

Phosphate Compounds in Respiring Mycelium of *Merulius lacrymans* (Jacq.) Fr.

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Incubation experiments with ^{32}P labelled orthophosphate have been carried out with the Cartwright strain of *Merulius lacrymans*, grown as shake cultures. Phosphate compounds found to be labelled with ^{32}P in experiments lasting up to two hours, were: pyrophosphate, ADP, ATP, GTP and UDP. On paper chromatograms, several unidentified radioactive spots were also seen. Poly-metaphosphate was identified in the residue after TCA extraction. In the barium insoluble fraction a metabolically inert phosphate compound occurred, which was believed to be tri- or tetraphosphate. This overlapped the ATP elution peak on column fractionations. Time-course experiments showed that pyrophosphate became more rapidly labelled than ATP. Both respiration and ^{32}P incorporation were strongly inhibited by 10^{-4} M 2,4-dinitrophenol.

Little information seems to be available regarding the occurrence of adenosine triphosphate (ATP) or other phosphate compounds in the Basidiomycete group of fungi. Nord and Sciarini¹ studied the presence of phosphorylation processes in *Merulius niveus* and concluded that "phosphorylation does not constitute an integrate part of the phase sequence of carbohydrate degradation by wood-rotting fungi".

As part of an investigation of the biochemistry and physiology of the wood-decomposing Basidiomycete *Merulius lacrymans*, it was therefore considered of interest to study the presence of phosphate compounds and their metabolic activity.

MATERIALS AND METHODS

The Cartwright strain of *M. lacrymans* from *Centraalbureau voor Schimmelcultures*, Baarn, Holland, has been used throughout these investigations. The fungus was grown submerged in 200 ml portions of medium in 500 ml Erlenmeyer flasks, on a reciprocal shaker at room temperature (18—22°C). The medium consisted of: glucose, 20 g; peptone,

10 g; KH_2PO_4 , 1.5 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 1.5 g; thiamine, 1 mg per liter. The flasks were covered with aluminium foil, and were inoculated from a surface culture or a previous shake culture which had been disintegrated in a Waring blender running with low speed for less than 1 min. The cultures were harvested after 8–10 days of growth. pH in the medium had then fallen to about 2.5. Inoculation on agar plates revealed no presence of bacteria. The mycelium was separated from the substrate by filtering through folded filter paper. The filter was washed thoroughly by a mixture of 0.01 M KCl and 0.01 M MgCl_2 , and the mycelium finally suspended in the same solution. pH in the suspension was about 4.5. Respiration measurements were carried out either by gas analysis, utilizing a Scholander 1/2 ml Gas Analysis Apparatus, or by Warburg technique.

For isolation and identification of phosphate compounds, the suspension of washed mycelium was incubated with carrier-free ^{32}P -labelled orthophosphate with vigorous shaking in order to obtain a high rate of respiration. To the respiring suspension, trichloroacetic acid (TCA) was added to a final concentration of 5 % and the shaking continued for a couple of minutes. The suspension was then cooled and centrifuged and the extract treated with barium acetate. The barium insoluble compounds were separated by centrifugation, treated with Dowex 50 in hydrogen form until dissolved, neutralized with KOH, centrifuged with high speed to remove mucous material and fractionated on a Dowex 1, X8 formate column according to Hurlbert *et al.*² Metaphosphate was isolated from the residue after the TCA precipitation as described by Klungsøyr *et al.*³ Paper chromatography was carried out on Whatman No. 1 filter paper washed with 1 % oxalic acid. The solvent systems used, were: *n*-propanol, NH_3 , H_2O (6:3:1) and *tert*-butanol, formic acid, H_2O (8:3:4). The papers were developed with the phosphate reagent of Hanes and Isherwood⁴. Radioautography of two-dimensional chromatography was carried out using Kodak X-ray film.

RESULTS

Separation and identification of phosphate compounds. A considerable number of phosphate compounds became labelled by incubation of the mycelial suspension with ^{32}P labelled orthophosphate. Fig. 1 shows a paper chromatogram of the TCA extract from an experiment when the incubation time was 2 h. Five hundred ml suspension was here incubated with $800 \mu\text{C } ^{32}\text{P}$. Ten ml of the TCA extract was freeze-dried after removal of the TCA with ether in a continuous liquid-liquid extraction apparatus, and the residue dissolved in 1 ml water. Ten μl of this solution was placed on the paper. Only a few

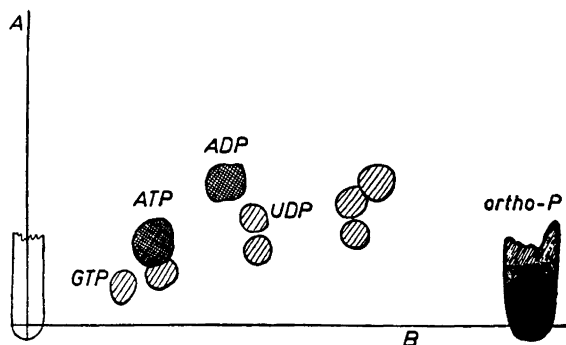


Fig. 1. Paper chromatogram of a TCA extract of *Merulius* mycelium, incubated with radioactive orthophosphate for 2 h. The degree of shading indicates the amount of radioactivity in the spots. Solvent systems: A, *n*-propanol, ammonia, water (6:3:1) and B, *tert*-butanol, formic acid, water (8:3:4).

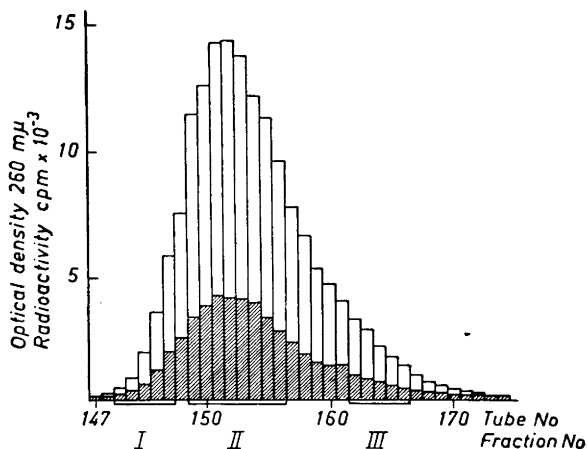


Fig. 2. Rechromatography of ATP from *Merulius* mycelium. The fractions marked I, II and III were lyophilized and analysed separately. Open columns: radioactivity in counts per min $\times 10^{-3}$. Shaded columns: optical density at 260 $m\mu$.

of the compounds have been identified, but there are definite radioactive spots for ATP, ADP, GTP and UDP.

From the main portion of the extract, the Ba insoluble phosphates were fractionated on an ion exchange column. Here fractions were obtained containing pyrophosphate and ADP. A fraction containing radioactivity and adenine like material absorbing at 260 $m\mu$ was obtained at the elution volume characteristic for ATP. The whole fraction was pooled, lyophilized and analyzed. The molar ratios were 4, 1 and 1, for acid labile phosphate, ribose and adenine, respectively. The substance was rechromatographed on the formate column, Fig. 2, and the tubes containing radioactivity and absorbing at 260 $m\mu$ were pooled in 3 fractions, lyophilized and analyzed separately for purine, acid labile P and radioactivity. Each fraction was examined by paper chromatography. The paper chromatography showed the presence of ATP in fractions 1 and 2, and of GTP in fraction 3, all radioactive. All three fractions gave a spot at the starting point, trailing in the direction of the ammoniacal system.

Table 1. Analysis of the three fractions of the rechromatographed triphosphate peak shown in Fig. 2.

Fraction number	I	II	III
Optical density ratio 260 $m\mu$ /275 $m\mu$	0.428	0.422	0.676
Radioactivity Cpm/ μ mole purine	9 220	9 100	8 250
Acid labile P/purine	4.7	3.05	9.15

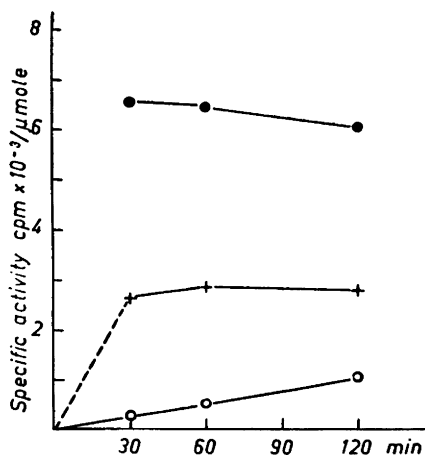


Fig. 3. The specific radioactivity in orthophosphate, pyrophosphate and ATP as a function of incubation time. The values are given as counts per min per μ mole phosphorus in orthophosphate and pyrophosphate, and per $2 \times \mu$ mole purine in ATP. The third phosphate in ATP is probably somewhat radioactive, so that the specific activities calculated for this compound are maximal values. ● : orthophosphate, + : pyrophosphate, ○ : ATP.

This spot contained no radioactivity, and is probably given by the phosphate compound(s) that overlaps the ATP and GTP peaks from the column. The amounts of acid labile P per μ mole of purine differed widely in the three fractions (Table 1), while the radioactivities per μ mole purine were the same in all of them. These results demonstrate the presence of acid labile, ribose and purine free phosphate contaminants in the isolated ATP and GTP. These metabolically inert phosphate compounds might be low molecular polyphosphates, for instance triphosphate or tetrathosphate.

About three μ moles acid labile phosphate per μ mole adenin has been obtained repeatedly on the most highly purified ATP from different batches of mycelium in this fungus. This lead to the tentative conclusion that the fungus contained adenosine tetrathosphate (AQP) instead of ATP. Paper chromatography demonstrated, however, that this was not the case. Spots corresponding with ATP were consistently found, while spots corresponding with AQP (a preparation from Sigma Chem. Corp. was used as reference sample) could not be detected.

Metaphosphate. A phosphate-containing compound was isolated from the TCA insoluble residue, which was not dialyzable, gave a purple to pink colour with toluidine blue and was converted to orthophosphate by hydrolysis

Table 2. The effect of 2,4-dinitrophenol on respiration and radioactive phosphate incorporation in *Merulius* mycelium. Experimental time one hour.

	Respiration rate μ l O ₂ per mg dry weight and hour	³² P incorp., %
Control	5.5	1.10
10 ⁻⁴ M DNP	1.8	<0.01

with normal sulfuric acid at 100°C for 15 min. This polymetaphosphate was present in the fungus in comparatively large amounts. The incorporation during respiration, of ^{32}P labelled orthophosphate into the TCA insoluble fraction of the cells was negligible.

The time course of the incorporation process. An experiment was carried out in which samples of mycelium incubated with ^{32}P labelled orthophosphate were analyzed after 30, 60 and 120 min of respiration. The barium insoluble fractions of the TCA extracts were fractionated as usual, and the radioactivity in the fractions determined. The results are shown in Fig. 3. It will be seen that the specific activity in pyrophosphate increases rapidly, while that in ATP does so more slowly, and remains during the experimental period lower than that of pyrophosphate.

The effect of dinitrophenol on the incorporation. 2,4-Dinitrophenol is a potent inhibitor of oxygen uptake in *M. lacrymans*, and was also found to inhibit phosphate incorporation. Results from one experiment are shown in Table 2. The experiment was carried out in Warburg flasks, and the cells killed by adding TCA from the side arm at the end of the experimental period, which lasted one hour.

DISCUSSION

The chemical identification of ATP in *M. lacrymans* met with some difficulties. Whereas paper chromatography gave definite evidence to the presence of ATP, the column fractionation technique used obviously did not separate between ATP and some metabolically inactive phosphate which we believe to be tri- or tetraphosphate. For this reason too high values for acid labile and total phosphate were found in the ATP preparations.

The time course of ^{32}P incorporations from orthophosphate follows closely that earlier described by Klungsoyr *et al.*³ for *Acetobacter suboxydans*. The only difference seems to be that the reaction goes considerably more slowly in *M. lacrymans*; something which probably is connected with its metabolic activity as a whole.

As far as these investigations go, they show that *M. lacrymans* contains a system for generating energy-rich phosphate bonds operating during active respiration and with ATP as the main store for such bonds. The phosphate metabolism does not seem to differ much from that of other microorganisms.

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