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γ-L-[^{3}H]-Glutaminyl-4-[^{14}C]hydroxybenzene (GHB): biosynthesis and metabolic fate after applying on *Agaricus bisporus*

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Abstract

γ-L-[^{3}H]-Glutaminyl-4-[^{14}C]hydroxybenzene (doubly labelled GHB), a phenolic amino acid characteristic of the *Agaricus* genus, was synthesized by feeding direct precursors such as 4-[U-^{14}C]aminophenol and L-[U-^{3}H]glutamic acid to mushroom gills. Radiolabelled compounds, namely mannitol, glutamine and doubly labelled GHB, were applied onto different tissues of growing sporophore and mycelium to follow their possible translocation into fruit body. Following contact with mushroom enzymes, doubly labelled GHB was immediately cleaved into its two components; the aromatic moiety was mainly consumed and lost as CO_2. This result was confirmed by applying 4-[U-^{14}C]aminophenol on both growing mushroom and sterile mycelium, which led to the recovery of the radiolabel as $^{14}CO_2$.

Keywords: γ-L-[^{3}H]-Glutaminyl-4-[^{14}C]hydroxybenzene (GHB); Biosynthesis; Metabolism and translocation; *Agaricus bisporus*

1. Introduction

γ-L-Glutaminyl-4-hydroxybenzene (GHB), a phenolic amino acid characteristic of *Agaricus* genus, was first discovered in *Agaricus hortensis* (Jadot, Casimir & Renard, 1960); its structure, synthesis and physicochemical properties are now well established (Jadot et al., 1960; Weaver, Rajagopalan & Handler, 1971a; Weaver, Rajagopalan, Handler & Byrne, 1971). It has been shown to accumulate in *A. bisporus* mycelium and fruit bodies where it is almost confined to gills and skin (Rast, Stüssi & Zobrist, 1979; Rast, Stüssi, Hegnauer & Nyhlén, 1981; Stüssi & Rast, 1981; Oka, Tsuji, Ogawa & Sasaoka, 1981; Foret & Arpin, 1991; Soulier, Foret & Arpin, 1993; Jolivet, Voiland, Pellon & Arpin, 1995).

The GHB biosynthetic pathway has also been elucidated. Labelled shikimic acid was incorporated almost only into the aromatic moiety, *via* the shikimate–chorismate pathway (Rast et al., 1979, 1981; Stüssi & Rast, 1981; Sasaoka, Ogawa, Tsuji & Bando, 1980; Tsuji, Bando, Ogawa & Sasaoka, 1981). Using [3-^{3}H] and [1,6-^{14}C] shikimic acid, it has been clearly proved that shikimate becomes aminated at the 4-position during *p*-aminobenzoic acid biosynthesis (Tsuji et al., 1981). The latter metabolite then undergoes decarboxylation and hydroxylation by a FAD-dependent monooxygenase, 4-aminobenzoate hydroxylase, to form 4-aminophenol (or *p*-aminophenol (PAP); the GHB aromatic moiety) (Tsuji, Ogawa, Bando & Sasaoka, 1986; Tsuji, Oka, Kimoto, Hong, Natori et al., 1996). Then, γ-glutamyltransferase (GGT) transfers the glutamyl part to 4-aminophenol to give GHB (Gigliotti & Levenberg, 1964).

GHB function in mushroom is not yet well understood. It has been suggested that spore dormancy could result from the presence of respiratory inhibitors originating from this compound (Vogel, McGarry, Kemper & Graham, 1974; Vogel, Kemper, McGarry & Graham, 1975): the product of tyrosinase-mediated oxidation of GHB inhibited both mitochondrial respiratory enzymes and protein synthesis in mushrooms. Similar effects were observed *in vitro* with rat liver mitochondria and ribosomes, as well as with intact bacteria, bacterial ribosomes and DNA polymerase

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(Graham, Tye & Vogel, 1977). Other authors showed that GHB could be considered as a precursor of spore wall melanins (Rast et al., 1979, 1981; Stüssi & Rast, 1981; Oka et al., 1981). Indeed, in the presence of tyrosinase and oxygen, GHB is readily oxidized into the corresponding diphenol (GDHB) and *ortho*-quinone (GBQ), which polymerizes into melanins (Weaver et al., 1971a,b; Rast et al., 1979, 1981; Stüssi & Rast, 1981; Szent-Gyorgyi, Chung, Boyajian, Tishler, Arison et al., 1976). Elementary composition and IR spectrum of GHB-melanin, obtained *in vitro* by enzymatic oxidation of GHB, most closely match those of the bioproduct (Pierce & Rast, 1995). The physiological role of GHB could, therefore, be concerned with (photo)-protection of mushroom spores.

However, the question remains open about its fate in the mycelium. Is it a stock molecule used *in situ*, or else is it translocated (either as a whole or after hydrolysis) into the sporophore? To answer this question, a doubly labelled [^{14}C,^{3}H]GHB has been biosynthesized after preliminary experiments with stable isotopes. The radioactive GHB, as well as other ^{14}C-labelled mushroom compounds (D-mannitol and L-glutamine), were then applied to mycelium and on several parts of the carpophore. Then, their fates have been followed in fruit bodies by autoradiography and radioactivity counting.

2. Results and discussion

2.1. Biosynthesis of labelled GHB

Gill tissues, known to be a prevalent site of GHB accumulation (Rast et al., 1979, 1981; Stüssi & Rast, 1981; Foret & Arpin, 1991; Soulier et al., 1993; Jolivet et al., 1995) were fed with several labelled precursors. Crude gill extracts obtained as described in Section 3 were submitted to successive chromatographies on exchange ion resins; this purification process was validated with unlabelled GHB, by TLC and HPLC analysis, and by UV and ^{1}H NMR spectrometry (see Section 3). Gill biosynthetic abilities and incubation conditions were first tested with stable isotopes, namely [^{15}N]ammonium acetate and D-[1-^{13}C]glucose. Experimental parameters and results obtained from purified labelled GHB are summed up in Table 1; they clearly prove that gills contain the full enzymic equipment required to synthesize GHB. Incorporation rate was higher in isolated gills than in the ones attached to intact mushrooms: 3.0 and 1.8%, respectively, while the total amount of GHB per g of gills was lower in isolated gills: 0.46 *versus* 1.01 mg g^{-1} for the intact mushrooms. This shows that anabolism is more active in intact gills. Further experiments were therefore carried out with radiolabelled precursors applied on gills attached to intact mushrooms.

More direct precursors were chosen for radiolabelling both aromatic and glutamyl moieties of GHB. 4-Amino[U-^{14}C]phenol ([^{14}C]PAP) was used to label the aromatic part; whereas 4-aminophenol is a tyrosinase substrate and oxidizes spontaneously in the presence of oxygen (Boekelheide, Graham, Mize, Anderson & Jeffs, 1979), this small phenol can easily cross the membrane and be incorporated into GHB (Table 2). The glutamyl part was labelled either with L-[U-^{14}C]glutamine or L-[U-^{3}H]glutamic acid, leading to the same incorporation yield (Table 2). Since glutamate is not a GGT substrate (Gigliotti & Levenberg, 1964), it should be quickly transformed into glutamine or another γ-glutamyl derivative, such as the phenylhydrazine agaritine (another aminoacid specific of *Agaricus* genus), before the transfer of the glutamyl moiety occurred (Fig. 1).

Table 1
Labelled GHB synthesized by gills after a 5 hr incubation with stable isotope-containing precursors. Results are expressed as μg of [U-^{15}N]GHB or [9,11-^{13}C]GHB g^{-1} of gills

Assay	1	2
Precursors	$CH_3COO^{15}NH_4$	$CH_3COO^{15}NH_4$ and D-[1-^{13}C]Glc
initial ^{15}N (mg)	26.25	7.50
initial ^{13}C (mg)	—	6.50
Conditions	5 hr on gills in situ	5 hr on separated gills in a petri dish
Amount of gills (g)	37.75	34.80
GHB total (mg)	38.0	15.9
δ ‰		
^{15}N	2 484	2 964
^{13}C	—	115
μg [U-^{15}N]GHB g^{-1} gills	9.26	5.02
μg [9,11-^{13}C]GHB g^{-1} gills	—	2.95
Incorporation		
^{15}N	1.8%	3.0%
^{13}C	—	1.5%

Table 2
Preparation of radiolabelled GHB. Gills were incubated with the specified precursors, for the indicated times. Results are expressed as GHB specific radioactivities (mCi mol^{-1}) and as percentages of incorporation

Precursors	Incubation time (hr)	GHB (mmol)	GHB (mCi mol^{-1})	Incorporation (%)
[^{14}C]Gln(5 μCi)	4	21.0	1.4	0.6
[^{14}C]Gln(5 μCi)	12	9.3	11.0	2.1
[^{14}C]Gln(5 μCi)	24	14.0	13.0	3.6
[^{14}C]Gln(5 μCi)	30	7.0	7.4	1.0
[^{3}H]Glu (5 μCi)	4	23.0	1.7	0.8
[^{3}H]Glu (5 μCi)	12	24.2	5.0	2.4
[^{3}H]Glu (5 μCi)	24	7.9	20.0	3.1
[^{14}C]PAP (5 μCi)	12	25.0	22.0	10.8
[^{14}C]PAP (5 μCi)	24	7.5	21.0	3.2
[^{14}C]PAP (100 μCi) and [^{3}H]Glu (1000 μCi)	12	25.2	378.8 [^{14}C] and 176.8 [^{3}H]	9.5 [^{14}C] and 0.4 [^{3}H]
[^{14}C]PAP (100 μCi) and [^{3}H]Glu (1000 μCi)	24	16.6	207.2 [^{14}C] and 335.1 [^{3}H]	3.4 [^{14}C] and 0.6 [^{3}H]

Optimal incubation time depended on the precursor. With glutamine and glutamic acid, the best incorporations (3.6 and 3.1%, respectively) were obtained after a 24 hr incubation, while a 12 hr incubation was enough to achieve the highest value (10.8%) when 4-aminophenol was used as the precursor. The rather low values obtained with glutamine/glutamic acid could be explained by the dilution of these exogenous aminoacids with the unlabelled endogenous ones, which is not the case with the 4-aminophenol present in the cells only as a trace amount.

Double labelling of GHB was achieved by incubating both precursors (4-amino[U-^{14}C]phenol and L-[U-^{3}H]glutamic acid) during 12 and 24 hr; two sets of doubly radiolabelled GHB, with different $^{3}H/^{14}C$ ratios (0.47 and 1.62 for 12 and 24 hr incubation, respectively) were obtained and used for further translocation studies.

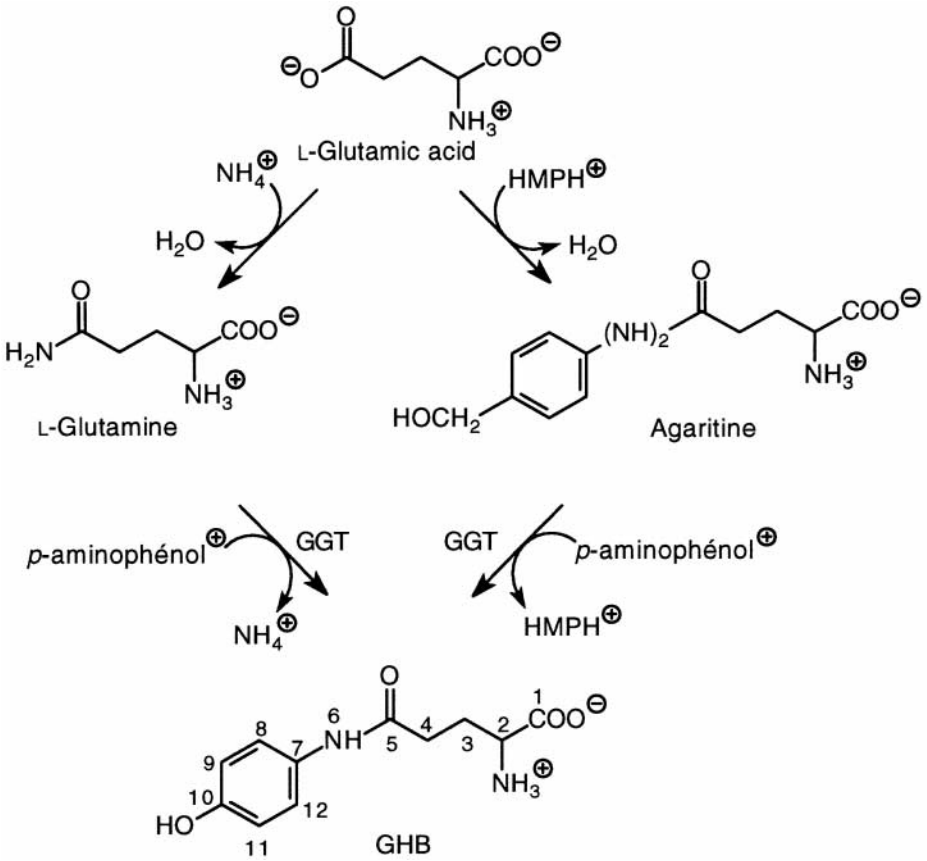

Fig. 1. Proposed scheme of γ-glutamyl transferase (GGT)-mediated GHB synthesis from glutamine and glutamic acid. HMPH: *p*-hydroxymethyl phenylhydrazine.

2.2. Transport of labelled metabolites into the sporophore

Before applying the doubly labelled GHB, general translocation processes (pathways, speed and accumulation sites) were examined in the whole mushroom by feeding carpophores with L-[^{14}C]glutamine and D-[^{14}C]mannitol, metabolites always found in high amounts in the mushroom (Foret & Arpin, 1991). Their courses were followed by autoradiographies (three to five day exposure times), leading to the following interesting information:

1. Although glutamine is charged, mannitol is not, the same behaviour was observed for both compounds, showing a dilution of radioactivity with increasing distances from the initial application zone. These metabolites could be swept away in-between hyphae, and within the cell wall, by diffusion and mass flow due to evaporation process. However, this could be insufficient to explain the movement of solutes and active transport should therefore be taken into account (Hammond & Wood, 1985).
2. Two preferential pathways could be distinguished. The first, which leads from the stipe directly to the gills and, *via* the flesh, to the skin, was especially obvious after applying the labelled soln onto the mycelium. The second pathway passed successively through the annulus into the skin and then the flesh, as shown clearly after applying labelled compounds at the mushroom periphery (stipe base, pellicle). Both routes had been previously observed following administration of [^{32}P]Pi and [1-^{14}C]mannitol, respectively (Kritskii, Kulaev, Mairova, Fais & Belozerskii, 1965; Rast, 1966). These two routes were not mutually exclusive: when the labelled soln had been absorbed through the stipe base, radioactivity was found in the skin even after veil excision (results not shown). In this case, radioactivity migrated upwards through the stem and across the gills, reaching eventually the skin.
3. L-[U-^{14}C]Glutamine and D-[1-^{14}C]mannitol accumulated preferentially in specific tissues, such as the gills. Such an accumulation could be explained in a straightforward way by the need of energy and metabolites required to form basides and spores (Kritskii et al., 1965). Skin and annulus were also found to be heavily labelled; this could result from metabolite concentration following evaporation. On the contrary, the upper part of the flesh was only slightly labelled; a possible explanation could rest on the histological organization of the hyphae during sporophore development (Moore, 1994).
4. The progress of [1-^{14}C]mannitol applied onto the stipe base of a set of identical mushrooms was monitored by locating radioactivity at different times. The translocation rate was evaluated at least as 1 cm hr^{-1}, in agreement with that previously found (Rast, 1966).

2.3. Transport and metabolism of exogenous labelled GHB

Doubly radiolabelled GHB was applied on growing mushrooms and the radioactivity of each isotope was quantified. Previous results obtained with labelled L-glutamine and D-mannitol were confirmed although no accumulation site could be distinguished, neither by autoradiography nor by liquid scintillation counting. The most outstanding result was a dramatic increase of the ^{3}H/^{14}C ratio measured in each tissue, from 5 to nearly 30-fold with respect to the GHB initial ratio (Table 3). The highest values were found in the gills and increased with increasing times. This clearly proved that GHB was cleaved and that the two resulting parts had different fates; the aromatic moiety was likely consumed and lost as CO_2, while the glutamyl moiety was incorporated into proteins or remained as free aminoacid. According to further analyses (results not shown), the fate of the glutamyl residue markedly depended on the tissue: ^{3}H was detected almost only in the insoluble fraction of the skin and in the soluble fraction of the flesh. These results corroborate previous observations showing that free glutamine and glutamic acid amounts were lower in the skin that in the flesh (Foret & Arpin, 1991).

Mineralization of the GHB aromatic part was confirmed by feeding *A. bisporus* gills and sterile mycelium with [U-^{14}C]PAP. The $^{14}CO_2$ amounts released after three days ranged between 10 and 24.5% of the initial radioactivity (Table 4), showing that *A. bisporus* owns all the equipment required for aromatic ring opening and then uses the resulting product as an energy source. This ability had been emphasized in *Neurospora crassa,* where catechol can easily be opened owing to a dioxygenase activity, yielding protocatechuate and then β-ketoadipate; afterwards, the latter compound is hydrolyzed into succinate and acetate usable

Table 3
Specific isotope counting in several mushroom tissues, after feeding the mycelium with doubly labelled GHB (initial ^{3}H/^{14}C ratio = 1.56)

Tissues	dpm [^{14}C]×1000	dpm [^{3}H]×1000	^{3}H/^{14}C
Mycelium	176.4	1040.6	5.90
Stipe base	5.0	41.0	8.25
Stipe (upper part)	2.0	20.6	10.49
Stipe (outside)	8.1	53.9	6.68
Flesh (lower part)	1.1	11.9	10.29
Flesh (upper part)	0.2	4.6	25.60
Gills	0.2	4.7	26.14
Skin	0.3	4.0	14.97

Table 4
Amounts of $^{14}CO_2$ released during incubation of *A. bisporus* mycelium or gills with 4-amino[U-^{14}C]phenol for 24, 48 and 72 hr. The results, expressed as dpm, are the average values of three determinations (s.d. 0.6%)

Incubation time (hr)	24	48	72
Mycelium			
- dpm [$^{14}CO_2$] × 1000	2 781	4 487	5 381
- % of initial radioactivity	12.6%	20.4%	24.5%
Gills			
- dpm [$^{14}CO_2$] × 1000	1 279	1 857	2 208
- % of initial radioactivity	5.8%	8.4%	10.0%

in energy metabolism (Haslam, 1974). In *A. bisporus*, when [U-^{14}C]shikimate was used as a GHB precursor, 99% of the radioactivity was found in the aromatic part, while 94% of the radioactivity from [U-^{14}C]benzoic acid was recovered in the glutamyl moiety (Rast et al., 1981; Stüssi & Rast, 1981). So, when radiolabelled GHB was incubated with the mushroom, the aromatic part was used, as any aromatic substrate, to produce energy and new compounds; this explains the traces of ^{14}C found in all tissues, even in spores.

Our results clearly show the lack of transport of exogenous GHB from the mycelium to the gills. This molecule is synthesized and used *in situ* in several tissues (mycelium, gills and skin) of the mushroom; its role within the mycelium remains unclear.

Due to the immediate hydrolysis of exogenous GHB, it seems likely that endogenous GHB should be protected from the cytosolic enzyme attacks. So, mushroom GGT might be a membrane-bound enzyme, such as the one found in *Lentinus edodes* (Iwami, Yasumoto, Nakamura & Mitsuda, 1975) or in mammals (Tate & Meister, 1982), releasing GHB directly into protective vesicles. Consequently, the mushroom GGT characteristics—including its active site—are now under investigation.

3. Experimental

3.1. Biological material

GHB biosynthesis was studied with fresh *A. bisporus* C45 (Le Lion) fruit bodies, stage 3, cap diameter 4–6 cm, incubated upside down in a beaker. After removing the veil, the soln of labelled GHB was laid down onto gills, the surface of which was slightly scratched to facilitate the absorption. At the end of incubation, gills were removed, weighted and GHB was extracted.

Transport experiments were carried out in triplicate with fresh carpophores bearing attached mycelium. After complete absorption of the labelled soln put on several mushroom parts, mushrooms were replaced for 3 to 5 days in the bed layer, within a moist enclosure at 18°C.

Mineralization experiments were carried out by applying labelled [U-^{14}C]PAP soln on both carpophores and sterile mycelium grown on rich compost medium. When mycelium had colonized 2/3 of the petri dishes, the labelled soln was applied on the periphery of growing mycelium from 4 petri dishes. Carpophores and petri dishes were put separately into sterile boxes with a beaker containing 3 ml ethanolamine as a $^{14}CO_2$ trap.

3.2. Labelled material

Stable isotopes (^{15}N and ^{13}C) were firstly used: $^{15}NH_4OAc$ and D-[1-^{13}C]glucose with an enrichment of 99% (Euriso-top). Radioisotopes (^{3}H and ^{14}C) were then tested: 4-amino[U-^{14}C]phenol (83 Ci mol^{-1}), L-[U-^{3}H]glutamic acid (53 000 Ci mol^{-1}), D-[1-^{14}C]mannitol (57 Ci mol^{-1}) were from Amersham and L-[U-^{14}C]glutamine (225 Ci mol^{-1}) was from the CEA. Radioactivity amounts used are defined in Tables 2–4.

3.3. GHB purification

Gills were ground ×3 in a mortar, with sand and 5 ml of 0.5% (w/v) $NaHSO_3$ in 1% HOAc. Lysates were filtered and centrifuged for 20 min at 10 000*g*. Supernatants were pooled and the crude extract was taken to dryness; the dry material was dissolved into 5 ml distilled H_2O and then laid on an anion exchange column (45×2.4 cm). The column was eluted with H_2O (1 ml min^{-1}), allowing a coarse separation of GHB. Fractions containing the monophenol were pooled and taken to dryness. Dry residue was suspended and laid on a cation exchange column (45×2.4 cm) also eluted with H_2O (1 ml min^{-1}), giving pure GHB. The exchange ion resins were prepared as described by Redgwell (1980); the anion exchanger QAE-Sephadex A-25 was activated for 2 days in 0.5 M HCO_2Na and, after filtration, stored in 0.05 M HCO_2Na. The cation exchanger, SP-Sephadex C-25, was activated for 2 days in 0.5 M $(NH_4)_2SO_4$ and after filtration, for one day in 7% HCO_2H; it was then stored in 1% HCO_2H. After each purification step, a sample (200 µl) was picked off for UV and radioactivity analyses. Purity was monitored by TLC on silica gel 60 F_{254} (Merck 5554) with BuOH–HOAc–H_2O (60:15:25) (Datta & Hoesch, 1987), and by HPLC using a Nucleosil C18 column (250×4.6 mm) eluted with 2% MeOH in 0.4% HOAc (0.8 ml min^{-1}) (Jolivet et al., 1995).

The UV spectrum of purified GHB was identical to that of standard GHB (λ_{max}: 245 nm (4.03)). ^{1}H NMR spectrum (200 MHz, D_2O): δ 2.22 (2H, *m*, H-3), 2.61

(2H, *m*, H-4), 3.83 (1H, *t*, H-2), 6.90 (2H, *d*, $J = 8.9$ Hz, H-9 and H-11), 7.28 (2H, *d*, $J = 8.9$ Hz, H-8 and H-12). For numbering see Fig. 1.

3.4. Stable isotope analysis

Elementary analysis was carried out at the Centre d'Analyse de Solaize (Rhône). GHB δ ‰ was calculated for both ^{15}N and ^{13}C as indicated below:

$$\delta‰ = \left[\frac{\frac{^{15}N}{Ntotal}\text{sample}}{\frac{^{15}N}{Ntotal}\text{standard}} - 1 \right] \times 1000$$

and

$$\delta‰ = \left[\frac{\frac{^{13}C}{Ctotal}\text{sample}}{\frac{^{13}C}{Ctotal}\text{standard}} - 1 \right] \times 1000,$$

where $\frac{^{15}N}{Ntotal}$ standard $= 0.3702$ and $\frac{^{13}C}{Ctotal}$ standard $=$ 1.098 were determined with unlabelled GHB purified in the same way.

3.5. Radioactivity analysis

Aliquots (200 µl) were mixed with 5 ml scintillation liquid (Packard) and radioactivity was quantified using Wallac 1400 DSA model (Pharmacia, Wallac). Efficacity curves versus external standard ratio had been established previously with ^{3}H and ^{14}C-labelled soln measured respectively in the ^{3}H and in the ^{14}C windows. In the case of a double labelling, the radioisotope ^{14}C was counting in the ^{3}H window. Therefore, a third efficacity curve had to be drawn and the value of ^{3}H disintegrations (n_H) was calculated with the following formula:

$$n_H = \frac{n_h' - n_c' \frac{E_2}{E_1}}{E_3},$$

where E_1 and E_2 were the efficacities in ^{14}C counted, respectively, in the ^{14}C and in the ^{3}H windows, E_3 was the efficacity in ^{3}H counted in the ^{3}H window and n_c' and n_H' were the values of disintegrations measured in the ^{14}C and ^{3}H windows, respectively.

GHB-treated mushrooms were dissected into several tissues (stipe base, external part of the stipe including veil, upper part of the stipe, lower and upper parts of the cap flesh, gills and skin) for radioactivity counting. Small pieces were first digested overnight at 50°C in Soluene-350 (1 ml for 500 mg). Digested soln (1 ml) was added to 10 ml of scintillation liquid (Emulsifier Scintillator Plus, Packard) prior to radioactivity counting.

Autoradiographies were performed with dry mushroom slices (1 mm thick), previously soaked for 1 hr in pure HOAc and then for 2 hr in HOAc containing 10% (w/v) 2,5-diphenyloxazole, and clamped for one week on a BioMax MR film (Kodak).

$^{14}CO_2$ released in the receptacle was trapped using ethanolamine. Ethanolamine/CO_2 soln (3 ml) was added to 10 ml Hionic–Fluor and 8.5 ml Methyl Cellosolve (Packard).

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