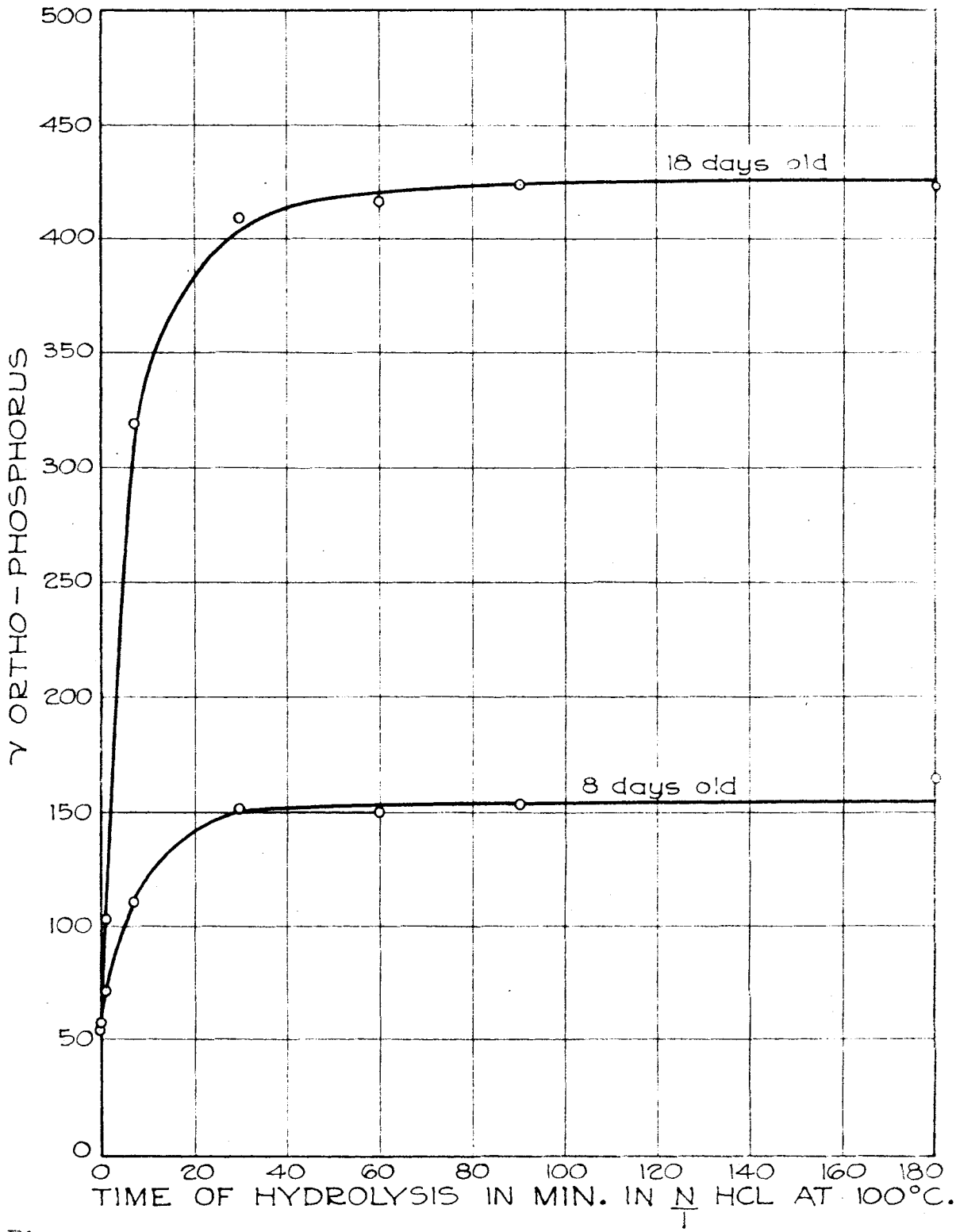


Fig. 6. Hydrolysis curves of acid-soluble phosphorus obtained from mycelia of Chaetomium funicola at two different ages.

be expected on the basis of previous studies on phosphorus intake by living growing cultures. This increase of total acid-soluble phosphorus is observed to consist of increases in each of the phosphorus fractions. However, these fractions do not increase in proportional amounts. Expressed as percent of the total phosphorus, the data show that the pyrophosphate phosphorus fraction increased from 26.0 percent to 54.6 percent while the organic phosphate and inorganic orthophosphate phosphorus fractions decreased from 27.6 to 11.2 percent and 46.4 to 34.3 percent respectively. These changes agree in the main with the observation made by Vorbrodt on Aspergillus niger at different periods of incubation that the mineral phosphorus in the mycelium increases (118).

The inter-relationships of the acid-soluble phosphorus fractions in mycelial preparations of Chaetomium funicola.

Introduction. To determine the inter-relationship of the acid-soluble phosphorus fractions present in the mycelium of C. funicola and also the probable role of this phosphorus in the respiratory dissimilation of carbohydrates, several possible methods of investigation present themselves, namely: (1) to determine the behavior of the different phosphorus fractions in the mycelium in growing cultures under different conditions; (2) to determine the behavior of the different phosphorus fractions in the mycelium on autolysis; (3)

to determine the behavior of the phosphorus with enzymic preparations of the mycelium when actively dissimilating carbohydrates. In the present work only the second and third of these methods were considered for C. funicola, namely, autolysis and enzymic preparations.

Literature review. The autolysis of fungal tissue has been the subject of very little study, particularly with respect to phosphorus. Perhaps the only observation made on this question was that of Schnücke (100) who found young mycelia of Aspergillus niger to be higher in total phosphorus content than older mycelia. On the other hand, fungi such as Oidium lactis, Penicillium W 11 and Dematium pullulans produced no recognizable excretion of phosphorus with age (100).

The mineralization of phosphorus on autolysis of other tissues has been observed for liver (35, 99, 108), "magenmucosa", spleen, kidney, pancreas, heart and striated muscle (110), brain (2), nerves (34), blood (97), yeasts (58) and bacteria (123). Sveringhaus (108) observed the liberation of phosphorus from liver breis preserved with toluene. Rona and Mislowitzer (99) obtained a similar increase in inorganic phosphorus from liver and postulated that autolysis of phosphorus compounds was essentially the conversion of colloidal phosphorus to inorganic phosphorus through the following steps: colloidal phosphorus → (intermediate) → difficultly hydro-

lyzable phosphorus → easily hydrolyzable phosphorus → inorganic phosphorus. The easily hydrolyzable phosphorus was considered to consist essentially of hexosemonophosphate and hexosediphosphate. With rabbit and dog liver, Barrenscheen, Pany and Berger (7) observed that the inorganic phosphorus increase paralleled the falling off of glycogenolysis. They observed that the inorganic phosphate arose not only from pyrophosphate but also from the difficultly hydrolyzable acid-soluble phosphorus fractions. Flock (35) obtained complete breakdown of the pyrophosphate fraction with autolysis of dog liver in 15 minutes and the subsequent slow increase of inorganic phosphorus was brought about by a breakdown of the more stable acid-soluble esters. Teorrel and Norberg (110) considered the inorganic phosphorus increase to arise in part from bodies such as the difficultly hydrolyzable acid-soluble phosphorus and in part from lipoid phosphorus, but mainly from the former. The acid-soluble fraction was observed never to decompose completely. Phosphatid phosphorus decomposition was observed to be slower than that of the acid-soluble phosphorus since the latter is considered to be a step in the production of the inorganic phosphorus form.

The relationship between inorganic phosphorus liberation and the vital processes within the cell have been quite extensively investigated with blood cells. While Rona and Iwasaki (97) first considered inorganic phosphorus liberation

from blood cells to be a process independent of glycolysis and autolysis, subsequent work (98) showed that an equilibrium occurs within the cell between the phosphorus in organic combinations and that in inorganic form. Addition of sugars such as glucose, fructose, mannose, or galactose retarded inorganic phosphorus liberation. Rona and Iwasaki (98) postulated that these sugars caused the equilibrium to be shifted in the direction of phosphorus in organic combination (esterification), while substances like disaccharides, pyruvic and lactic acids, sodium fluoride and haemolysin, as well as hydrogen-ion concentrations greater than pH 7.3, were unfavorable to esterification and caused the equilibrium to be shifted to the side of inorganic phosphorus formation. A close relationship between inorganic phosphorus liberation and glycolysis was observed by Engelhardt and Ljubimowa (29). Jost (42) observed inorganic phosphorus to be taken up by blood cells at the same time that sugar was being split and found that the phosphorus taken up was converted mainly to diphosphoglyceric acid, thus confirming an earlier observation of Lawaczek. Since the formation of this acid occurred anaerobically, Jost postulated its formation to proceed through an intermediary triose phosphate.

Barrenscheen and Vásárhelyi (8) emphasized the intermediate and essential role of pyrophosphate in blood glycolysis, observing that the pyrophosphate content of the

blood from various animals increased as the glycolytic power of the blood increased. The following conversions were postulated: inorganic phosphorus - easily hydrolyzable intermediate phosphate (pyrophosphate) - difficultly hydrolyzable phosphorus; which were subsequently made into reversible reactions (5) based on the observations that haemolysin, sodium fluoride, oxalate, narcotics, ether, chloroform and propyl alcohol caused a lowering of glycolytic activity with an increase of inorganic phosphorus entirely from the pyrophsphate fraction. Engelhardt (27, 28) observed a similar role of the pyrophosphate fraction with respiration in nucleated erythrocytes. Addition of respiratory poisons such as cyanide, carbon monoxide and urethane caused an increase in the inorganic phosphorus fraction from the pyrophosphate fraction. Similar increases were observed when the nucleated blood cells were subjected to anaerobic conditions. On restoring such cells to aerobic conditions a partial inorganic phosphorus uptake occurred.

Experimental. In the following experiments, the acid-soluble phosphorus was studied in mycelial preparations of Chaetomium funicola in the presence and absence of glucose and various poisons which are known to interfere with certain enzymatic reactions. The object of these experiments was to determine whether the different acid-soluble phosphorus

fractions possessed similar inter-relationships as found in blood cells and in liver.

The methods used in these experiments for obtaining acid-soluble phosphorus and for the analyses of the extracts were described in general in the part on methods. Additional details are presented as the individual experiments are considered. Inorganic orthophosphorus was determined throughout by the method of King (44).

In table XVII are presented data of an experiment in which the effects of additions of glucose, sodium fluoride and toluene to mycelial preparations were studied.

The data show (Fig. 7) that inorganic orthophosphorus increased in all the experimental treatments. Glucose exerted a retarding action on the mineralization of phosphorus in the presence and in the absence of either sodium fluoride or toluene. Sodium fluoride exerted a retarding action and toluene favored the mineralization of phosphorus in the absence and in the presence of added glucose. The pyrophosphate fraction increased and then decreased with time, considerably less increase occurring in the presence of toluene.

Table XVIII contains the results of an experiment in which the entire acid-soluble phosphorus fraction was considered. Inorganic orthophosphorus increased with time as also did the total acid-soluble phosphorus. As before, on addition of toluene a greater phosphorus mineralization oc-

Table XVII. Effect of glucose, sodium fluoride and toluene on the behavior of inorganic orthophosphorus and pyrophosphate phosphorus in autolysis.

Flask No.	Additions to mycelial preparations	Phosphorus fraction	Y Phosphorus per 3.0 cc. of extract: Time of autolysis in days 1/6	3	9	20	31
1	None	ortho-P	48.7	60.6	84.4	95.1	97.9
		pyro-P	32.1	48.5	44.3	34.6	38.9
2	Glucose	ortho-P	48.2	59.3	76.6	89.4	91.2
		pyro-P	36.2	40.7	49.2	37.3	35.5
3	NaF	ortho-P	47.7	56.0	64.3	70.2	68.1
		pyro-P	38.1	46.1	47.9	44.5	41.7
4	Glucose + NaF	ortho-P	48.4	59.3	73.9	78.7	68.1
		pyro-P	37.0	42.1	48.9	56.4	41.7
5	Toluene	ortho-P	49.0	65.5	100.0	116.1	112.0
		pyro-P	38.0	41.3	39.9	38.4	28.2
6	Glucose + toluene	ortho-P	47.1	68.8	97.4	111.0	106.8
		pyro-P	37.3	34.5	42.1	34.2	29.7

Mycelium grown eight days in petri dishes containing Czapek-Dox medium with five percent glucose.
Mycelium ground and made to 200 cc.
Quantities of materials used per flask, 30 cc.
Mycelial preparation, 10 cc. 0.2 M NaF, 10 cc. 20 percent glucose, 0.5 cc. toluene and total volume made to 50 cc. with water. Temperature 30°C. Samples allowed to extract for four days.

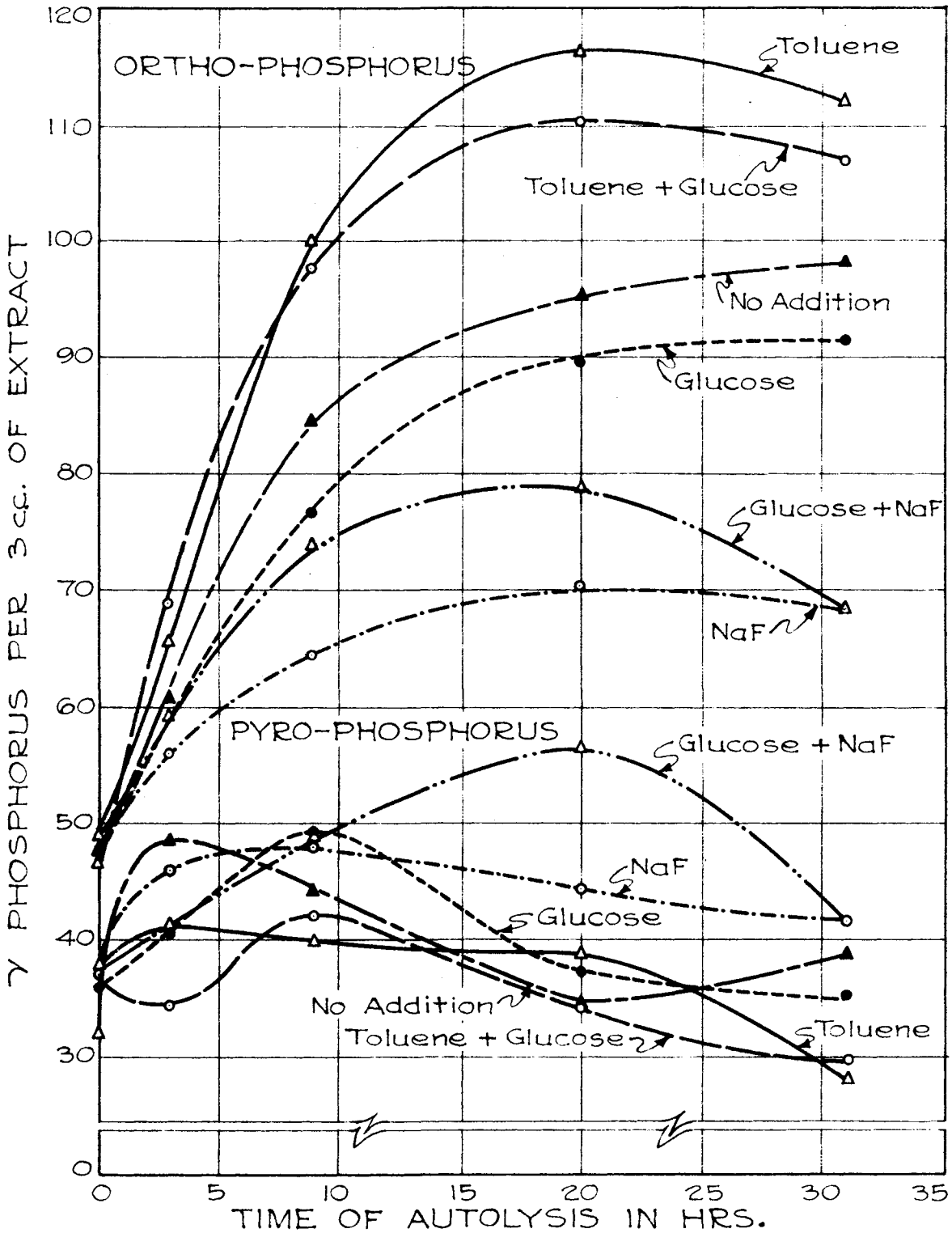


Fig. 7. Effect of autolysis of mycelial preparations of Chaetomium funicola on two acid-soluble phosphorus fractions.

Table XVIII. Effect of sodium fluoride, toluene and glucose on the different acid-soluble phosphorus fractions in autolysis.

Flask No.	Additions made to the mycelial preparation	Phosphorus fraction	γ Phosphorus per 2.5 cc. of extract: Time of autolysis in hours: 1/6	2.5	5	8	12	17	24
1	NaF	P_{total}	114.5	115.0	121.9	133.2	128.8	137.5	141.5
		$P_{0'}$	34.0	40.4	44.8	50.6	61.5	68.7	70.9
		$P_{7'-0'}$	19.9	12.2	12.2	12.9	8.0	4.3	7.3
		$P_{30'-7'}$	10.7	5.7	6.7	3.4	4.1	2.5	0.7
		$P_{180'-30'}$	2.2	6.6	3.0	3.1	7.5	4.1	6.5
		$P_{total-180'}$	43.2	43.6	48.3	51.0	49.7	46.9	38.5
2	NaF + glucose	P_{total}	114.5	115.0	121.9	133.2	128.8	137.5	141.5
		$P_{0'}$	34.1	42.2	48.4	56.0	72.1	84.2	91.6
		$P_{7'-0'}$	18.8	23.4	13.6	14.0	4.0	2.1	3.0
		$P_{30'-7'}$	10.6	-2.0	9.0	6.1	5.4	3.1	2.2
		$P_{180'-30'}$	4.5	3.3	1.5	1.8	4.3	2.8	5.4
		$P_{total-180'}$	47.1	46.1	59.4	55.3	43.0	45.3	39.3
3	Toluene	P_{total}	119.0	121.0	135.1	145.0	147.8	150.0	155.5
		$P_{0'}$	38.5	52.9	68.3	88.4	101.5	115.2	125.5
		$P_{7'-0'}$	22.2	14.5	13.2	1.4	10.5	9.5	10.8
		$P_{30'-7'}$	9.3	5.2	12.5	14.2	-9.2	-1.7	--
		$P_{180'-30'}$	3.5	3.4	9.7	1.2	5.1	-4.7	04.2
		$P_{total-180'}$	45.5	45.0	32.4	39.8	30.7	30.0	23.4
4	Toluene + glucose	P_{total}	117.2	132.3	136.0	145.0	147.0	149.5	150.0
		$P_{0'}$	38.4	57.5	72.9	91.9	110.8	113.5	126.0
		$P_{7'-0'}$	17.3	14.0	10.5	2.3	9.2	13.6	10.3
		$P_{30'-7'}$	10.6	7.0	3.1	11.0	-0.5	-4.6	-1.3

Table XVIII. Continued.

$P_{180'-30'}$	--	1.1	2.0	2.0	-1.5	0.9	-0.5
$P_{total\ -180'}$	--	52.7	36.5	36.8	25.0	26.1	15.5

Mycelium from 80 petri dishes made to 240 cc. of mycelial preparation.
To the flask were added 60 cc. of mycelial preparation, 30 cc. of 10 percent glucose, 12 cc. of 0.2 M NaF, 1 cc. toluene and water to make total volume to 120 cc., temperature 30°C. Samples allowed to extract for five days before centrifugation.

curred than in the presence of sodium fluoride. Glucose exerted no significant action on these phosphorus fractions. The pyrophosphate decreased in amount with autolysis. The decrease with pyrophosphate phosphorus occurred more slowly in the presence of sodium fluoride than in the presence of toluene. In the presence of the latter, this fraction decreased to a low level in approximately eight days and then increased. In the presence of sodium fluoride the difficultly hydrolyzable phosphorus fraction ($P_{total} - 180'$) increased in amount for approximately 12 days of incubation and then decreased. In the presence of toluene, this same fraction continually decreased. The amounts of the more easily hydrolyzable organic phosphorus fractions ($P_{30'} - 7'$ and $P_{180'} - 30'$) were small and together exhibited considerable fluctuations. The $P_{30'-7'}$ phosphorus fraction decreased in the presence of sodium fluoride alone, but in the presence of glucose and sodium fluoride together, fluctuations occurred. Similar fluctuations occurred in the presence of toluene.

In the previous two experiments the length of time allowed for the extraction of the acid-soluble phosphorus was from four to five days at 0.5°C. To determine the effect of time of extraction and concentration of trichloracetic acid on the results of phosphorus analysis, the following experiment was performed: mycelial preparations of C. funicola (mycelium grown six days) were added to four flasks to which

were further added two volumes of water to each flask and toluene to make one percent of the final volume. Two of the flasks were placed at 30°C. for 24 hours and different concentrations of trichloracetic acid were added after the lapse of this time to make the desired concentration of the acid. The other two flasks were treated with the acids immediately. All four flasks were placed at 0.5°C. and samples (mycelium with solution) were removed after different times. The samples were centrifuged and the centrifugate returned to the cold room till analyzed. The results of the analyses obtained are shown in table XIX.

The data show inorganic orthophosphorus to remain relatively constant for the different periods of extraction. Organic phosphorus on the other hand showed a definite increase in one day of extraction at 0.5°C. with mycelial preparations not subjected to a period of 24 hours of incubation at 30°C. These results are in accord with those of Kay (43) who obtained similar behavior with various animal tissues. This author considered the additional organic phosphorus extracted to be derived from the lipoids as glycerophosphate.

In view of the necessary precaution in the use of trichloracetic acid, an experiment was performed in which the time of extraction allowed was one hour. Table XX contains the results of the experiment planned to compare the action of different respiratory and glycolytic poisons.

Table XIX. Effect of time of extraction, concentration of trichloracetic acid and previous treatment of mycelial preparation on phosphorus distribution.

Flask No.	Treatment of mycelial preparation previous to addition of trichloracetic acid	Final concentration of trichloracetic acid, in percentage	Phosphorus fraction	γ phosphorus per 2.0 cc. of extract						
				Time of extraction in days						
				0	1	2	3	3 1/2	4 1/2	5 1/2
1	None	5	Inorg-P	28.4	28.1	28.9	28.1	--	--	27.8
			Org-P	91.8	107.0	122.0	125.2	--	--	127.0
2	24 hours at 30°C.	5	Inorg-P	78.2	77.5	75.7	--	77.8	77.5	--
			Org-P	160.0	160.0	156.0	--	164.4	158.0	--
3	None	10	Inorg-P	28.1	28.1	27.5	27.8	--	--	26.6
			Org-P	86.0	104.6	102.0	105.2	--	--	108.2
4	24 hours at 30°C.	10	Inorg-P	59.5	59.5	60.2	--	61.0	59.6	--
			Org-P	116.0	117.0	117.4	--	112.8	112.8	--

Table XX. Effect of other poisons on the behavior of the different phosphorus fractions.

Flask No.	Poisons added to mycelial preparation	Time of autolysis, hours	γ Phosphorus per 2 cc. of extract: Total	Inorganic: ortho-P	Inorganic: pyro-P	$P_{30'-7'}$	$P_{180'-30'}$	$P_{total-180'}$
1	M/50 NaF	0	40.1	15.6	9.1	2.3	2.4	10.7
		2.5	52.1	21.3	14.1	0.3	-0.5	16.9
		12	89.4	38.5	21.0	1.8	1.2	26.9
2	M/1000 KCN	0	44.2	14.2	12.8	2.4	0	17.2
		2.5	69.5	24.6	17.0	-0.6	2.4	26.5
		12	89.4	44.7	24.0	-0.1	2.4	18.3
3	0.57 M ethyl urethane	0	46.3	14.5	10.8	2.5	-0.5	19.0
		2.5	69.5	25.9	17.6	0	-3.8	29.8
		12	89.4	43.6	20.9	0.7	0	24.3
4	M/100 Phlorrhizin	0	46.3	14.1	15.3	0	--	16.9
		2.5	70.2	27.5	14.1	0	--	28.6
		12	89.4	44.0	21.5	0.3	--	23.6
5	None	0	41.7	13.9	14.2	-1.2	0.9	13.9
		2.5	78.2	25.2	15.1	1.6	-3.7	40.3
		12	86.2	45.5	22.5	1.1	-0.4	17.5

Mycelium grown six days in 80 petri dishes with five percent glucose in the medium. Mycelium ground and diluted to 240 cc. To the flasks added: 40 cc. of mycelial preparation; 20 cc. of four percent glucose; 0.8 cc. of toluene; 2.7 cc. of M/10 iodoacetic acid, pH 6.7; 16 cc. of M/10 NaF, pH 6.85; 8 cc. of M/100 KCN, pH 6.75; 8 cc. of phlorrhizin, (0.4722 gms. in 10 cc.), and water to make the total volume 80 cc. Extraction of the mycelium in samples allowed for only one hour's time.

The data show very little difference between the various poisons. As in the other experiments, the total phosphorus, orthophosphorus and the difficultly hydrolyzable phosphorus fractions increased with time of autolysis. In addition, there occurred an increase in the pyrophosphate fraction. During the first two and one-half hours of autolysis, sodium fluoride retarded the increase of total acid-soluble phosphorus and inorganic phosphorus. Potassium cyanide, ethyl urethane and phlorrhizin likewise retarded the increase of the total acid-soluble phosphorus fraction but to a less extent than sodium fluoride. No appreciable difference in the effects of these poisons was observed.

From the results of the foregoing experiments (tables XVII, XVIII and XX) the situation obtained by using mycelial preparations is that of autolysis. Were the mycelial preparations carrying on any significant amount of respiratory activity, the effect of potassium cyanide and urethane would have perhaps resulted in an increased liberation of inorganic phosphorus as has been found by Engelhardt (27) for blood cells. On the other hand, should a fermentative activity be solely in operation, sodium fluoride (52, 53) as well as phlorrhizin (65, 66) would have resulted in an increased liberation of inorganic phosphorus because of their hindering action on glycolysis.

The effects of the added substances on the liberation

of inorganic phosphorus obtained in these experiments can be attributed to the action of enzymes concerned in autolysis. Sodium fluoride is known to retard the action of phosphatase enzymes (52, 53) while toluene is generally considered to be a plasmolyzing agent, effecting the liberation into solution of the various enzymatic constituents.

The observed increase in the pyrophosphate fraction in experiments 17 and 18 indicates the intermediate position of this fraction between inorganic orthophosphorus and organic phosphorus present in the mycelium of C. funicola. However, the relation of the pyrophosphate fraction to the acid-soluble phosphorus constituents is not clear. In table XX the data show inorganic phosphate, pyrophosphate and organic phosphate fractions to be materially increased simultaneously. In table XVIII, on the other hand, the data show that during the first two and one-half hours of incubation the total acid-soluble phosphorus fraction remains approximately the same while an increase in inorganic orthosphosphorus occurs. The source of this increase in phosphorus is derived mainly from the pyrophosphate fraction. This result is supported also by other experiments not included here.

It is further observed from table XVIII that the decomposition of the various acid-soluble phosphorus fractions is accompanied by an increase in the pyrophosphate fraction. This observed increase, however, is only in the presence of

toluene and not in the presence of sodium fluoride, a phosphatase inhibiting poison.

Carbonyl compounds in relation to the intermediate mechanism of glucose dissimilation by Chaetomium funicola.

The formation of carbonyl compounds in glucose degradation is recognized in the various schemes proposed for the mechanism of the process. Their formation in the dissimilation of glucose by fungi required demonstration and identification for a clearer understanding of the respiration process. Several means, based on their combining capacity with carbonyl reacting reagents, are available for their demonstration. In the present work the investigations of these compounds were made by utilizing their binding capacity with sodium bisulphite and 2:4-dinitrophenylhydrazine. Growing cultures and mycelial preparations were made.

Formation of carbonyl compounds in growing cultures. In Part I of the present work the analysis of the volatile neutral fraction revealed the production of very little if any volatile neutral compounds. Iodoform, 2:4-dinitrophenylhydrazine, Schiff's and the silver mirror tests were negative. In the absence of volatile neutral compounds an analysis of the entire medium was undertaken.

Sodium bisulphite binding capacity of the medium. For

the purpose of finding the sodium bisulphite binding capacity of the medium the liquid filtrate was analysed. The cultures used were part of the experiment reported in Part I with the different initial concentrations of glucose. The method used was that of Clift and Cook (20). The results obtained for the bound sodium bisulphite are shown in table XXI.

Table XXI. Sodium bisulphite binding capacity of the medium.

Time of incubation of culture in days	cc. of 0.005 N I_2 corresponding to the bound sodium bisulphite in 5 cc. of media filtrate. Concentration of glucose in percent				
	2.5	5.0	7.5	10.0	12.25
17	0.45	0.67	0.97	1.26	1.26
26	0.25	0.70	1.26	1.33	1.37
47	0.20	0.38	1.45	1.20	0.85

The data show that in no case was the amount of sodium bisulphite bound very large. The presence of and the increase in bound sodium bisulphite observed is attributed largely to the binding capacity of glucose alone as was determined by using different concentrations of glucose corresponding to the initial values. However, the occurrence of sulphite binding at 47 days of incubation for cultures on 2.5 and 5.0 percent initial concentrations of glucose could not be that caused by glucose since at this time glucose analyses for these cultures revealed almost zero amounts of glucose.

Formation of 2:4-dinitrophenylhydrazine derivatives. To the culture filtrate (400 cc.) obtained from C. funicola grown for six days on Czapek-Dox medium in petri dishes, were added 100 cc. of a one percent solution of 2:4-dinitrophenylhydrazine in hot 2 N HCl. The abundant reddish-orange amorphous precipitate (A) obtained after four hours of standing was separated and the filtrate allowed to stand for a further period of three days when a second precipitate (B) was obtained. The latter was of an orange color and granular in appearance. The two precipitates A and B were washed several times each with 2 N HCl and water. Treatment with sodium carbonate solution to remove the acid constituents of the precipitates was not followed because with such treatment an immediate blackening occurred with no appreciable removal of soluble material. The precipitates were further washed with 95 percent cold ethyl alcohol. Treatment with hot 95 percent ethyl alcohol produced with precipitate A a reddish solution in which was dissolved only a very small amount of material that precipitated out on cooling. The hot alcohol-insoluble residue was yellowish-brown; gave a violet color with alcoholic KOH (on evaporation produced a brown residue); with sodium carbonate solution a violet color; contained an ethyl acetate-soluble fraction which on removal of the solvent left an orange-red residue that turned a violet color with alcoholic

KOH and a brown residue on the evaporation of the alcohol. Treatment of precipitate B with hot 95 percent alcohol produced an orange-red solution in which was contained a considerable amount of orange-red material. With alcoholic KOH and sodium carbonate the same color formation and reaction as in the hot alcohol insoluble fraction of precipitate A were produced. Because of the similarity of the hot alcohol-insoluble residue of precipitate A and hot soluble fraction of precipitate B, precipitates A and B were bulked and the whole subjected to a solvent fractionation process with melting point determinations of the residues of the different fractions. It was hoped that possibly some fraction of the combined precipitates would yield characteristics common to some known derivative of 2;4-dinitrophenylhydrazine. In no case were the amounts of materials obtained sufficient for further analyses than melting point determinations. The following is the step-wise procedure followed, together with the results of the melting point determinations and color with alcoholic KOH.

1. Hot alcohol insoluble. Extracted with hot nitrobenzene.
   2. Hot nitrobenzene insoluble - small orange sphaerocrystals; colored alcoholic KOH violet. Melted at 260-268°C. leaving a residue of metallic grey sheen which did not disappear at 330°C. Repeatedly extracted three times with glacial acetic acid.

3. Glacial acetic acid insoluble - residue washed four times with ethyl alcohol and obtained an orange-yellow residue; m.p. 255°-275°C. leaving a residue of metallic-green sheen. Extracted with ethyl acetate three times.

4. Ethyl acetate insoluble - residue yellow-orange, sphaerocrystals darkened in color at 250° C. and melted at 257°C., leaving a residue of metallic-grey sheen at each crystal. Treated with pyridine (chloroform and absolute ethyl alcohol would do nothing).

5. Pyridine insoluble - yellowish-grey sphaerocrystals aggregated in clumps; did not melt but changed color at 280°C. No color with alcoholic KOH. Residue obtained on ashing.

5.5. Pyridine soluble - solution deep red; residue yellow, melting from 230°C - extracted with acetone.

6. Acetone insoluble - amorphous orange-red mass containing some of the crystals found in 5; violet color produced with alcoholic KOH; melted at 259°-262°C. Washing with acetone produced m.p. 258°-260°C. melting to a brown liquid.

6.6. Acetone soluble- solution red, residue

yellowish-orange amorphous flakes melting at 253°-255°C. to a brown liquid. Evaporation of a small amount of the acetone solution in a test tube, obtained long course crystals m.p. 245°C. and 259°-262°C. Extracted with ethyl acetate.

7. Ethyl acetate insoluble --.

7.7. Ethyl acetate soluble - solution yellow, residue metled at 238°-241°C. to a brown liquid in which were dissolved other materials. Extracted with acetone.

8. Acetone insoluble - yellow, m.p. 259°-261°C.; violet color, with alcoholic KOH.

8.8. Acetone soluble - solution yellow, residue melted at 238°-245°C. (mixture).

4.4. Ethyl acetate soluble - solution yellow, yellow sphaerocrystals m.p. 235°-240°-245°C. (mixture).

3.3. Glacial acetic acid soluble - water added to the solution and obtained a precipitate which was recrystallized four times in a similar manner and finally washed with alcohol; reddish-brown crystals, turned color at 210°C. and metled completely at 240°-242°C. to a clear brown liquid; violet color with alcoholic KOH. The wash alcohol yielded crystals

melting at 242°-245°C.

2.2. Hot nitrobenzene soluble - cooled.

9. Cool nitrobenzene insoluble - small orange sphaerocrystals; violet color with alcoholic KOH, m.p. 271°-273°C.; residue metallic grey sheen.

9.9. Cool nitrobenzene soluble. Fractional precipitation by evaporation.

10. Nitrobenzene difficultly soluble - orange-yellow crystals; melting at 265°-268°C. to produce a brown liquid.

10.10. Nitrobenzene readily soluble - red sphaerocrystals, melting at 249°-255°C. to a brown liquid and at 253°-256°C. to a brown liquid.

1.1. Hot alcohol soluble - Residue extracted with cold alcohol.

11. Cold alcohol insoluble - orange sphaerocrystals; m.p. 160°-240°C.; metallic grey residue formed. Extracted with glacial acetic acid.

12. Glacial acetic acid insoluble --

12.12. Glacial acetic acid soluble - by addition of water the solution obtained a precipitate; centrifuged, washed once

with alcohol and dried in air: orange-yellow crystals, softened at 200°C. and melted at 246°C. producing a brown liquid. Material appeared homogeneous.

11.11. Cold alcohol soluble. Fractional precipitation by evaporation of alcohol.

13. Evaporation of alcohol to one-half volume - small, round, red crystals, m.p. 162°-165°C., violet with alcbhoic KOH.

13.13. Evaporation of alcohol to one-fourth volume - orange red crystals, m.p. 135°C. and 216°C.

13.13.13. Evaporation of alcohol to completion. Orange-yellow crystals (powdery), m.p. 75°-80°C., 71°-74°C., violet color with alcoholic KOH. Red sphaerocrystals, m.p. 110°C. and approximately 230°C. Brownish red color with alcoholic KOH.

It is obvious from the above material that a large number of melting point ranges are obtained. On the basis of solubility in hot ethyl alcohol, the compounds may be devided into those possessing high and low melting points. The identity of any of these compounds is not known.

However, of interest in this connection are the 2:4-dinitrophenylhydrazones of the following: glyceraldehyde, 167°C., 265°C. (15), dihydroxyacetone, 163°C. (15), pyruvic acid, 216°C. and acetaldehyde m.p. 164°-165°C. (18, 101).

It may be mentioned that Matthiessen and Vorwerk (73) obtained from blood and urine a number of different 2:4-dinitrophenylhydrazones whose melting points were located over a wide range, from 81°C. to 245°C. and over 300°C. From aqueous extracts of pine wood rotted by Merulius lachrymans Boswell (14) obtained a number of different 2:4-dinitrophenylhydrazones that produced violet color with alcoholic KOH.

Formation of carbonyl compounds in mycelial preparations. Methylglyoxal, pyruvic acid and acetaldehyde assume an important place in the Neuberg scheme of carbohydrate dissimilation (81, 82). Their place in fungal metabolism is not clear. While acetaldehyde is frequently found to be a product of the dissimilation of glucose by fungi, the same cannot be said of the other two compounds. However, their formation is assumed, which assumption is supported by the isolation of methylglyoxal by Suthers and Walker (107) from Aspergillus niger preparations.

In the present work, the formation of methylglyoxal, pyruvic acid and acetaldehyde were sought from mycelial preparation of Chaetomium funicola.

First trial. The mycelium was grown for 12 days on 150 cc. of medium in five two-litre erlenmeyer flasks. The mycelium was washed, pressed and divided into two. One-half was stored over night at 0.5°C. while the other half was dried in vacuo over concentrated sulphuric acid. These mycelia were portioned out equally into two flasks each, the contents of the flasks being as follows:

Moist mycelium.	Flask number 1	2
2/3 M phosphate buffer, pH 6.8, cc.	5	5
2.5 percent sodium hexosediphosphate, cc.	20	0
20 percent glucose, cc.	0	10
Toluene, cc.	2	2
Water, cc.	15	25

Dried mycelium. Contents of the flasks the same as for the moist mycelium. With both the moist mycelium and dry mycelium of this first trial, residual 2:4-dinitrophenylhydrazine derivatives were obtained in two hours after the addition of this compound. The residue did not show any indications of melting. With alcoholic KOH, the residue formed a violet blue color characteristic for and presumeably of the bis-(2:4-dinitrophenylhydrazone) of methylglyoxal.

Second trial. The contents of the flasks were the same as in trial 1. The mycelium used was grown for 18 days on Czapek-Dox solution containing 10 percent sucrose and allowed to remain in contact for six hours with the same solution but in the absence of glucose. The mycelium was used immediately after washing.

Only yellow colloidal solutions were obtained on the addition of 2:4-dinitrophenylhydrazine after standing for three hours.

Third trial. In this trial Aspergillus niger was used in addition to Chaetomium funicola. The contents of the flasks were the same as in trial 1. The mycelia used were:

(a) C. funicola: mycelium grown for 12 days on Czapek-Dox medium in five two-litre erlenmeyer flasks; washed and kept in moist but pressed condition for 14 days at 0.5°C.

(b) Aspergillus niger: mycelium obtained from a gluconic acid fermentation; stored moist at 0.5°C. for six days.

The precipitates formed on standing for three hours after the addition of 2:4-dinitrophenylhydrazine were fractioned according to the method of Simon and Neuberg (101). The results obtained are presented in a tabular form, as follows:

Observations	Chaetomium funicola		Aspergillus niger	
	Glucose present: Na H.D.P.* absent	Glucose absent: Na H.D.P. present	Glucose present: Na H.D.P. absent	Glucose absent: Na H.D.P. present
1. Precipitate formed.	Abundant	Abundant	Very slight	Very slight
Color of precipitate.	Yellow	Brown	Dirty yellow	Dirty yellow
2. Sodium carbonate extract				
a) Color of solution	Reddish brown	Reddish brown	Colorless	Colorless
b) Addition of HCl.	Yellow precipitate. m.p. 200°-220°C.	Yellow precipitate, m.p. 200°-220°C.	No precipitate	No precipitate
c) Color precipitate with alcoholic KOH	Red	Red	--	--
3. Alcoholic extract	Reddish brown, m.p. approx. 150°C.	Reddish brown, m.p. approx. 150°C.	--	--
Color with alcoholic KOH	Blood red	Blood red	--	--
4. Residue				
a) Color and abundance	Dirty grey, abundant	Reddish, abundant	None	Dirty grey
b) Color with alcoholic KOH	Faint blue violet	Deep blue violet	None	Very faint blue violet

Fourth trial. An attempt was made in this trial to obtain enough product to fractionate and to purify the constituents of the 2:4-dinitrophenylhydrazine derivatives. Five flasks were prepared to contain the same constituents as trial 1. However, in all these flasks only glucose was added and not sodium hexosediphosphate. After 48 hours of incubation, trichloracetic acid was added to a five percent concentration and allowed to stand overnight.

Three days were allowed for the abundant formation of 2:4-dinitrophenylhydrazine precipitates. These were combined and fractioned. The results obtained are as follows:

Fraction	Recrystallization medium	Melting point	Color with alcoholic KOH
Methylglyoxal	Nitrobenzene	295°C.	Blue violet
Pyruvic acid	Alcohol	211°-214°C.	Reddish brown
Acetaldehyde	Alcohol	167°C.	Reddish brown

No mixed melting points were determined because the amount of material obtained was small.

Simon and Neuberg (101) give the following characteristics for the same hydrazones:

	Melting point	Color with alcoholic KOH
Methylglyoxal	298°C.	Blue violet
Pyruvic acid	216°C.	Reddish brown
Acetaldehyde	164°-165°C.	Reddish brown

Campbell (18) found 168°C. for acetaldehyde and 218°C. for pyruvic acid.

Availability of certain acids as carbon sources.

A good test for an intermediate in the dissimilation of carbohydrates is that the tested compound should be decomposed or utilized with the same ease and velocity as the glucose molecule itself. Since Chaetomium funicola did not accumulate any detectable amount of acid material in the medium the question arises as to whether the acids such as the dicarboxylic acids which normally arise and accumulate with many fungi (9, 10, 54, 92, 93), are formed and immediately utilized or whether they are not formed at all.

To obtain an answer to the question, cultures were prepared in 250 cc. erlenmeyer flasks on 50 cc. Czapek-Dox medium containing as carbon various organic acids. These acids were introduced into flasks in amount of 0.8 grams carbon previously adjusted to a pH of 6.4 by the addition of sodium hydroxide. The results obtained are presented in table XXII.

Only notes on the relative amounts and types of growth were obtained because in no case except that on glucose, was the mycelial development sufficient to make dry weight determinations. After a period of 43 days of incubation it was observed that development occurred only on fumaric, lactic and succinic and tartaric acids as well as on glucose.

Table XXII. Development of C. funicola on different acids as sources of carbon.

Organic acid	Development after 43 days	Further development 12 days after the addition of glucose
Acetic acid	None	No effect
Citric acid	None	No effect
Fumaric acid	Germination and very slight growth	Good surface growth as islets over the entire surface
Glucose		
Lactic acid	Gelatinous growth throughout, slight	Increased development on surface only as islets, less than with fumaric acid.
Malic acid	Germination and very slight growth	Growth throughout the medium, slight.
Maleic acid	None	No effect
Malonic acid	None	No effect
Succinic acid	Germination and very slight growth	Growth throughout the medium, slight
Tartaric acid	Germination and very slight growth	Growth throughout the medium, slight

Development on lactic acid was greater than on any of the other acids, being gelatinous and throughout the medium, whereas in the others only germination occurred. Because of this retarded growth of the fungus on these acids, at the time of 43 days of incubation 0.2 grams glucose in 10 cc. of water were added to each flask. This addition was hoped to reveal the unavailability of the acids to the fungus as well as to detect any toxic effect. The results obtained show that with additions of glucose no development was induced in those flasks in which the fungus failed to develop in the presence of only the acids, namely, in acetic, citric, maleic and malonic. However, with the other acids development was furthered by glucose in proportion to the amount of growth in its absence.

From the above results it is probable that maleic, malonic, citric and acetic acids are not formed or readily formed by C. funicola. The production of acetic acid observed by Lovell (61) is in contradiction to this observation, but Raistrick et al (94) observed no formation of volatile acids by their Chaetomium sp. Malonic and maleic acids are known (1) to be poisons of the reversible Szent-Gyorgyi oxidation-reduction system in animal tissues. Since development occurs on lactic, succinic, fumaric and malic acids support is had for glucose by C. funicola dissimilation to be according to the Chrzaszcz-Tiukow hypothesis.

DISCUSSION

Fungi in general are strongly aerobic organisms which do not develop under strictly anaerobic conditions (12). Their strong oxidative powers in the presence of an ample air supply endow them with the property of converting the sugar molecule completely to carbon dioxide, water and mycelium (12). Under certain conditions, certain of these fungi cause the formation and accumulation of various products such as benzenoid and non-benzenoid ring compounds and various pigments (92, 93), as well as compounds of the type of dicarboxylic acids. Because of its strong oxidative tendencies Chaetomium funicola is obviously a good representative of the group.

The oxidative breakdown of the glucose molecule by C. funicola is a slow process, the rate being dependent on its own synthetic activity. This relationship is not affected by altering the composition of the medium as to nitrogen, concentration of glucose, and concentration of phosphorus, as well as the initial pH of the medium; glucose decomposition occurring approximately in proportion to the amount of mycelium formed. The formation and accumulation of products in the medium are evident processes in the later stages of

development of the fungous cultures. The factors responsible for this accumulation are not known. In one experiment with 2.5 percent glucose, the mycelial weight decreased after 18 days in culture and the medium showed the formation of white crystalline material that separated out on concentrating the medium. Autolytic decomposition of the mycelium cannot entirely account for the accumulation of these compounds, however, because analyses show the fungus to continue to utilize glucose in a linear manner. Rather, it appears that they are normal products of the respiratory dissimilation of carbohydrates.

The nature of the crystalline material formed in the medium is not known. From the change in the hydrogen-ion concentration, these products appear to be mainly of the neutral type, since acidic compounds form only in the later stages of development.

The occurrence of an "anaerobic" phase in glucose breakdown by C. funicola is quite likely. The failure of C. funicola to grow in the presence of monoiodoacetate points to the occurrence of oxidation-reduction reactions in this fungus similar to those types of reactions presented in the anaerobic breakdown of carbohydrates by yeasts. Likewise the marked reduction of growth in the presence of sodium fluoride would indicate a necessary participation of the complete phosphatase enzyme system in carbohydrate metabolism (39).

Support for this phase is further given by the occurrence of acid-soluble phosphorus fractions in the mycelium similar to those of other living organisms such as yeasts.

The necessity of phosphorus for the development of C. funicola is characteristic of living organisms in general. Its significance is not yet clear. While Vorbrodt (118, 119) considered mineral phosphorus to have an important role in the mycelium of Aspergillus niger, Nord et al (86, 87, 88) have considered phosphorylation to be a minor process in the dissimilation of carbohydrates by Fusarium oxysporum and F. lini. These latter authors grew these fungi under conditions of nearly complete anaerobiosis, and considered only the medium. As has been shown in the present work, the site of phosphorus activity is probably entirely within the mycelium and not in the medium. Therefore, an investigation of the phosphorus in the mycelium became necessary in order to arrive at an understanding of the functional role of phosphorus with the fungi.

The demonstration in the present work of the occurrence of pyrophosphate phosphorus and other organic phosphorus fractions in the acid-soluble constituents of the mycelium of Chaetomium funicola and other fungi stands out as significant evidence for the concept of phosphorylation as a process in carbohydrate breakdown with these fungi. The failure to demonstrate the formation of phosphoglyceric acid as well as

significant phosphorus uptake by mycelial preparations cannot be considered evidence against phosphorylation in carbohydrate dissimilation by this organism. The difficulties encountered in such trials are similar to those observed by others for top yeasts when a separation of the enzymes from the cells cannot be readily effected (84). Likewise the use of mycelial preparations introduce certain difficulties (50).

The nature of the acid-soluble organic phosphorus fraction in the mycelium is at present obscure. It is certain that if either hexosediphosphate or Neuberg-ester are present they must be present in very small amounts. Since the organic phosphorus is mainly of the difficultly hydrolyzable type, one must consider for its composition the Embden and Robison esters, phosphoglyceric acid, diphosphoglyceric acid and possibly other esters. The inclusion of one or several of these compounds is not impossible and indeed would not be surprising if so demonstrated, particularly since recent work (47, 48, 49) has shown the formation of difficultly hydrolyzable phosphorus esters by oxidative phosphorylation where phosphoglyceric acid is the main ester formed.

Pyrophosphate phosphorus has been found to occur in many living organisms and tissues (51, 56, 57, 58, 59). No similar observation, with the exception of that of Nord (87), seems to have been made with filamentous fungi. Since Nord

et al (87) postulate the presence of adenosinetriphosphate and adenylic acid in the mycelium of Fusarium oxysporum and F. lini and their method was that of deamination by muscle tissue preparations, the logical conclusion from the presence of pyrophosphate in Chaetomium funicola as well as with the other fungi examined would be that the phosphorus thus demonstrated occurs as adenosinetriphosphate in the mycelium of these fungi.

The close positive relationship between the pyrophosphate content and glycolysis have been repeatedly observed for nucleated and non-nucleated blood cells (27, 28, 30). The functional role of the pyrophosphate in the mycelia of fungi examined, might be considered that of a co-phosphorylase and interpreted in a manner similar to that applied to many other tissues (67, 74, 91). von Euler and Brandt (30) and Engelhardt (27, 28), believe it permissible to consider adenosine-triphosphate as a constituent of the co-ferment complex of respiration.

The demonstration in the present work for the formation of carbonyl compounds such as acetaldehyde, pyruvic acid and methylglyoxal cannot be considered evidence against the phosphorylation mechanism of carbohydrate dissimilation by these fungi. Recent work has shown methylglyoxal to arise from one of the phosphorylated intermediate compounds of carbohydrate dissimilation in a secondary process,

while pyruvic acid arises from phosphopyruvic acid.

SUMMARY

1. Chaetomium funicola Cke. was studied for the manner of glucose breakdown in Czapek-Dox medium and for the probable intermediate mechanism of such breakdown, especially the role of phosphorus.

2. On Czapek-Dox medium with glucose as the sole source of carbon the carbon of the glucose decomposed was converted mainly to carbon dioxide and mycelium for approximately the first 18 days of incubation. Subsequent to this time there occurred an accumulation in the medium of non-volatile products.

3. Non-volatile acids were not formed in appreciable amounts.

4. Volatile compounds were formed in only small amounts during the entire period of 33 days of incubation. The formation of volatile acids as determined by titration and volatile neutral compounds were negligible in amounts. Steam distillation carried over small amounts of materials in the volatile acid fraction.

5. The hydrogen-ion concentration of the medium moved progressively towards the alkaline side for 18 to 26 days of incubation, subsequent to which a decrease occurred.

6. Ether extraction of the concentrated culture medium removed a brown liquid fat. Crystalline materials that had different melting points were also removed.

7. Aged cultures on 2.5 percent initial glucose in the medium produced small amounts of a white crystalline material that separated out on concentration of the medium.

8. Changing the glucose concentration of the medium from 2.5 to 12.5 percent revealed glucose utilization to follow a linear path, the rate of utilization being higher with higher concentrations of glucose. The mycelial weights increased uniformly at all concentrations of glucose for approximately the first 18 days of incubation subsequent to which a break occurred. At lower concentrations of glucose (2.5 percent) the mycelial weights decreased whereas with higher concentrations of glucose the increases in mycelial weight continued.

9. Changing the initial pH of the medium revealed that C. funicola grows readily over a wide range of pH but fails to grow at an initial pH of 2.12. Growth was reduced more at an initial pH of 2.90 than at 8.68. In all cases, the pH of the medium was maintained on the alkaline side or was shifted in that direction. Glucose utilization was approximately proportional to the amount of mycelial growth.

10. Ammonium salts were more ready sources of nitrogen than were nitrate salts. With the former salts the medium was

soon rendered sufficiently acid to retard further mycelial development. Nitrite nitrogen was not available and probably toxic.

11. Increasing the concentration of dihydrogen potassium phosphate in the medium above the normal (one gram KH_2PO_4 per one litre of Czapek-Dox medium) resulted in lower amounts of mycelial growth and slightly increased glucose utilization. A concentration of KH_2PO_4 one-fourth of normal resulted in a slightly lower glucose utilization without affecting the amount of mycelium formed.

12. Agitation and aeration of the medium caused C. funicola to grow throughout the medium but did not accelerate glucose utilization; only 18.3 percent glucose being utilized in 18 days of incubation.

13. C. funicola failed to grow on Czapek-Dox medium containing monoiodoacetate concentrations of M/1000 or greater. With concentrations of M/10,000 growth was reduced by 50 percent.

14. In the presence of M/500, M/200 and M/50 sodium fluoride concentrations in Czapek-Dox medium, the growth of C. funicola was 26, eight and three percent of the normal, while at M/10 concentration the fungous spores failed to germinate.

15. Phosphoglyceric acid could not be isolated from mycelial preparations of C. funicola by following the method used for yeasts and bacteria, even when the conditions and

the amount and kinds of materials were varied.

16. Phosphorus uptake by mycelial preparations were very small. At times a liberation of inorganic phosphorus occurred.

17. Development of living cultures on different concentrations of phosphorus in the medium and different initial pH values revealed the amount of phosphorus removed from the medium to be approximately proportional to the amount of mycelial growth. Organic phosphorus formation in the medium was very slight.

18. The fungi Fusarium lini Bolley, F. oxysporum Schl. v. cubense (E.F. Sm.) Wr. et Rg., F. bulbigenum Cke. et Mass. v. niveum (E.F. Sm.) Wr., Chaetomium funicola Cke. and Aspergillus niger v. Tieghm were analyzed by Lohmann's method for the different acid-soluble phosphorus fractions contained in the mycelium.

19. Inorganic orthophosphorus was found to be present in only small amounts, constituting with the different fungi 15 to 25 percent and possibly less of the total acid-soluble phosphorus.

20. A pyrophosphate fraction was found to be present with all the fungi, its percentage of the total acid soluble phosphorus being with A. niger, 22.7 percent; Chaetomium funicola, 49.7 percent; Fusarium lini, 39.0 percent; F. cubense, 58.4 percent and F. niveum, 51.8 percent. Its identity with adeno-

sinetriphosphoric acid was considered.

21. Phosphorus in organic combination was found to constitute approximately 30 percent of the total acid-soluble phosphorus with Fusarium and Chaetomium spp., while greater than 50 percent with Aspergillus niger. This phosphorus is characterized by extreme difficulty of hydrolysis in N/1 HCl at 100°C., resembling in this respect the Embden or Robison esters.

22. Phosphagen phosphorus was not found.

23. Mycelium of Chaetomium funicola 18 days old was found to contain greater amounts of pyrophosphate phosphorus than mycelium six days old.

24. Autolysis caused the mineralization of phosphorus. The inorganic phosphorus formed arose from the pyrophosphate, acid-soluble organic phosphate and colloidal phosphate phosphorus fractions. The effect of poisons on autolysis was studied. No definite inter-relationship was observed between the various phosphorus fractions using mycelial preparations.

25. Carbonyl compounds were found to be present in the medium. While sodium bisulphite did not show any binding capacity with constituents of the medium, 2;4-dinitrophylhydrazine yielded a large number of fractions with different melting points. Methylglyoxal, pyruvic acid and acetaldehyde were demonstrated in mycelial preparations containing glucose

and sodium hexosediphosphate either together or alone.

26. Fumaric, lactic, malic, succinic and tartaric acids were found to be carbon sources for C. funicola whereas acetic, citric, maleic and malonic were not.

27. The relationship of the above findings to phosphorylation as an intermediate mechanism of carbohydrate dissimilation by C. funicola is discussed.

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