Studies on the Metabolism of Mould Fungi

1. PHOSPHORUS METABOLISM IN MOULDS

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Phosphorus, together with nitrogen, sulphur, magnesium and organically bound carbon, has been one of the few elements long recognized as indispensable for the growth and metabolic activities of the mould fungi (Lafar, 1904). Much later other elements, such as iron, copper, zinc, manganese, molybdenum and gallium, were added to the list of essential nutrients (Foster, 1939). It has been repeatedly shown that many moulds, including numerous species of *Aspergillus*, *Penicillium* and *Rhizopus*, are unable to grow unless small amounts of phosphate are present in the nutrient medium.

The first systematic study of the phosphorus metabolism in moulds was carried out by Vorbrodt (1920, 1926, 1928), who demonstrated that growing mycelia of *Aspergillus niger* take up orthophosphate from the culture media and store it partly in the inorganic form and partly in the form of new compounds. More recently, Michel-Durant (1938) observed that the uptake of orthophosphate by *A. niger* is insignificant at the early stage of mycelial growth but that it assumes bigger proportions at the stage of the formation of the conidia. According to this author, there are three groups of phosphorus compounds in the mycelium, a small 'lipoid fraction' and two ethanol-insoluble fractions, one of which is soluble in trichloroacetic acid ('phosphore organique soluble indéterminé') and the other acid-insoluble, represented by the non-extractable residue.

The paucity of data concerning the chemical nature of the phosphorus compounds in moulds and their metabolic function is in striking contrast to the wealth of information available regarding phosphorus compounds in yeast, bacteria, higher plants and animal tissues. It seemed, therefore, desirable to investigate the nature and behaviour of phosphorus compounds in moulds, and to examine the possibility of the existence in moulds of a link between the phosphorus metabolism and other processes, such as the metabolism of carbohydrate. (Preliminary communication, Mann, 1943.)

EXPERIMENTAL

The stock cultures used for inoculation were grown on beerwort-agar slopes and were subcultured once a month. The majority of the experiments was

carried out with *A. niger* v. Tiegh. (National Collection of Type Cultures (N.C.T.C.) no. 594), but occasionally other moulds were used, such as *A. niger citricus* Neuberg (N.C.T.C. no. 1692), *A. niger* Thaysen (N.C.T.C. no. 603), *Penicillium oxalicum* Currie-Thom (N.C.T.C. no. 983) and *P. notatum* Westling-Fleming (N.C.T.C. no. 4222). The culture media were composed of 10% glucose, 0.5% NaNO_3 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, with varying amounts of K_2HPO_4 , dissolved in tap water. 50–300 ml. portions of the medium were used and distributed in crystallizing dishes to a depth not exceeding 1.5 cm. The inoculum amounted to approximately 1 mg. of spore material (dry wt.) per 100 ml. medium. The mycelia were usually grown at 30° under aseptic conditions.

The disappearance of glucose from the medium of the growing culture was followed quantitatively by the method of Benedict. The total amount of organic acids produced and excreted by the mycelium into the medium was assessed by titration with 0.1 N-NaOH in presence of phenolphthalein as indicator. Owing to the presence of gluconic acid in lactone form, a rather long time was required to reach the end-point of titration. Besides gluconic acid, the two other main organic acids produced were citric acid and oxalic acid. Citric acid was determined gravimetrically as pentabromoacetone and oxalic acid as calcium oxalate.

Before performing any phosphorus analysis of the mycelium, the medium was siphoned from beneath the mycelium and replaced by the same volume of water. Care was taken not to submerge any part of the surface of the mycelium. After two more changes of water at 8 hr. intervals, the mycelium was spread evenly on a glass plate and quickly divided with a sharp knife into several parts. Some of the material was used for the estimation of the dry weight (at 105°) and for the determination of the total content of phosphorus, and the remainder was thoroughly extracted with cold trichloroacetic acid. This extract, which contained the *total acid-soluble phosphorus*, was used for the estimation of: (1) the true inorganic phosphorus determined as MgNH_4PO_4 ; (2) phosphorus determined as phosphate which reacts directly with ammonium molybdate, P_0 ; (3) the phosphorus which appears as orthophosphate after 7 and 30 min. hydrolysis with N-HCl, P_7 and P_{30} ; (4) phosphorus as the total phosphate after incineration with H_2SO_4 and HNO_3 , P_{tot} (Lohmann & Jendressik, 1926).

Nitrogen was estimated by the Kjeldahl procedure. The assays for nicotinic acid, riboflavin and aneurin were very kindly undertaken by Dr E. Kodiček and Dr Y. L. Wang of the Nutritional Laboratory, Cambridge.

Growth and metabolism of Aspergillus niger in presence of varying concentrations of phosphate

With 0.002% K_2HPO_4 or less in the culture medium no growth or only a very poor growth of *A. niger* was observed and conidia appeared very late, if at all. With 0.02% K_2HPO_4 , thin mycelia appeared 2 days after sowing and developed within the next 2 days into strong, white, smooth pellicles. Usually on the 4th day of growth the first black conidiophores made their appearance. With 0.2–0.5% K_2HPO_4 the growth was still quicker, and within 4 days a very thick mycelium was produced which, instead of being white and smooth, showed a creased surface and was of a distinctly yellowish colour, especially at the surface in contact with the medium. No conidiophores were present at that stage. They usually appeared on the 6th or 7th day of growth.

Both the rate at which phosphate was absorbed from the medium as well as the concentration which it finally reached in the mycelium were found to depend on the initial amount of K_2HPO_4 in the medium. With 0.02% K_2HPO_4 in the medium, the rate of disappearance was so rapid that after 3 days of growth the medium was completely depleted of phosphorus; the final concentration of phosphorus reached in the mycelium was about 0.3% P (dry wt.). With 0.2–0.5% K_2HPO_4 in the medium, the rate of absorption of phosphate by the growing mycelium was somewhat slower during the first days of growth and reached a higher level only at

the preconidial stage; the final concentration of phosphorus in the mycelium was 1–2% P (dry wt.).

Effect of phosphate concentration on metabolism. By varying the concentration of phosphate in the medium it was possible to influence markedly both the carbohydrate and the nitrogen metabolism of the mould, as well as its respiration. Table 1 illustrates the differences between two cultures, A and B, grown in the presence of 0.02 and 0.2% K_2HPO_4 , respectively. The culture B grew faster than the culture A; it utilized glucose more rapidly and turned a higher proportion of it into citric acid. In addition, it was capable of building up a much larger store of nitrogen than the phosphorus-deficient culture A. The nitrogen stored in the mycelium, derived from the nitrate of the medium, was found to be entirely of organic nature; neither nitrate nor free ammonia could be detected. Approximately one-half of the mycelium-N could be extracted with trichloroacetic acid or with other deproteinizing reagents. Among the N-containing substances thus extracted were aneurin, riboflavin and nicotinic acid. It is of considerable interest to note that in response to an increased concentration of inorganic phosphate in the medium, the formation of all three vitamins was enhanced to such an extent as to compare favourably with rich vitamin sources such as liver or yeast. The high riboflavin content is responsible for the yellow colour of the phosphorus-rich mycelium, and also, it may have some bearing on the fact that such a mycelium, when separated from its medium and transferred to water, continued to respire for several days much more vigorously

Table 1. *Effect of varying concentration of orthophosphate on the metabolism of A. niger*

	A grown in presence of 0.02% K_2HPO_4	B grown in presence of 0.2% K_2HPO_4
Mycelium (5 days old)		
Appearance	Thin, white, smooth, first conidiophores	Thick, yellowish, no conidiophores
Dry weight	460 mg.	1092 mg.
Q_{O_2} of intact mycelium	6.2	11.4
Q_{O_2} of mycelium pulp	0.4	1.9
Q_{O_2} of pulp + 1% glucose	30.0	19.0
Total nitrogen, mg.	8.1	23.7
Total phosphorus, mg.	1.5	12.1
Magnesium, mg.	3.8	6.8
Aneurin, μ g.	3.2	19.0
Riboflavin, μ g.	16.1	78.7
Nicotinic acid, μ g.	19.4	302.0
Medium (50 ml.)		
Appearance	Colourless	Yellow
Glucose, %	3.7	0.1
Glucose oxidase activity; 2 ml. medium + 10 mg. glucose, pH 7, 30°, take up in 15 min.	120 μ l. O_2	30 μ l. O_2
Total phosphorus, mg.	0.0	6.3
Titratable total acidity, ml. N-acid	15.0	16.0
Citric acid, ml. N-acid	3.8	7.9
Oxalic acid, ml. N-acid	0.05	0.0

than the P-deficient mycelium. Grinding of the mycelia with water caused a pronounced fall in the O_2 uptake, but even then, the pulp obtained from the mycelium B had a much higher rate of O_2 uptake than that prepared from mycelium A. When, however, glucose was added to the pulp, the O_2 uptake increased more in A than in B. This was due to a higher activity of glucose oxidase in the mycelium A.

The absorption of phosphate from the medium and the formation of phosphorus compounds in the mycelium

Absorption of phosphate. The experiments recorded in Table 2 were carried out with *A. niger* v. Tiegh. in crystallizing dishes containing 50 ml. of the medium described, with 0.2% K_2HPO_4 and grown at 30°. Thin mycelia usually appeared 24 hr. after sowing, and the production of gluconic and citric acid, at the expense of glucose, soon followed. At the same time, the absorption of phosphate from the medium began to be noticeable. Occasionally the rate of the absorption of phosphate by the growing mycelium was rather slow during the first days of growth and reached a higher velocity only at the preconidial stage. In other instances, however, the absorption proceeded rapidly from the beginning of the growth and was well marked at an early stage of growth, several days before the appearance of the first conidiophores.

Formation of phosphorus compounds in the mycelia. In order to follow the fate of the absorbed phosphate, a study was made of the content and the distribution of phosphorus compounds in the mycelia. A large

proportion of the phosphorus which accumulated in the mycelia, sometimes as much as 90%, could be extracted by grinding the mycelia thoroughly with trichloroacetic acid. Only a small proportion of the acid-soluble phosphorus, 10–20% in all, behaved like ordinary orthophosphate, and the value of the phosphorus determined directly (P_0) equalled that in the magnesium-ammonium-phosphate precipitate. A large proportion of the total acid-soluble phosphorus, as assessed by incineration with H_2SO_4 and HNO_3 , broke down completely to orthophosphate after a 7 min. hydrolysis with 1N-HCl at 100°, and thus behaved like a typical 'easily acid-hydrolyzable phosphorus compound'. Only a small proportion of the phosphorus present in the mycelium was found to occur in a 'difficultly hydrolyzable' form.

The formation of the phosphorus compounds in the mycelium usually reached its peak at the time when the first conidiophores were appearing. At the same time the medium was already depleted of 50–90% of the orthophosphate originally present, as well as of glucose, and contained large quantities of gluconic acid and citric acid. When the concentration of these two acids had reached its maximum, oxalic acid was still absent, and could be found only later, after the mycelium had lived for several days at the expense of the first two organic acids. As soon as the reserve of gluconic and citric acid was exhausted, the process of autolysis set in, heralded by a decrease in the dry weight of the mycelium and accompanied by a rapid decomposition of the phosphorus compounds in the mycelium and the reappearance of orthophosphate in the medium.

Table 2. *Phosphorus metabolism in growing cultures of A. niger*

(P_0 =phosphorus determined as phosphate which reacts directly with ammonium molybdate; P_7 =the phosphorus which appears as orthophosphate after 7 min. hydrolysis with N-HCl; P_{30} =the phosphorus which appears as orthophosphate after 30 min. hydrolysis with N-HCl; $P_{tot.}$ =phosphorus as the total phosphate after incineration with H_2SO_4 and HNO_3 .)

		Mycelium					Medium (50 ml.)					
Age (days)	Appearance	Dry wt. (g.)	Trichloroacetic acid extracts mg. P				Glucose (g.)	Total acidity (ml. N-acid)	P_0 (mg.)	$P_{tot.}$ (mg.)	Citric acid (ml. N-acid)	Oxalic acid (ml. N-acid)
			P_0	P_7	P_{30}	$P_{tot.}$						
0		—	—	—	—	—	5.0	0.0	17.8	17.8	—	—
I 2	Thin, continuous	0.506	0.4	1.6	—	—	—	3.2	—	—	0.6	0.0
3	White, strong	1.056	1.5	5.0	5.5	6.5	1.1	15.0	10.5	10.5	3.9	0.0
5	First conidia	1.340	1.5	9.0	9.5	11.0	0.1	18.0	5.0	5.0	6.9	0.0
9	Many conidia	1.728	1.1	12.3	12.9	14.0	0.0	5.5	0.0	0.0	5.5	0.1
14	Autolysis	0.932	2.6	4.8	—	—	0.0	0.3	8.0	9.6	0.0	0.3
II 2	Thin, continuous	0.352	0.2	1.5	1.5	1.8	1.1	6.5	16.0	16.0	—	—
4	White, strong	0.760	0.7	5.7	6.1	10.0	0.3	12.5	—	—	—	—
6	Few conidia	1.356	2.3	9.0	10.3	12.0	0.0	7.5	0.0	0.0	—	—
14	Autolysis	0.816	4.6	6.3	6.3	6.8	0.0	0.0	6.0	6.0	—	—
III 3	White, strong	0.320	1.5	5.0	—	—	1.1	13.0	—	—	4.0	0.0
5	First conidia	0.792	0.7	8.6	—	—	0.0	15.5	—	—	4.6	0.0
8	Many conidia	1.150	0.6	10.0	—	12.0	0.0	8.0	0.0	0.0	5.0	0.0
12	Onset of autolysis	0.920	—	—	—	—	0.0	2.0	0.3	0.5	1.0	1.0
19	Autolysis	0.510	—	—	—	—	0.0	0.0	11.0	12.0	0.0	Trace

Table 3. *Formation of an easily acid-hydrolyzable phosphorus compound in moulds*

Species	Mycelium			Medium (50 ml.)		
	Dry wt. (g.)	Trichloroacetic acid extracts (mg. P)		Glucose (g.)	Acidity (ml. N-acid)	*P _o (mg.)
		*P _o	*P ₇			
<i>A. niger</i> v. Tiegh.	1.196	2.7	9.4	0.0	9.5	2.5
<i>A. niger citricus</i> Neuberger	1.008	0.6	1.3	0.8	11.5	12.5
<i>A. niger</i> Thaysen	1.252	5.3	6.6	0.0	1.5	2.5
<i>P. oxalicum</i>	0.624	0.2	3.0	3.3	0.75	10.0
<i>P. notatum</i>	0.960	1.3	4.7	Trace	10.5	10.0

* See note to Table 2.

The formation of easily hydrolyzable phosphorus from orthophosphate is not restricted to one strain of *A. niger* but was found also with other moulds (Table 3). However, the amounts of the readily hydrolyzable phosphorus formed by these moulds were smaller than with *A. niger* v. Tiegh.

Effect of oxygen and respiratory inhibitors on phosphorus metabolism. The absorption of phosphate from the medium and the formation of phosphorus compounds in the mycelium were found to depend strictly on the presence of oxygen. In this respect the phosphorus metabolism of moulds differs substantially from the various phosphorylation processes which occur in yeast and in animal tissues. Cyanide and azide (0.001N), iodoacetate (0.001N) and fluoride (0.005N) were found to have a powerful inhibitory effect on the respiration as well as on the carbohydrate metabolism of the moulds; at the same time these inhibitors strongly suppressed the phosphorus metabolism.

The effect of these inhibitors could best be studied on mycelia grown directly in the cups of Barcroft differential manometers (Table 4).

A. niger was sown on 5 ml. medium. During the early stages of growth the shaking of the manometers had to be

avoided owing to the great fragility of the fresh mycelium. With a continuous mycelium gentle shaking could be safely applied, but it had little effect on the extent of the oxygen consumption owing to the fact that the mycelium respired mainly on the surface. After 4 days of growth at 30° the manometer cups containing the pellicles were attached to the manometers and the course of the respiration was followed quantitatively. The oxygen uptake of the mycelia was measured at 30°, and expressed in terms of

$$Q_{O_2} = \frac{\mu\text{l. } O_2 \text{ taken up}}{\text{mg. dry wt.} \times \text{hr.}}$$

On the addition of inhibitors the absorption of phosphate from the medium came suddenly to a standstill. The action of the inhibitors on the respiration and the phosphate absorption could be reversed provided the mycelium had not been left in contact with the poison for more than a few hours. Otherwise an autolysis set in and the phosphorus compounds formed in the mycelium broke down rapidly with the liberation of free phosphate.

In order to demonstrate the effect of oxygen on the phosphate metabolism it was found convenient to grow mycelia for several days on media deficient in phosphate (0.01–0.02 % instead of 0.2 % K_2HPO_4), but otherwise composed as described above. If these mycelia were then transferred to 0.1 % KH_2PO_4 in

Table 4. *Effect of respiratory inhibitors on the phosphorus metabolism*

Mycelium grown on 5 ml. medium in Barcroft manometer cups; 4 days at 30°.

Addition to the medium		Analyzed after (hr.)	Mycelium				Medium	
Substance	Concentration (M)		Dry wt. (mg.)	Q_{O_2}	*P _o (mg.)	*P ₇ (mg.)	*P _o (mg.)	*P ₇ (mg.)
—	—	—	90	12.7	0.07	0.60	0.08	0.08
Iodoacetate	0.001	3	90	3.6	0.09	0.60	0.09	0.09
	0.001	24	82	1.6	0.09	0.40	0.30	0.32
Fluoride	0.005	3	—	5.8	—	—	—	—
	0.005	24	80	2.3	0.09	0.40	0.10	0.10
	0.01	3	—	4.8	—	—	—	—
	0.01	24	80	1.9	0.05	0.40	0.20	0.25
	0.02	3	—	1.3	—	—	—	—
	0.02	24	60	0.5	0.05	0.40	0.20	0.25
Azide	0.001	3	—	2.8	0.07	0.50	0.10	0.10
	0.001	24	65	1.3	0.09	0.40	0.30	0.32
Cyanide	0.001	3	90	1.5	0.08	0.55	0.10	0.10

* See note to Table 2.

the presence of oxygen, they quickly utilized the inorganic phosphate and built up from it the easily hydrolyzable phosphorus compound (Fig. 1, curve I). Anaerobically, there was no change in the content of KH_2PO_4 in the medium (Fig. 1, curve II). When, after a period of anaerobic incubation, oxygen was let in, the mycelium responded immediately with renewed absorption of phosphate. However, the absorption never reached the same level as with mycelia which had not been exposed to anaerobic conditions. The reason is that even a short period of anaerobiosis has a general adverse effect on the

Fig. 1. The absorption of phosphate from the medium in presence and absence of oxygen

metabolic activities of the mycelium. A prolonged lack of oxygen was found to lead invariably to autolysis.

When mycelia were transferred to neutralized solutions of sodium pyrophosphate or of certain

sugar-phosphoric acid esters instead of to orthophosphate, the process of phosphate absorption was slower because it was preceded by the breakdown of the phosphoric compounds into orthophosphate (Table 5). Glucose had a slightly delaying effect on the process of phosphate absorption.

Metaphosphatase

It is essential to use trichloroacetic acid for the successful extraction of phosphorus compounds from the mycelium. When the mycelium was ground with water instead of with trichloroacetic acid, the acid-hydrolyzable phosphorus compounds of the pulp broke down within a short time to orthophosphate. They could be preserved, however, either by heating the pulp to 100° and keeping it at this temperature for a few minutes or by rapidly dehydrating the pulp with acetone and converting to a dry powder. No losses in the acid-soluble phosphorus occurred during the acetone treatment which, however, did remove all acetone-soluble substances including some acetone-soluble, difficultly hydrolyzable phosphorus compound. The distribution of phosphorus, nitrogen and magnesium, between the acetone extract, the acetone powder and the trichloroacetic acid extract from the acetone powder of one mycelium, is illustrated by the scheme shown on p. 344.

The assumption that the breakdown of phosphorus compounds in the mycelium ground with water might be due to the presence of a phosphatase was strengthened by the observation that if such a pulp was brought into contact with a variety of phosphoric acid derivatives, it decomposed them quickly into orthophosphate.

The phosphatase contained in the mycelium could be extracted with water without any loss of activity. The extract (1 ml.; 12 mg. dry wt.), when acting upon 2 ml. substrate (Na salts; 1.2 mg. P) for 3 hr. at pH 5 and 30° , converted to orthophosphate 70–98% of the following compounds: pyrophosphate, metaphosphate, hexametaphosphate, α -phosphoglycerol, β -phosphoglycerol, Cori ester (1-phosphoglucose), Embden ester (6-phospho-hexose),

Table 5. *Metabolism of pyrophosphate and sugar-phosphoric acid esters by the mycelium*

Mycelium grown in manometer vessel. On the fifth day medium replaced by 5 ml. of:	Incubation at 30° (hr.)	Q_{O_2} of mycelium	Media		
			*P _o (mg.)	*P _i (mg.)	Acidity (ml. N-acid)
Water	0	8.3	0.0	0.0	0.0
	16	6.3	0.05	0.05	0.25
Sodium pyrophosphate (4 mg. P)	0	9.2	0.0	4.0	0.0
	16	10.0	2.5	3.0	0.14
K salt of Cori ester (6 mg. P)	0	9.4	0.0	6.0	0.0
	16	12.8	3.9	4.5	0.16
Na salt of Embden ester (6 mg. P)	0	8.6	0.0	0.1	0.0
	16	11.9	2.5	2.5	0.08

* See note to Table 2.

Mycelium (3.6 g. dry wt.)	
1.08% P, 2.32% N, 0.555% Mg	
Acetone extract	Acetone powder
0.023% P, 0.045% N, 0.01% Mg	1.05% P, 1.87% N, 0.550% Mg
	Trichloroacetic acid extract (80 ml.)
	0.85% P (0.024% P _o , 0.790% P _i)
	0.825% N, 0.525% Mg

Harden-Young ester (1:6-diphospho-fructose), 3-phosphoglycerate, adenosine triphosphate (all three atoms of phosphorus), muscle adenylate, yeast adenylate, aneurin diphosphate and phytate.

The outstanding property of this polyphosphatase, and one which sets it apart from other known phosphatases, is the ease with which it acted on metaphosphate. This enzyme may therefore be referred to as a *metaphosphatase*.

The only instance where metaphosphatase activity has been previously described in moulds is the observation made by Kitasato (1928 *a, b*), who found that preparations of 'Taka-phosphatase' from *A. oryzae* are active towards metaphosphate. He had to use, however, as much as 1 g. of Taka-phosphatase and incubate this preparation for 20 hr. with 250 mg. sodium metaphosphate in order to obtain 10% of the phosphorus in the form of orthophosphate. Compared with the metaphosphatase of *A. niger* the activity of 'Taka-phosphatase' appears to be negligible.

Metaphosphatase in metabolism fluid. The examination of the culture media separated from the mycelia of *A. niger* revealed that they too possessed considerable metaphosphatase activity; 2 ml. medium separated from a 4-day-old mycelium and incubated for 5 hr. with 2 ml. sodium metaphosphate (0.6 mg. P) converted it completely to orthophosphate. Incidentally, the presence of the phosphatase in the medium provides an explanation for the observation that organic phosphorus compounds added to the culture media are first dephosphorylated and then absorbed as orthophosphate.

Purification of metaphosphatase. In order to purify the metaphosphatase and to obtain the enzyme free from glucose oxidase (always present alike in the mycelium and the medium), the aqueous extracts from the mycelia were dialyzed, the enzyme was precipitated with 3 vol. of cold acetone, re-dissolved in water and finally purified by adsorption on tricalcium phosphate gel and alumina C₇. In this manner it was possible to obtain metaphosphatase preparations practically free from glucose oxidase. In addition, it was found that 0.01 M-sodium nitrate had a strong inhibitory effect on the activity of glucose oxidase at pH 5 (but not at pH 7), whereas it did not affect the activity of metaphosphatase at all. The optimum activity of metaphosphatase oc-

curred at pH 3.7-4.2. A purified enzyme preparation (60 µg.) incubated with a solution of sodium metaphosphate or pyrophosphate (0.3 mg. P) for 2 hr. at pH 5 and 30° converted both compounds quantitatively to orthophosphate.

DISCUSSION

Unlike the animal tissues and yeast cells, the metabolic activities of the mould fungi, especially those of the acid-producing species like *Aspergillus* and *Penicillium*, depend to a large extent on the presence of oxygen. *Aspergillus niger*, for instance, will oxidize sugar very efficiently in the presence of oxygen to non-volatile organic acids such as gluconic, citric and oxalic acid, whereas anaerobically it scarcely ferments sugar at all. This phenomenon was well known to the early investigators, and Duclaux in his *Traité de Microbiologie* (1898) very appropriately referred to the process of oxalic acid formation in moulds as 'respiration oxalique', a term which is more suitable than the later 'oxalic fermentation' or 'oxidative fermentation'. The experiments described in this paper demonstrate for the first time that in addition to the carbohydrate metabolism, the phosphorus metabolism of *A. niger* is also strictly aerobic in character. In this respect the moulds differ again from yeast, where the presence or absence of oxygen is said to be of little importance so far as the absorption of phosphate is concerned (Mullins, 1942). The intimate relationship between the phosphorus metabolism and the respiration of *A. niger* is best demonstrated by the fact that various inhibitors of mould respiration, such as cyanide, azide, iodoacetate and fluoride, also stop phosphorus metabolism. This fact indicates a connexion between the metabolism of carbohydrate and that of phosphorus compounds in moulds. Moreover, an increase in the content of phosphate in the culture medium enhances both the utilization of carbohydrate and the formation of organic acids, particularly citric acid. However, the increased production of citric acid may also be due to changes in the nitrogen metabolism.

Mycelia grown in the presence of high phosphate concentrations have a much higher nitrogen metabolism than those grown on phosphate-deficient media and are capable of building up a considerable reserve of various valuable nitrogenous substances.

Another point which emerged is that the mycelia of various moulds convert a large proportion of the absorbed orthophosphate into acid-hydrolyzable phosphorus compounds. One of these compounds breaks down completely to orthophosphate after a 7 min. hydrolysis with $N-HCl$ at 100° , and thus behaves like pyrophosphate. Another compound occurs in the 'difficultly hydrolyzable' fraction.

It is interesting to note that *A. niger*, which contains an abundant store of phosphorus compounds in the mycelium, also possesses a highly active phosphatase. This enzyme acts optimally under the acid conditions prevailing in the mould culture. It attacks a variety of derivatives of phosphoric acid, among them metaphosphate, a fact which will be commented upon in the next paper of this series.

SUMMARY

1. Mould fungi with a predominantly aerobic carbohydrate metabolism have also a characteristic aerobic phosphorus metabolism. They absorb orthophosphate aerobically and convert it into a number of phosphorus compounds.

2. The rate with which phosphate is absorbed and utilized depends on its initial concentration in the culture medium. Cultures grown in the presence of large concentrations of phosphate develop more rapidly than those grown in low concentrations,

have a higher respiratory quotient, utilize glucose more rapidly, produce more citric acid, have a higher nitrogen metabolism and exhibit a substantially increased content of certain vitamins.

3. Cyanide and azide ($0.001N$), iodoacetate ($0.001N$) and fluoride ($0.005N$) strongly inhibit the respiration of these moulds, and bring the phosphorus metabolism to a standstill.

4. A large proportion of the orthophosphate absorbed aerobically is converted to acid-hydrolyzable compounds. One of these compounds breaks down to orthophosphate after a 7 min. hydrolysis with $N-HCl$, and thus behaves like a typical 'easily hydrolyzable phosphorus compound'. Another compound occurs in a 'difficultly hydrolyzable' form.

5. The formation of phosphorus compounds in the mycelium reaches its peak at the preconidial stage. During autolysis these compounds are decomposed and excreted into the medium as orthophosphate.

6. The occurrence of a metaphosphatase in both the mycelium and the culture medium of *Aspergillus niger* is described. The enzyme has been purified and separated from glucose oxidase. Whereas the activity of glucose oxidase is considerably reduced by $0.01N-NaNO_3$, the phosphatase activity remains intact.

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Studies on the Metabolism of Mould Fungi

2. ISOLATION OF PYROPHOSPHATE AND METAPHOSPHATE FROM *ASPERGILLUS NIGER*

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The mycelium of *Aspergillus niger* contains a large proportion of its phosphorus in the form of compounds which can be easily extracted with trichloroacetic acid and which yield orthophosphoric acid after acid hydrolysis. In order to obtain more definite information with regard to the chemical nature of these compounds an attempt was made to isolate them from the mycelium.

EXPERIMENTAL

The first step in the purification procedure was the extraction of the mycelia with trichloroacetic acid. This was followed by precipitation with $Pb(NO_3)_2$, by means of which two distinct fractions were obtained which will be referred to as 'Pb fraction I' and 'Pb fraction II'. The first fraction precipitated