Table II. Concentration of Costunolide and Artemorin in Various Liqueurs Sold under the Common Denomination of Genepi

sample	concn, μg/100 mL	
	costunolide	artemorin
1	86.6	57.8
2	33.0	18.2
3	466	230
4		
5	149	83.2
6		
7	338	*a*
8	598	320
9	250	*a*
10		
11	608	326
12	203	99.6

[a] Not detectable as it is covered with other peaks.

sesquiterpene lactone, is only slightly bitter, all the other compounds we isolated are intensely bitter, particularly artemorin (V), which, due also to its concentration, should be considered the main bitter principle of the plant.

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Evidence for the Occurrence and Formation of Diazonium Ions in the *Agaricus bisporus* Mushroom and Its Extracts

Allan E. Ross,* Donald L. Nagel, and Bela Toth

N^{β}-[(+)-γ-Glutamyl]-4-(hydroxymethyl)phenylhydrazine (agaritine), a component of the common cultivated commercial mushroom *Agaricus bisporus*, is hydrolyzed to the 4-(hydroxymethyl)benzenediazonium ion, a carcinogen in mice, by an enzyme system present in the mushroom. This system, together with others, may be responsible for a 0.6-ppm level of the 4-(hydroxymethyl)benzenediazonium ion in the mushroom. A second diazonium ion is generated in acid extracts of *A. bisporus* from a precursor of unknown structure in the mushroom.

The common cultivated mushroom of commerical *Agaricus bisporus* contains relatively large amounts of the hydrazide N^{β}-[(+)-γ-glutamyl]-4-(hydroxymethyl)-phenylhydrazine (agaritine) (Levenberg, 1960, 1961). Kelly et al. (1962) reported a 0.04% yield of agaritine, based on the fresh weight of the mushroom. Using an HPLC assay system, we have found between 0.1 and 0.3% (wet weight) of agaritine in locally purchased mushrooms (unpublished data). Accordingly, an average-sized mushroom (15 g) could contain 45 mg of agaritine.

Levenberg (1961) also described an enzyme that catalyzes the hydrolysis of agaritine to glutamate and 4-(hydroxymethyl)phenylhydrazine (**2**) (Figure 1), and in 1963 Gigliotti isolated and partially characterized an enzyme that converts **2** to the 4-(hydroxymethyl)benzenediazonium ion (**3**). The enzyme is specific for hydrazines and, therefore, inactive on hydrazides. The presence of enzymes capable of generating a diazonium ion from agaritine supports the finding of **3** in the fresh mushroom by Levenberg (1962). In that report, however, **3** was poorly characterized.

To date, tumors have been induced in laboratory animals by 55 hydrazine derivatives (Toth, 1975, 1980), including **3** which, as the tetrafluoroborate salt, caused tumors in the subcutis and skin of mice (Toth et al., 1981). Two other compounds, closely related to **2**, *N'*-acetyl-4-(hydroxymethyl)phenylhydrazine and a reduction product of **2**, 4-methylphenylhydrazine, both significantly increased the incidence of lung and blood vessel tumors in mice (Toth et al., 1977, 1978) (Table I). *N'*-Acetyl-4-(hydroxymethyl)phenylhydrazine, like agaritine, contains the $HOCH_2(C_6H_4)$–NH–NH–CO– substructure. These recent carcinogenicity findings on compounds closely related to agaritine or, in the case of **3**, present in the mushroom motivated an investigation of the fate of agaritine in mushroom extracts and the confirmation and quantitation of **3** in the fresh mushroom.

MATERIALS AND METHODS

Mushrooms. Common commercial mushrooms, *A. bisporus*, were purchased from local food stores and used within 2 days of purchase.

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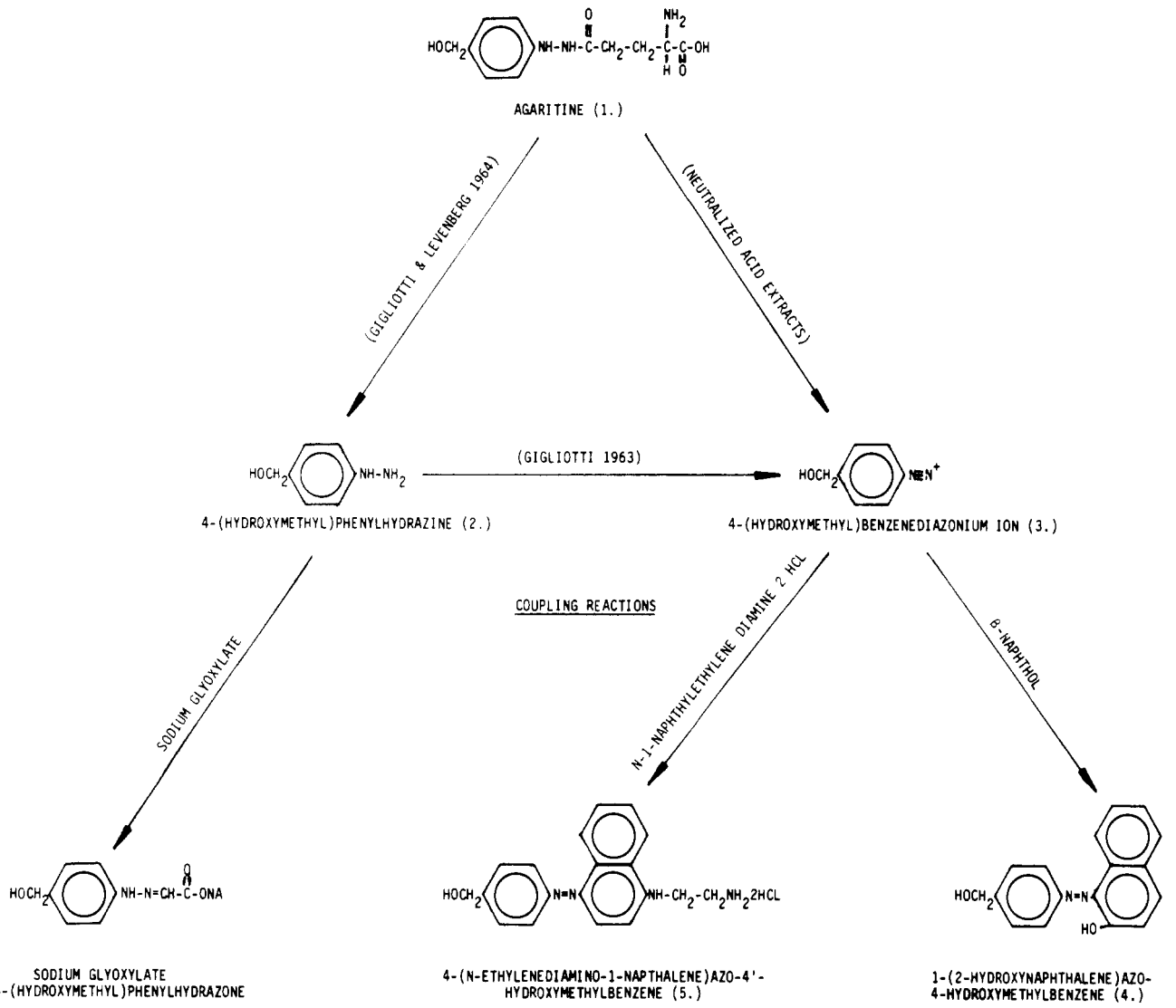
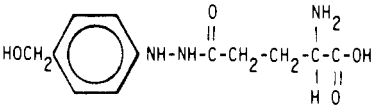
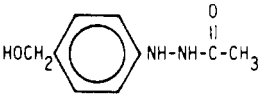
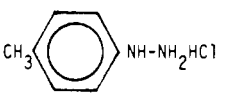
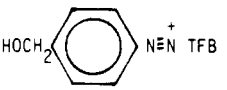


Figure 1. Enzymic catabolism of agaritine.

Table I. Carcinogenicity of Agaritine-Related Compounds

compound	structure	tumor site in mice
agaritine		negative[a]
N'-acetyl-4-(hydroxymethyl)phenylhydrazine		lung and blood vessel[b]
4-methylphenylhydrazine hydrochloride		lung, blood vessel, and subcutis[c]
4-(hydroxymethyl)benzenediazonium tetrafluoroborate		subcutis and skin[d]

[a] B. Toth, unpublished experiments. [b] Toth et al. (1978). [c] Toth et al. (1977). [d] Toth et al. (1981).

Reagents. β-Naphthol and *p*-aminobenzoic acid were obtained from Aldrich Chemical Co. (St. Louis, MO). The *p*-aminobenzoic acid was reduced to *p*-aminobenzyl alcohol with hydrogen and platinum oxide in absolute ethanol at 30 psi and recrystallized from ethyl ether, mp 62–63 °C [lit. mp 64 °C (Tiele and Dimroth, 1899)]. Phenylhydrazine was obtained from Fisher Scientific Co. (Fair Lawn, NJ), and *N*-naphth-1-ylethylenediamine dihydrochloride (NED) and 4-methylphenylhydrazine were from Eastman Organic Chemicals (Rochester, NY). The latter two compounds were recrystallized from water and ethanol, respectively, before use. *N'*-Acetyl-4-(hydroxymethyl)-phenylhydrazine and agaritine (Figure 1) were prepared as previously described (Toth et al., 1978; Wallcave et al., 1979). Nitrosyl tetrafluoroborate was purchased from Alpha Chemical Co. (Danvers, MA) and washed consecu-

tively with glacial acetic acid–chloroform (2:1), chloroform, and anhydrous ethyl ether. All washings were done with the solvents precooled to 0 °C.

Synthesis of 4-(Hydroxymethyl)benzenediazonium Tetrafluoroborate. A 1.75-g (1.75-mmol) samples of nitrosyl tetrafluoroborate was suspended in 15 mL of ethyl acetate maintained at 0 °C. The solution was cooled to −20 °C, and a solution of *p*-aminobenzyl alcohol (1.23 g, 1.0 mmol) in 15 mL of ethyl acetate was added in one portion. The mixture was stirred for 1 h, 15 mL of ethyl acetate was added, and stirring was continued for 0.5 h. The off-white precipitate was filtered under nitrogen pressure, washed with ethyl acetate (3 × 25 mL), and dried (yield 2.0 g, 90%; mp 51–52 °C). Recrystallization was from methanol–ethyl acetate (mp 61–62 °C). ^{1}H NMR (Varian CFT-20) (D_2O) δ 8.53 (d, J = 8.3 Hz, 2 H), 7.90 (d, J = 8.3 Hz, 2 H), 4.89 (s, 2 H), 4.66 (br s, 1 H); IR (KBr) (Beckman 1R9) 2275 cm^{-1} (N≡N); UV (H_2O) 274 nm (ε 25 000). Anal. Calcd for $C_7H_7BF_4N_2O$: C, 37.84; H, 3.15; N, 12.61. Found: C, 38.13; H, 3.20; N, 12.49.

Synthesis of 2-Hydroxy-1-naphthaleneazo-4′-(hydroxymethyl)benzene (4). We undertook the following synthesis to provide a standard for the isolated adduct formed in the mushroom extract by reaction of **3** with added β-naphthol.

A solution of 144 mg of β-naphthol in 15 mL of 10% NaOH was vigorously stirred at 5 °C. A cooled equimolar solution of the tetrafluoroborate of **3** was slowly added. After 0.5 h, the solution was neutralized and filtered, and the precipitate was recrystallized from ethanol: mp 162–163 °C; UV (95% ethanol) 228 nm (ε 3.24 × 10^4), 482 (ε 1.55 × 10^4); UV (ether) 465 nm (ϵ_{465} 1.25 × 10^4). Anal. Calcd for $C_{17}H_{14}O_2N_2$: C, 73.37; H, 5.36; N, 10.07. Found: 73.39; H, 5.17, N, 10.13. The proton and C^{13} NMR spectra were consistent with the assigned structure (Figure 1).

Synthesis of 4-[(2-Aminoethyl)amino]-1-naphthalene)azo-4′-(hydroxymethyl)benzene (5). To provide a standard for the isolated adduct formed in the mushroom extract by the reaction of **3** with added NED, we undertook the following synthesis.

A 520-mg sample of NED was dissolved in a minimal amount of 5 N HCl. A solution of the tetrafluoroborate of **3** (440 mg) in water was added with stirring. After the solution was allowed to stand for 0.5 h, the precipitate was filtered, washed with consecutive portions of ethyl acetate and ether, and dried under vacuum, mp 177–178 °C. A satisfactory elemental analysis was obtained (±0.3% C, H, and N). Spectral characteristics were consistent with the assigned structure (Figure 1). UV (0.1 M HCl) 555 nm (ε 5.2 × 10^4).

Chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates (Brinkmann Instruments, Inc.) and developed with the following solvent systems: I, ether–hexane (8:2); II, methylene chloride–tetrahydrofuran (9:1); III, 1-butanol–acetic acid–water (4:1:1).

Gas chromatography (GC) was performed on a Varian 3700 with a 6-ft glass column containing 4% SE-30 on Chromasorb W. The flow rate was 15 cm^3 of He/min, the column temperature was 240 °C, and the effluent was monitored with a flame ionization detector.

Analysis of *A. bisporus* for Arenediazonium Ions. *In Neutralized Extracts.* The mushrooms were chopped and then homogenized in a Waring blender with 2 volumes of 0.1 N HCl. After centrifugation, the supernatant (at pH 2.2) was neutralized to pH 7 with 25% NaOH and the precipitate, which formed on cooling to 0 °C, removed by a second centrifugation. The supernatant was then treated with a 2% solution off β-naphthol in methanol (10 μL/mL). After various times, the entire reaction mixture, or aliquots thereof, was saturated with NaCl and extracted with 2 volumes of ether for UV assay. The ether layer was concentrated to dryness in vacuo or in current of N_2, and the bright orange residue was dissolved in a minimal amount of methanol for GC (see above) or TLC (system I or II) analysis.

For isolation of enough coupled azo compound for positive determination of its structure, the neutralized extract from 220 g of mushrooms, after incubation with β-naphthol, was extracted with ether. The residue, following removal of the ether, was dissolved in 0.4 mL of methanol and applied to a 20 × 20 cm silica gel TLC plate (1 mm). An analytical standard of **4** was spotted alongside the extract. After development with system II, the orange band at the same R_f (0.56) as the synthetic standard **4** was scraped off, packed into a small column, and eluted with ether. After removal of the ether in a current of nitrogen, the residue was dissolved in methanol for spectral analysis. The total yield was 0.4 mg of **4** which calculates to 0.9 μg of the diazonium ion **3**/g of mushroom (wet weight). GC on a 6 ft × 2 mm glass 4% SE-30 column (details given above) showed a peak at 18 min identical with that of the retention time of the authentic standard. The electron impact mass spectra (A.E. I. MS 902) of the authentic and isolated compounds were virtually identical (see Figure 2).

In Acidic Extracts. The 0.1 N HCl supernatant described in the previous section was treated with a 2% aqueous solution of NED (10 μL/mL). After various times, the entire reaction mixture, or aliquots thereof, was made highly alkaline (pH >13) with 25% NaOH and extracted with ether (2 × 2 volumes). The ether was removed and the residue taken up in a minimal amount of methanol for TLC (system III). The two major spots were scraped off into 1 mL of 0.1 M HCl, the silica gel was removed by centrifugation, and optical density was determined at 555 nm.

The R_f of the smaller spot (0.56) was identical with that of compound **5**. The large spot (R_f 0.44) is of unknown structure. Mass spectrographic data was inconclusive, although the molecular weight seems near that of **5**. The UV spectrum is almost identical with that of **5**, and accordingly the compound was monitored at 555 nm.

Precipitation of the Catalytic Activity. Cold acetone (3 volumes) was added to the above-described neutralized extract (1 volume) at about 4 °C. The precipitate that formed overnight at −20 °C was collected by centrifugation and dissolved in 0.1 volume of water. Twice this volume of cold acetone was added, and the precipitate was collected by centrifugation. This second precipitate was dissolved in 0.1 volume of water and further diluted (×10) for activity measurements with agaritine and other hydrazides and hydrazines as substrates. β-Naphthol was the trapping agent for diazonium ions.

Incubation Conditions. Each incubation mixture contained 0.1 mL of the solution of the second precipitate, 2.5 mg of a substrate, 0.1 mL of a 2% solution of β-naphthol in methanol, and water to 5 mL. The incubation was at 37 °C for 24 h. At 2, 4, 6, and 24 h, 0.5-mL samples were removed, saturated with NaCl, and extracted with 2 mL of ether. The optical density of the ether layer was determined at 460 nm.

RESULTS AND DISCUSSION

Diazonium ion **3** may exist in mushroom extracts either because (1) it is stable enough to be a normal mushroom constituent and released into the extract on homogenization or (2) it forms from a precursor by chemical or enzyme

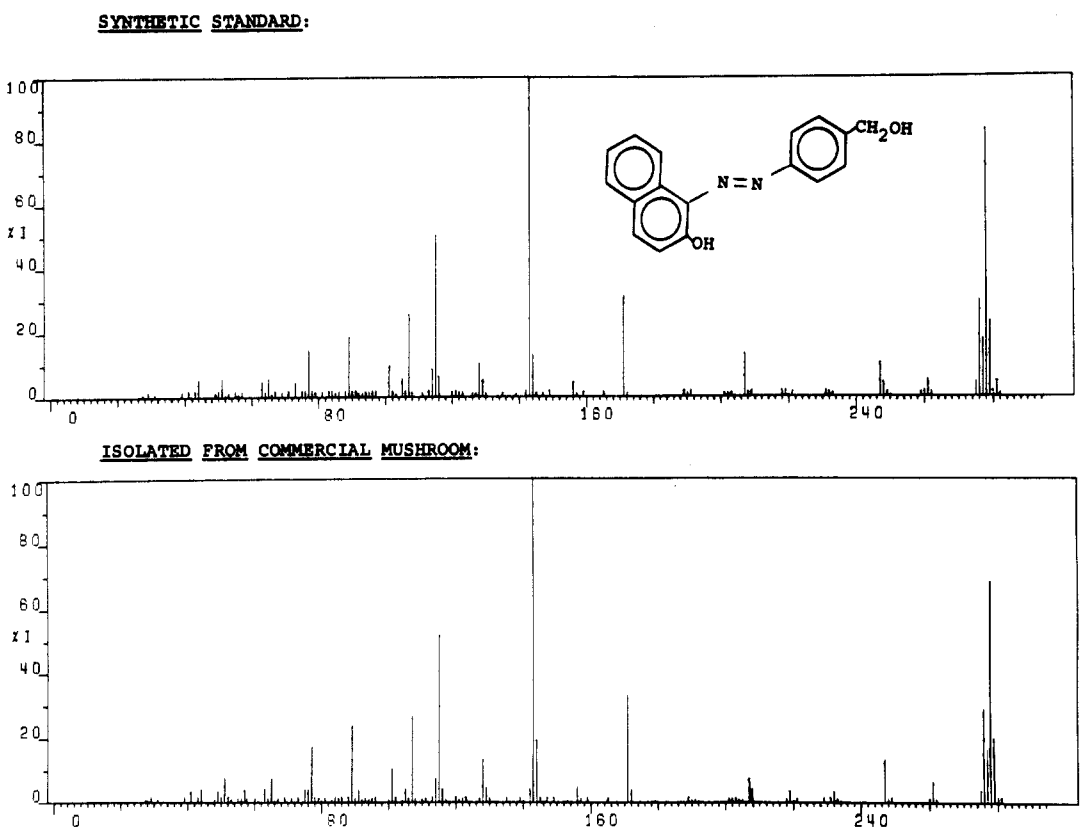


Figure 2. Mass spectra of 2-hydroxy-1-naphthaleneazo-4′-(hydroxymethyl)benzene.

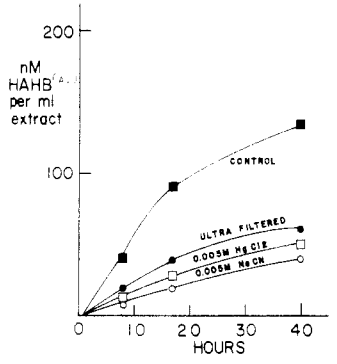


Figure 3. Enzyme-produced 4-(hydroxymethyl)benzenediazonium ion from in situ agaritine in neutral mushroom extracts. 5 mL of neutralized extract plus 50 μL of 2% β-naphthol in MeOH. 1-mL samples saturated with NaCl and extracted with 2 mL of ether. Assay = optical density of ether layer at 460 nm. A_1 = 2-hydroxy-1-naphthaleneazo-4′-(hydroxymethyl)benzene.

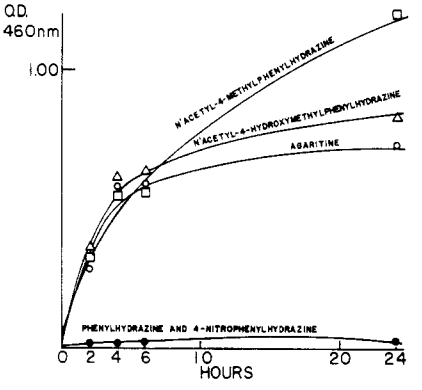


Figure 4. Specificity of mushroom enzyme system for hydrazides. Incubation mixture: 0.1 mL of acetone precipitate, concentrate, 2.5 mg of substrate, 0.05 mL of 2% β-naphthol in methanol, and water to 5 mL, shaken in water bath at 37 °C. Assay: 0.5-mL samples saturated with NaCl and extracted with 2 mL of ether; optical density of the ether solution at 460 nm.

action. As mentioned previously, Gigliotti and Levenberg (1964) reported an enzyme system in mushroom extracts capable of splitting agaritine (1) to the unstable 4-(hydroxymethyl)phenylhydrazine (2) and glutamic acid. Gigliotti (1963) also reported an enzyme system capable of catalyzing generation of the diazonium ion 3 from 2 that was specific for hydrazines. Thus, the two above systems combined could generate 3 from agaritine.

To establish that the presence of 3 in our extracts was due to enzyme action, we compared the formation rate of the azo compound 5 in a neutralized extract containing β-naphthol with that in the same preparation containing (a) 0.005 M NaCN or (b) 0.005 M $HgCl_2$ or (c) ultrafiltered through a membrane with a M_r 50 000 cutoff (Amicon Corp., MA). The results are shown in Figure 3. After 40 h, inhibition due to NaCN, $HgCl_2$, or ultrafiltration was 75%, 67%, and 61%, respectively, and is likely due to enzyme inactivation, with NaCN and $HgCl_2$, or to enzyme removal, with ultrafiltration. The most obvious substrate for this enzyme system is agaritine, present in these extracts in concentrations of up to 2 mg/mL. Acetone precipitates of these extracts were tested for enzymatic properties as described under Materials and Methods with the following compounds as substrates: synthetic agaritine, N′-acetyl-4-(hydroxymethyl)phenylhydrazine (6), N′-acetyl-4-methylphenylhydrazine (7), phenylhydrazine (8) and 4-nitrophenylhydrazine (9). Incubation mixtures containing 8 and 9 generated no azo compounds. The formation rate of azo compounds from diazonium ions is shown in Figure 4. All the hydrazides containing the R–NH–NH–CO–R group acted as substrates for the enzyme system in the acetone precipitate to form diazonium ions. N′-Acetyl-4-methylphenylhydrazine was a better substrate than the others, and TLC (system I) of ether extracts of these incubation mixtures demonstrated, as

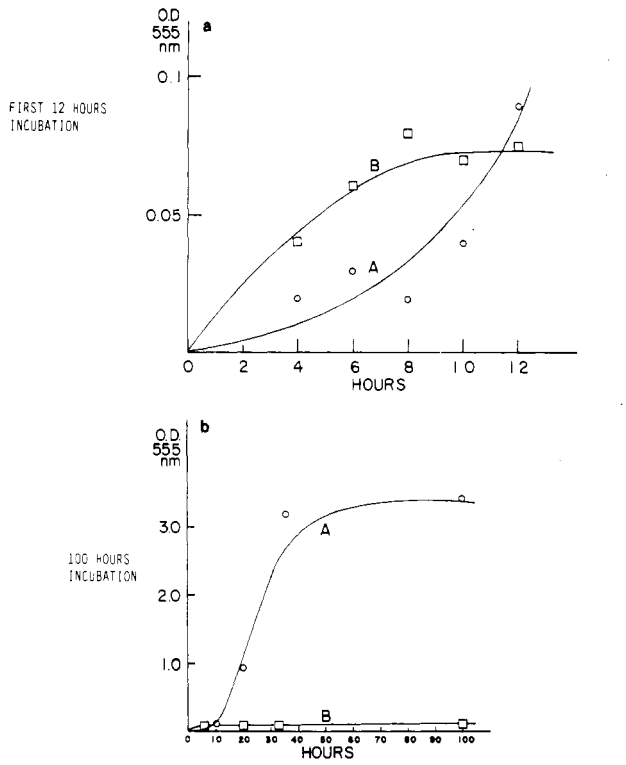


Figure 5. Formation of azo compounds from diazonium ions in acidic extracts of *A. bisporus*. 50-mL samples taken from the 0.1 M HCl extract of 420-g mushrooms and brought to pH >13 with 25% NaOH. The coupled azo compounds were extracted into ether (2 × 2 volumes), the ether was removed, and the residue was dissolved in 0.1 mL of methanol. 5-μL samples were spotted on a silica gel TLC plate. After development with butanol–acetic acid–water (4:1:1), the spots were scraped into 1 mL of 0.1 M HCl, and the optical density was determined at 555 nm. (○) Major product A (R_f 0.44); (□) minor product B (R_f 0.56).

expected, that agaritine and **6** generate the same diazonium ions and that the azo compounds formed with β-naphthol have the identical R_f with the authentic azo compound.

We have presented evidence that *A. bisporus* contains an acetone-precipitable enzyme system capable of catalyzing oxidation of hydrazides, including agaritine, to their related diazonium ions. This system is inactive on hydrazines and is therefore different from that described by Gigliotti (1963). However, the system we have isolated may break down agaritine via the unstable hydrazine **2**. To test for this, we incubated the enzyme solution with agaritine and sodium glyoxylate. Generation of **2** would result in formation of sodium glyoxylate 4-(hydroxymethyl)-phenylhydrazone (Gigliotti and Levenberg, 1964) (Figure 1). We found no evidence for formation of this compound under these conditions. Thus, *A. bisporus* appears to have two enzyme systems capable of generating the diazonium ion **3** from agaritine. One system, whereby agaritine is oxidized to **3** via the unstable hydrazine **2**, has been reported (Gigliotti, 1963; Gigliotti and Levenberg, 1964), and the other in which agaritine is oxidized to **3**, without intermediate formation of **2** (Figure 1), is described herein.

Diazonium Ions in Acidic Extracts of *A. bisporus*. Since there appear to be two enzyme systems in the mushroom that are capable of generating the diazonium ion **3** from agaritine, as well as relatively high concentrations of this substrate, it is possible that **3** is an intermediate in the metabolic pathways involved in the formation or breakdown of agaritine. Thus, at any one time, there could be a steady-state level of **3** in the mushroom. After adding NED to acid extracts of mushrooms and incubating for 6 h, Levenberg (1962) reported formation of an azo compound that had identical paper chromatographic properties with those of the authentic **5**, indicating the presence of **3** in the extracts.

We found that if 0.1 M HCl extracts (pH 2.2) of *A. bisporus* were reacted longer (18–100 h), two azo compounds could be extracted into ether and separated by TLC eluting with system III. The smaller component had an R_f (0.56) identical with that of synthetic **5** and is presumed to be due to the presence of, or generation of, **3** in the extract. The second azo compound formed a much larger spot on the TLC plate (R_f 0.44) and is attributed to the presence of, or the generation of, a second diazonium ion in the extract. These two azo compounds are also formed in extracts from mushrooms homogenized with 0.5, 1.0, and 2.0 M HCl (pHs 0.96, 0.63, and 0.35, respectively) and in a 0.1 M HCl extract filtrate after ultrafiltration through a membrane with a M_r 5000 cutoff. Enzymatic generation of these two diazonium ions is therefore unlikely. The production rate of these two azo compounds was followed in a 0.1 M HCl extract containing NED. The results are shown in Figure 5. In Figure 5a, the lesser product B, with the same R_f as synthetic **5**, is formed relatively more rapidly over the first 6–8 h, as previously observed by Levenberg (1962). If NED is added to dilute solutions (0.5–1 μg/mL) of authentic **3**, the reaction takes 5 h to completion. It is possible, therefore, that compound **5** was formed in these acid extracts of *A. bisporus* by the chemical combination of NED with the **3** present in the mushroom at time of extraction in a concentration of about 1 μg/g. Also, the acid extract may cause an unknown precursor to decompose to **3**. Under these extraction conditions, agaritine does not decompose to **3**.

The slow initial formation rate of major component A in this extract suggests that, rather than a free diazonium ion being present, an unknown diazonium ion is formed from a precursor by the acid conditions of the extraction technique. If A has a similar M_r to azo compound **5**, commercial mushrooms may contain a compound capable of generating at least 20 μg/g (wet weight) of a diazonium ion under acidc conditions that mimic closely the conditions in the human stomach.

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