Mycol. Res. **99** (7): 859–866 (1995) *Printed in Great Britain*

Glucose catabolic pathways in *Lentinula edodes* determined with radiorespirometry and enzymic analysis

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Carbohydrate metabolism in different tissues was studied using radiorespirometry and enzymic analysis. The ^{14}C-1/^{14}C-6 ratios in CO_2 respired from specifically labelled glucose fed to *Lentinula edodes* tissues grown on a chemically defined medium ranged from 2·5 (vegetative mycelium) to 14·9 (young lamellae). This reflects the relative activity of the pentose phosphate pathway (PPP), very high in basidiome but low in mycelium, the highest ratio being recorded in tissues which are biosynthetically most active (young lamellae), requiring the reducing power of NADPH generated through the PPP. Extensive conversion of ^{14}C-3, 4-labelled glucose to $^{14}CO_2$ in the mycelium underlined the important role of the Embden–Meyerhof–Parnas pathway (EMP) in that tissue. A ^{14}C-1/^{14}C-6 ratio of 14·8 for young lamellae grown on woodchips indicates that the growth medium did not influence the pathway used in the basidiome. Ratios for the young pileipellis and stipe were 8·0 and 10·4 respectively. A ratio of 3·6 for *Coprinus cinereus* gills confirms the comparatively lesser importance of the PPP in this organism.

Enzymic determination corroborated the metabolic pattern deduced from radiolabelling. Activity of PPP enzymes (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) were three times as high in the basidiome compared with the mycelium, highest activity being in the young pileus. EMP enzymes (fructose 1,6-bisphosphate aldolase and glucose 6-phosphate isomerase) were more active in mycelium than in fruit body, and also more active than PPP enzymes within the mycelium itself. Within the basidiome, specific activities of EMP and PPP enzymes were about the same. Enzymic activity in mature pileus of *C. cinereus* resembled the pattern in the mycelium rather than the basidiome of *L. edodes*; EMP enzymes were very much more active than PPP. The positive correlation between high PPP activity and accumulation of large amounts of mannitol in the basidiome is discussed.

The button mushroom, *Agaricus bisporus* (J. E. Lge) Imbach, has attained the high yield and reliability of production enjoyed today through development of carefully controlled environmental growth conditions, applied and fundamental research being combined to give high technology cultivation. Unfortunately this happy state does not extend to the shiitake mushroom industry.

Shiitake (*Lentinula edodes* (Berk.) Pegler), a white-rot saprotroph, has traditionally been cultivated on hardwood logs (Ito, 1978) with oaks being natural substrates and the preferred species for cultivation (San Antonio, 1981). Even in Japan, which produces 70% of the world's total shiitake mushroom crop, most of the mushrooms are produced by the traditional bed log technique. However, the long growth period before cropping, the premium on growing space and increasing shortage of hardwoods, have caused many producers to turn to the synthetic log method. This method involves cultivation on sawdust, woodchips or lignocellulosic wastes contained in plastic bags under environmentally controlled conditions. This technique was first reported in the late 1970s (Fuzisawa, Maedai & Hattori, 1978; Mee, 1978; Fuzisawa & Hattori, 1979) and has since increased tremendously in importance. It has proved to be commercially much more successful than the traditional method of cultivation; yields as high as 145% (biological efficiency – fresh weight yield per unit D.W. substrate) harvested over a 6-month period have been reported (Royse, 1985).

Factors affecting yield and size variation in synthetic and sawdust cultures have been studied extensively (Terashita, Kono & Murao, 1980; Han *et al.*, 1981; Kawamura, Goto & Nakamura, 1983; Leatham, 1985; Royse, 1985; Royse, Schisler & Diehle, 1985; Matsumoto, 1988; Tan & Chang, 1989*a*, *b*), but basic studies on the physiology and biochemistry of *L. edodes* are lacking, although detailed information on these aspects is crucial to effective manipulation of the crop.

In the first part of this study (Tan & Moore, 1992), convenient methods were devised for *in vitro* cultivation of mycelium and fruiting bodies of *L. edodes* within a short period of time in the laboratory. This is essential as *L. edodes* is a very fastidious organism with respect to fruiting and normally has a comparatively long vegetative growth phase before fruiting is initiated on the natural substrate. Tan & Moore (1992) identified suitable strains and devised techniques for laboratory cultivation of *L. edodes* on chemically defined liquid, or on woodchip-medium which could produce fruit bodies reliably within 28 d.

In this report, biochemical studies investigated basic

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carbohydrate metabolism in *L. edodes* with a comparison of glucose dissimilation in mycelia and basidiome tissue of *L. edodes* at different developmental stages. Two approaches were adopted, an *in vivo* radiorespirometric method using glucose labelled at different carbon positions, and an *in vitro* enzymic analysis.

MATERIALS AND METHODS

Cultures, media and cultivation techniques

The strain of *L. edodes* used was Le-11 (Tan & Moore, 1992). It was routinely maintained on Potato Dextrose Agar (PDA) at 25 °C and sub-cultured from a stock kept at 5°. Tan & Moore (1992) described methods for mycelial propagation and fructification on a defined medium (called LM, recipe in Leatham, 1983). All cultivation was done in crystallizing dishes placed in a Stewart Plastics seedling propagator set. Fruiting cultures were incubated at 25° in an illuminated incubator on a 9 h light (200–400 lx) and 15 h dark cycle. After 28 d the cultures were transferred to another incubator with the same illumination but with a fluctuating temperature regime of 12 h at 14° and 12 h at 22°. *L. edodes* fruit bodies were also produced on a woodchip medium, which was modified from that of Tan & Chang (1989*a*); it consisted of (in dry weight percentages): woodchips (75%), wheat flour (10%), wheat bran (10%), yeast (2%), $CaSO_4$ (2%), $CaCO_3$ (1%). The ingredients were mixed in a 1:2 (w/w) ratio with tap water and sterilized in crystallizing dishes by autoclaving at 15 p.s.i. for 1 h. Woodchip medium was inoculated with homogenized 21-d-old mycelia grown on LM. The inoculated medium was then subjected to the same treatment for growth and fruiting as with the LM except the incubation time for vegetative growth (spawn run) was about 8 wk.

The *Coprinus cinereus* strain used was a wild-type dikaryon ('Meathop') originally isolated from Meathop Hill in Cumbria, U.K. (Moore, Liu & Kuhad, 1987). It was kept as a stock culture on *Coprinus* complete medium (CM; Moore & Pukkila, 1985). To obtain fruit bodies, cultures were grown on horse dung which had been autoclaved at 103 kn m^{-2} for 30 min. After inoculation, the medium was incubated at 37° in the dark for 3 d, then the culture was transferred to an illuminated incubator set at 26°. Illumination was provided by fluorescent lights of about 500 lx intensity with a daylength cycle of 16 h light/8 h darkness (Moore & Pukkila, 1985). Primordia appeared 5–6 d after inoculation, maturing into fruit bodies 3–4 d after that.

Determination of metabolic fate of glucose

Specifically radiolabelled glucose was used to establish the catabolic fate of glucose in various tissues of *L. edodes* (mycelium and basidiomes grown on LM and woodchips medium) and *C. cinereus* (basidiomes grown on horse dung). The assay medium was LM supplemented with specifically labelled glucose. For each of the [1-^{14}C]-, [2-^{14}C]-, [3, 4-^{14}C]- and [6-^{14}C]-glucose-supplemented media tested, four replicates were set up and the production of $^{14}CO_2$ over regular time intervals was determined.

Assay of $^{14}CO_2$. A simple radiorespirometric apparatus was devised to monitor $^{14}CO_2$ liberation from labelled glucose. It consisted of a plastic disposable ('Universal') tube containing 4 ml of LM minus glucose medium. Resting on the bottom of this tube was a disposable pipette tip heat-sealed at its point and serving as a support for an Eppendorf tube containing KOH. Trials showed that a volume of 0·2 ml KOH was sufficient to trap all the CO_2 evolved. The KOH traps were replaced every 30 min or 60 min according to the experiment. Specifically labelled glucose was added to the medium with a microsyringe to start the experiment. If mycelium was used as the inoculum it was prepared as follows. Mycelial scrapings from fresh agar slants of *L. edodes* were inoculated into liquid LM and incubated at 25° for 14 d in disposable Petri dishes. Mycelial mats were lifted out of the dishes and macerated at half-speed on an Atomix homogenizer for 60 s. It was re-inoculated into fresh LM in disposable dishes and grown for 21 d at 25° in stationary culture after which the mycelium was harvested by filtration through Whatman No. 1 filter paper on a Buchner funnel and washed twice with distilled water before weighed quantities were used as inoculum for the radiorespirometric tests. If fruit body tissues were used, pieces were excised with a surgical blade, weighed and floated in the test medium. Preliminary experiments showed that 5 mg D.W. of cells ml^{-1} of radiolabelled medium was most suitable as an inoculum. As a value of 10·5% was obtained for the dry/wet weight ratio of the tissues, a fresh tissue concentration of 50 mg ml^{-1} was used in all the tests.

The radiorespirometer tubes were incubated at 25° on a gyratory shaker at 250 rpm. After the required time period, the Eppendorf containing KOH was removed with forceps and inserted into plastic scintillation vials containing 20 ml scintillant (Optiphase Hisafe II, from Pharmacia Limited). After mixing with a vortex mixer the vials were counted with a Beckman β scintillation spectrometer for 5 min. A fresh trap was replaced on to the plastic support in the radiorespirometer tube. Pilot experiments showed that the KOH traps were so efficient at scavenging $^{14}CO_2$ that negligible loss of $^{14}CO_2$ occurred during trap replacement.

^{14}C Inventory

Proper functioning of the apparatus and experimental set-up described above was established by allowing mycelia to metabolize 0·1 μCi of [1-^{14}C]-, [2-^{14}C]-, [3, 4-^{14}C]- or [6-^{14}C]-glucose for 20 h after which the distribution of ^{14}C between CO_2, tissue and medium was audited. Upon termination of the experiment, radiorespirometer vessels containing the mycelium were cooled in ice for about 10 min (Zagallo & Wang, 1967), then mycelium was separated from the medium by centrifugation at 3000 ***g***. The tissue was washed twice with 4 ml distilled water and recentrifuged. Supernatants from the original and the two washings were combined, and 1 ml was mixed with 14 ml of Optiphase Hisafe II scintillation fluid. The washed mycelial tissue was mixed with 15 ml of scintillation fluid, macerated and stirred on a vortex mixer before its radioactivity was counted to determine the ^{14}C of the cells. Hourly counts from the $^{14}CO_2$ liberated (and trapped in KOH as described above) during the 20 h incubation were pooled to

Table 1. Inventory of ^{14}C in mycelial cultures of *L. edodes* 20 h after administration of [^{14}C]glucose

Fraction	[1-^{14}C]glucose		[2-^{14}C]glucose		[3,4-^{14}C]glucose		[6-^{14}C]glucose	
	DPM*	%	DPM	%	DPM	%	DPM	%
^{14}C administered	246321	100	223546	100	227611	100	236834	100
^{14}C recovered								
Culture medium	33327	13·5	44352	19·8	28269	12·4	57859	24·4
Tissue	108236	43·9	103211	46·2	97031	42·6	122040	51·5
Cumulative $^{14}CO_2$	100015	40·6	63668	28·5	91532	40·2	48627	20·5
Total	241578	98·1	211231	94·5	216832	95·3	228526	96·5

*DPM = disintegrations per minute.

give a total $^{14}CO_2$ count. Counts from the medium, cells and CO_2 were combined to give the total ^{14}C activity recovered (shown in Table 1).

Enzyme determinations

Preparation of extracts. All operations were carried out at 5° in the cold room with apparatus pre-chilled in crushed ice. Mycelium was harvested by filtration through Whatman No. 1 filter papers, weighed, washed with distilled water and, if not used immediately, stored at −20°. Fruit bodies were decapitated, and the caps and stipes weighed and stored separately at −20°, if not used immediately.

Extracts of soluble proteins were prepared by grinding in liquid nitrogen. The fine powder obtained was transferred to a micro-homogenizer and the relevant extraction buffer (the same as that used in the assay for the enzyme) added. The tissue was then homogenized for a few minutes at 5°. The homogenate was centrifuged for 20 min at 10000 *g* at 0°. The cell-free extract was dialysed overnight at 5° against buffer solution, concentrated by freeze-drying, and stored at −20° until required.

Enzyme assays. All assays were linked to pyrimidine nucleotide oxidation/reduction which was measured spectrophotometrically. Assay procedures were optimized from the following sources:

Fructose 1,6-bisphosphate aldolase (D-Fructose 1,6-bisphosphate D-glyceraldehyde 3-phosphate lyase, E.C. 4.1.2.13), procedure followed Willnow (1984).

Glucose 6-phosphate isomerase (D-Glucose 6-phosphate ketolisomerase, E.C. 5.3.1.9), assay modified from Bergmeyer, Grabl & Walter (1983*a*).

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ 1-oxidoreductase, E.C. 1.1.1.49) (G6PD), assay procedure from Deutsch (1984).

6-Phosphogluconate dehydrogenase (6-phospho D-gluconate: $NADP^+$ 2-oxidoreductase (decarboxylating), E.C. 1.1.1.44) (6PGD), after Bergmeyer *et al.* (1983*b*).

No inhibitions were encountered in tests in which commercially available pure enzymes were added to the complete assay mix (medium + extract).

Protein determination. Protein in the extract was precipitated with 10% (w/v) trichloroacetic acid overnight at 4°, recovered by centrifugation, dissolved in NaOH and measured using the Lowry method. Bovine serum albumin was used as the standard. The specific activity of the two EMP and PPP enzymes were determined from the various tissue extracts. For each enzyme, the mean value was established from 6 replicate determinations.

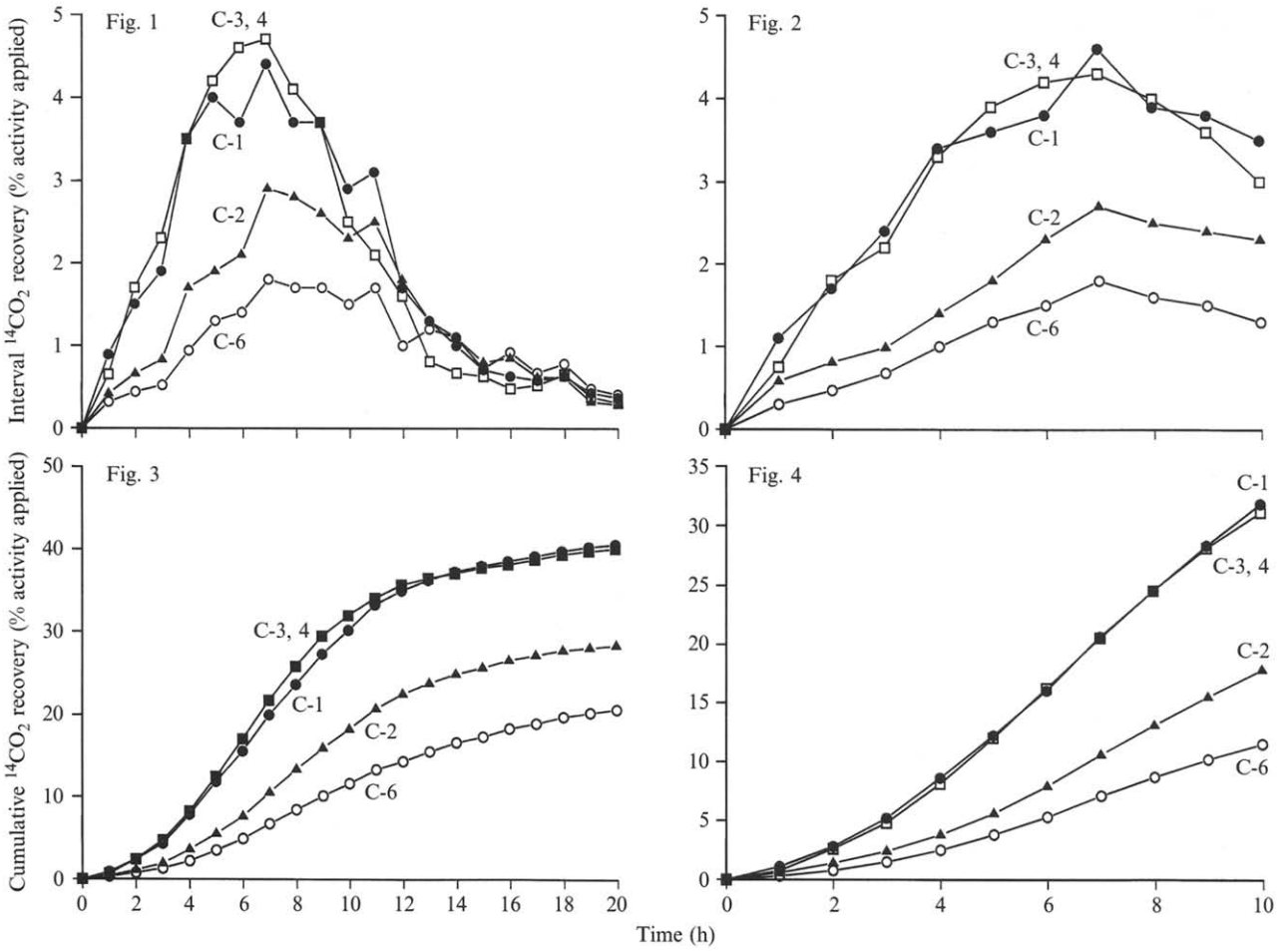
Chemicals. All reagents were of Analar grade and apart from radiolabelled glucose were obtained from Sigma Chemical Company Limited, Poole, U.K. or BDH, Poole, U.K. Glucose universally labelled, and labelled at C-1 and C-6 was purchased from Amersham International plc, Buckinghamshire, U.K., while C-2 and C-3,4 labelled glucose were bought from New England Nuclear, DuPont (U.K.) Limited, Stevenage, Hertfordshire, U.K.

RESULTS AND DISCUSSION

Radiorespirometry

The basis for the determination of which glucose catabolic pathway is operative is the labelling pattern of the $^{14}CO_2$ evolved from ^{14}C-glucose. In the pentose phosphate pathway (PPP), C-1 of glucose is released first as CO_2, C-6 only being released following further metabolism or recycling. In the Embden–Meyerhof–Parnas pathway (EMP), C-1 and C-6 of the original glucose are both released as CO_2 following pyruvate oxidation. This is because triose phosphates, derived from fructose-1,6 bisphosphate following aldolase action, equilibrate in a reaction catalysed by triose phosphate isomerase. Both C-1 and C-6 of the original glucose thus become the methyl group of pyruvate. When pyruvate is subsequently oxidized (in the tricarboxylic acid cycle), this would be the last carbon atom to be released as CO_2, whereas CO_2 derived from carbons 3 and 4 of glucose would be the first to be released. Hence, feeding tissues with glucose labelled in the different carbons could show the relative contribution of each pathway to glucose dissimilation by the amounts of $^{14}CO_2$ released from the different carbons (Duffus & Duffus, 1984).

The C-1/C-6 ratio is especially important. If glucose oxidation occurs solely via the PPP, the C-1/C-6 ratio will be very high as virtually only $^{14}CO_2$ from C-1 is released. If intermediates are recycled, then the ratio would decrease as $^{14}CO_2$ from C-6 is released. If EMP is the major pathway, then the ratio will be about 1 since the rate of release of $^{14}CO_2$ from C-1 and C-6 will be the same because both of these carbon atoms are incorporated in the methyl of pyruvate. A high amount of $^{14}CO_2$ from carbons 3 and 4 would also be



Figs 1–4. Recovery of $^{14}CO_2$ from metabolism of specifically labelled glucose by vegetative mycelia of *Lentinula edodes*. Figs 1 & 3 describe a group of experiments which were incubated for 20 h, the experiments described in Figs 2 & 4 were incubated for 10 h. In Figs 1 & 3 each point represents the amount of $^{14}CO_2$ collected in the KOH trap over the preceding 1 h time interval (hence, 'interval recovery'), the traps being changed at the end of each interval. Figs 2 & 4 show the accumulation of $^{14}CO_2$ with time. Each point is the mean of four replicate radiorespirometer vessels.

expected. If both PPP and EMP are operating simultaneously, the C-1/C-6 ratio will increase as the contribution of PPP increases, and decrease as the contribution of EMP increases. C-1/C-6 ratios cannot set a precise value for the contribution of each pathway because of recycling in the PPP and removal of intermediates, such as triose phosphate, by other biochemical processes (Duffus & Duffus, 1984).

^{14}C Inventory

The complete inventory of the labelled carbon atoms in the respiratory $^{14}CO_2$, cells, and incubation media observed at the end of a 20 h experiment is presented in Table 1. A greater than 95% recovery was obtained in all cases. A large proportion of the administered radioactivity was incorporated into the tissue (43–52%), a lesser amount was metabolized as $^{14}CO_2$ (21–41%) while 12–24% remained in the medium.

Determination of the metabolic fate of glucose in different tissues of L. edodes *and* C. cinereus

In all the radiorespirometric experiments, the total production of $^{14}CO_2$ was measured over a specific time interval (= 'interval production') and expressed as a percentage of the activity (dpm) of the radiolabelled glucose fed to the radiorespirometer vessel at the start. Cumulative production was also calculated, and the C-1/C-6 ratio was calculated for all the experiments.

Mycelia of L. edodes *grown on LM*

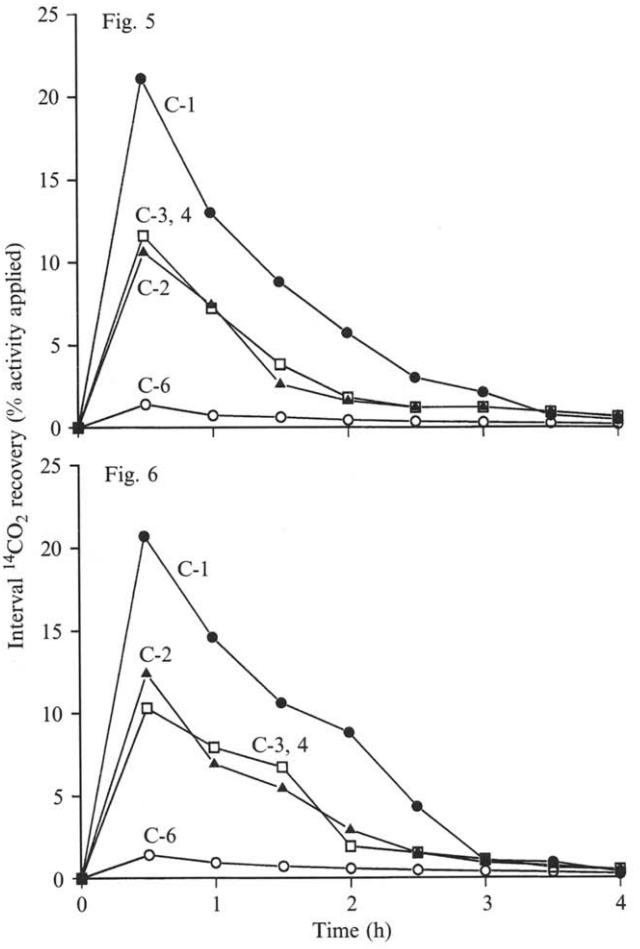
Results from the two experiments exhibited the same pattern of utilization of the [^{14}C]glucose (Figs 1–4). The combustion pattern consisted of two phases, an assimilatory phase and a depletion phase similar to that observed in baker's yeast and *Escherichia coli* (Wang *et al.*, 1956, 1958), but different from the results reported for filamentous organisms (*Aspergillus niger*, *Penicillium chrysogenum*, *P. digitatum*) and pseudomonads such as *Pseudomonas saccharophila* and *P. reptilivora* (Wang *et al.*, 1958) where only one phase was observed. The first phase in *L. edodes*, that of assimilation, was marked by the rapid combustion of C-3,4 and C-1. Peak evolution of $^{14}CO_2$ from all the differently labelled carbon atoms occurred 7 h after the labelled glucose was fed. The mean C-1/C-6 ratio was 2·5 at peak hour production and also at the end of one Relative Time Unit (1 RTU). RTU is defined as the time required in a given

Table 2. Ratios of C-1 $^{14}CO_2$ to C-6 $^{14}CO_2$ at time of peak $^{14}CO_2$ production and after utilization of labelled glucose observed with different tissues of *L. edodes* and *Coprinus cinereus*

	C-1/C-6 ratio at time of peak $^{14}CO_2$ production	Time to reach peak $^{14}CO_2$ production (h)	C-1/C-6 ratio at end of RTU*	Length of RTU (h)
L. edodes mycelia	2·5	7	2·5	10
L. edodes pileipellis of young basidiome	8·0	1·5	7·5	4
L. edodes lamellae of young basidiome	14·9	0·5	12·6	4
L. edodes lamellae of young basidiome grown on woodchips†	14·8	0·5	12·3	4
L. edodes lamellae of mature basidiome	8·6	1	7·7	6
L. edodes stipe of young basidiome	10·4	1	10·5	4
Coprinus cinereus gills of basidiome	3·6	6	2·9	10

* RTU is defined as the time required for an organism to consume all or most of the intact labelled substrate originally added (Wang *et al.*, 1958).

† Result of a single experiment; other entries are the means of two independent experiments.



Figs 5, 6. Recovery of $^{14}CO_2$ from metabolism of specifically labelled glucose by young lamellae of *Lentinula edodes* basidiomes. Each point is the mean of four replicate radiorespirometer vessels and shows the amount of $^{14}CO_2$ collected in the KOH trap over the preceding 30 min interval. Tissue in experiments shown in Fig. 5 was grown on the defined LM medium. Tissue used for experiments shown in Fig. 6 was grown on the woodchips medium.

experiment for an organism to consume all or most of the intact labelled substrate originally added (Wang *et al.*, 1958). The end of the time unit is usually indicated by the rapid decrease in CO_2 recovery from C-1 or C-3(4) of glucose which are known to be the first products of decarboxylation via the currently known pathways. The C-1/C-6 ratio at both the peak hour recovery and at the end of 1 RTU was 2·5, indicative that the PPP was operative, but this is a low value. The extensive conversion of C-3,4 in the early phase of incubation reflected the very important role played by the EMP in the mycelia of this organism.

The prompt and impressive appearance of $^{14}CO_2$ from C-3,4-labelled glucose undoubtedly reflected the conversion of pyruvate, arising from glucose via the EMP to acetate and CO_2, whereas the preferential combustion of C-1 over that of C-6, with a peak C-1/C-6 ratio of 2·4, indicated the occurrence of a 1–5 cleavage via the PPP (Cohen, 1951). The moderate appearance of C-2 during the assimilation phase indicates that this cleavage was followed by some, though not extensive, pentose-cycle reactions since the latter would result in a rapid conversion of these C-2 atoms into CO_2 (Wang *et al.*, 1958).

In the depletion phase, there was a higher rate of combustion of the C-2 of glucose as compared to C-6. This could be due to the fact that much of the acetate formed via the EMP route (methyl group equivalent to C-1 and C-6, carboxyl group equivalent to C-2 and C-5) was diverted into the synthesis of amino acids and other cellular constituents. Some of these compounds probably existed in a transitory state prior to their transformation into more stable cell constituents and therefore subject to re-routing to respiratory functions during the depletion phase. Several of these amino acids could also break down via the tricarboxylic cycle in the absence of exogenous carbon sources. The higher rate of combustion of C-2 (acetate carboxyl) as compared to C-6 (acetate methyl) of glucose is in line with this assumption. It could be inferred from the experiments with *L. edodes* mycelia, that the glucose was utilized by a combination of both the EMP and the PPP. In the operation of the PPP, it appears that there was no extensive oxidation of glucose via the pentose cycle although phosphogluconate decarboxylation served as a source of pentose.

Basidiome tissues of L. edodes

C-1/C-6 ratios obtained from experiments with basidiome tissues were all very much higher than observed in mycelia (Table 2). Furthermore, peak hour production of CO_2 occurred very much earlier and the RTU was very much shorter (Table 2). Presumably the faster rate of glucose metabolism reflects greater demand for biosynthetic activities in the expanding basidiome and the high C-1/C-6 ratios indicate increased importance of the PPP. Evolution of CO_2 from glucose labelled at C-3,4 was generally much lower than that observed in the case of mycelia and was almost equal to that recovered

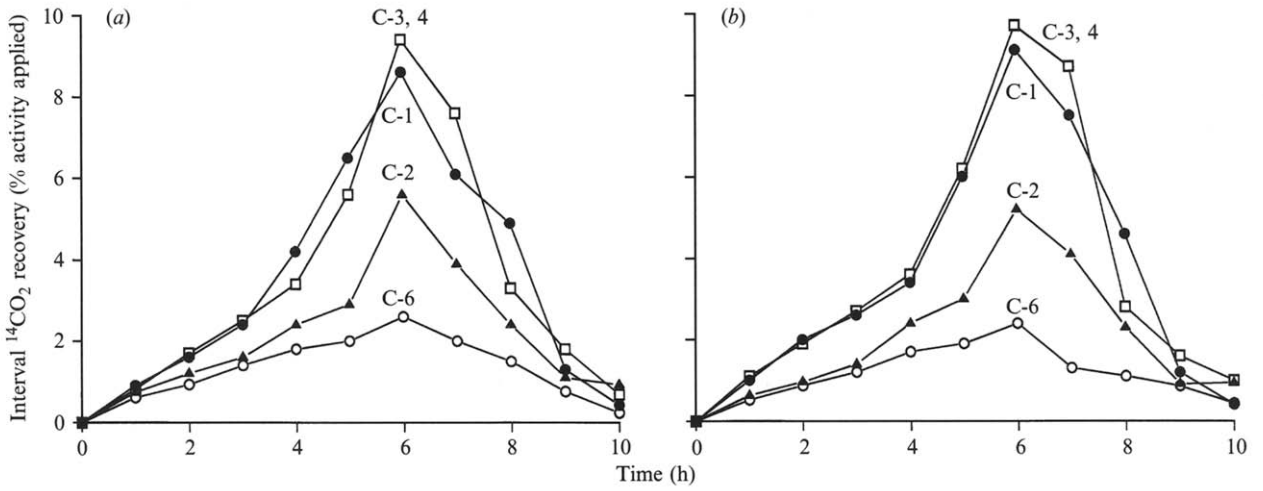


Fig. 7. Recovery of $^{14}CO_2$ from metabolism of specifically labelled glucose by mature gills of *Coprinus cinereus* basidiomes. Each point is the mean of four replicate radiorespirometer vessels and shows the amount of $^{14}CO_2$ collected in the KOH trap over the preceding 1 h interval. Experiments in the two plots used different crops of basidiomes, but were otherwise identical.

Table 3. Specific activity (μmol substrate used mg protein^{-1} min^{-1}) of enzymes extracted from different tissues of *L. edodes* and *C. cinereus*

	Mycelia (21 d old, *L. edodes*)	Young cap (*L. edodes*)	Mature cap (*L. edodes*)	Young stipe (*L. edodes*)	Mature cap (*C. cinereus*)
Fructose 1,6-bisphosphate aldolase	1·08 ± 0·05	0·42 ± 0·05	0·31 ± 0·03	0·38 ± 0·04	1·10 ± 0·08
Glucose 6-phosphate isomerase	1·06 ± 0·08	0·45 ± 0·05	0·32 ± 0·03	0·34 ± 0·04	1·77 ± 0·17
Glucose 6-phosphate dehydrogenase	0·14 ± 0·02	0·45 ± 0·05	0·36 ± 0·03	0·42 ± 0·04	0·17 ± 0·02
6-Phosphogluconate dehydrogenase	0·09 ± 0·01	0·39 ± 0·03	0·30 ± 0·02	0·32 ± 0·02	0·11 ± 0·02

Entries are means ± S.D. of six independent determinations.

from C-2. This implies that the EMP played a less important role in the basidiome than it did in mycelia.

Comparison of basidiomes grown on LM or woodchips media (Figs 5, 6) showed that there was no major difference in the metabolic pathways utilized by *L. edodes* whether it was grown on a chemically defined medium (LM) or on the organic (woodchips) medium. Evidently, the cultivation medium did not materially affect the pathway utilized in glucose metabolism in the basidiome. This is particularly interesting in view of the supposed role of the pattern of metabolism seen in *L. edodes* basidiomes in providing for mannitol synthesis (see below) because the carbon source in the medium has been shown to be important in influencing the type of polyol formed in vegetative cultures of a number of yeasts and filamentous fungi (Vining & Taber, 1964; Adler & Gustafsson, 1980; Jennings, 1984; Ellis, Grindle & Lewis, 1991). Our observations imply that the metabolic shift in the basidiome is a true developmental one which is beyond the influence of the substrate.

Basidiome tissues of C. cinereus

A C-1/C-6 ratio of 3·6 was observed with a peak at 6 h, and a mean ratio of 2·9 at an RTU of 10 h (Table 2). These low ratios resemble those obtained from metabolism by the mycelia of *L. edodes* and suggest that metabolism of the *Coprinus* basidiome is very different from that of the basidiome of *L. edodes*. The PPP did not play a major role in *Coprinus*. Recovery of $^{14}CO_2$ from [3,4-^{14}C]glucose was very high (Fig. 7), even slightly higher than that from [1-^{14}C]glucose, signifying the important role played by the EMP in *C. cinereus*.

Enzyme determinations

Four key enzymes were assayed, two of these, fructose 1,6-bisphosphate aldolase ('aldolase'; catalysing the conversion of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) and glucose 6-phosphate isomerase ('G6P isomerase'; catalysing the isomerization of glucose 6-phosphate to fructose 6-phosphate) are principal enzymes of the EMP. The other two, glucose 6-phosphate dehydrogenase ('G6PD'; governing the conversion of glucose 6-phosphate to 6-phosphogluconolactone) and 6-phosphogluconate dehydrogenase ('6GPD'; responsible for the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate) play major roles in the PPP. Six replicate determinations of the specific activity were made for each of the enzymes. The results, summarized in Table 3, are subject to the general fidelity of the extraction procedure in preserving the intra-cellular ratios of the enzymes assayed.

The three-fold higher activity of the PPP enzymes, G6PD and 6PGD, in the basidiome tissues compared with mycelia would be compatible with a requirement for enhanced reducing power in the form of NADPH for biosynthetic activities in basidiome tissues. A similar increase has been seen for G6PD in *A. bisporus* (Hammond, 1977). Evidence for the stimulation of G6PD during initiation of periodic fruit body growth in *A. bisporus* has also been reported by Minamide &

Hammond (1985). Higher activity of these enzymes in the young cap and stipe compared with a mature cap of *L. edodes* is again suggestive of greater biosynthesis activity in young tissues.

The outcome from both enzymic (= *in vitro*) and radiorespirometric (= *in vivo*) determinations is, therefore, the same: the PPP becomes the major route of glucose catabolism in fruit body tissue of *L. edodes*.

The major function of the PPP is to furnish reduced pyrimidine nucleotides for NADPH-requiring biosynthetic reactions (Lehninger, 1975), the NADPH being generated during the oxidation of glucose-6-phosphate to 6-phosphogluconolactone and the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate. The most decisive evidence for the role of NADPH in biosynthesis came from the studies of *Neurospora crassa* mutants which have an abnormally low level of NADPH corresponding with an altered enzyme, glucose-6-phosphate dehydrogenase and a different morphology from that of the wild type (Brody, 1970).

Change in the carbohydrate metabolism of the fungus as it grows and differentiates has been reported in *Serpula lacrimans* (Brownlee & Jennings, 1981), but our results with *L. edodes* are most similar to those obtained with *A. bisporus* in which a greater proportion of glucose oxidation occurs via the PPP in the basidiome than in the mycelium. This has been correlated in *A. bisporus* wih the biosynthesis of the reserve and osmoregulatory carbohydrate, mannitol, in huge amounts in the basidiome (up to 50% dry weight) but not in the mycelium (3–4%) (Hammond & Nichols, 1976, 1977; Hammond, 1977). Synthesis of mannitol in *A. bisporus* is mediated by an NADPH-dependent mannitol dehydrogenase using fructose as substrate (Edmundowicz & Wriston, 1963) and the NADPH is obtained through the PPP (Dutsch & Rast, 1972). In filamentous fungi, there is good evidence that the PPP is generally involved in polyol metabolism and the level of activity of the PPP may quantitatively determine the level of polyol synthesis (Holligan & Jennings, 1972).

Accumulations of mannitol have also been found in *L. edodes*, but only in those tissues with a high PPP activity (Tan & Moore, 1994). GLC comparison of the mannitol contents of various tissues of *L. edodes* used the same strain (Le-11) cultivated under the same conditions as used here. Mycelia (low PPP activity) contained only 1·3% (D.W./D.W.) mannitol whereas basidiome tissues (high PPP activity) contained 20–30%.

Glucose metabolism in the basidiome of *L. edodes* thus resembles that in *A. bisporus* but contrasts with that reported for *C. cinereus*. In *C. cinereus*, it has been claimed that the EMP represents the major route of sugar catabolism in both the basidiome and mycelium, with the PPP playing only a minor role and being completely dispensed with in early basidiome development (Moore & Ewaze, 1976). The major role of the EMP in the *C. cinereus* basidiome, in sharp contrast to the situation in *L. edodes* and *A. bisporus*, was confirmed in the experiments reported here (Tables 2, 3). Interestingly, the total polyol content of the basidiome in *C. cinereus* did not exceed 6% of the dry weight and declined in concentration as the basidiome developed (Darbyshire, 1974), and neither mannitol (Tan & Moore, 1994) nor NADP-linked mannitol dehydrogenase (Rao & Niederpruem, 1969) have been detected in *C. cinereus* although a low concentration of mannitol (1·2%) was found in the vegetative cells of *C. friesii* (Pfyffer & Rast, 1980).

The contrasts between *C. cinereus* on the one hand and *L. edodes* and *A. bisporus* on the other, serve to emphasize the correlation between mannitol accumulation and high activity of the PPP. Mannitol possibly acts as a store of reducing power and osmoregulatory agent, controlling the influx of water necessary for cap expansion, although it must be emphasized that basidiome growth in *L. edodes* occurs by hyphal multiplication rather than the hyphal inflation evident in *Agaricus* (see discussion in Tan & Moore, 1994). However, if the PPP does affect regulation of water influx (through its influence on mannitol content) there could be commercial implications in researching means to manipulate the PPP in the basidiome (and, consequently, its water content) since 90% of the fresh shiitake mushroom consists of water.

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(*Accepted 22 November 1994*)

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