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INHIBITION OF PYRUVATE METABOLISM IN SOME FILAMENTOUS FUNGI UNDER HIGH PRESSURES OF OXYGEN WITH SPECIAL REFERENCE TO *MUCOR RACEMOSUS* AND *ASPERGILLUS NIGER*

SOALIHA AHMED*

Department of Botany, University of Exeter, Exeter, England.

Abstract

Variability in the degree of inhibition of pyruvate oxidase system under high pressures of oxygen has been observed. In *Aspergillus niger* only 6% of the total carbohydrate used appeared as pyruvate in the medium, whereas in *Mucor* about 50% of the total carbohydrate used appeared as pyruvate in the medium. Studies with radioactive glucose revealed that in *Mucor sp.* the inhibition of pyruvate oxidase system is virtually complete, whereas *in A. niger* there is only partial inhbition of pyruvate oxidation allowing some pyruvate to enter the TCA Cycle.

Introduction

Inhibition of pyruvate oxidase system under High Pressures of Oxygen (HPO) has been shown in micro-organisms by many workers (Webley, 1954; Wolin, Evans & Niven, 1955; Dilworth, 1962; Pritchard, 1966). Inhibition of pyruvate metabolism under HPO has also been observed in higher animals (Dickens, 1946; Thomas & Neptune, 1963) and in higher plants (Barker & Mapson, 1955). Microorganisms are more resistant to HPO (Caldwell, 1963; Gottleib, 1966; McAllister, Stark, Norman & Ross, 1963) compared with the tissues of higher animals and plants (Caldwell, 1964; Bean, 1945). Preliminary studies on the survival of *Aspergillus niger* and *Mucor racemosus* revealed that *Aspergillus niger* is much more resistant to HPO than *M. racemosus*. A comparative study on the inhibition of pyruvate oxidase system in these two organisms has been carried out using radioactive glucose under HPO.

Materials and Methods

In the present investigation the following species of fungi have been used:

(a) *Mucor racemosus* Fresen. (CMI 35716).

(b) *Mucor plumbeus* Bon. (CMI 107722).

(c) *Aspergillus niger* Van Tiegh. (E 10).

(d) *Aspergillus oryzae* (Ahlb.) Cohn. (E 126).

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*Present address:—Department of Botany, University of Karachi, Karachi, Pakistan.

(e) *Rhizopus oryzae* Went and Geerl. (CMI 40564).

(f) *Fusarium solani* (Mart) App. et Wr. (CMI 68412).

(g) *Penicillium chrysogenum* Thom' (E 129).

(h) *Neurospora crassa* Shear & Dodge (CMI 24298).

(i) *Sordaria fimicola* (Rob) Ces. & de Not. (E 24).

CMI = Commonwealth Mycological Institute.

E = Fungus Collection, Dept. of Botany, University of Exeter, England.

Growth of fungus cultures

The cultures were grown on glucose glutamate medium (Pritchard, 1965). After four days growth the fungus pads were replaced on a sterile solution of glucose. Before replacement the fungus pads were first rinsed with sterile deionised water and then carefully floated on the replacement medium.

High Presure Oxygen treatment

The pressure vessels were a modification of the vessel described by Caldwell (1956). A pressure of 10 atmospheres (atm) of pure oxygen (HPO) was used for all the experiments described in this paper. Initial readings were taken just before subjecting the cultures to HPO.

Determination of keto-acids

Keto-acids were determined by the method of Isherwood & Cruickshank (1954) using certain modifications of Niavis (1956). This method is based on the chromatographic separation of 2-4 dinitrophenyl hydrazone and colorimetric estimation of separated hydrazone. All the keto-acids determinations were carried on the medium only, because most of the amount accumulated is excreted into the medium and a very small amount remains in the mycelium (Pritchard, 1966).

Determination of sugar

The method used for quantitative determination of reducing sugars was that described by Hagedorn & Jenson (1923). This method is based on the reduction of potassium ferricyanide to potassium ferrocyanide by means of reducing sugar present in this extract and iodometric titration of the remaining ferricyanide by means of sodium thiosulphate.

Feeding labelled glucose

Uniformly labelled glucose was obtained from the Radiochemical Centre, Amersham & 8 μc of ^{14}C glucose was added to each culture flask with a micropipette.

Extraction of the mycelium

The filtered mycelium was dropped into boiling 80% ethanol and the boiling was allowed to continue for 1-2 minutes. Thereafter the mycelium was homogenised

in the ethanol. The resulting solution was evaporated to dryness. The dried solution was redissolved in a known volume of 60% ethanol and used for two dimensional chromatography. The culture filterates were used for keto acid analysis.

Chromatography

Two dimensional chromatography was carried-out on Whatman No. 1 filter paper. The alcoholic extract was applied quantitatively with the help of a micro-pipette. The solvents used were:

(1) Phenol: Water, 100:40.

(2) Butanol: Propionic acid: Water, 47: 23: 30.

Radioautography

For radioautography Ilfox non screen radiofilm was used. The chromatograms were left in contact with the film in darkness for two weeks. Thereafter it was developed with I.D. 19 developer for about 10-15 minutes and then washed in running water for 30 minutes and dried.

Detection of spots on chromatograms

The chromatograms were sprayed with 1.66 gm. pthalic acid and 0.9 ml. aniline, dissolved in butanol, ethanol and water in a ratio of 48:48:4. for sugar and with 0.3% ninhydrin in solution in 95% ethanol for amino acids.

Determination of radioactive counts

Counts were made directly on two dimensional chromatogram, after tracing the radioactive spots from radioautographs by a Geiger Muller thin-end window counter. For determining the radioactive peaks of keto-acids on chromatograms, a 11/4" wide strip of chromatogram was run through a Tracer lab 4 Scanner from which a recorded tracing of the count rate was obtained.

Results

A preliminary survey was carried-out on a heterogenous group of filamentous fungi to see the effect of HPO on pyruvate oxidase system. The main aim of the experiment was to see how the accumulation of pyruvate in a glucose replacement medium during HPO treatment varied in different fungi.

The results obtained are shown in Table 1. Very little carbohydrate was utilised in *N. crassa* and *S. fimicola.* This may be due to the deficiency of some vitamin in the medium which are required by these fungi. Owing to this great variability in the degree of inhibition of pyruvate metabolism two contrasting species *M. racemosus* and *A. niger* were selected for further studies.

TABLE 1. Carbohydrate utilization and pyruvate production by some filamentous fungi after 24 hours sojourn in 10 atm. of pure oxygen.

Species	Carbohydrate Used mg.	Pyruvate Produced mg	Pyruvate Produced / Carbohydrate used × 100	
Mucor racemosus	98.0	49.0	50.0	(1.28)*
Rhyzopus oryzae	45.0	16.97	35.5	
Aspergillus oryzae	70.0	14.68	20.0	
A. niger	47.5	3.15	6.25	(0.484)*
Sordaria fimicofa	11.25	2.30	18.1	
Neurospora crassa	21.25	1.234	4.7	
Penicillium chrysogenum	155	4.105	2.5	
Fusarium solani	112	0.894	0.32	

*Figures in brackets indicate the percentage of carbohydrate used which appeared as pyruvate in air control samples.

Glucose methabolism of *A. niger* under 10 atmosphers of pure Oxygen

There was no large accumulation of pyruvate after 4 days treatment with HPO (Fig. 1). Cultures treated for 7 days showed a slight increase in the amount of pyruvate. Glucose utilisation was quite rapid upto a period of 48 hours both in air

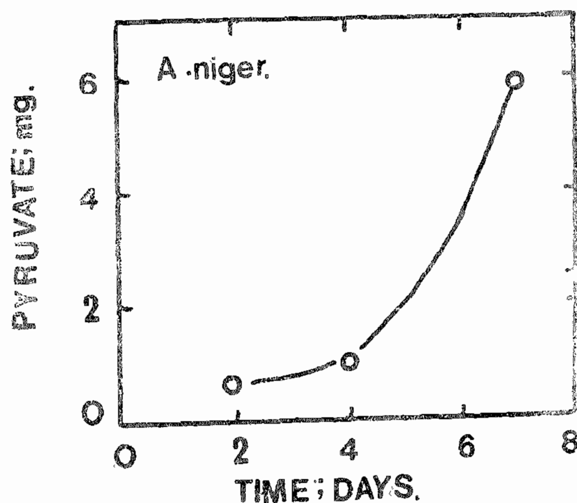

Fig. 1. Changes in pyruvate content of the medium of *Aspergillus niger* during a 7 day sojourn in 10 atm. of pure oxygen.

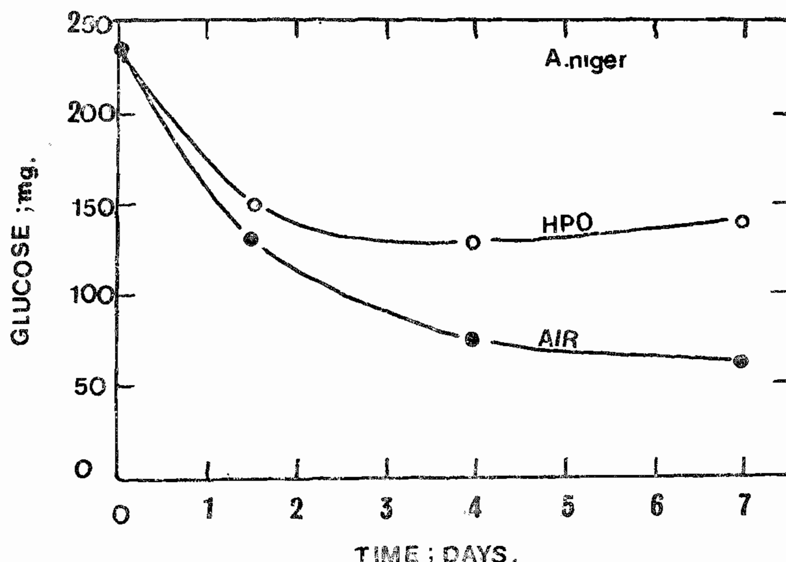

Fig. 2. Utilization of glucose by *Aspergillus niger* during a 7 day sojourn in 10 atm. of pure oxygen and in air.

and oxygen (Fig. 2). After further treatment glucose values remained more or less stationary.

Glucose metabolism of *Mucor* sp under HPO

As also reported by Dilworth, (1962) and Pritchard, (1966) there was a large accumulation of pyruvate in the medium (Fig. 3). Carbohydrate utilisation was also found to be very rapid during this period. The expreiment was discontinued after 4th day because of the early death of the fungus under HPO.

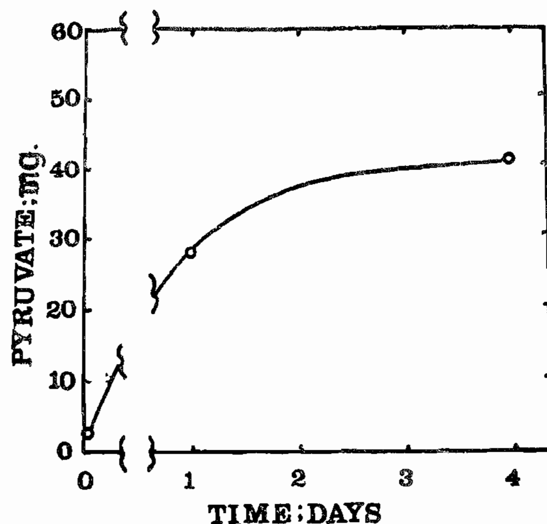

Fig. 3. Changes in the pyruvate content of the medium of *Mucor* sp. during a 4 day sojourn in 10 atm. of pure oxygen (—) 24 hrs. gap between the time of initial reading and subjecting the fugus to HPO.

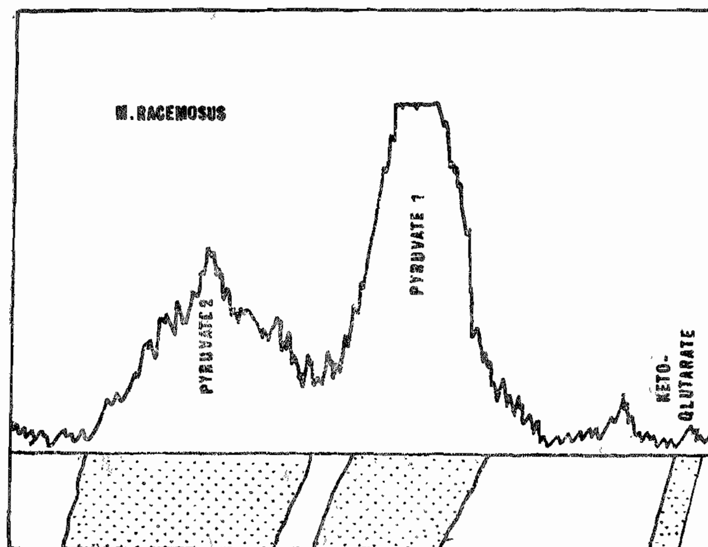

Fig. 4. Tracing made by chromatogram scanner showing distribution of radioactivity on a keto acid chromatogram obtained from the medium of HPO treated culture of *M. racemosus*. (The dotted zone represents the position of two sterioisomeric hydrazone bands formed by Pyuvic acid on the chromatogram, which is also indicated as pyruvate 1 & pyruvate 2 in radio active peaks).

Comparative metabolism of *A. niger* and *M. racemosus* under HPO

To compare further the metabolism of *A. niger* and *M. racemosus*, a short term experiment was carried-out using ^{14}C lebelled glucose. The result obtained are shown in Figs. 4-6. In *M. racemosus* most of the radioactivity accumulated in pyruvate (Fig. 4). In this species tests made for the presence of amino acids mostly

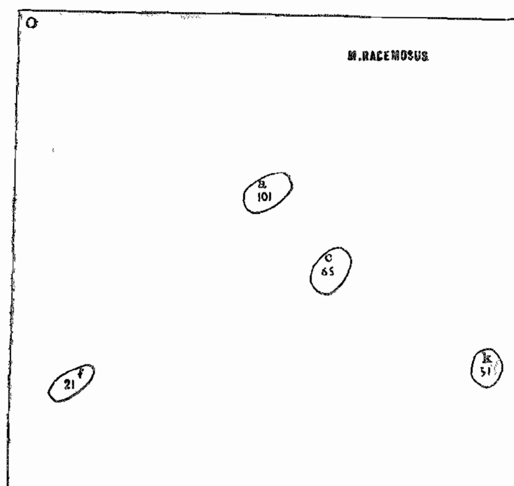

Fig. 5. Autoradiograph of alcoholic extracts of *M. racemosus* mycelium treated for 24 hrs. with 10 atm. of pure oxygen in the presence of 14C glucose. The nos. in circle represent the radio-active counts. (k—alanine; f—citric acid; a & c some unknown compound).

gave negative results, only alanine (Spot K in Fig. 5) showed a small amount of radioactivity. In *A. niger*, most of the radioactivity accumulated in some unknown compounds (Spot a,c and d in Fig. 6). These spots gave negative results for amino-acid and sugar and their nature could not be determined. However, the presence of radioactive glutamic acid, aspartic acid organic acids and citric acid (f) indicates that the inhibition of pyruvate oxidation in *A. niger* is not complete at 10 atm. A tentative indentification of spot 'a' with glucose is possible but colouration with aniline pthalate was very weak.

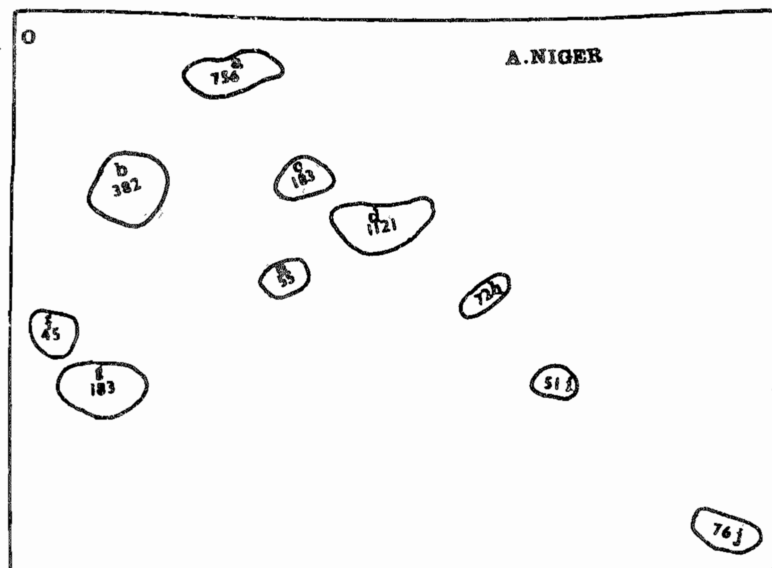

Fig. 6. Autoradiograph of Alcholic extract of *A. niger* mycelium treated for 24 hrs. with 10 atm. of pure oxygen in presence of ^{14}C glucose. The nos. in circle represent the radio active counts (b—aspartic acid; f—citric acid; g—malic acid; e—glutamic acid, a,c,d,g,h,i,j, some unknown compound).

The present experiments confirm two main points. (i) The virtually complete of pyruvate oxidase system of *Mucor* with the resulting accumulation of pyruvate in the medium. (ii) Only partial inhibition of pyruvate oxidation in *A. niger* allowing some pyruvate to enter tricarboxylic acid cycle and only small accumulation of pyruvate in the medium.

Discussion

The complete inhibition of pyruvate oxidation in *Mucor* sp. is in agreement with the results obtained by Pritchard (1966). Analytical and isotopic studies revealed that glucose metabolism of *A. niger* under HPO was quite different from that of *Mucor* species. In this species, only 6% of the total carbohydrate used appeared as pyruvate (Table 1), whereas in *Mucor*, about 50% of the total carbohydrate used, appeared as pyruvate in the medium. Radioactive studies confirmed these results and revealed that some of the pyruvate enters into the TCA Cycle in *A. niger* (Fg. 6), whereas in the *Mucor* sp the pyruvate produced accounted almost completely for the carbohydrate used. Studies made to investigate the degree of inhibition of

pyruvate metabolism in different fungi supported the previous observations (Pritchard, 1966) that it varies from genus to genus and sometimes within the species of the same genus, as observed in the case of *A. niger* and *A. oryzae*.

A possible reason for this difference in behavior may be due to the differences in the ability of organism to produce hydrogen peroxide from oxygen. It has been suggested that oxygen poisoning maybe due to indirect inhibition of some enzyme system as a result of formation of hydrogen peroxide under HPO (Mann & Quastel, 1946; Dimmick, Heckley Hollis, 1961, Gerschman et. al., 1958; Ahmed, 1969). Therefoe it seems probable that degree of inhibition of pyruvate oxidase system might vary with the amount of H_2O_2 produced. The smaller degree of inhibition of pyruvote oxidase in *A. niger* compared to *Mucor* sp suggests that probably the peroxide generation system of *A. niger* is less efficient than that of *Mucor* sp. An alternative explanation may possibly be in structural differences between different fungi in that the cell wall may constitute a more effective diffusion barrier in some fungi than in others. If oxygen toxicity is due to accumulation of H_2O_2, the oxygen sensitivity of an organism will depend upon its ability to increase catalase level in order to cope with the higher rate of H_2O_2 formation when it is treated with HPO (Pritchard & Hudson, 1967; Ahmed & Pritchard, 1970). This would seem to be a likely explanation for the degree of inhibition of pyruvate oxidase system in different fungi.

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