

Phenylhydrazines in the Cultivated Mushroom (*Agaricus bisporus*)

- occurrence, biological properties, risk assessment and recommendations

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By Andersson, H.C. and Gry, J.

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Preface

The Nordic Committee of Senior Officials for Food Issues is an advisory body of the Nordic Council of Ministers which co-ordinates Nordic work in the field of food and nutrition. The Committee has given the Nordic Working Group on Food Toxicology and Risk Evaluation (NNT) the responsibility to promote co-operation and co-ordination among Nordic countries in matters relating to food toxicology and risk assessment.

Assessment of health risks connected with exposure to naturally occurring toxicants in foodstuffs has become an important area for NNT in the recent years. A series of Nordic reports based on the work performed by the Nordic project group on inherent natural toxicants in food plants and mushrooms has been published:

Gry, J. and Pilegaard, K. (1991) Hydrazines in the Cultivated Mushroom (*Agaricus bisporus*). Vår Föda 43;Supplement 1.

Uggla, A. and Busk, L. (1992) Ethyl carbamate (urethane) in alcoholic beverages and foodstuffs - A Nordic View. Nordiske Seminar- og Arbejdsrapporter 1992:570.

Størmer, F.C., Reistad, R. and Alexander, J. (1993) Adverse health effects of glycyrrhizic acid in licorice. A risk assessment. Nordiske Seminar- og Arbejdsrapporter 1993:526.

Andersson, C., Slanina, P. And Koponen, A. (1995) Hydrazones in the false morel. TemaNord 1995:561.

Søborg, I., Andersson, C. and Gry, J. (1996) Furocoumarins in Plant Food - exposure, biological properties, risk assessment and recommendations. TemaNord 1996:600.

Gry, J. and Andersson, H.C. (1998) Nordic seminar on phenylhydrazines in the Cultivated Mushroom (*Agaricus bisporus*). TemaNord 1998:539.

Andersson, C. (1999) Glycoalkaloids in tomatoes, eggplants, pepper and two Solanum species growing wild in the Nordic countries. TemaNord 1999:599.

Andersson, H.C. (2002) Calystegine alkaloids in Solanaceous food plants. TemaNord 2002:513.

Andersson, C., Wennström, P. and Gry, J. (2003) Nicotine in Solanaceous food plants. TemaNord 2003:531.

There is a large and still increasing consumption of the cultivated mushroom in the Nordic countries. The Nordic project group on inherent natural toxicants in food plants and mushrooms has followed the scientific information on the toxicity of *Agaricus bisporus* and the phenylhydrazine derivatives occurring in the mushroom since the end of the 1980s. In 1991 the project group reviewed the available data and found it not to be of adequate standard to evaluate the human risk at the time. In 1996 the project group organised a Nordic Seminar on Phenylhydrazines in the Cultivated Mushroom (*Agari-*

cus bisporus) in Kolding, Denmark, to promote the exchange of information between chemists and toxicologists active in the field of *Agaricus bisporus* research.

The present report is an updated review of the phenylhydrazines in the cultivated mushroom (*Agaricus bisporus*) - occurrence, biological properties, risk assessment and recommendations – (Databases searched: TOXLINGE, BIOSIS, MEDLINE and FSTA, June 2003 included) prepared by the Nordic project group on inherent natural toxicants in food plants and mushrooms.

The *Project Group* consisted of the following members:

Jørn Gry (co-ordinator)	Danish Veterinary and Food Administration	Denmark
Henrik Frandsen	Danish Veterinary and Food Administration	Denmark
Christer Andersson	National Food Administration	Sweden
Tore Aune	University of Veterinary Medicine	Norway
Jan Alexander	National Institute of Public Health	Norway
Anja Hallikainen	National Food Agency	Finland
Torkell Johannesson	Department of Pharmacology, University of Iceland	Iceland

The present report was initiated in 1996 and has been prepared by Christer Andersson¹, Sweden, and Jørn Gry², Denmark, after very thorough discussions in the project group and finally adopted by NNT.

The NNT acknowledges Dr. S. Clausen, Chemistry Department, Royal Veterinary and Agricultural University, Denmark, and Dr. H. Frandsen, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration, Denmark, for the chemical synthesis of agaritine and related phenylhydrazine derivatives. The synthesis was financed by a grant from the Nordic Council of Ministers.

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1. Sammanfattning

Under perioden 1961 till 1985 påvisades fyra olika fenylhydraziner eller närbesläktade föreningar i den odlade champinjonen, *Agaricus bisporus*. Ett av dessa ämnen, agaritin (N^2 -[γ -L-glutamyl]-4-hydroxymetylfenylhydrazin) förekommer i störst mängd än de övriga fenylhydrazinerna. Halter mellan 80 och 1 700 mg/kg har återfunnits i den färska svampen. Vanligen ligger dock nivån i intervallet 200 till 500 mg/kg färskvikt. Utöver agaritin, innehåller den odlade champinjonen små mängder 4-karboxyfenylhydrazin (10-11 mg/kg färskvikt), β -N-[γ -L-(+)-glutamyl]-4-karboxyfenylhydrazin (42 mg/kg färskvikt) och 4-hydroxymetylbenzendiazonium joner (HMBD) (0.6-4 mg/kg färskvikt).

Studier av försöksdjur har visat att agaritin och/eller dess nedbrytningsprodukter absorberas snabbt och i påtaglig omfattning från mag-tarmkanalen. Däremot kan ingen oförändrad agaritin påvisas i blodet. Enzymet γ -glutamyl transpeptidas förekommer i cellmembranen i flera av kroppens vävnader och tros vara det enzym som klyver agaritin till glutaminsyra och 4-hydroxymetylfenylhydrazin. Fenylhydrazinet omvandlas därefter till HMBD, som antas vara det ämne som ger upphov till reaktiva radikaler i vävnaderna. Radikalerna kan sedan orsaka skador på makromolekyler. Måttliga mängder DNA-skador har påvisats i olika vävnader från försöksdjur som exponerats oralt för agaritin och HMBD, vilket skulle kunna tyda på att fenylhydrazinerna är genotoxiska carcinogener. Denna tolkning stöds av observationen att såväl svampextrakt, som agaritin och de andra fenylhydrazinerna i *A. bisporus*, inducerar mutationer i bakterier testade *in vitro*, om än i liten omfattning. Dessutom har *in vivo* studier på möss visat att agaritin inducerar genmutationer i transgena försöksdjur och 4-hydroxymetylbenzendiazonium jonen mikrokärnor i perifera lymfocyter.

Endast ett fåtal hydrazinderivat är kända från naturen. Industrin har emellertid syntetiserat ett större antal hydrazinföreningar för egna ändamål. Eftersom flertalet av de nära ett hundra hydraziner som studerats (cirka 85%) är giftiga och/eller cancerframkallande i försöksdjur, inledde Toth och medarbetare en serie undersökningar för att utvärdera huruvida fenylhydrazinerna i den odlade champinjonen, till dessa närbesläktade ämnen och svampen i sig uppvisade carcinogen aktivitet. I tre av fyra långtidstudier utvecklades tumörer i möss som utfodrades med rå, ungsbakad eller frystorkad *A. bisporus*. I den fjärde studien, med ungsbakade champinjoner, men med en annan (mer balanserad) utfodring av svampen, erhöles ingen ökning i tumörincidens. Inte heller erhöles någon tumörinduktion i två bristfälliga långtidsstudier utförda på råttor. Antalet råttor var alltför lågt för att en mindre ökning i tumörincidens skulle kunna påvisas. Dessutom är det tänkbart att behandlingen av svampfodret (ordinarie torkning och malning, alternativt tvättning kombinerat med tryckkokning, homogenisering och blandning med kranvatten) kan ha resulterat i en signifikant nedbrytning av de potentiellt aktiva fenylhydrazinerna. Tre av de fyra fenylhydraziner eller närbesläktade ämnen som förekommer i *A. bisporus* - 4-karboxyfenylhydrazin, β -N-[γ -L-(+)-glutamyl]-4-karboxyfenylhydrazin and HMBD - framkallade tumörer när höga doser gavs till möss (Swiss) oralt (tvångsmatning eller i dricksvattnet), eller när ämnena injicerades under huden. Den fjärde fenylhydrazinen, agaritin, visade sig inte ge upphov till tumörer när ämnet gavs i dricksvattnet eller injicerades under huden. Denna oväntade upptäckt har påtagligt försvårat

tolkningen av de data som erhållits vid studierna med fenylyhydraziner. Emellertid skall påpekas att inga försiktighetsåtgärder vidtogs i cancerstudierna för att skydda agaritinet mot en eventuell oxidativ nedbrytning i vattenlösningen. Det har nämligen nyligen visats att agaritin lätt bryts ned i väl syresatta vattenlösningar.

Det måste understrykas att de långtidsstudier av cancerinduktion som denna riskbedömning bygger på, kan kritiseras av flera skäl. En och samma musstam (Swiss) har använts i samtliga studier. Musstammen skiljer sig från flertalet musstammar som används i cancerstudier genom att inte vara inavlad. Det faktum att mössen får para sig fritt, resulterar i en större variation i känslighet mellan de olika mössen än vad som skulle vara fallet för djuren i inavlade stammar. Dessutom har studierna ofta använt sig av olämpliga kontrollgrupper - antingen historiska kontroller eller kontrollgrupper som inte startats samma dag som de behandlade grupperna. I stället för att genomföra cancerstudierna på vanligt sätt under en förbestämd tidsperiod (vanligen 2 år), fick studien pågå till dess att djuren dog av hög ålder eller allvarlig sjukdom (inklusive tumörsjukdom). Cancerstudier använder sig vanligen av tre dosgrupper. De aktuella studierna har oftast haft endast en dosgrupp. Några av studierna har kritiserats för att fodertillförseln inte varit balanserad. Vanligen studeras ett större antal biokemiska och toxikologiska parametrar parallellt med huvudvariabeln (tumörinduktion) för att bättre kunna utvärdera erhållna resultat. Sådana observationer har endast bristfälligt rapporterats i de publicerade studierna med odlad champinjon och dess fenylyhydraziner.

Trots att flertalet cancerstudier på mus gav positivt utslag finns inga tecken på att vare sig *A. bisporus* eller fenylyhydrazinerna i svampen är embryotoxiska eller teratogena i däggdjur vid biologiskt relevanta doser. Det skall dock påpekas att försöksuppläggningsen på de få studier som finns tillgängliga inte uppfyller de krav som ställs i moderna riktlinjer för hur sådana studier skall utföras. Det skall också påpekas att ytterst få toxicitetstudier utförts med fenylyhydrazinerna i den odlade champinjonen vid sidan av cancerstudierna på försöksdjur.

Den uppskattade årskonsumtionen av odlad champinjon (*Agaricus bisporus*) per capita varierar mellan de nordiska länderna - från ungefär 0.6 kg per person i Finland till 2.4 kg per person i Sverige. Även marknadsandelen färsk champinjon varierar mellan de olika länderna, från 33% till 70%. Resten utgörs av konserverad champinjon.

Eftersom per capita intaget är en uppskattning av medelintaget och inte tar hänsyn till det faktum av svamp endast konsumeras av en del av populationen, blir per capita intaget för lågt för att beskriva intaget hos dem som verkligen konsumerar svamp. En mindre dansk studie tyder på att endast hälften av befolkningen konsumerar odlad champinjon och att 5% av befolkning konsumerar fem gånger så mycket som medelintaget och 0.1% av befolkningen trettio gånger mer än medelintaget.

Med undantag för agaritin är vår kunskap dålig vad gäller hur lagring och tillagning av champinjon påverkar halten av fenylyhydraziner i den konsumerade svampen. För agaritin vet vi att halten reduceras i odlad champinjon både under lagring och tillredning. Hur mycket agaritin som försvinner beror på sättet svampen hanterats. Vanliga tillagningsmetoder halverar mängden agaritin. Konserverad champinjon innehåller högst 10% av den mängd som återfinns i färsk svamp.

Med utgångspunkt från angivna data kan per capita exponeringen för agaritin (som en markör för fenylyhydrazinerna i odlad champinjon) i de nordiska länderna uppskattas. Exponeringen ligger någonstans i intervallet mellan 48 och 788 mg agaritin/år, vilket

motsvarar ett dagsintag på mellan 2.1 och 36 µg/kg kroppsvikt. Exponeringen påverkas av mängden svamp som konsumeras (nationalitet), halten av agaritin i den färska svampen (200-500 mg/kg) och huruvida man föredrar färsk (33-70%) eller konserverad svamp. En enstaka svamprätt med 75 g färsk *A. bisporus* resulterar i en exponering på cirka 15-33 mg agaritin, vilket motsvarar 250-550 µg/kg kroppsvikt.

Trots att cancerstudien med agaritin på (Swiss) möss var negativ, används sex andra cancerstudier baserade på oral tillförsel (tvångsmatning, i dricksvattnet eller i fodret) av odlad champinjon eller enskilda ämnen (tvångsmatning, i dricksvattnet eller i fodret) till Swiss möss i kombination med det uppskattade intaget av *A. bisporus* i de nordiska länderna för att göra en kvantitativ riskbedömning av den odlade champinjonen. Tre av långtidsstudierna utfördes med *A. bisporus per se*, medan de andra tre genomfördes med de fenylyldraziner och närbesläktade ämnen som förekommer i svampen. Vid beräkningen av cancerrisken användes en linjär extrapolering från mus till människa som baserades på flera antagen redovisade i rapporten, men där de viktigaste var att metabolismen och känsligheten hos mus och människa är jämförbar.

Sammantaget tyder de svaga mutagena effekterna *in vitro* och *in vivo* (agaritin och HMBD) och de carcinogena effekterna hos (1) tre av de fyra fenylyldrazinerna i *A. bisporus* och (2) den färska, ungsbakade och frystorkade svampen *per se* i Swiss albino möss, att *A. bisporus* är svagt cancerframkallande i försöksdjur och följaktligen kan innebära en risk för konsumenter. Den uppskattade livstidsrisken för en nordisk medelkonsument av färsk (eller frystorkad) *A. bisporus* är cirka 200×10^{-6} (50×10^{-6} för finska konsumenter), medan intaget av en motsvarande mängd tillagad champinjon resulterar i en hälften så stor cancerrisk. I realiteten kommer medelkonsumenten att förtära en blandning av färsk och tillagad svamp. Vissa matlagningsmetoder kommer att reducera fenylyldrazininnehållet i svampen påtagligt, medan andra former för matberedning har mindre uttalad effekt. Kunskaperna om hur vanlig tillagning påverkar halten av andra fenylyldraziner än agaritin i champinjon är ännu dåligt känd. Av de fenylyldraziner och besläktade ämnen som förekommer i champinjonen och studerats i cancerstudier har HMBD högst carcinogen potential. Detta är också vad man kan vänta sig mot bakgrund av den förväntade verkningsmekanismen.

Det bör ånyo påpekas att den vanligaste fenylyldrazinen i *A. bisporus*, agaritin, inte framkallade tumörer i försöksdjuren när ämnet gavs i dricksvatten. Av den anledningen har ingen riskbedömning gjorts för agaritin. Emellertid kan man förvänta sig att den studieuppläggning som använts i åtminstone en av cancerstudierna riskerat att en signifikant mängd av agaritin i dricksvattnet brutits ned.

1. Summary

During the period 1961 to 1985 four different phenylhydrazines or related compounds were detected in the cultivated mushroom, *Agaricus bisporus*. Of these, β -N-[γ -L-(+)-glutamyl]-4-hydroxymethylphenylhydrazine, also called agaritine, occurs in high amounts. A level between 80 and 1 700 mg/kg has been reported in fresh mushrooms. The average value found in mushrooms on the market is between 200 and 500 mg/kg fresh weight. In addition to agaritine, the cultivated mushroom also contains small quantities of 4-(carboxy)phenylhydrazine (10-11 mg/kg fresh weight), β -N-[γ -L-(+)-glutamyl]-4-(carboxy)phenylhydrazine (42 mg/kg fresh weight) and 4-(hydroxymethyl)benzene diazonium ions (HMBD) (0.6-4 mg/kg fresh weight).

Studies in experimental animals have shown that agaritine, and/or its degradation products, are to a significant degree and rapidly absorbed from the gastro-intestinal tract, but in blood no unchanged agaritine can be detected. The enzyme γ -glutamyl transpeptidase, present in various tissues of the body, is believed to be the enzyme responsible for cleaving agaritine into L-glutamic acid and 4-hydroxymethylphenylhydrazine. The latter compound is subsequently transformed to HMBD, which is the substance believed to give rise to reactive radical species able to damage macromolecules. A low frequency of DNA damage has been observed in various tissues of experimental animals exposed orally to agaritine and HMBD, an observation indicating that these compounds may be genotoxic carcinogens. This interpretation is supported by the observation that mushroom extracts, as well as agaritine and the other phenylhydrazines occurring in *A. bisporus*, induce a low frequency of mutations in bacterial test systems *in vitro*. Furthermore, *in vivo* studies on mice have revealed that agaritine induces gene mutations in transgenic animals and that the 4-(hydroxymethyl)benzediazonium ion induces micronuclei in peripheral lymphocytes.

In addition to the limited number of naturally occurring hydrazine derivatives, of which the phenylhydrazines in the cultivated mushroom is one example, the industry has produced a number of synthetic compounds for industrial use. Since most (around 85%) hydrazines studied (about one hundred) are toxic and/or carcinogenic in experimental animals, Toth and co-workers undertook the work to assess the possible carcinogenic activity of the phenylhydrazines and related compounds in *A. bisporus*, as well as of the mushroom itself. In three, out of four, long-term carcinogenicity studies tumours were induced in various tissues of mice fed raw, baked or freeze-dried *A. bisporus*. In the fourth study, with baked mushrooms but with another (more balanced) feeding schedule, the increase in tumour incidence was not significant. No tumours were induced in two long-term studies in rats. But in these rather inadequate studies the number of animals was too low to detect a relatively small increase in tumour incidence. Furthermore, the processing (pre-treatment) of the mushrooms (ordinary drying and milling, or washing, combined with pressure cooking, homogenising and mixing with tap water) might well have resulted in a significant degradation of the potentially active phenylhydrazines. Three of the four phenylhydrazines and related compounds known to occur in *A. bisporus* - 4-(carboxy)phenylhydrazine, β -N-[γ -L-(+)-glutamyl]-4-(carboxy)phenylhydrazine and HMBD - were carcinogenic at high doses when administered orally (by

gavage or in drinking water) and also when injected subcutaneously in Swiss mice. The fourth - and major - phenylhydrazine, agaritine, was found not to be carcinogenic when given in drinking water or by subcutaneous injection, a finding that has significantly muddled the interpretation of the carcinogenicity data. However, no precautions were taken in these studies to protect against a possible oxidative degradation of agaritine in water. Agaritine has recently been shown to be vulnerable to such degradation in aqueous solution.

It must be underpinned that the long-term carcinogenicity studies on which the risk assessment has been made, might be criticised for several reasons. Only a single mouse strain, the Swiss mouse, has been used in the studies. In contrast to most mouse strains, which are inbred, the Swiss mouse is a randomly bred strain. This results in a heterogeneity in sensitivity between animals. The studies have often used improper controls – either a historical control group, or a control group that was not started at the same time as the exposed group. In stead of carrying out the carcinogenicity studies in the normal way of exposing the animals for a predetermined time period (usually 2 years), the studies were continued until the mice died or were found in poor condition. Standard carcinogenicity studies have three dose groups but these studies have usually used only a single dose group. Furthermore, some of the studies have been criticised for resulting in an unbalanced diet. In general a number of biochemical and toxicological endpoints are studied besides the main end-point (tumours) in order to be able to interpret the data. Such studies have rarely been reported in the carcinogenicity studies with the cultivated mushroom and its phenylhydrazines.

In contrast to the many positive carcinogenicity studies, there are no indications that *A. bisporus* or the phenylhydrazines occurring in the mushroom are embryotoxic or teratogenic in mammals at biologically relevant doses. However, it should be stressed that the design of the few studies available also in this case did not follow modern guidelines for these types of study. It should also be stressed that very few toxicity studies other than carcinogenicity studies have been performed with the phenylhydrazines in the mushroom.

The estimated annual per capita intake of the cultivated mushroom (*Agaricus bisporus*) varies between the Nordic countries - being 0.6 kg in Finland having the lowest intake among Nordic countries and 2.4 kg in Sweden with the highest intake. Also the habit of using fresh mushrooms for dietary purposes varies between countries. The market share of fresh mushrooms was between 33% and 70%. The rest being preserved mushrooms, mainly sold in cans.

Since the per capita intake is an estimation of average intake and does not take into consideration the fact that mushrooms usually are consumed only by a part of the population, the per capita consumption is too low to express the consumption among mushroom eating consumers. Danish data, based on a limited consumer study, indicate that only around 50% of the population consume the cultivated mushroom, and that 5% of the population consume five times the median intake and 0.1% thirty times the median intake.

Except for agaritine, the data on the influence of storage and food processing on the amount of phenylhydrazines in the consumed mushroom are scanty. The agaritine level in the cultivated mushroom is reduced both during storage and processing. The magnitude of the reduction depends on conditions during storage and food preparation. Nor-

mal cooking procedures about halves the agaritine content. Especially canned mushrooms contain less than 10% of the agaritine level in fresh mushrooms.

Taken together, these data allows an estimation of the per capita exposure to agaritine (as a marker for the phenylhydrazines in the mushroom) in the Nordic countries. The estimated exposure in the Nordic countries is somewhere between 48 and 788 mg agaritine/year, corresponding to a daily exposure of between 2.1 and 36 µg/kg body weight. The exposure being influenced by the quantity of mushrooms consumed (nationality), the amount of agaritine in the fresh mushroom (200-500 mg/kg) and the preference of using fresh instead of processed mushrooms (33-70%). A single mushroom dish with 75 g fresh *A. bisporus* would result in an exposure to 15-33 mg agaritine, that is, 250-550 µg/kg body weight.

Despite the carcinogenicity study with agaritine in mice being negative, six other carcinogenicity studies in the same species, with oral application (by gavage, in drinking water or in the diet), were considered in combination with an estimated human intake to *A. bisporus* in the Nordic countries for the sake of performing a quantitative risk assessment of the cultivated mushroom. Of these studies, three were long-term studies with *A. bisporus per se*, and three were long-term studies with the phenylhydrazines and related compounds occurring in the mushroom. The calculation of the cancer risk used a linear extrapolation from mice to man, based on a number of assumptions described in the main text, of which the most important being the metabolism and sensitivity of the two species being identical.

Taken together the weak mutagenic effect *in vitro* and *in vivo* (agaritine and HMBD) and the carcinogenic effects of three of the four phenylhydrazines occurring in *A. bisporus*, as well as of the fresh, baked and freeze-dried mushroom *per se* in Swiss albino mice all suggest that *A. bisporus* is weakly carcinogenic in animals and accordingly might impose a risk for the consumer. The estimated lifetime human cancer risk of an "average" Nordic consumer of fresh (or freeze-dried) *A. bisporus* is around 200×10^{-6} (50×10^{-6} for Finnish consumers), whereas intake of a corresponding amount of household processed mushrooms results in a cancer risk only about half as high. In reality an average consumer will consume a mixture of fresh and processed mushrooms. Some types of processing (i.e. canning) will reduce the phenylhydrazine content of the mushroom substantially, whereas other types of processing seem to have a much less pronounced effect. However, the influence of standard cooking procedures on the content of other phenylhydrazines of the cultivated mushroom than agaritine is not very well studied. Of the phenylhydrazines and related compounds investigated in carcinogenicity studies and occurring in the mushroom, HMBD had the highest carcinogenic potency. This finding agrees with what is expected based on the anticipated mechanism of action.

It should also be noticed that the major phenylhydrazine in *A. bisporus*, agaritine, did not induce tumours when administered in the drinking water. Therefore, no risk assessment has been made for agaritine. However, it may be anticipated that due to the study design, agaritine could have been significantly degraded in the drinking water in one of these studies.

2. Introduction

In 1991 The Nordic Working Group on Food Toxicology and Risk Evaluation (NNT) reviewed the available data on phenylhydrazines naturally occurring in the cultivated mushroom (*Agaricus bisporus* L.), as well as data on the potential toxicity of these constituents and of the mushroom itself (Gry and Pilegaard, 1991).

The conclusion of this review was that the cultivated mushroom, the most consumed commercial mushroom in the world, contains substantial amounts of phenylhydrazines, about 500 mg per kg raw mushroom. Agaritine is the major phenylhydrazine in *A. bisporus* (of many mycologists anticipated to be the same species as *A. hortensis* and *A. brunnescens*) but the mushroom also contains other phenylhydrazine derivatives. The review further concluded that “the concentration of these hydrazines may change during storage and processing and may vary depending on the strain and cultivation conditions. The degradation products have not been identified”. It continued: “The published toxicological studies give rise to serious concern as to a possible human health risk from consumption of the cultivated mushroom. As no epidemiological data are available and as the long-term carcinogenicity studies with the mushroom and its hydrazine constituents are not of an adequate standard, it is not possible to evaluate the human risk at present” (Gry and Pilegaard, 1991). In agreement with the Nordic project group Subramanian (1995) in a subsequent review concluded: “We certainly need more data on the agaritine content in mushroom species and their possible biological effects”.

Based on the recommendations given in the Nordic report from 1991, a series of toxicological and chemical studies on the cultivated mushroom and its phenylhydrazine constituents were recently performed in the Nordic countries. The aim was to provide updated data for an assessment of whether the consumption of the cultivated mushroom constitutes a human risk.

To promote the exchange of information between chemists and toxicologists active in the field of *Agaricus bisporus* research, especially American, English, Czech and Nordic researchers, the “Nordic project group on inherent natural toxicants in food plants and mushrooms” organised a seminar in Kolding, Denmark, August 1996 (Gry and Andersson, 1998). The seminar was the first step in updating the review on “Phenylhydrazines in the Cultivated Mushroom (*Agaricus bisporus*)”.

The present report builds on all the scientific information that has been published on the toxicity of *Agaricus bisporus* and the phenylhydrazine derivatives occurring in the mushroom. It also draws on the studies performed within this project and the scientific information and recommendations given at the Nordic seminar on phenylhydrazines in the Cultivated mushroom (Gry and Andersson, 1998).

3. Identity, physical and chemical properties, and analytical methods

The commonly eaten cultivated mushroom of commerce in the Western hemisphere, *Agaricus bisporus*, belongs to the Agaricaceae family, the meadow mushrooms. The species-determining name *bisporus* was derived from a latin word meaning "with two spores". *A. bisporus* was in reality derived from several different species and its continual propagation over many centuries has resulted in several (close to 200) commercial strains which have no exact counterpart in nature (Toth, 2000a). *Agaricus campestris* (Field Mushroom or Meadow Mushroom) may be the most closely related of any of the common species encountered in North America (Toth, 1983).

3.1. Identity

During the course of an investigation into the metabolism of α -keto acids in soluble extracts of basidiomycetes, Levenberg (1961) isolated a glutamin-containing compound from the press-juice of *A. bisporus*. He characterized the compound physically and chemically as β -N-[γ -L-(+)-glutamyl]-4-hydroxymethylphenylhydrazine and gave it the trivial name agaritine. This was an important observation, since it was the first reported occurrence of a phenylhydrazine derivative in natural products. Subsequently other investigators isolated agaritine from the mushroom using a improved method and confirmed its structure by synthesis (Daniels et al., 1961). The early investigators also established the level of agaritine to be fairly high in the cultivated mushroom (Kelly et al., 1962). The chemical structure of agaritine is given in Figure 1.

Agaritine is not the only nitrogen-nitrogen bond-containing substance that has been found in mushrooms. It is not even the only phenylhydrazine derivative. Already 1962 Levenberg demonstrated the occurrence of the 4-(hydroxymethyl)benzene-diazonium

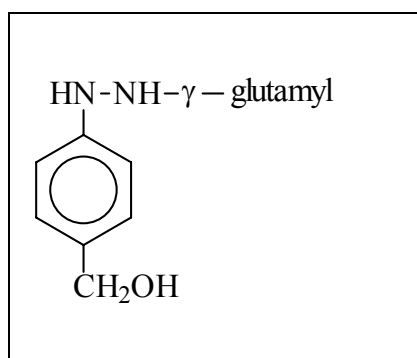


Fig. 1 The molecular structure of agaritine (β -N-[γ -L-(+)-glutamyl]-4 hydroxymethylphenylhydrazine).

ion (Figure 2) in the basal-stalk section of the stipe of *A. bisporus*. This compound was not detected in the cap and upper portion of the stipe.

Already in connection with the identification of agaritine in the cultivated mushroom a highly active enzyme that catalyses the cleavage of agaritine to 4-(hydroxymethyl)-phenylhydrazine (Figure 2) and L-glutamic acid was identified (Levenberg, 1961). The enzyme was partially purified (26-fold) from soluble *A. bisporus* extracts and was shown to irreversibly catalyse hydrolysis of the γ -L-glutamyl-hydrazide bond of agaritine. Hydrolysis and γ -glutamyl transfer were shown to be common functions of the same enzyme protein (Gigliotti and Levenberg, 1964). This degradation of agaritine to 4-(hydroxymethyl)phenylhydrazine and L-glutamic acid by the enzyme which subsequently was given the name agaritine γ -glutamyl transferase has been confirmed by Baumgartner et al. (1998). The early observations lead to the speculation that a link exists between agaritine and the 4-(hydroxymethyl)benzenediazonium ion.

Properties that differentiates this agaritine degrading enzyme from other glutamotransferases and glutamyltranspeptidases are: a) its inability to effect transfer to amino acids such as glycine, phenylalanine, leucine and aspartic acid, b) its ability to transfer to water, and c) its ability to utilize aromatic hydrazine and their γ -L-glutamyl derivatives as acceptor and donor substrates, respectively. The product of the enzymatic agaritine hydrolysis, 4-(hydroxymethyl)phenylhydrazine is very unstable and has never been detected in *A. bisporus*.

Subsequently it was demonstrated that two enzyme systems capable of generating the 4-(hydroxymethyl)benzenediazonium ion from agaritine occur in the mushroom. One system is the one mentioned above, in which agaritine is oxidised to the diazonium ion via the unstable 4-(hydroxymethyl)phenylhydrazine. In the other system agaritine is oxidised to the diazonium ion without intermediate formation of 4-(hydroxymethyl)phenylhydrazine (Ross et al., 1982a). These systems (together with others) may be responsible for producing the low level (0.6-4 ppm) of 4-(hydroxymethyl)benzenediazonium ion that has been detected in *A. bisporus*.

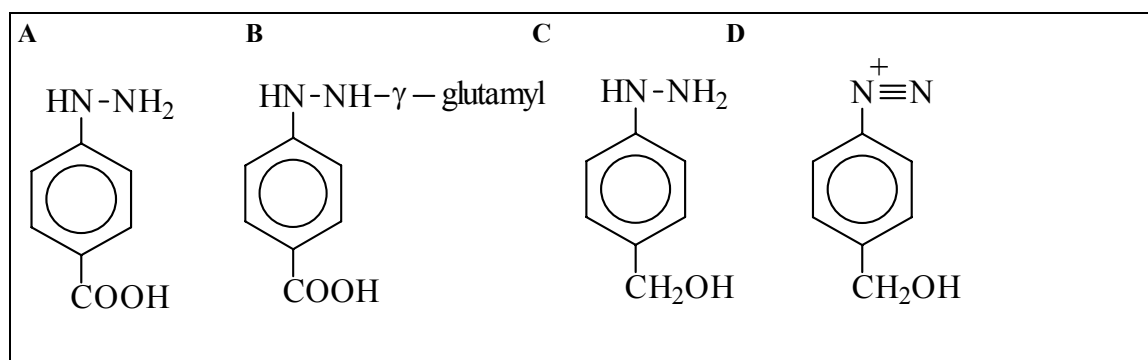


Fig. 2 Other phenylhydrazine derivatives than agaritine, or related compounds occurring in *Agaricus bisporus*. (A) 4-(carboxy)phenylhydrazine; (B) β -N-(γ -L(+)-glutamyl)4-(carboxy)phenylhydrazine; (C) 4-(hydroxymethyl)phenylhydrazine; and (D) 4-(hydroxymethyl)benzenediazonium ion.

LaRue (1977) postulated 4-(carboxy)phenylhydrazine and β -N-[γ -L-(+)-glutamyl]-4-carboxyphenylhydrazine (Figure 2) to be possible biosynthetic precursors of agaritine. Both these substances were subsequently identified in *A. bisporus* by Chauhan and co-workers (1984, 1985).

3.2. Physical and chemical properties

Most of the hydrazines described above are sensitive to oxidation. In particular the enzymatically produced degradation product of agaritine, 4-(hydroxymethyl)phenylhydrazine easily decomposes to the 4-(hydroxymethyl)benzenediazonium ion by elimination of water. The physicochemical properties of agaritine is shown in Table 1. It can be noted that agaritine is very soluble in water.

Studies recently performed within this project have revealed that the stability of chemically synthesised agaritine in aqueous solution is dependent on the oxygen tension in the solution. In these studies the stability of agaritine dissolved in argon purged Milli Q water and stored under argon at room temperature was compared with the stability of agaritine at room temperature when dissolved in i) tap water (Søborg, Denmark) and stored in open vials, ii) tap water added 2 mM dithiotreitol and stored in closed vials, and iii) 50% ethanol and stored in closed vials. Figure 3 shows that agaritine is stable for at least 48 hours when stored under argon in argon purged water. However, dissolved in tap water agaritine seems quite unstable. The stability was concentration-dependent. At a concentration of 0.6 mg/ml almost 70% of agaritine was lost during 24 hours storage at room temperature. No agaritine could be detected after 48 hours. At a concentration of 0.3 and 0.15 mg/ml nearly all agaritine is lost during the first 24 hours of storage at room temperature. In parallel experiments (data not shown) we investigated the stability

Table 1. Chemical and physical properties of agaritine (Levenberg, 1961).

Agaritine = β-N-[γ-L-(+)-glutamyl]-4-hydroxymethylphenylhydrazide	
Synonyms:	L-glutamic acid-5-[2-(4-hydroxymethyl)phenylhydrazide]
IUPAC System. Name:	L-glutamic acid, 5-[2-(α -hydroxy- <i>para</i> -tolyl)hydrazide]
CAS registration No.:	2757-90-6
Molecular formula:	C ₁₂ H ₁₇ N ₃ O ₄
Molecular weight:	267.3
Chemico-physical characteristics:	Neutral colourless crystals
Optical rotation:	$[\alpha]^{23}_{D+60}$ (c = 1.1 in water)
Spectroscopy data:	λ_{max} (water) 237.5 nm (ϵ 12 000) and 280 nm (ϵ 1 400). Infrared and nuclear magnetic resonance spectral data have been reported
Solubility:	Very soluble in water; practically insoluble in most anhydrous organic solvents
Stability:	Stable in solid state; sensitive to oxidation, particularly in aqueous solution
Melting point:	203-208°C (decomposes without melting)

of methanolic and aqueous (distilled water) solutions of agaritine stored at 4 and 22°C for 0-36 days. At 20 mg/ml of water, the only concentration studied in this experiment, agaritine was significantly more stable than in the experiment described above. The temperature during storage had very little influence on the stability. Only 45.8% of

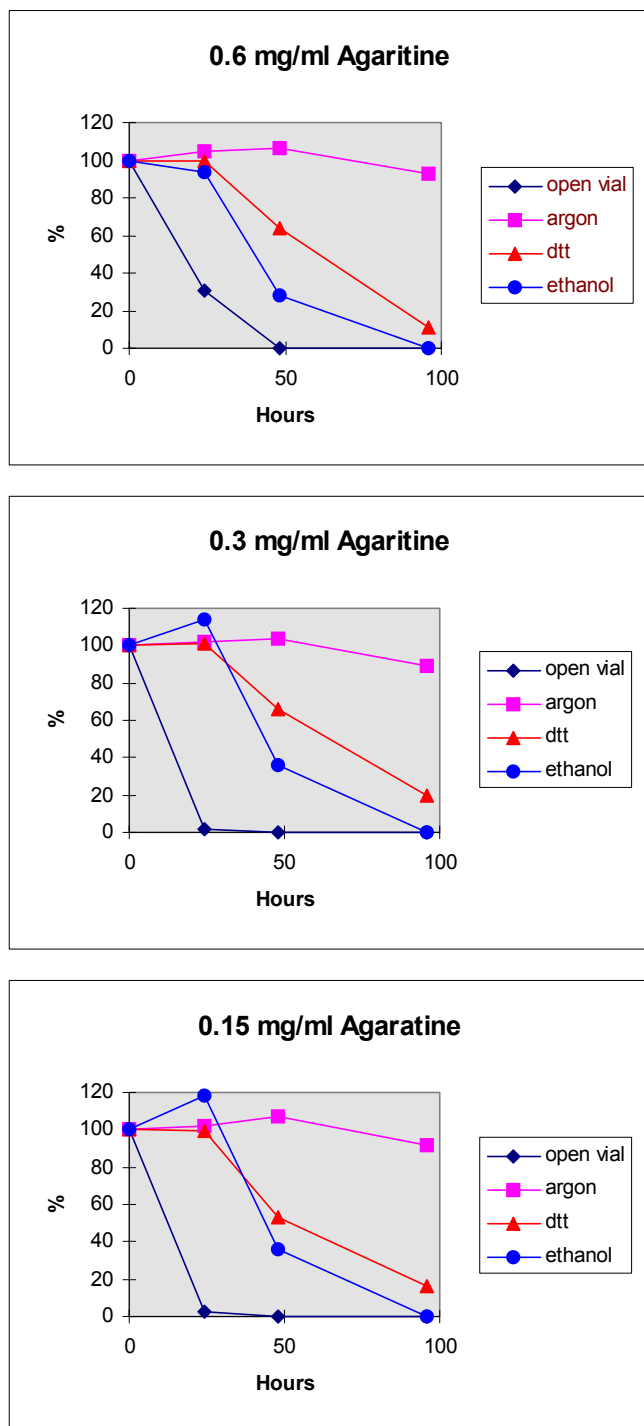
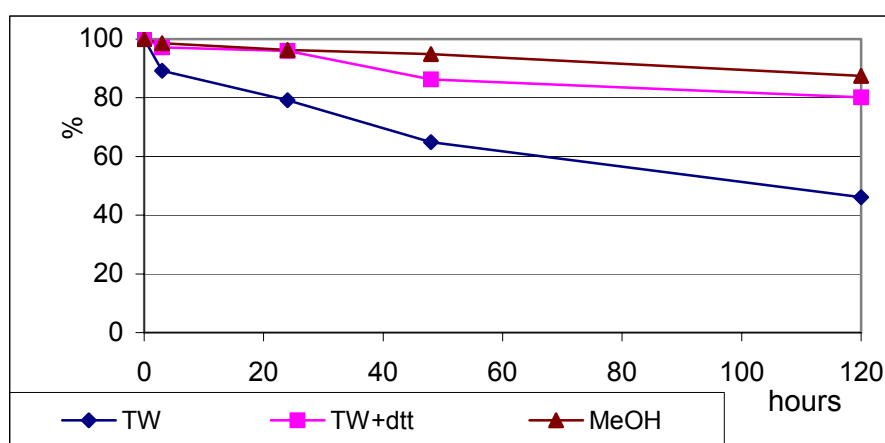


Fig. 3. Recovery of agaritine after storage in an open vial of tap water (◆) or in a closed vial (under argon) of either argon purged Milli Q water (■), tap water added 2 mM dithiotreitol (dtt) (▲) or 50% ethanol (●). The concentration of agaritine was 0.6, 0.3 or 0.15 mg/ml, respectively.

the compound had been degraded during the first 14 days of storage in distilled water. The degradation in methanolic solution was less, only 7.8% being degraded during this time period. It is not known whether the difference in stability of agaritine observed in these two studies is due to different concentrations of agaritine being used or that the different types of water contained different amounts of oxygen or other compounds of importance for the degradation. The observation on stability described above may have bearing on the interpretation of many experimental studies (Frandsen and Gry, unpublished results).

In order to study the stability of agaritine in more details the “Nordic project group on inherent natural toxicants in food plants and mushrooms” in co-operation with Czech colleagues carried out a series of experiments. It was confirmed that the stability of agaritine solutions is highly influenced by the oxygen tension and the type/purity of solvent (Hajšlová et al., 2002). In Figure 4 is shown the stability of agaritine (0.3 mg/ml) over 5 days when dissolved in tap water a 2 mM solution of dithiotreitol in tap water or in

a) closed vials



b) open vials

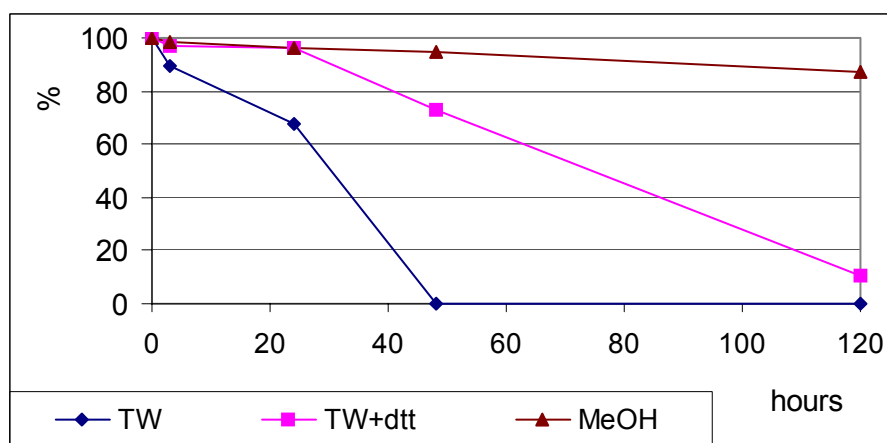


Fig. 4. Degradation of agaritine (0.3 mg/ml) in various media (TW, tap water; dt, 2 mM dithiotreitol in TW; MeOH, methanol) in (a) closed vials and (b) open vials.

methanol and kept in closed (Figure 4a) and open (Figure 4b) vials at ambient temperature, respectively. The data in Figure 4 are the means from two parallel experiments. As can be seen, a successive drop in agaritine content occurred during storage in all tested media. However, the stability of aqueous solutions was significantly lower than that of methanolic solutions. Agaritine solutions were more stable in closed vials (Figure 4a).

The quickest degradation of agaritine occurred in tap water. Already after 48 h. no agaritine could be detected in tap water solutions stored in open vials. In closed vials, slightly < 50% of the compound remained after 120 h. Addition of dtt to the tap water improved the stability of the analyte, particularly when the solution was stored in closed vials. However, also in the presence of dithiotreitol nearly all agaritine was degraded after 120 h in open vials (around 11% remaining). Agaritine was most stable in methanol, around 87-88% of the compound remained after 120 h both when stored in open and closed vials. The increased stability of agaritine (0.3 mg/ml) in Milli Q water and Milli Q water purged with oxygen free gas, N₂, was also confirmed (data not shown). In both solutions, agaritine was degraded to a similar degree as in methanol, > 75% remaining after 120 h. The stability was slightly higher in Milli Q water purged with N₂ (Hajšlová et al., 2002).

The influence of pH on the degradation rate of agaritine in aqueous extracts of *A. bisporus* at ambient temperature was also studied as shown in Figure 5. Agaritine was degraded more quickly at pH 1.5 than at pH 4.5 especially during the first days of incubation. It was relatively more stable at pH 6.8. After an incubation of 24 h > 50% of the compound was degraded at pH 1.5 but only around 18% was at pH 6.8. The reduced stability of agaritine at low pH was confirmed in a study on agaritine in simulated gastric fluid (pH 1.2). In Table 2 it is shown that the breakdown of agaritine was faster both in extracts of fresh and of cooked mushrooms, compared to the pure solution of agaritine in simulated gastric fluid. Rather surprisingly, the rate of degradation of agaritine was faster in the two mushroom extracts than in the pure solution of agaritine,

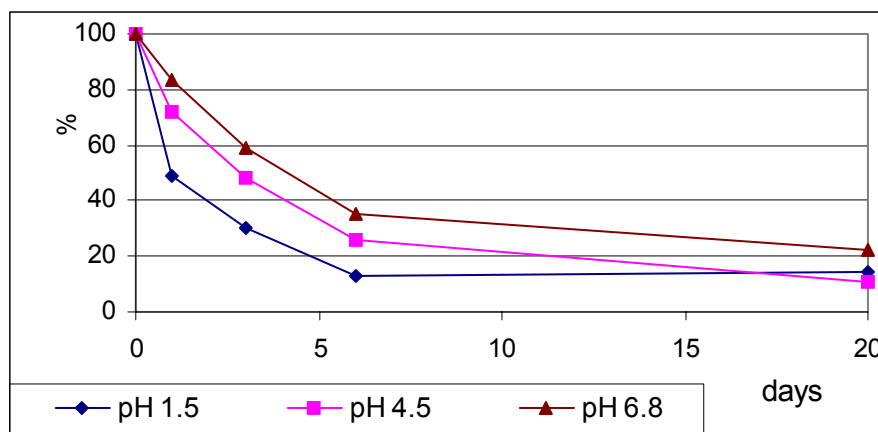


Fig. 5. Degradation of agaritine at various pHs (the agaritine content in fresh mushrooms was 197.7 mg/kg).

Table 2. Degradation of agaritine in simulated gastric fluid (% of original content).

Time (days)	Pure solution of agaritine	Agaritine extract from fresh mushrooms	Agaritine extract from cooked mushrooms
0	100.0	100.0	100.0
1	83.4	50.1	82.1
2	73.6	36.9	56.7
5	60.7	27.2	38.6
8	53.8	11.7	21.4
16	32.1	n.d.	n.d.
22	14.2	n.d.	n.d.

Original content of agaritine in standard solution (100%) was 0.02 mg/ml, whereas it was 230.2 mg/kg in the “fresh mushrooms” and 183.9 mg/kg in the “cooked mushrooms”, respectively.

although the degradation was slower in the extract of boiled mushrooms compared to the extract of the fresh mushrooms. It was speculated whether a mushroom constituent, coextracted with agaritine, could have catalysed the degradations (Hajšlová et al., 2002).

In assessing the possible risks associated with the consumption of the cultivated mushroom it is important to include an estimation of the degradation of agaritine, especially at the low pH in the stomach. The different stability of agaritine at various pH, as demonstrated above, is also of importance when extrapolating data obtained in experimental animals to man. The pH of the human stomach varies considerably, but it is commonly around pH 2. The pH of the rodent stomach, on the other hand, is higher, usually around pH 5 (Hajšlová et al., 2002).

Finally, the breakdown of agaritine in tap water in closed vials was studied for five days. At the end of the experiment only 37% of agaritine remained. The gradual drop in concentration of agaritine with storage time was accompanied by the simultaneous formation of at least four unknown degradation products. At the end of the incubation (five days) the solution contained one of the four degradation products in higher concentration (43%) than the remaining agaritine (37%) (Hajšlová et al., 2002).

It can be concluded that agaritine is very unstable in aqueous solutions, especially if oxygen has not been carefully excluded, and at low pH.

Many toxicological studies performed with extracts of the cultivated mushroom and with agaritine *per se* set out to explore whether this constituent can give rise to adverse effects on living systems and they have given variable and not easily interpretable results. Since few, if any, studies have carefully taken care of protecting agaritine solutions from oxidative degradation, it is highly likely that agaritine degradation has contributed to the variable results. Thus, in some experimental studies the exposure to agaritine might have been much lower than intended. Further, the experimental material may have contained unidentified degradation products of agaritine with unknown toxic potential.

3.3. Chemical synthesis

Methods to chemically synthesise agaritine, β -N-[γ -L-(+)-glutamyl]-4-carboxy-phenylhydrazine, and 4-(hydroxymethyl)benzenediazonium ion in the form of the tetrafluoroborate have been published (Wallcave et al., 1979; Kelly et al., 1982; Ross et al., 1982a; Chauhan and Toth, 1984; Datta and Hoesch, 1987). The method to synthesise agaritine (Wallcave et al., 1979) have been found to be insufficient and the method has, therefore, subsequently been modified by others (Walker et al., 1997; Frandsen, 1998).

3.4. Analytical methods

The first methods for agaritine analysis, from the early 1960's, were based on gravimetric and enzymatic assays. For example, Kelly et al. (1982), after a lengthy purification of agaritine from the mushroom found 360 mg agaritine per kg fresh weight. Levenberg (1961, 1964) used an enzymatic assay where agaritine was cleaved to 4-(hydroxymethyl)phenylhydrazine and L-glutamic acid. In the presence of excess glyoxylic acid, the 4-(hydroxymethyl)phenylhydrazine was converted to a phenylhydrazone which was subsequently detected spectrophotometrically at 325 nm.

In 1978 Issenberg developed a non-published high performance liquid chromatographic (HPLC) procedure for the analysis of agaritine (Speroni and Beelman, 1982). This procedure was modified and employed by Liu and others (1979, 1982) at the Pennsylvania State University, USA, to determine agaritine in fresh and processed mushrooms. Several difficulties were experienced with the procedure, which was based on methanol extracts of mushroom tissue being directly injected into the chromatograph. Below baseline resolution occurred between the agaritine peak and an unidentified peak immediately preceding it, making quantification of agaritine difficult. The phenolic material present in the methanol extracts also had the potential to influence the chromatography.

Subsequently workers from the same Institute described a sensitive HPLC for analysis of agaritine (Speroni and Beelman, 1982). Freeze-dried mushrooms were extracted with methanol, extracts evaporated to dryness, and the residue resuspended in the mobile phase (0.005N NaH_2PO_4 , pH 4.25) and subsequently passed through a C_{18} reverse-phase SepPak. The mobile phase was pumped through a cation-exchange column at a fixed speed and agaritine monitored at 237 nm. Recoveries of agaritine standards were high (>90 %) and the limit of detection 0.006 μg . To maintain the reliability of this procedure frequent calibration with agaritine standards are needed. Free amino acids that strongly absorb in the UV was shown not to interfere with the agaritine peak.

4. Biosynthesis

Only a few studies have investigated the biosynthesis of agaritine experimentally, and these have come to partly different conclusions. The first investigators to study agaritine biosynthesis in *Agaricus bisporus* were Schütte et al. (1972) who analysed the incorporation of radiolabelled compounds into agaritine. No incorporation of 3-¹⁴C-labelled tyrosine and phenylalanine was observed. However, incorporation rates of 0.14 %, 0.68 %, and 4.1 % were obtained with [U-¹⁴C]shikimic acid, [2-¹⁴C]glutamic acid, and *p*-aminobenzoic acid-[3,5-³H], respectively. Thus, *p*-aminobenzoic acid seems to a precursor to agaritine. However, the conclusion has lately been criticized by Baumgartner et al. (1998), who believes that Schütte et al. (1972) actually studied the synthesis of γ -glutaminy-4-hydroxybenzene (see below).

Schütte et al. (1972) also tried to determine the origin of the hydrazine part of the molecule by growing mushrooms supplied with ¹⁵N-labelled glutamic acid and glutamin-(CO¹⁵NH₂). These studies showed that both the α -amino group and the amid-nitrogen from both labelled compounds were incorporated into agaritine.

Based on the information presented by Schütte et al. (1972) on the biosynthesis of agaritine and the chemical analytical data showing several phenylhydrazine-derivatives and arylbenzenediazonium ions to occur in *A. bisporus* and related species, La Rue (1977) proposed that agaritine biosynthesis and degradation occur in the genus *Agaricus* as shown in Figure 6. As the biosynthetic part of this scheme is based on the data of Schütte et al. (1972), the scheme has been questioned by Baumgartner et al. (1998).

The intermediary compound in Figure 6, 4-(carboxy)phenylhydrazine, has been detected in *Agaricus bisporus* at much lower levels than agaritine (Chuan et al., 1984). This observation indicates that either the biosynthesis of agaritine proceeds through alternate pathways not involving 4-(carboxy)phenylhydrazine or that a rapid conversion of this compound to other intermediates involved in agaritine biosynthesis occurs (Chauhan et al., 1984). Turner (1983) proposed agaritinal (Figure 7) as a possible intermediate in agaritine biosynthesis. This aldehyde has not been found in *Agaricus bisporus*, but it has been isolated from several wild-growing *Agaricus* species that also containing agaritine (Chulia et al., 1988; Stijve and Pittet, 2000).

It seems likely, according to Baumgartner et al. (1998), that the biosynthetic scheme suggested by Schütte et al. (1972) is for γ -glutaminy-4-hydroxybenzene and not agaritine (β -N-(γ -glutamyl)-4-hydroxymethylphenylhydrazine), and that there is no *de novo* synthesis of the aromatic residue of agaritine in the *Agaricus bisporus* fruit body. In their hands, 4-aminobenzoic acid was no precursor of agaritine in fruit bodies detached from the mycelium but an excellent precursor for γ -glutaminy-4-hydroxybenzene. In the discussion on their findings, Baumgartner and co-workers refers to personal communications with K. Sasaoka that had made a similar observation. Instead of the desglutamyl moiety of agaritine being produced in the fruit body, these investigators show data supporting the view that the agaritine present in the fruit body is transported to this location from the vegetative hyphae.

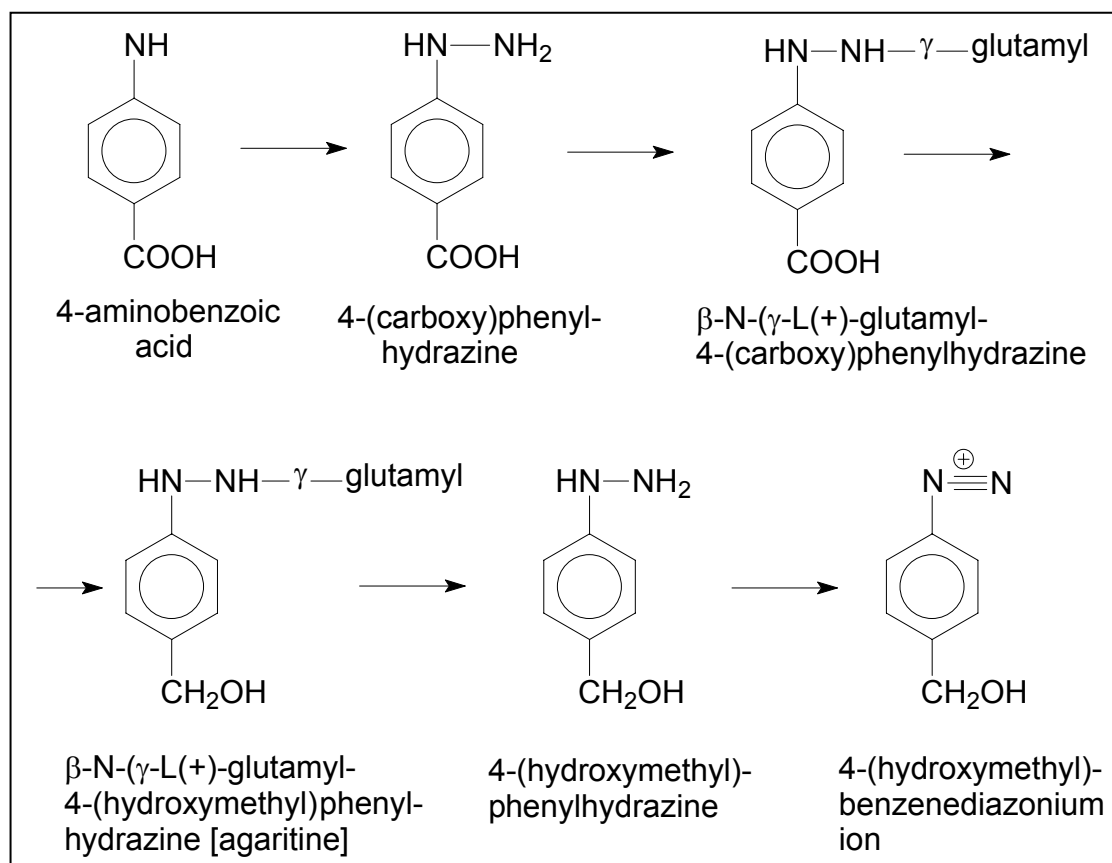


Fig. 6. A suggested but questioned biosynthetic route for agaritine in *Agaricus* mushrooms, and a presumed route of degradation.

Baumgartner et al. (1998) speculate that the assembly of agaritine takes place in the vegetative hyphae in contact with the wheat straw compost, using specific metabolic activities of representatives from three biologically different groups of organisms. At this location the assembly of agaritine requires both the aryl and the hydrazide moieties from exogenous sources (e.g. resulting from the breakdown of lignin by the fungus and

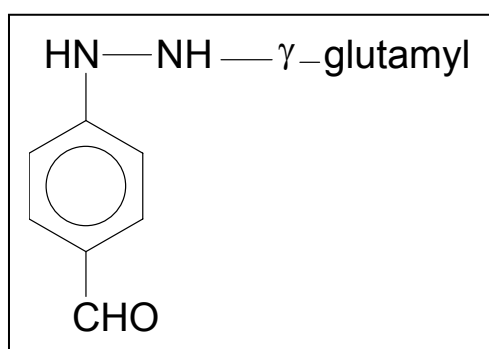


Fig. 7. Chemical structure of agaritinal.

the diazotrophic activity of a bacterial commensal in the substratum). The investigators reported that rhizomorph contained 2.1 and 2.6 mg/g dry weight of agaritine and γ -glutaminy-4-hydroxybenzene, respectively, when *A. bisporus* was grown on substratum, but only 1 mg/g dry weight when they tried to decontaminate hyphae from soil.

As mentioned in the introduction, the agaritine catabolite 4-(hydroxymethyl)benzenediazonium ion seems to be formed by two routes. One system, whereby the diazonium ion is generated enzymatically via the unstable compound 4-(hydroxymethyl)phenylhydrazine, and another in which the diazonium ion is generated directly from agaritine (Gigliotti and Levenberg, 1964; Ross et al., 1982a). Since leakage of agaritine into water was found to be negligible by Baumgartner et al. (1998), these authors suggest that its decrease in harvested mushrooms must be due to catabolism. Most probably, this catabolism embodies an oxidative transformation of the desglutamyl moiety of agaritine, occurring either on the intact molecule, or following removal of the glutamate residue (Baumgartner et al., 1998).

Why *Agaricus* mushrooms produce agaritine is not known. Since agaritine has been shown to inhibit mono- and diphenolase activity, Espin et al. (1998) have suggested that agaritine could play a role *in vivo* as an endogenous regulator of the mushroom polyphenol oxidase activity and thereby of the amounts of *o*-quinone formed. However, this does not seem to be a likely explanation.

On the other hand, it could very well be that a continuous degradation of agaritine to 4-(hydroxymethyl)phenylhydrazine occurs in *A. bisporus*, and results in a concomitant production of 4-(hydroxymethyl)benzenediazonium ions, which *in vivo* inhibits competitive fungi, since the production apparently increases with the age of the mushroom, making up for its growing vulnerability to attack by moulds. A few findings supports this hypothesis (Stijve et al., 1986):

- a) unlike other higher fungi, *Agaricus* species are seldom if ever found covered with mould growth;
- b) wild-growing species and strains contain much more agaritine than cultivated ones. The former may need more protection than the latter which grow in a protected environment;
- c) *Agaricus* species without agaritine produce other fungistatic compounds: the xanthodermi subgroup contains appreciable quantities of phenol, and the nitroamino acids in members of the sylvaticus subgroup may have a similar function.

5. Occurrence

5.1. The content of agaritine and related compounds in fresh mushrooms

Despite the evidence that agaritine is not anticipated to be the ultimate biologically active component in the cultivated mushroom, analytical work has nevertheless tended to concentrate on monitoring this compound, rather than its precursors, metabolites or decomposition products, presumably because of the relatively large amount of agaritine present in the mushroom, and the relative ease with which the analysis can be carried out. Agaritine could therefore be regarded as an indicator of the likely presence of potentially toxic phenylhydrazines. The natural occurrence of hydrazines and related compounds in mushrooms and plants has been reviewed by Toth (2000a).

Several factors, including strain of *A. bisporus* studied, cultural practice used in production, stage of cropping-cycle (flush), maturity and harvest may all interact to influence agaritine content. Without carefully controlling each of these variables, it is difficult to determine their individual influence on agaritine production. In the studies mentioned below, in which the agaritine content has been analyzed of the cultivated mushroom, this control has generally not been done.

Table 3 shows the amount of agaritine in fresh samples of *A. bisporus*, either obtained directly from mushroom growers or purchased in stores on the local market. Since the level of agaritine determined in *A. bisporus* is dependent both on the storage time and the conditions during storage (see next section), it is not surprising that slightly higher agaritine contents have been found in mushrooms obtained directly from the growers.

The overall range of agaritine levels that have been found in fresh *A. bisporus* are between 80 and 1 730 mg/kg fresh weight, with average values between 200 and 500 mg/kg. The three studies that have presented data only on a mg/kg dry weight basis (Levenberg, 1964; Speroni and Beelman, 1982; Speroni et al., 1983) are in agreement with the rest of the studies. The latter method of presenting the content of agaritine gives figures that are approximately 10 times higher than when giving the content in mg/kg fresh weight (Liu et al., 1982; Fischer et al., 1984).

According to some authors, the difference in agaritine content reported can not be explained by different parts of the fruiting body being studied in different investigations as there is no specific localisation of agaritine in the sporophores (Foret and Arpin, 1991). On the other hand, although Soulier and co-workers (1993) detected agaritine throughout the whole fruit body at fairly similar levels, the stipe base and hymenium contained significantly deviating levels of agaritine, lower levels in the stipe base and higher levels in the hymenium. This observation was recently confirmed within this project. We analysed various parts of the fruit body of cultivated *A. bitorquis* for the presence of agaritine and found the skin of the cap to contain 352 mg/kg (16% of the whole mushroom), the cap with its skin 254 mg/kg (56% of the whole mushroom), gills 254 mg/kg (12% of the whole mushroom), and the stem 194 mg/kg (16% of the whole mushroom),

respectively. The agaritine content of the whole mushroom was 249 mg/kg fresh weight (Schulzová et al., 2002).

One of the studies in Table 3 demonstrated a 4-fold difference in agaritine content among 14 lots of fresh mushrooms from 10 different growers, the range being 330-1 730 mg/kg fresh weight (Liu et al., 1982). Included in the analysis were five different strain types: white, off-white, golden white, cream, and brown. No indication of strain influence on mean values of agaritine concentration was apparent in the data, an observation that subsequently has been confirmed by others (Speroni et al., 1983; Fischer et al., 1984; Stijve et al., 1986). In the study of Speroni and co-workers (1983), one strain (PSU-351 with brown colour phenotype) had significantly higher agaritine levels than the remaining seven strains, 5 100 mg agaritine per kg mushroom (on a dry weight basis) as compared to 1 700-2 800 mg/kg. It was hypothesised that this strain, which was only recently isolated from nature, contains higher agaritine levels because it had not yet lost its inherited ability to inhibit growth of certain fungi. It is possible agaritine functions *in vivo* as an antimycotic agent. Also no relationship could be found between mushroom colour and agaritine level.

The observations referred to above were partly confirmed in our project. We purchased 28 different samples of fresh cultivated mushrooms with a cup diameter of 4-6 cm from the retail market in Prague, Czech Republic, 25 samples being *A. hortensis* (*A. bisporus*) and the other three *A. bitorquis* (Schulzová et al., 2002). There was no significant difference in agaritine content between species. The agaritine content of the 28 samples varied between 165 and 457 mg/kg, the average being 272 ± 69 mg/kg fresh weight (10th percentile 201 mg/kg, median 267 mg/kg and 90th percentile 356 mg/kg). However, some samples from early flushes obtained directly from growers contained significantly more agaritine (700 mg/kg fresh weight – cap diameter 4 cm) (Schulzová et al., 2002). This observation has also been made by Sharman et al. (1990).

According to Levenberg (personal communication, Kelly et al., 1962), agaritine is predominantly found in the fruiting bodies of young mushrooms - the concentration diminishing with age and increase in size of the fruiting bodies. This observation has been confirmed by several investigators (Chiarlo et al., 1979; Fischer et al., 1984; Schulzová et al., 2002), whereas others have been unable to confirm the finding (Sharman et al., 1990; Foret and Arpin, 1991). For example, Fisher et al. (1984) compared the agaritine level in young ($\varnothing$ cap 1.5-2 cm), medium age ($\varnothing$ cap 5-6 cm) and old ($\varnothing$ cap 7.5- cm) mushrooms of a white strain. They found the level in young mushrooms to be 629 mg/kg, in medium age mushrooms 438 mg/kg, and in old mushrooms 271 mg/kg (Fischer et al., 1984). It should be noted that in many of the studies presented in Table 3 it has not been tried to standardise the measurements of agaritine by specifying the size of the mushrooms analysed. No indication of a seasonal trend in agaritine content has been observed in *A. bisporus* (Liu et al., 1982).

The observation that smaller mushrooms contain higher amounts of agaritine than bigger ones was, however, confirmed by Stijve and co-workers (1986) who analysed the agaritine content of two wild-life *Agaricus* species, *A. arvensis* and *A. augustus*, in various stages of development. Fruiting bodies belonging to the same mycelium were picked, lyophilised within 24 hours, and analysed. The results indicated indeed a dramatic decrease in agaritine content with the age of the mushroom (measured as weight of the mushroom and diameter of the cap). It was suggested that the breakdown is due

Table 3. Agaritine content of fresh *Agaricus bisporus* determined in methanol extracts of the mushroom. The mushrooms were either obtained fresh from growers (g) or purchased at the local market (m). If the samples were not freeze-dried shortly after being obtained (which does not influence the agaritine level), they were analysed within 24 hours.

Mushroom sample (g = grower; m = market)	Diameter of cap (cm)	Analytical method	Content (mg/kg fresh weight)		Reference
			Average	Range	
2-3 days old mushrooms (g)	not stated	gravimetric	~ 400	-	Kelly et al., 1962
2-3 days old mushrooms (g)	not stated	gravimetric	~ 220	-	Daniels et al., 1961
1 fresh sample (g)	not stated	gravimetric	~ 3 300*	-	Levenberg, 1964
2 fresh samples (m)	not stated	HPLC	440; 720	170-1 170	Ross et al., 1982b
14 fresh samples of different strains (g)	3.0-4.5	HPLC	880	330-1 730	Liu et al., 1982
1 fresh sample (g)	not stated	HPLC	2 190*	-	Speroni and Beelman, 1982
8 fresh samples of different strains (g)	2.5-5.0	HPLC	-	1 700-5 100*	Speroni et al., 1983
2 fresh samples of different strains (g)	5.0-6.0	HPLC"	304±6.0; 438±2.5	- -	Fischer et al., 1984
11 fresh samples (m)	2.0-5.5	HPLC"	368±45	94-629	Fischer et al., 1984
1 fresh sample	not stated	?	228	-	Hashida et al., 1990
2 fresh samples of different strains (g)	not stated	HPLC""	~ 180	80-250	Sharman et al., 1990
5 fresh samples of different strains (m)	not stated	HPLC TLC		160-650	Stijve et al., 1986
1 fresh sample within a Nordic project (g)	not stated	HPLC	340	-	Andersson et al., 1994
2 fresh sample (m)	not stated	HPLC	212; 229	-	Andersson et al., 1999
5 fresh sample	not stated	HPLC	820	630-984	Burini et al., 1999

* given as mg/kg dry weight; " agaritine standard 94.5 % pure; "" agaritine standard 95 % pure.

to a constant enzymatic degradation of agaritine to 4-(hydroxymethyl)phenylhydrazine in the mushroom.

Table 4 shows which other phenylhydrazine derivatives or arylbenzenediazonium ions than agaritine that have been detected in *A. bisporus*. The table has tabulated all observations of these compounds available in the literature. It is obvious that the occurrence of 4-(carboxy)phenylhydrazine, β -N-[γ -L-(+)-glutamyl]-4-carboxy-phenylhydrazine and 4-(hydroxymethyl)benzenediazonium ion in *A. bisporus* requires confirmation. Czech chemists recently extracted *A. bisporus* with methanol and analysed the extract for β -N-[γ -L-glutamyl]-4-carboxyphenylhydrazine, β -N-[γ -L-glutamyl]-4-formylphenylhydrazine, β -N-[γ -L-glutamyl]-4-methylphenylhydrazine, and 4-methylphenylhydrazine (Schulzová and Hajslová, personal communication). Low quantities of β -N-[γ -L-glutamyl]-4-carboxyphenylhydrazine, and β -N-[γ -L-glutamyl]-4-methylphenylhydrazine, and traces of β -N-[γ -L-glutamyl]-4-formylphenylhydrazine was detected in the fresh extract but the amounts were reduced upon storage of the extract at room temperature. After 18 days levels were very low, but at this point in time small quantities of 4-methylphenylhydrazine was detected for the first time. There are substantial evidence that the 4-(hydroxymethyl)benzenediazonium ion exists in the fungus, although the investigators could not be certain whether it came through the metabolism of agaritine or if it exists as an independent entity (Levenberg, 1962). In addition to the compounds mentioned in Table 4, the presumed precursor to agaritine, 4-aminobenzoic acid, has been detected in *A. bisporus* at a level of 10 mg/kg fresh weight (Toth et al., 1997a).

In addition to the observations presented in Table 4, Stijve et al. (1986) analyzed for 4-(carboxy)phenylhydrazine in fresh samples of the cultivated mushroom, but could not detect any. This was perhaps not surprising since the detection limit was high in this study, around 100 mg/kg dry weight. In the same study the investigators were also unable to detect 4-aminobenzoic acid and N-[γ -L-glutamyl]-4-(carboxy)phenylhydrazine with similar limit of detection.

Shortly after having detected agaritine in *A. bisporus*, Levenberg (1964) examined boiled press-juice extracts from as many as 45 representative genera of basidiomycetes. As part of this survey boiled press-juice preparations from the fruit bodies of 15 different species belonging to the genus *Agaricus* were tested. Table 5 summarises the information available on which mushroom species do, and which do not contain agaritine. Of the 15 *Agaricus* species tested by Levenberg (1964), 10 species (*A. pattersonii*, *A. xanthodermus*, *A. argentatus*, *A. campestris*, *A. comtulus*, *A. crocodilinus*, *A. edulis*, *A. hortensis*, *A. micromegathus* and *A. perrarus*) contained agaritine in quantities comparable to those found in *A. bisporus*. Five other species, all belonging to the *sylvaticus* subgroup (*A. benesii*, *A. sterlingii*, *A. subrutilescens*, *A. sylvaticus*, and *A. sylvicola*) were devoid of the compound. Three members of the *Agaricus* genus (*A. campestris*, *A. diminutivus* and *A. hortensis*) were found to contain additional substances active in the enzymatic assay. However, the products formed in these cases had different spectral characteristics than those resulting from agaritine itself. These latter species were believed to contain other aromatic hydrazine derivatives of L-glutamic acid of as yet undetermined structure (Levenberg, 1964). The above scientific names for the different *Agaricus* species studied are cited from the corresponding publications. The nomenclature for a large number of *Agaricus* species has been dealt with in a study on the occurrence

Table 4. Content of arylbenzenediazonium ions and other phenylhydrazines than agaritine in *Agaricus bisporus* determined in methanol extracts of the mushroom. The mushrooms were either obtained fresh from growers (g) or purchased at the local market (m). If the samples were not freeze-dried shortly after being obtained (which does not influence the agaritine level), they were analysed within 24 hours.

Compound	Mushroom sample (g = grower; m = market)	Diameter of cap (cm)	Content (mg/kg fresh weight)	Reference
4-(carboxy)phenylhydrazine	1-2 days old mushrooms (g)	not stated	10.7±2.0	Chauhan et al., 1984
β-N-(γ-L(+)-glutamyl)4-(carboxy)phenylhydrazine	1-2 days old mushrooms (g)	not stated	42±3 16	Chauhan et al., 1985 Toth et al., 1997a
4-(hydroxymethyl)benzene diazonium ion	not stated	not stated	identified but not quantified	Levenberg, 1962
4-(hydroxymethyl)benzene diazonium ion	1-2 days old mushrooms (m)	not stated	0.6 4	Ross et al., 1982a Toth et al., 1997a

of agaritine in wild *Agaricus* species (Schulzova et al., manuscript submitted for publication).

Outside the genus *Agaricus*, agaritine has hitherto only been detected in two species, shiitake and the oyster mushroom. Very low levels, 0.82 mg/kg were found in shiitake (*Lentinula edodes*) by Hashida et al. (1990), whereas other investigators were unable to detect agaritine in fresh as well as canned shiitake (Stijve et al., 1986; Hajšlova et al., 1998). Italian investigators claim that fresh oyster mushroom (*Pleurotus ostreatus*) contains between 501 and 867 mg/kg (Burini et al., 1999). However, Czech investigators were unable to confirm this finding (Schulzová, personal communications).

Besides *A. bisporus*, four other *Agaricus* species known to harbour agaritine, have also been shown to have enzymatic γ -glutamyltransferase activity comparable to the activity in *A. bisporus*. These species are *A. edulis*, *A. pattersonii*, *A. perrarus*, and *A. xanthodermus*. No enzyme with this activity could be detected in *A. sterlingii* and *A. subrutilescens*, species that do not contain agaritine, and in a number of mushroom species belonging to other genera (Gigliotti and Levenberg, 1964).

Three wild-growing *Agaricus* species have been shown to contain nitrogen-nitrogen bond-containing compounds other than agaritine. The poisonous mushroom *A. xanthodermus* has been shown to contain leucoagaricone, γ -glutamyl-N'-4-(hydroxy)phenylhydrazine, and 4-(hydroxymethyl)benzenediazonium ions, the latter isolated in the form of its stable sulfonate (Hilbig et al., 1985; Dornberger et al., 1986), the edible *A. silvaticus* small quantities of β -nitraminoalanine and its decarboxylation product N-nitroethylenediamine (Chilton and Hsu, 1975), and the edible *A. arvensis*, *A. campestris*, *A. macrosporus*, *A. perrarus* and *A. subperonatus* β -N-(γ -glutamyl)-4-formylphenylhydrazine (agaritinal, Figure 7) (Chulia et al., 1988; Stijve and Pittet, 2000). According to Stijve and Pittet (2000) the level of agaritinal could be substantial in some of these mushrooms (above 1 g/kg). In addition *A. hortensis* has been found to contain the γ -(*p*-hydroxy)anilide of glutamic acid (Jadot et al., 1960).

5.2. Influence of storage and processing

The post-harvest storage and processing of *A. bisporus* may strongly influence the agaritine content of the mushroom. The influence of storage and processing on the level of other phenylhydrazines in the mushroom has been dealt with in one study (see section 5.2.5. Dry baking).

The most recent data on the influence of storage and processing on the content of agaritine in cultivated *Agaricus* mushrooms, based on joint studies between a Czech group of food scientists and the "Nordic project group on inherent natural toxicants in food plants and mushrooms" are summarized in Table 6 (Hajšlová et al., 1998; Andersson et al., 1999; Schulzová et al., 2002).

5.2.1. Refrigerating

In one study, post-harvest storage of mushrooms for 5 days at 2°C or 12°C reduced agaritine levels by 68 %. There was no loss of agaritine during the first three days; the largest decrease occurred between day 4 and 5 (Liu et al., 1982). In a similar experiment,

Table 5A. Mushrooms reported to contain agaritine.

Species	Reference	Species	Reference
<i>Agaricus abruptibulbus</i>	Stijve and Pittet, 2000	<i>Agaricus macrosporus</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Agaricus argentatus</i>	Levenberg, 1964	<i>Agaricus micromegathus</i>	Levenberg, 1964
<i>Agaricus arvensis</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Agaricus niveolutescens</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Agaricus augustus</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Agaricus nivescens</i>	Stijve and Pittet, 2000
<i>Agaricus bisporus (incl. var.)</i>	Stijve and Pittet, 2000	<i>Agaricus pattersonii</i>	Levenberg, 1964
<i>Agaricus bitorquis</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Agaricus perrarus</i>	Levenberg, 1964; Stijve and Pittet, 2000*
<i>Agaricus blazei</i>	Stijve and Pittet, 2000; Stijve et al., 2003	<i>Agaricus porphyrocephalus</i>	Stijve and Pittet, 2000
<i>Agaricus campestris (incl. var.)</i>	Levenberg, 1964; Stijve and Pittet, 2000*	<i>Agaricus semotus</i>	Stijve and Pittet, 2000
<i>Agaricus comtulus</i>	Levenberg, 1964; Stijve and Pittet, 2000	<i>Agaricus silvicola</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Agaricus crocodilinus</i>	Levenberg, 1964	<i>Agaricus subperonatus</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Agaricus edulis</i>	Levenberg, 1964; Stijve et al., 1986	<i>Agaricus vaporarius</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Agaricus excellens</i>	Stijve et al., 1986	<i>Agaricus xanthodermus</i>	Levenberg, 1964; Stijve et al., 1986
<i>Agaricus hortensis</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Lentinus edodes</i>	Hashida et al., 1990
<i>Agaricus liliceps</i>	Stijve and Pittet, 2000		

*both in Stijve et al., 1986 and in Stijve and Pittet, 2000

Table 5B. Mushrooms reported not to contain agaritine.

Species	Reference	Species	Reference
<i>Agaricus benesii</i>	Levenberg, 1964	<i>Agaricus placomyces</i>	Stijve and Pittet, 2000
<i>Agaricus bernardii</i>	Stijve and Pittet, 2000	<i>Agaricus sterlingii</i>	Levenberg, 1964
<i>Agaricus cupreobrunneus</i>	Stijve and Pittet, 2000	<i>Agaricus subrutilescens</i>	Levenberg, 1964
<i>Agaricus fuscofibrillosus</i>	Stijve and Pittet, 2000	<i>Agaricus sylvaticus</i>	Levenberg, 1964; Stijve and Pittet, 2000*
<i>Agaricus geesterani</i>	Stijve and Pittet, 2000	<i>Agaricus xanthoderma</i>	Stijve and Pittet, 2000
<i>Agaricus haemorrhoidarius</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Agrocybe aegerita</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Agaricus hortensis</i>	Stijve and Pittet, 2000	<i>Amanita caesarea</i>	Stijve et al., 1986
<i>Agaricus langei</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Amanita rubescens</i>	Stijve et al., 1986
<i>Agaricus meleagris</i>	Stijve et al., 1986	<i>Armillaria edodes</i>	Hashida et al., 1990
<i>Agaricus phaeolepidotus</i>	Stijve and Pittet, 2000	<i>Armillaria mellea</i>	Stijve et al., 1986

*both in Stijve et al., 1986 and in Stijve and Pittet, 2000

Table 5B. Mushrooms reported not to contain agaritine.

Species	Reference	Species	Reference
<i>Auricularia auriculajudae</i>	Stijve and Pittet, 2000	<i>Lepista nuda</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Auricularia polytricha</i>	Stijve and Pittet, 2000	<i>Leucoagaricus pudicus</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Boletus edulis</i>	Stijve et al., 1986	<i>Lycoperdon gemmatum</i>	Stijve et al., 1986
<i>Calocybe gambosa</i>	Stijve et al., 1986	<i>Macrolepiota procera</i>	Stijve et al., 1986
<i>Cantharellus cibarius</i>	Stijve et al., 1986	<i>Macrolepiota rhacodes</i>	Stijve et al., 1986
		<i>Marasmius oreades</i>	Stijve et al., 1986
<i>Cantharellus tubaeformis</i>	Stijve et al., 1986	<i>Micropsalliota brunneosperma</i>	Stijve and Pittet, 2000
<i>Clitopilus prunulus</i>	Stijve et al., 1986	<i>Morchella conica</i>	Stijve et al., 1986
<i>Coprinus comatus</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Morchella vulgaris</i>	Stijve et al., 1986
<i>Coprinus atramentarius</i>	Stijve et al., 1986	<i>Pholiota nameko</i>	Stijve and Pittet, 2000
<i>Craterellus cornucopioides</i>	Stijve et al., 1986	<i>Pleurotus citrinopileatus</i>	Stijve and Pittet, 2000
<i>Fistulina hepatica</i>	Stijve and Pittet, 2000	<i>Pleurotus eryngii</i>	Stijve and Pittet, 2000
<i>Flammulina velutipes</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Pleurotus ostreatus</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Grifola frondosa</i>	Stijve and Pittet, 2000	<i>Pleurotus salmoneostramineus</i>	Stijve and Pittet, 2000
<i>Hericium erinaceus</i>	Stijve and Pittet, 2000	<i>Russula cyanoxantha</i>	Stijve et al., 1986
<i>Hirneola auricula-judae</i>	Stijve et al., 1986	<i>Russula virescens</i>	Stijve et al., 1986
<i>Hydnum repandum</i>	Stijve et al., 1986	<i>Sarcodon imbricatum</i>	Stijve et al., 1986
<i>Hygrophorus marzuolus</i>	Stijve et al., 1986	<i>Sparassis crispa</i>	Stijve and Pittet, 2000
<i>Hypholoma capnoides</i>	Stijve and Pittet, 2000	<i>Sparassis laminata</i>	Stijve and Pittet, 2000
<i>Hypholoma sublateritium</i>	Stijve and Pittet, 2000	<i>Stropharia rugosaannulata</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Hypsizigus tessulatus</i>	Stijve and Pittet, 2000	<i>Suillus granulatus</i>	Stijve et al., 1986
<i>Hypsizigus ulmarius</i>	Stijve and Pittet, 2000	<i>Suillus luteus</i>	Stijve et al., 1986
<i>Kuehneromyces mutabilis</i>	Stijve et al., 1986; Stijve and Pittet, 2000	<i>Tricholoma equestre</i>	Stijve et al., 1986
<i>Lactarius deliciosus</i>	Stijve et al., 1986	<i>Tricholoma portentosum</i>	Stijve et al., 1986
<i>Laetiporus sulphureus</i>	Stijve and Pittet, 2000	<i>Tricholoma terreum</i>	Stijve et al., 1986
<i>Langermannia gigantea</i>	Stijve et al., 1986	<i>Tuber melanosporum</i>	Stijve et al., 1986
<i>Leccinum griseum</i>	Stijve et al., 1986	<i>Volvariella volvacea</i>	Stijve et al., 1986; Stijve and Pittet, 2000
<i>Lepista nebularis</i>	Stijve et al., 1986	<i>Xerocomus chrysenteron</i>	Stijve et al., 1986

*both in Stijve et al., 1986 and in Stijve and Pittet, 2000

two batches of *Agaricus bisporus* purchased from a local store with average agaritine contents of 440 and 720 mg/kg, respectively, were stored in the refrigerator (4°C) in polyethylene bags for 2 weeks. After one week one of the batches had lost 2 % of its agaritine content, whereas the other batch had lost 47 %. The corresponding figures after two weeks were 36 and 76 %, respectively (Ross et al., 1982b).

Since it is normal for mushrooms to be stored for periods equivalent to a week at 2°C in fresh-market channels, it is probable that consumers are exposed to lower agaritine levels than are found in freshly harvested mushrooms. From a practical standpoint, the use of extended post-harvest storage as a means to reduce agaritine in fresh mushrooms would not be desirable since deterioration in quality would most likely occur (Liu et al., 1982).

A similar reduction in the agaritine content during storage at low temperature was demonstrated also in *Agaricus hortensis* (Schulzová et al., 2002). The fresh mushroom put into the refrigerator contained 393 mg agaritine per kg fresh weight. After 6 days in the refrigerator (5 °C) the agaritine content was reduced by 25%, after 10 days by 40% and after 14 days by 50% (Table 6).

5.2.2. Freezing and thawing.

Freezing and thawing *A. bisporus* results in a significant reduction in agaritine content of the mushroom. Liu et al. (1982) observed about 20 % of the original agaritine content in thawed mushrooms, whereas Stijve et al. (1986) only observed 2 % of the original agaritine content. The method of freezing - quickly in liquid nitrogen or slowly at -20°C - did not influence the end result. The agaritine levels were 23-74 % lower in *A. bisporus* that had been rapidly frozen in liquid nitrogen than in mushrooms that had been immediately freeze-dried (equivalent to fresh mushrooms) (Sharman et al., 1990). Liu and colleagues suggested that the majority of the loss in agaritine occurred during thawing, when disruption of the cellular structures separating the degrading enzymes from the substrate would allow the enzymatic breakdown of agaritine not otherwise possible (Liu et al., 1982). It is not known whether any breakdown of agaritine occurred during the 30 days of storage at -25°C.

When *A. bitorquis*, containing 249 mg agaritine/kg, was stored for one week at -18°C, the agaritine content was reduced by 25% when analysed after extraction with methanol without thawing the sample and by 48% after thawing for one hour at room temperature (or by 33% after thawing 0.5 minutes in microwave oven). After storage of *A. bitorquis*, containing 261 mg agaritine/kg, at -18 °C for one month without thawing, the agaritine content was reduced by 41% and after thawing for one hour at room temperature by 77%, as shown in Table 6 (Schulzová et al., 2002).

5.2.3. Freeze-drying.

Freeze-drying or lyophilisation seems to have no influence on the agaritine content of *A. bisporus* (Liu et al., 1982). In accordance with this observation, two Swiss studies found high levels of agaritine in lyophilised samples of the cultivated mushroom, the levels being between 2 110 and 7 800 mg/kg dry weight (Fischer et al., 1984; Stijve et al., 1986).

Table 6. Influence of storage and household processing on the agaritine content of cultivated *Agaricus* mushrooms (Hajšlová et al., 1998; Andersson et al., 1999; Schulzová et al., 2002).

Process	Conditions (°C)	Time	Amount of agaritine remaining in the mushroom (%) ¹
Storage:			
Refrigerating	5	6 days	75
Refrigerating	5	14 days	50
Drying	25	24 hours	82
Drying	50	7.5 hours	76
Drying	40-60 ²	7 hours	81
Freezing without thawing	-18	7 days	75
Freezing with thawing	-18	7 days	52
Freezing without thawing	-18	30 days	59
Freezing with thawing	-18	30 days	23
Freeze drying	-51	18 hours	~100
Household processing:			
Boiling	~100	5 minutes	44
Boiling	~100	60 minutes	12
Dry baking	200	10 minutes	77
Dry baking	200	20 minutes	57
Dry baking	200	30 minutes	24
Deep frying	150	10 minutes	50
Deep frying	170	5 minutes	52
Frying (whole mushroom caps, oil)	150	10 minutes	43
Frying (sliced, oil)	~100	5 minutes	34
Frying (sliced, butter)	~100	5 minutes	69
Microwave heating	1000 W, 2450 MHz ³	15 seconds	59
Microwave heating	1000 W, 2450 MHz ³	30 seconds	49
Microwave heating	1000 W, 2450 MHz ³	60 seconds	35
Canned mushrooms	-	-	< 10

¹ 100% = agaritine content in fresh mushrooms before processing

² 40 °C for two hours, then 60 °C for five hours

³ 20 gram sliced mushrooms

Freeze-drying of *A. hortensis*, containing 410 mg agaritine/kg, at -51 °C for 18 hours did not influence the content of agaritine. The content in the dried lyophilised mushroom was around 4000 mg/kg dry weight (Schulzová et al., 2002).

5.2.4. Drying.

Very low levels of agaritine have been reported in dried Italian *A. bisporus* (pumizia), whereas extremely high amounts have been detected in dried sliced mushrooms on the market in UK, 6 520 mg/kg dry weight (Sharman et al., 1990). Unfortunately, it is not known how these two samples have been produced. As mentioned above, freeze-drying seems to have negligible effect on the agaritine content of the cultivated mushroom.

Schulzová et al. (2002) followed the agaritine content of *A. hortensis* during drying at ambient temperature (25 °C), at 50 °C and at programmed elevated temperature (40 °C for two hours followed by 60 °C). The drying of the sliced mushrooms was continued until the material obtained constant weight. The agaritine content was reduced by 18-24% (Table 6).

5.2.5. Dry baking.

Because in some countries humans consume *A. bisporus* mostly in baked form, e.g. in the USA, the amounts of agaritine in baked mushrooms have been investigated. Gannett and Toth (1991) analysed for agaritine and other hydrazine derivatives in extracts from fresh or baked (10 minutes at 225°C) mushroom. Baking reduced the level of agaritine by 27 %, 4-(carboxy)phenylhydrazine by 10 % and β -N-[γ -L-(+)-glutamyl]-4-carboxy-phenylhydrazine by 23 % when compared to the fresh extract. In another experiment, the fresh or baked homogenate of *A. bisporus* was analysed for 4-(hydroxymethyl)-benzenediazonium ion. Baking reduced the content by 41 %. Ganett and Toth (1991) concluded that dry baking reduced the content of the four nitrogen-nitrogen bond-containing compounds by about 25 % only.

Dry-baking was also studied by Schulzová et al. (2002). *A. bitorquis*, containing 340 mg agaritine/kg, was heated on baking paper for 0 to 45 minutes at 200 °C. After 10 minutes baking, the agaritine content was reduced by 23%, after 20 minutes by 43%, after 30 minutes by 76 % and after 45 minutes by 89%, but in the latter case, after 45 minutes baking at 200 °C, the organoleptic quality of the mushrooms was unsatisfactory (Table 6).

5.2.6. Boiling.

The loss of water-soluble compounds from mushroom tissue is a result of shrinkage and extraction, and has been carefully studied by Biekman et al. (1996), using mannitol as an example of such compounds.

In an American investigation blanching of two different strains of *A. bisporus* in boiling water for 5 minutes reduced the agaritine content by 57 % and 75 %, respectively. At the same time these two strains lost 20 % and 24 % in fresh weight, usually referred to as shrinkage (Liu et al., 1982). Similarly, when two fresh samples of *A. bisporus* containing 290 and 295 mg/kg agaritine, respectively, were blanched for 5-7 min, the agaritine content in the mushrooms were reduced to 181 and 169 mg/kg, respectively. After the blanching the brine contained 116 and 134 mg/kg agaritine, respectively, giving a total amount of 297 and 303 mg/kg in the two samples (Fischer et al., 1984). In agree-

ment with these observations Japanese investigators have reported a significant decrease in agaritine content of *A. bisporus* boiled at 100°C for 10 minutes (Hashida et al., 1990).

Also, Schulzová et al. (2002) studied the effect of boiling on the content of agaritine in the cultivated mushroom. Sliced fruit bodies of *A. bitorquis*, containing 360 mg agaritine/kg, were boiled in closed jars in steam. Similar results as shown in Figure 8 were obtained, when whole mushrooms were used instead of sliced mushrooms. As can be seen in Figure 8, boiling resulted in a significant progressive decrease of agaritine in solid mushrooms, whereas the concentration in the aqueous solution increased during the first 15 minutes. No further increase in the agaritine content of the boiling water was observed thereafter. The most pronounced changes occurred during the first minute of boiling. The solid mushrooms had lost 55% of their agaritine after 5 minutes of boiling and about 90% after 60-120 minutes of boiling. The total amount of agaritine in the system was also reduced during cooking, being about 75% of the original amount after 5 minutes and about 60% of the original amount after 60 minutes of boiling.

5.2.7. Canning.

In the case of low-fat foods such as brine-packed mushrooms, destruction or prevention of the germination of the spores of *Clostridium botulinum* and some thermophilic bacteria causing economic spoilage is of primary importance during the canning process. Speroni and co-workers (1985) have studied the thermal degradation kinetics of agaritine in model systems and agaritine retention in canned mushrooms, and Sastry et al. (1985) the agaritine retention in mushrooms during the canning process. The former investigators studied the effect of pH and heating on agaritine degradation in buffer, mushroom puree and canned mushrooms. This was done by warming up agaritine with citric-phosphate buffer (in ampoules) or agaritine and mushroom puree buffered with the same salts to specified temperatures (101-133°C) during a predetermined time intervals. Additionally, cans containing quartered mushrooms with brine and buffer were stillretored using five thermal process schedules with equivalent lethalties for

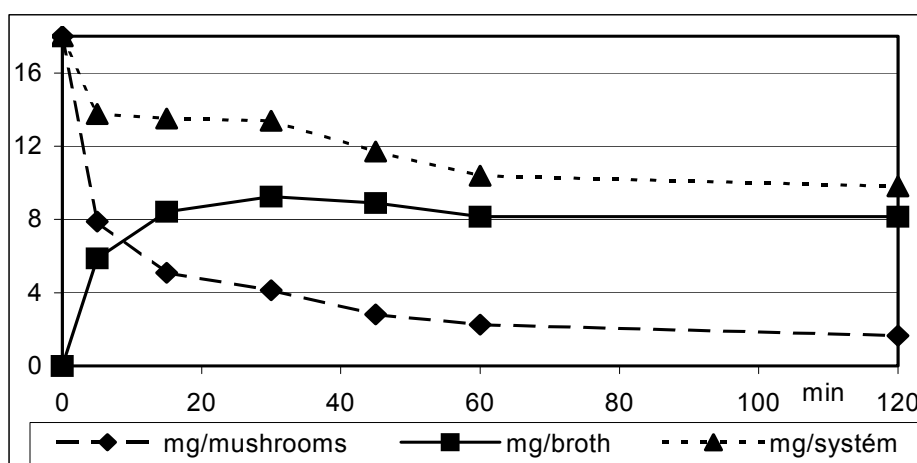


Fig. 8. Amount of agaritine (mg) remaining in mushrooms (*A. bitorquis*), cooking broth and the whole system (showing the extent of agaritine degradation during cooking) after boiling in water for up to 120 minutes.

Clostridium botulinum. In both the buffer and puree systems, agaritine degradation could be modelled by first-order kinetics. The results of both investigations indicated that high temperature-short time treatment during the canning process resulted in the greatest agaritine retention in the mushroom part (Sastry et al., 1985). The removal of agaritine by blanching would be especially desirable because any breakdown products of agaritine formed during the thermal process would not accumulate in the can. Furthermore, would any of the breakdown products be carcinogenic, it may be more desirable to optimise the blanching operation to remove agaritine from the mushrooms before retorting and then use high temperature-short time processing to actually minimise the destruction of agaritine and formation of hazardous breakdown products in the can. One of the first measurements of the agaritine level in mushrooms *per se* as well as in mushroom products was performed by American investigators (Ross et al., 1982b). They found no agaritine in canned *A. bisporus*, but 440 mg/kg in fresh mushrooms. It was suggested by these investigators that agaritine is decomposed by the temperatures and pressures that are used during food processing.

Recently, the Nordic project group on inherent natural toxicants in food plants and mushrooms” in collaboration with food chemists in the Czech Republic analysed 35 different trademarks of canned mushroom products for agaritine, 25 of which were based on cut mushrooms and 10 on whole mushrooms (Andersson et al., 1999). These cans were produced in the Netherlands (18 products), China (6 products), France (4 products), India (3 products), Spain (2 products), the Czech Republic (1 product) and Sweden (1 product). On average whole mushrooms contained 14.9 ± 6.7 mg agaritine per kg product whereas cut mushrooms contained 18.1 ± 7.8 mg/kg. There was no statistically significant difference between these two values. Agaritine levels in brine were generally slightly less than the amount detected in the canned mushrooms.

Table 7 summarises published measurements on agaritine content of canned *Agaricus bisporus*. It is evident that relatively low agaritine levels are found in canned products, usually less than 10 % of the agaritine level detected in fresh mushrooms (Liu et al., 1982; Stijve et al., 1986; Hajslövä et al., 1998; Andersson et al., 1999). A comparison of concentration values in mushrooms and brine indicates a near-equilibrium situation between solid and liquid phases. This equilibration is probably established during can storage.

5.2.8. Pan-frying

In the Nordic countries frying of the cultivated mushrooms in butter or vegetable oils is a much more common cooking procedure than dry baking. Therefore, the influence of frying sliced *A. hortensis* (298 mg agaritine/kg) in these fats for 5 minutes on the agaritine content of the mushroom was investigated by the joint Czech-Nordic research group (Schulzová et al., 2002). The temperature of the vegetable oil and butter, respectively, was around 100 °C during the entire frying period, the mushroom slices being stirred continuously during the process. After frying for 5 minutes in vegetable oil, the agaritine content had been reduced to 34% of the original agaritine content in fresh mushrooms, but only 0.6% of the original agaritine content could be recovered in the oil. Frying, sliced mushrooms in butter for 5 minutes reduced the agaritine content to 69% of the agaritine content in fresh mushrooms before processing. Only 2.8% of the original agari-

Table 7. Reported levels of agaritine in canned *Agaricus bisporus*.

Agaritine content (mg/kg fresh weight) mushrooms brine		Number of samples	Reference
31 (1-64)	57 (3-104)	11	Fischer et al., 1984
28 (20-33)	9 (6-11)	12	Sharman et al., 1990
undetectable	undetectable	3	Ross et al., 1982
29 (23-35)	34 (25-43)	2	Stijve et al., 1986
65 (40-90)	115 (80-150)	2	Liu et al., 1982
undetectable	not determined	not given	Hashida et al., 1990
17.2 (<3-30)	11.0 (<3-30)	35	Andersson et al., 1999

tine content was recovered in the butter. In another experiment whole mushrooms caps of *A. bitorquis* was fried at around 150 °C in a pan with vegetable oil for 10 minutes (5 minutes on each side). The mushrooms originally contained 338 mg/kg agaritine. Ten minutes of frying reduced the agaritine content by 57% (Table 6) (Schulzová et al. 2002).

5.2.9. Deep-frying

Schulzová et al. (2002) studied the influence of deep-frying at 150 and 170 °C in vegetable oil on the agaritine content of *A. hortensis* (the original amount was 429 mg/kg). The agaritine content was reduced to approximately 50%, either after 10 minutes of deep-frying at 150 °C or after 5-7 minutes at 170 °C (the optimal cooking time in both cases with respect to organoleptic properties) (Table 6).

5.2.10. Microwave heating

The influence of microwave heating on the agaritine content of *A. bitorquis* has been investigated. Twenty gram of sliced mushroom (original content of agaritine 276 mg/kg) was heated in the microwave oven (1000W, 2450 MHz). After 15 seconds the agaritine content was reduced by 41%, after 30 seconds by 51%, after 45 seconds by 61% and after 60 seconds by 65% (Schulzová et al., 2002).

5.3. Other products containing the cultivated mushroom

Table 8 shows the reported levels of agaritine in miscellaneous types of *A. bisporus* products on the market - some products contain high levels and others low. In one of the first measurements of agaritine content of mushrooms and mushroom products, the compound was undetectable in mushroom soup (Ross et al., 1982b). Low agaritine levels in ready-to-eat mushroom soup have been confirmed by whereas Andersson and co-workers (1999) observed somewhat higher agaritine levels in some mushroom soups based on a dry powder. However, in the later study the amount of agaritine in the ready-to-eat product never exceeded 30 mg per portion. On the other hand, there are products that contain higher agaritine levels than others, but such products can not easily be identified by the consumers.

Table 8. Reported levels of agaritine in miscellaneous types of *Agaricus bisporus* products.

Type of product	Agaritine content (mg/kg fresh weight)	Reference
Frozen mushrooms	330	Ross et al., 1982b
Mushrooms sautéed in olive oil at 300°C (7 min.)	300	Ross et al., 1982b
Sliced mushrooms and mushroom powder used as ingredients for dehydrated soups	100 - 250*	Stijve et al., 1986
Pasta sauce (n=4)	1.6-15.3	Andersson et al., 1999
Mushroom soup	0	Ross et al., 1982b
Mushroom soup	<5	Sharman et al., 1990
Mushroom soup (n=3)	1.8-62.8	Andersson et al., 1999
Mushroom sauce	3.9	Andersson et al., 1999

* recalculated, assuming 90% water in mushroom Sharman and co-workers (1990),

As fresh mushrooms in general have a short shelf life compared to most vegetables, the industry have tried to develop mushroom products, with longer shelf lives, that could be exported to distant destinations. This could be done by removing water and adding inhibitors such as salt to the mushrooms. *Agaricus bisporus* has recently been explored for snack development by using osmotic dehydration in concentrated salt solution (Brennan and Salmier, 1998). The laboratory studies have been promising, but nothing is known about the safety of the product. For example, it is not known to what extent the processing influence the phenylhydrazines in the mushroom.

5.4. Influence of cultivation, including genetic modification

Commercial mushroom growing entails several production steps, which may affect agaritine concentration in the harvested mushroom. Mushroom composting involves a type of fermentation whereby the original starting material is chemically and biologically altered to provide the mushroom mycelium with the proper nutrients needed for growth. Mushroom compost types are commonly classified according to the starter material used. "Natural composts" consist mainly of wheat straw-bedded horse manure. They are generally the most widely used and least expensive compost types. "Synthetic composts" consist largely of hay and crushed corn cobs and are generally any compost where horse manure is not the major ingredient. "Blended composts" are made from mixtures of "natural" and "synthetic" composts. Most type of composts, in addition, also requires nitrogen supplements, Gypsum to aid in pH control, and often many other ingredients.

Mushrooms grown in synthetic compost had higher agaritine levels than mushrooms obtained from natural or blended compost (Speroni et al., 1983). On a dry weight basis two samples of mushroom grown on synthetic compost contained approximately 4 100 and 7 500 mg/kg agaritine, respectively, whereas mushrooms grown on blended com-

post contained 2 700 and 3 100 mg/kg and mushrooms grown on natural compost 2 400 and 2 700 mg/kg, respectively. Within a given flush, the mushrooms harvested from synthetic composts were always higher in agaritine concentration than mushrooms from natural or blended composts. Actual isolation of the compost constituents affecting the agaritine levels will require additional research.

Spawning refers to the inoculation of the compost with the mycelium of a selected strain of *A. bisporus*. Earlier it was believed that mycelium contains substantially less agaritine than the fruiting bodies (Speroni et al., 1983), but later studies have shown this not to be the case. Soulier and coworkers (1993) detected agaritine in 17 out of 20 samples of distal mycelia and 19 out of 20 samples of proximal ones, with a mean agaritine content (depending on strain type) around 135 mg per litre cell solution. These observations indicate that the mycelium can be considered as an active site of synthesis of agaritine and its presence within this tissue cannot result from a translocation from fruiting bodies. On the contrary, Baumgartner et al. (1998) have suggested that agaritine is transported from the vegetative hyphae of the mycelium to the fruit body.

During the cropping or harvesting period, rhythmic 3- to 5-d harvests, which constitute a single flush or break, are followed by periods when few or no mushrooms are available for harvest. Growers usually harvest from at least three but generally no greater than eight flushes per crop before aborting the mushroom bed. Later flushes produce smaller yields than earlier flushes. At the same time there is a strong tendency for agaritine concentrations of mushrooms to increase with the later flushes (Speroni et al., 1983).

A few pesticides, for example chlorfenoviphos, chlorpyrifos, cyromazine, diflubenuron, methoprene and prochloraz are used to control competing insects and fungi during *Agaricus* cultivation. It is not known whether the use of these pesticides influence the agaritine content of the mushrooms.

Commercially grown strains of *A. bisporus* on occasion produce sectors that display undesirable phenotypes. Such sections show altered compost colonisation, have reduced yield and produce inferior quality mushrooms. Genetic studies of such inbreeding depression sectors have revealed loss of heterozygosity at specific loci, and changes in deheterokaryotization, alteration in the frequency of somatic recombination, chromosome loss, chromosomal length polymorphisms and possible chromosome translocations, as well as changes in copy number of the ribosomal DNA repeats (Horgen et al., 1996). Inbreeding depression, as defined by eight different fitness components, have been reported in *A. bisporus* (Xu, 1995), but the relevance of inbreeding depression to *A. bisporus* breeding in general, and the agaritine content of the mushroom in particular, is not yet known.

Little genetic variation exists between cultivated strains of *A. bisporus*. During the last decade new prospects for *A. bisporus* strain improvement has been anticipated (Kerrihan, 1993), and considerable efforts have been devoted to development of molecular biological techniques, especially a transformation system for the mushroom. The unavailability of a practical gene transfer system has been the single largest obstacle precluding the use of molecular approaches for the genetic improvement of mushrooms. Although several investigators have shown considerable interest in developing useful transformation technologies for mushrooms (Royer and Horgen, 1991; Challen et al., 1991; Li and Horgen, 1993; Challen and Elliott, 1994; van der Rhee et al. 1996a,

1996b), no methods have been generally used due to low transformation efficiency or lack of utility and convenience. The most marked obstacles for developing a satisfactory transformation system has been attributed to factors such as the generation of false positives, low level of integration of transforming DNA, poor expression of foreign sequences, DNA modifications after integration or low competence of certain mushroom strains (Mikosch et al., 2000). An important step to a more successful transformation system was taken when De Groot et al. (1998) succeeded in transforming *Agaricus bisporus* and other mushrooms with an *Agrobacterium*-mediated fungal transformation system. The advantage with this system was that it was more convenient than the previous protoplast-based schemes (van der Rhee et al., 1996a, 1996b). Its disadvantage was a low efficiency. The situation was substantially improved when Chen et al. (2000) recently reported successful transformation of fruiting body tissue by *Agrobacterium tumefaciens*-mediated methods. The choice of promoter, strain of *A. tumefaciens*, and type of fruiting body tissue were critical for optimal transformation efficiency. Transgenic vegetative cultures were obtained in less than 2 weeks, and fruiting bodies 8 weeks later under controlled environmental conditions. A similar system for transformation of vegetative mycelium of *A. bisporus* has also been reported (Mikosch et al., 2001).

A reduction in the endogenously regulated browning reaction (Wichers and van Leeuwen, 1996) is an obvious goal for research on transformation of *A. bisporus*, but a reduction in agaritine content could also be a task for future investigations. It is clear that turnover of glutamate/glutamine has a central position in fungal nitrogen metabolism. Whether the nitrogen metabolism in *A. bisporus* has any bearing on the agaritine level or turnover (biosynthesis/degradation) of agaritine is not known (Baars et al., 1995). As quite a large number of genes have been isolated, sequenced and mapped on all the 13 chromosome pairs of *Agaricus bisporus* (a total of 41 genes in year 2000) (Whiteford and Thurston, 2000), there is now a more promising future for the transformation of this species.

5.5. Conclusion on occurrence

Commercial mushroom growing entails several production steps (factors) which may influence the agaritine concentration in the harvested mushroom. Factors having influence include strain-type of *A. bisporus*, cultural practice employed in production, stage of cropping-cycle (flush), and maturity of mushrooms at harvest. Since the amount of agaritine measured in *A. bisporus*, in addition, depends on how long and under which conditions the mushrooms are stored after harvest, it should be kept in mind that the agaritine level in "fresh" mushrooms purchased in local stores may be lower than the levels in fresh mushrooms obtained directly from mushroom producers.

Table 9 summarises available data on estimated average agaritine content of fresh *A. bisporus* and marketed products containing this mushroom. High amounts of agaritine are found in fresh and freeze-dried mushrooms, and possibly also in frozen *A. bisporus*. However, there are indications that the levels are reduced upon thawing of the mushrooms. The level of agaritine in mushrooms dried at room temperature is insufficiently investigated.

Table 9. Estimated average agaritine content of *A. bisporus*, fresh and processed mushrooms. References are given in the main text.

Type of mushroom product	Estimated agaritine content (% of fresh weight) - in relation to content in fresh mushroom
Fresh	200-500 mg/kg
Refrigerated after the first few days	about 75-100%
Refrigerated after one week	about 50-75%
Refrigerated after two weeks	about 50%
Frozen without thawing	about 50-75%
Frozen with thawing	about 25-50%
Freeze-dried	no influence on the agaritine content
Dried	about 75-80%
Dry-baked	about 25-75% (depending on time)
Boiled for 5 minutes	extracts about 50% ¹⁾
Boiled for 60 minutes	extracts about 90% ²⁾
Pan-fried	about 50%
Deep-fried	about 50%
Microwave-heated for up to 60 seconds	about 50%
Canned	less than 10%

¹⁾ Total amount in boiled mushroom and boiling water about 75% of original amount in the fresh mushroom; ²⁾ Total amount in boiled mushroom and boiling water about 60% of original amount in the fresh mushroom.

The information on how the agaritine content of *A. bisporus* is influenced by food production is limited. However, research devoted to these questions is ongoing. Dry baking of the mushroom, as it is done during pizza baking, reduces the agaritine content by about 25%. Wet processing of mushrooms, like boiling, reduces the agaritine levels by about 50%. Low quantities of agaritine are present in canned *A. bisporus*, usually less than 10% of the quantity in fresh mushrooms. Also microwave-heating may reduce the agaritine content by more than 50% within one minute.

In addition to the cultivated mushroom (*A. bisporus*), 10 wild *Agaricus* species (*A. patersonii*, *A. xanthodermus*, *A. argentatus*, *A. campestris*, *A. comptulis*, *A. crocodilinus*, *A. edulis*, *A. hortensis*, *A. micromegathus* and *A. perrarus*) contain agaritine in quantities comparable to those found in *A. bisporus*.

Since molecular biological techniques to transform *A. bisporus* were recently developed, many different types of modification of the mushroom strains can be foreseen in the future. One such modification could possibly lead to reduced levels of phenylhydrazines.

6. Production and consumption

6.1. Production

The cultivated mushroom, *Agaricus bisporus*, is the most extensively consumed species of mushroom worldwide (Royse, 1997). In 1991 37 % of the world production of cultivated edible mushrooms was *Agaricus bisporus* - the quantity produced being 1.6 million tonnes compared with 1.2 million tonnes in 1986 - an increase in production of 30 % during these five years. The most consumed mushroom species/genera and their production in 1986 and 1991 are listed in table 10. The major producers of the cultivated mushroom are the EU-countries, China and the USA. The European mushroom production and processing totalled 1 022 million kg in 1995 (Toth, 2000a).

6.2. Consumption

According to Moss and Mitchell (1994) eight Western countries account for 85 % of the worldwide cultivated mushroom consumption. The USA is the largest mushroom consumer with 30 %, Germany is second with almost 20 % , United Kingdom, France and Italy are each responsible for approximately 10 % of the worldwide consumption.

The annual *per capita* intake of the cultivated mushroom (fresh, canned and total) in eight Western countries is shown in table 11. In 1998-1999 the United States annual consumption of this fungus totalled 455 million kg (Toth, 2000a).

In the Nordic countries the total annual production of *Agaricus bisporus* is about 12 000 tonnes. Data on the production and estimated per capita intake of this mushroom in the five Nordic countries are given in table 12. As shown in this table the estimated annual *per capita* intake of the "fresh" (or frozen) mushroom varies between countries from 0.4 to 1.6 kg; for "preserved" (e.g. canned) mushroom from 0.2 to 1.6 kg and the "total" quantity of mushroom from 0.6 to 2.4 kg.

It should be underpinned that the estimated *per capita* intakes are based on information on production, export and import but do not take into consideration the vary different eating habits with respect to edible mushroom within the populations. This is for instance reflected in a recent Danish study on dietary habits in Denmark (Danskernes Kostvaner 1996, Andersen, 1998) where according to the replies to a questionnaire more than half of the adults had not eaten cultivated mushrooms, 5 % did eat more than five times the median intake and for instance 1 0/00 had eaten thirty times more than the median intake.

6.3. Conclusion on production and consumption

The cultivated mushroom is the most consumed mushroom worldwide, with the USA having the largest consumption. However, the annual *per capita* intakes are highest in Germany, Netherlands and Canada: 3.2, 2.9 and 2.7 kg respectively (1990).

Table 10. World production of cultivated edible mushrooms (fresh weight (x 1000 tonnes)) in 1986 and 1991 (Chang, 1993; Philips, 1981; Royse, 1997).

Species	English name	1986	1991
<i>Agaricus bisporus</i>	The cultivated mushroom Button Mushroom	1215	1590
<i>Pleurotus spp.</i>	Oyster Mushroom Oyster	169	917
<i>Lentinula edodes</i>	Shiitake	320	526
<i>Auricularia spp.</i>	Black Ear, Jew's Ear, Cloud Ear	119	465
<i>Volvariella volvacea</i>	Straw, Paddy Straw	178	253
<i>Flammulina velutipes</i>	Velvet Shank Winter Velvet Stem	100	187
<i>Tremella fuciformis</i>	Snow Fungus Silver Ear	40	140
<i>Hericium erinaceus</i>	Monkeyhead Bear's Head	-	66
<i>Pholita nameko</i>	Nameko Vicid	25	40
<i>Hypsizygus marmoreus</i>	Shimeji	-	32
<i>Grifola frondosa</i>	Hen of the Wood	-	8
Others		10	49
Total		2176	4273

Table 11. Annual consumption (kg per capita) of the cultivated mushroom (*Agaricus bisporus*) in some high-consuming Western countries in 1990 (Moss and Mitchell, 1994).

Country	Fresh mushrooms	Preserved mushrooms	Total
Germany	1.2	2.0	3.2
Netherlands	2.5	0.4	2.9
Canada	1.4	1.3	2.7
France	1.0	1.3	2.3
United Kingdom	2.0	0.20	2.2
USA	0.90	0.90	1.8
Italy	0.70	0.40	1.1

Table 12. Annual Nordic production and consumption (*per capita* intake) of the cultivated mushroom (*Agaricus bisporus*) in 1996 or 1997¹⁾

Denmark (1997)	Fresh (tonnes)	Preserved (tonnes)⁴⁾	Total (tonnes)
Production	8 510 ³⁾	96	8 606 ³⁾
Imported	2 051	4 020	6 071
Total	10 561	4 116	14 677
Exported	2 371	818	3 189
Available for consumption	8 190	3 298	11 488
Estimated <i>per capita</i> intake ²⁾	1.6 kg	0.63 kg	2.2 kg
Finland (1997)			
Production	1 500	-	1 500
Imported	0 300	1 100	1 400
Total	1 800	1 100	2 900
Exported	0 030	-	0 030
Available for consumption	1 770	1 100	2 870
Estimated <i>per capita</i> intake ²⁾	0.35 kg	0.22 kg	0.56 kg
Iceland (1996)			
Production	negligible	negligible	negligible
Imported	302	262	564
Total	302	262	564
Exported	negligible	negligible	negligible
Available for consumption	302	262	564
Estimated <i>per capita</i> intake ²⁾	1.1 kg	0.98 kg	2.1 kg
Norway (1996)			
Production	350	-	350
Imported	2 700	4 387	7 087
Total	3 050	4 387	7 437
Exported	0	-	-
Available for consumption	3 050	4 387	7 437
Estimated <i>per capita</i> intake ²⁾	0.70 kg	1.0 kg	1.7 kg
Sweden (1996/1997)			
Production	1 700	661	2 361
Imported	5 915	13 790	19 705
Total	7 615	14 451	22 066
Exported	0	661	661
Available for consumption	7 615	13 790	21 405
Estimated <i>per capita</i> intake ²⁾	0.86 kg	1.6 kg	2.4 kg

¹⁾ Production, import and export figures are provided from: Denmark: Danmarks Statistik, 1998; Finland: Hal-likainen, pers. communication, 1998; Iceland: Johannesson, pers. communication, 1998; Norway: Aune, pers. communication, 1998; Sweden: Andersson, pers. communication, 1998; ²⁾ Danish population: 1996: 5.216 x 10⁶, Finnish population: 1996: 5.099 x 10⁶, Icelandic population: 1996: 0.267 x 10⁶, Norwegian population: 1996: 4.348 x 10⁶, Swedish population: 1996: 8.816 x 10⁶ (Source: Norden i tal, 1997)

³⁾ Corrected for cultivated mushrooms not sold by the producer.

⁴⁾ The weight of dried, cultivated mushrooms are included in “**Preserved (tonnes)**” and is calculated as ten times the dried weight.

In the Nordic countries the annual *per capita* intakes are somewhat lower (1996/97): Sweden (2.4 kg), Denmark (2.2 kg), Iceland (2.1 kg), Norway (1.7 kg) and Finland (0.6 kg).

7. Toxicokinetics

7.1. Absorption, distribution and excretion

There are two investigations available on the absorption of agaritine in experimental animals. In one of these, albino Swiss mice from a randomly bred colony, weighing about 30 g, were given a single dose of 3 mg agaritine in 0.1 ml water by gavage (Ross et al., 1982b). The animals were anaesthetised and killed at various times after treatment. The blood and five sections of the stomach and gut were examined for agaritine content. Agaritine was found in all parts of the gastrointestinal tract (stomach, upper and lower small intestine, caecum and large intestine) from 15 minutes to 2 hours after treatment. The amount of dosed agaritine recovered was high (70 % or more) during the first 15 minutes after dosing but decreased thereafter, being 53 % at 30 minutes, and only 15-17 % at 1 h and 2 h after administration. No agaritine was detected in any part of the gut 3 hours after administration. Agaritine was not detected in the blood at any time (the detection limit was 30 µg/ml). The authors assumed that agaritine was not detected in the blood due to an inadequacy of the analytical techniques, but it could of course also be due to metabolism of the compound.

The other investigation was comprised of a series of separate experiments. In single dose balance studies agaritine was administered by gavage to male Sprague-Dawley rats and Swiss Hsd/Ola:HIN/s mice; both species received a total dose of 25 mg agaritine/kg body weight with ^{14}C -agaritine added at levels of 3 µCi and 0.6 µCi per animal for rats and mice, respectively (Walton et al., 2000). Urine and faeces were collected at 24 hour intervals for up to 96 hours (rats) and 120 hours (mice).

In rats similar amounts of radioactivity were excreted in urine and faeces (43.3% and 44.2% of the dose, respectively) mainly in the first 24 hours, and excretion was essentially complete after 48 hours. In mice, excretion of radioactivity occurred to a much greater extent in urine (75.8%) than in faeces (23.2%) and was more protracted than in the rat with significant amounts of radioactivity being excreted up to 96-120 hours. In particular, the faecal excretion appeared to be biphasic and prolonged, possibly suggesting some biliary excretion and even enterohepatic circulation (accounting for the more prolonged urinary excretion than in the rat). However, this remains to be investigated and would be somewhat unusual in that biliary excretion of xenobiotics is normally greater in rat than mouse (Walker et al., 1998; Walton et al., 2000). No unchanged agaritine, no N'-acetyl-4-(hydroxymethyl)phenylhydrazine, and no 4-(hydroxymethyl)-benzene diazonium ion was detected in the urine. There was also no evidence that glucuronide and sulfate conjugates were present. All radioactivity was linked to unidentified metabolites, mainly three different compounds. Although qualitative differences in the nature of the agaritine metabolites in the two animal species were not evident, quantitative differences could be discerned (Walton et al., 2000).

At termination of the single dose studies, 96 hours (rats) and 120 hours (mice) after dosing, significant residual activity was found in liver and kidney of the rat; the radiolabel being covalently bound to protein. When expressed in terms of protein, in the rat the

extent of binding was 1.1 and 1.0 pg/mg protein in the liver and kidney, respectively; the corresponding values for the mouse were 0.9 and 0.8 pg/mg protein. Electrophoresis indicated that this was non-specific binding and not associated with specific protein(s). Less radioactivity was determined in the intestine, spleen, heart and lungs (Walker et al., 1998; Walton et al., 2000).

Blood levels of radioactivity were determined in the rats and mice of this experiment. Radioactivity was very rapidly absorbed in the mouse, blood levels peaking after 30 minutes at about 8.5 µg agaritine equivalent/ml blood; excretion was similarly rapid and blood levels had fallen to 0.02 µg by 24 hours after dosing. This resulted in a half-life of 1 hour in mice. In the rat, maximum blood levels were achieved 3-4 hours after dosing and the peak concentration (2.3 µg agaritine equivalent/ml) was about a third of that in mouse. The half-life was 3 hours in the rat. The area under the curve (AUC) values were similar in both animal species, indicating that the extent of absorption was the same. The results also indicate that none of the radioactivity in blood is associated with unchanged agaritine, N'-acetyl-4-(hydroxymethyl)phenylhydrazine, or 4-(hydroxymethyl)benzene diazonium ion. The area under the curve (AUC) values, were similar in both animal species, indicating that the extent of absorption was the same (Walker et al., 1998; Walton et al., 2000).

In subsequent multiple dose studies animals received doses of unlabelled agaritine (25 mg/kg body weight) on three consecutive days prior to a fourth dose containing ¹⁴C-agaritine (3µCi for rats and 0.6µCi for mice). Following this protocol in the rat, urinary excretion of radioactivity was similar to that observed after a single dose (40.0%) but a greater proportion (55.7%) of radioactivity was excreted in faeces and it was more protracted, significant amounts being observed up to 96 hours after dosing. In the mouse, as in the single dose study, urinary excretion predominated (61.4%) but was proportionately somewhat less with more excreted in faeces (31.8%). In these studies, preliminary TLC analysis indicated the presence of three significant metabolites in urine, none of which retained the glutamate moiety; no unchanged agaritine was detected. Thus, repeated administration of agaritine to rats and mice did not alter the urinary metabolic profile and excretion of radioactivity (Walker et al., 1998; Walton et al., 2000).

The fact that no agaritine could be detected in the plasma and urine of rats and mice to which agaritine was administered orally by gastric gavage, could either indicate that agaritine is quickly degraded chemically or microbiologically in the stomach, or subject to an extensive first-pass effect during its passage through the gastrointestinal tract (Walton et al., 1997a). As γ-glutamyl transpeptidase activity in the gut is only about 1% of that present in the kidney (Orlowski and Meister, 1970), it is likely that other microbial or intestinal enzymes are involved, or that agaritine is chemically degraded in the gastrointestinal tract (Walton et al., 1997a). As high γ-glutamyl transpeptidase activity is present in the serum, at least in humans, it is conceivable that the hydrazine is rapidly metabolised in the blood.

Walton and co-workers (2000) also feed male Hsd/Ola:NIH/S mice *Agaricus bisporus* according to the feeding schedule used by Toth and Erickson (1986) when they demonstrated the carcinogenicity of the cultivated mushroom (see section 9.2.1). The mice were fed a normal balanced diet for 4 days/week and during the three remaining days were fed a diet consisting solely of locally obtained, finely diced *A. bisporus*; both diets were provided *ad libitum*. After four weeks each mouse was given by gastric gavage a single dose of [¹⁴C]agaritine (25 mg/kg body weight). The animals were then transferred

to metabolic cages, and urine and feces were collected every 24 hours. A group of five mice served as controls and were maintained on the control diet throughout the experimental period. Studies of the body weight during the experiment showed that the mice lost body weight during the three days they were given the mushroom feed, and quickly recovered it when they were given normal mice pellets. The average daily intake of mushrooms over the entire feeding period was 6.6 g per day per mouse. No difference in the excretion profile of [^{14}C]agaritine was seen between the animals fed the mushroom diet and those that were maintained on a balanced semisynthetic diet. Qualitatively and quantitatively, also the metabolic profile of [^{14}C]agaritine was not altered by the feeding of the mushroom diet.

7.2. Biotransformation

In vitro studies on the metabolism of agaritine have been performed with both purified enzymes, crude enzyme preparations and tissue slices. The metabolism of agaritine and the closely related arylhydrazines N'-acetyl-4-(hydroxymethyl)phenylhydrazine and 4-methylphenylhydrazine by mixed-function oxidases obtained from uninduced livers of Swiss mice was studied *in vitro* by Lawson and Chauhan (1985). 4-Methylphenylhydrazine hydrochloride and the N-acetyl-derivatives of 4-methylphenyl- and 4-(hydroxymethyl)phenylhydrazine, were all readily metabolised to diazonium ions that were stable enough to interact with a trapping agent, 2-naphthol, to give a coloured azo product. Their metabolism was inhibited by metyrapone and carbon monoxide. The glutamylated compound agaritine was poorly metabolised in this study, and the metabolism was not inhibited by metyrapone or carbon monoxide, suggesting that the minor breakdown in this case represented nonspecific decomposition, not metabolism.

The first step in the metabolism of agaritine instead seems to be the removal of the glutamyl group. Studies performed at pH 9.0 have demonstrated that pig's kidney γ -glutamyl-transpeptidase is capable of hydrolysing agaritine to 4-(hydroxymethyl)phenylhydrazine and L-glutamic acid (Ross et al., 1982b). In these studies sodium glyoxylate was used as a trapping agent for 4-(hydroxymethyl)phenylhydrazine, and the produced chromophore quantified by measuring the absorbance at 325 nm. The concentration of glyoxylate 4-(hydroxymethyl)phenylhydrazine increased with time due to generation of 4-(hydroxymethyl)phenylhydrazine from agaritine. The hog kidney enzyme has properties that are very similar to the properties of γ -glutamyltranspeptidase from the cultivated mushroom, but the activity *in vitro* of the mammalian enzyme at room temperature was 7-9 times higher than that of the mushroom enzyme.

γ -Glutamyl transpeptidase is a cell surface glycoprotein that cleaves γ -glutamyl amide bonds. The most abundant physiologic substrates for the enzyme are glutathione and glutathione-conjugated compounds. γ -Glutamyl transpeptidase initiates the cleavage of extracellular glutathione into its constituent amino acids, which can then be transported into the cell, and initiates the initial step in the conversion of glutathione-conjugated compounds to mercapturic acid (Hanigan, 1998; Taniguchi and Ikeda, 1998). Apparently it also removes the glutamyl moiety from agaritine. Purified γ -glutamyl transpeptidase from bovine kidney has been shown to rapidly convert agaritine into two metabolites; only 10% of agaritine remained following a 2 h incubation (Walton et al., 1997a). The same metabolites were generated when the purified enzyme was replaced with kidney homogenates from rats or mice (the former was more active than the second). The

major metabolite has been tentatively identified as 4-(hydroxymethyl)phenylhydrazine, whereas the minor metabolite is an unknown degradation product(s) of agaritine and possibly also of 4-(hydroxymethyl)phenylhydrazine. The minor metabolite was also formed in small amounts in the absence of the enzyme.

When the γ -glutamyl transpeptidase activity was determined in various tissues of rat and mice, using γ -glutamyl-7-aminomethylcoumarin as substrate in pooled tissue from 5 animals, rat kidney were shown to have higher activity than mouse kidney, 168 and 82 nmol/min per mg protein, respectively (Walton et al., 1997a). Hepatic preparations, namely homogenates, microsomes and cytosol, and lung homogenates from either rat or mouse failed to metabolise agaritine even after a 3 h incubation. γ -Glutamyl transpeptidase activity in homogenates from rat liver and lung was 1 and 11 pmol/min per mg protein respectively, and in mouse liver and lung 1 and 14 pmol/min per mg protein, i.e. orders of magnitude lower than the activity encountered in the kidney (Walton et al., 1997a).

In agreement with these observations, kidney preparations metabolically converted agaritine to product(s), which bound covalently to protein. Preparations from the rat were about three times more effective than preparations from the mouse (Walton et al., 1997a). Liver homogenates, cytosol or microsomes, from rats and mice, were unable to metabolise agaritine.

Thus, it seems likely that agaritine is able to produce reactive metabolites after first having been deglutamylated to the arylhydrazine 4-hydroxymethylphenylhydrazine by γ -glutamyl transpeptidase, and then further metabolised to the corresponding diazonium ion. Gannett et al. (1997) trapped aryl radicals during the metabolism of arylhydrazines by rat liver microsomes and microsomes from C50 cells (a mouse keratinocyte cell line). This observation show that arylhydrazines have the potential to give rise to aryl radicals, which subsequently may give rise to C8-arylguanine adducts, and could, at least partly, explain the genotoxicity of arylhydrazines and related compounds that are oxidatively metabolised to arenediazonium ions, the precursor to aryl radicals.

Other *in vitro* studies showed ram seminal vesicle prostaglandin(H)synthase to be able to metabolise agaritine, N'-acetyl-4-(hydroxymethyl)phenylhydrazine and 4-methylphenylhydrazine. However, since N'-acetyl-4-(hydroxymethyl)phenylhydrazine was much more efficiently metabolised than the two other compounds, acetyl groups seems to promote the enzyme-substrate interaction in this case (Lawson, 1987). Agaritine differs from 4-(hydroxymethyl)phenylhydrazine in the nature of the substituent on the hydrazine moiety and was poorly metabolised – agaritine has a glutamyl group instead of an active acetyl group. Also mouse lung prostaglandin(H)synthase and mouse lung cytochrome P-450 were shown to metabolise N'-acetyl-4-(hydroxymethyl)phenylhydrazine and 4-methylphenylhydrazine to reactive metabolites.

Price et al. (1996) used precision-cut rat, mouse and human liver slices and precision-cut rat and mouse lung slices to investigate the *in vitro* metabolism of [ring-U-¹⁴C]agaritine. Liver and lung slices were examined because these two tissues were sites of tumour formation in mice fed fresh *Agaricus bisporus* (Toth and Erickson, 1986). The protein content of liver slice tissue was greater than the protein content of lung slice tissue. Levels of cytochrome P-450 were greater in rat and mouse than in human liver slices. The cytochrome P-450 content of lung slices was not determined.

In the metabolism studies, tissue slices were incubated with 25 μ M radiolabeled agaritine for 0.5-4 hours. A time-dependent covalent binding of [14 C]agaritine metabolites to tissue slice proteins was observed in both liver and lung slices from all three species (Price et al., 1996). Covalent binding to liver slice proteins was much greater in mouse than in rat and human liver slices, whereas covalent binding was greater in mouse than in rat lung slices. Based on these *in vitro* studies the authors speculate whether the rat might be a better model for studying *Agaricus* toxicity in man than the mouse. γ -Glutamyl transpeptidase activity was greatest in human liver slices and lowest in mouse liver slices. However, no relationship was observed between tissue slice γ -glutamyl transpeptidase activity and [14 C]agaritine metabolite covalent binding. HPLC analysis of media samples from the mouse liver slice culture (which had the greatest rate of covalent binding), revealed agaritine and at least one major and two minor unidentified metabolites. The metabolism of [14 C]agaritine to polar products by mouse liver slices was time dependent.

7.3. Conclusion on toxicokinetics

No agaritine could be found in the gastro-intestinal tract of mice 3 h after administration of the compound by the oral route. When the toxicokinetics of agaritine was studied with radiolabelled compounds, radioactivity was rapidly absorbed and blood levels peaked within a few hours, the time point being dependent on the species investigated. The rate of clearance of the activity from blood was also dependent on the species. No unchanged agaritine was detected in blood.

Agaritine metabolites were excreted in urine and faeces. In the urine, three unidentified metabolites, all without the glutamate moiety, have been detected. Some days after an oral administration of radiolabeled agaritine, a significant residual activity was detected covalently bound to tissue proteins in internal organs such as the liver and the kidneys. *In vitro* studies with liver and lung tissue slices exposed to agaritine confirmed these observations. Since the covalent binding to liver slice proteins was much greater in mouse tissue than in rat and human tissue and the binding to proteins in the later two tissues were of similar magnitude, it has been suggested that rats might be a better experimental model system than the mouse for studying *Agaricus* toxicity in man. Further studies are required to confirm this suggestion.

Other *in vitro* studies, focusing on biotransformation of agaritine, have shown that purified γ -glutamyl transpeptidase, isolated from various tissues of many species, or kidney homogenate from rats and mice (which are rich in this enzyme) converts agaritine to two metabolites, of which the major have been tentatively identified as 4-(hydroxymethyl)phenylhydrazine. The γ -glutamyl transpeptidases used in these studies have very similar properties to the γ -glutamyl transpeptidase occurring in the cultivated mushroom.

The fact that no unchanged agaritine could be detected in the plasma and urine of rats and mice administered this compound orally or by gastric gavage, could indicate that agaritine is chemically or microbiologically degraded in the stomach, or subject to an extensive first-pass effect during its passage through the gastrointestinal tract. As γ -glutamyl transpeptidase activity in the gut is only about 1% of that present in the kidney it is likely that microbial or other intestinal enzymes are involved, or that agaritine is chemically degraded in the gastrointestinal tract. As high γ -glutamyl transpeptidase ac-

tivity is present in the serum, at least in humans, it is also conceivable that the hydrazine is rapidly metabolised in the blood.

8. Effects in short-term tests

8.1. In vitro studies on phenylhydrazine metabolism in the mushroom

Extracts of *Agaricus bisporus* metabolised ^{14}C -agaritine, producing three main unidentified metabolites. The metabolism was strongly stimulated by 2-mercaptoethanol (which restores metabolic activity of the mushroom γ -glutamyl transpeptidase). All three metabolites produced a weak mutagenic response in *Salmonella typhimurium* TA 104. By incubating agaritine with purified hog kidney γ -glutamyl transpeptidase, which converts agaritine to 4-(hydroxymethyl)phenylhydrazine, it could be demonstrated that none of the three metabolites of agaritine produced by the mushroom extract was identical to this compound. Addition of iodoacetic acid (an inhibitor of γ -glutamyl transpeptidase) to the incubation mixture of agaritine and mushroom extract, impaired the metabolism of agaritine to 4-(hydroxymethyl)phenylhydrazine by the mushroom homogenate. There were some indications that one of the three main metabolites could have been a hydrazone formed by reaction of 4-(hydroxymethyl)phenylhydrazine with an organic acid present in the mushroom. This could explain why 4-(hydroxymethyl)phenylhydrazine was not detected among the metabolites, but at present this is only a speculation (Walton et al., 2001).

Since tyrosinase (Seo et al., 2003) occur in *Agaricus bisporus* and this enzyme metabolise agaritine and N'-acetyl-4-(hydroxymethyl)phenylhydrazine *in vitro*, it has been suggested that agaritine to some extent is metabolised by tyrosinase in the mushroom *in vivo* (Walton et al., 1997b, 2001). This suggestion is compatible with recent reports that agaritine inhibits mushroom tyrosinase *in vitro* (Espin et al., 1998). Since commercial tyrosinase has been shown to be contaminated with laccase, the possibility that the metabolism of agaritine and N'-acetyl-4-(hydroxymethyl)phenylhydrazine was catalysed, at least in part, by laccase can not be ruled out (Walton et al., 2001). The metabolites produced by tyrosinase had a weak mutagenic effect in a bacterial test system (Walton et al., 2001).

8.2. Studies on DNA binding

To study the potential for generation of free radicals during food consumption, Goodman et al. (2002) used electron paramagnetic resonance spectroscopy to investigate free radical generation on maceration (simulating mouth chewing) of a range of vegetable products commonly consumed in an uncooked state. The radicals formed as a result of physical damage were trapped in 'spin-trapping' experiments using several different diamagnetic molecules. Various varieties of *Agaricus bisporus* were tested together with the vegetables. Clear signals were observed when the mushroom was macerated in the presence of 4-POBN (α -(pyridyl-1-oxide)-N-t-butyl-nitrone) and PBN (phenyl-t-butyl-nitrone), indicating the formation of large quantities of adducts of the radical from 4-hydroxymethylbenzene diazonium salts. Parallel studies on the vegetables showed

that saliva caused a major reduction in the intensity of the spin-trapped signal, suggesting the presence of radical scavengers in saliva (Goodman et al., 2002).

The 4-(hydroxymethyl)benzenediazonium ion (HMBD) is a highly reactive compound that is believed to be produced from agaritine in the mushroom or when agaritine is metabolised in mammals. It has chemical potential to interact with macromolecules such as DNA.

The first study showing a covalent binding of HMBD to DNA dates back to 1986 (Shepard et al., 1986). In this study tritium-labelled and unlabelled 4-hydroxy[methyl-³H]benzenediazonium ion were synthesised by the investigators and used to expose five female Crl:CD-1 (ICR)BR mice orally to 450-879 mg/kg tritium-labelled HMBD. After an exposure period of 6 h, mice were killed and DNA extracted from homogenated stomach, small intestine, large intestine/appendix and liver tissue. Much higher specific activity was found in stomach and small intestine DNA compared to the activity in large intestine/appendix or liver DNA. This observation might indicate that the compound acts as an activation-independent genotoxic carcinogen producing most DNA damage at the site of application. DNA from the studied tissues was enzymatically digested and nucleotides separated with HPLC. In the stomach 44 % of the eluted radioactivity was due to covalent adducts, whereas the corresponding figure in the small intestine was 16-38 %. This finding was interpreted as a demonstration that HMBD undergoes covalent binding to DNA *in vivo*, which could be the mechanism for 4-(hydroxymethyl)benzenediazonium ion-induced carcinogenicity (Shepard et al., 1986).

Subsequently, Japanese investigators studied *in vitro* the ability of HMBD to modify bases and sugar moieties occurring in DNA (Hiramoto et al., 1995a, 1995b). When the four deoxyribonucleosides were incubated together with HMBD at 37°C, pH 7.4, electron spin resonance spin-trapping techniques revealed the formation of 4-(hydroxymethyl)phenyl radicals and a reduction in the levels of all four nucleosides. The reduction in nucleoside levels was inhibited by ethanol and Cys. Major products of the reaction of deoxyguanosine (dGuo) and deoxyadenosine (dAdo) with HMBD were isolated and their structures established as 8-[4-(hydroxymethyl)phenyl]dGuo (8-HMP-dGuo) and 8-[4-(hydroxymethyl)phenyl]dAdo (8-HMP-dAdo), respectively. The same types of DNA adducts were isolated from calf thymus DNA treated with HMBD. These DNA damages could be demonstrated with ³²P-postlabeling. In addition to these major DNA adducts, other DNA adducts were formed at lower frequencies. These were not characterised further. Malonaldehyde was released from DNA treated with HMBD, which indicate that also the deoxyribose moieties of DNA were damaged. The formation of adducts in DNA was inhibited by ethanol or 2-mercaptoethanol. Taken together these results indicate that the carbon centred 4-(hydroxymethyl)phenyl radical generated from HMBD after removal of a nitrogen molecule can directly modify the base and the sugar moieties of DNA without generating oxygen-derived radicals (Figure 9). The inhibitory effect of ethanol was ascribed its scavenging activity for the carbon-centred radical. The inhibitory effect of Cys and 2-mercaptoethanol was found to be due to the formation of reversible adducts between HMBD and the SH compounds.

When plasmid supercoiled DNA was incubated with HMBD, the supercoiled DNA was converted into a nicked circular relaxed form and subsequently also into a linear form. Sequence analysis indicated that the reactive compound produced from HMBD cleaved

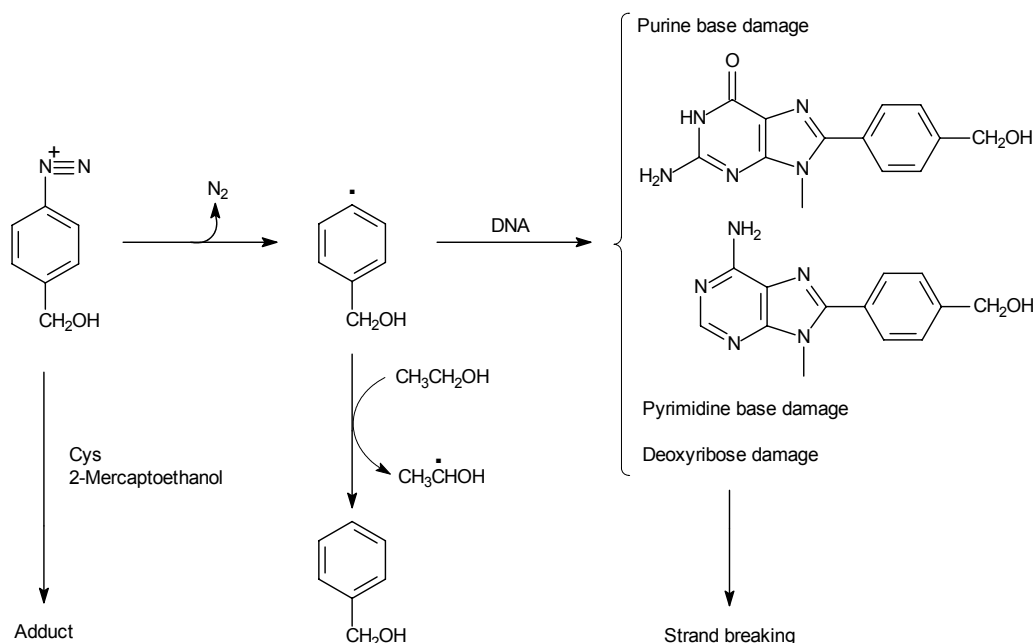


Fig. 9. Mechanism of 4-(hydroxymethyl)benzenediazonium ion (HMBD)-induced DNA damage.

the plasmid DNA strand non-specifically. HMBD also fragmented the intracellular DNA of *Escherichia coli*, and induced micronuclei in peripheral reticulocytes of mice exposed to this substance (Hiramoto et al., 1995a). These are probably lesions secondary to the damage of bases and sugar moieties in DNA.

Hiramoto and co-workers (1998) have followed up their *in vitro* studies by administering male ICR mice intraperitoneally with HMBD and searching for DNA adducts formed in the liver *in vivo* by the ^{32}P -postlabeling technique. As adducts could be demonstrated 24 h after administration but not 48 h after administration, the investigators suggested that the DNA lesion was repaired to a large extent.

The observations made by the Japanese group and referred to above, have been confirmed by American investigators (Lawson et al., 1995; Gannett et al., 1996). The American investigators also noted that HMBD had other mechanisms of action. The specific adducts formed between HMBD and DNA appears to depend on the structure of the reactive intermediate. Reduction of HMBD first produces an aryl diazenyl radical and, following elimination of nitrogen, aryl radicals. The two radical species, aryl diazenyl radicals and aryl radicals, can react at the C⁸ position of guanine and form C⁸-azo or C⁸-arylguanine adducts, respectively. In addition to the electron spin resonance signal identifying the 4-(hydroxymethyl)phenyl radicals, the American investigators also identified spin trapped hydroxyl radicals, indicating that hydroxyl radicals and aryl radicals are generated simultaneously. Furthermore, the American group early recognised that HMBD also appears to react with NH_2 groups in bases to form triazene derivatives (Gannett et al., 1987). For example, the reaction of HMBD with adenine resulted in a 1:1.8 mixture of triazene adducts where the terminal nitrogen of the 4-(hydroxymethyl)benzenediazonium ion and the 4-(formyl)benzenediazonium ion, re-

spectively, were bound to the N-2 position of adenine. The mechanism of the latter compound being formed is believed to be an oxidation *in situ* (Gannett et al., 1987). Together these observations suggests that either HMBD itself or the 4-(hydroxymethyl)phenyl radical may serve as the ultimate genotoxicant. C⁸-4-(Hydroxymethyl)phenylguanine has also been identified in C50 cells (an immortalised non-differentiated murine keratinocyte cell line) and primary mouse keratinocytes exposed to 10-100 µM HMBD (Lawson et al., 1995). Similar exposures of cultured Chinese hamster V79 cells to HMBD resulted in DNA single-strand breaks and DNA cross-links, which probably are secondary lesions in DNA.

The consequences of C⁸-arylguanine and other DNA adducts being formed are unknown. Gannett and co-workers (1996) have suggested that induction of C⁸-arylguanine may cause a change in conformation about the glycosidic bond from *anti* to *syn*, and therefore disrupt the normal guanine-cytosine base pair and give rise to mutations. In turn, the potential mutagenic lesion may be readily and rapidly repaired. Similarly C⁸-adenine adducts and triazene formation at N⁶ of adenine might be mutagenic lesions. If HMBD is continuously produced from agaritine in the cultivated mushroom or by the internal metabolism in a consumer of *Agaricus bisporus*, any of the lesions formed by the reaction of HMBD and DNA might be genotoxic, and possibly promote the induction of cancer.

Also agaritine and/or its metabolites have been shown to bind covalently to DNA *in vivo*. In these studies 94% pure synthesised ¹⁴C-U-ring-labelled agaritine was gavaged to four male and four female C57BL/6J/zur mice that were sacrificed 24 h later (Shepard and Schlatter, 1998). DNA was isolated from the liver, kidneys and stomach of the treated animals and the radioactivity determined. The exposure to ¹⁴C-ring-labelled agaritine resulted in measurable levels of radioactivity covalently associated with the DNA from all three sources. The highest specific activity was observed in the stomach, the first site of contact between agaritine and the tissues. The binding was substantially less to DNA of the other tissues. As the activity was five times higher in males than in females, the authors conclude that enzymatic processes are involved in either the activation of agaritine or the detoxification of the ultimate genotoxic agent. As DNA-binding is only one step in the complex process of carcinogenesis, it is not surprising that this sex difference did not occur in the mushroom carcinogenicity studies of Toth and co-workers reported in section 9.2.1. These DNA-binding studies, together with earlier studies on *in vivo* mutagenicity in transgenic mice by the same research group, identified agaritine to be a very weak genotoxic agent.

The ability of agaritine to damage the DNA of pancreatic acinar cells have been investigated both after *in vivo* and *in vitro* exposure (Curphey et al., 1987). The damage looked for were primary and secondary lesions detectable with the DNA elution technique of Kohn at 1 hour after exposure to agaritine. The ability of agaritine to induce lesions at other time points after exposure were not investigated. Doses to the experimental animals in the range 0.1-1 mmol/kg, which are doses at which established carcinogens are effective at the selected time point, failed to induce any observable DNA breaks after exposure of rats and hamsters *in vivo* as well as rat and hamster pancreatic tissue *in vitro*. According to the authors, the absence of DNA damage in the pancreases of either hamsters or rats receiving agaritine at moderately high doses suggests that this compound is not likely to be a pancreatic carcinogen.

In addition to the DNA-binding studies of agaritine or agaritine metabolites reported above, Price and co-workers (1996) have detected covalent protein binding after exposure to radiolabelled agaritine (see, section biotransformation).

8.3. Tests in microorganisms

As mentioned above, the ultimate carcinogenic metabolite of agaritine is believed to be the highly reactive and mutagenic 4-(hydroxymethyl)benzene diazonium ion. For this metabolite to be formed, the glutamyl moiety must be removed from the molecule to release the free phenylhydrazine that subsequently, presumably, is oxidised to the diazonium ion, most likely by the cytochrome P450-dependent mixed function oxidases (Ioannides, 1996).

No less than 20 different studies have investigated the mutagenic activity of either extracts of the cultivated mushroom (*Agaricus bisporus*) or of the purified hydrazine derivatives occurring in the mushroom in various strains of *Salmonella typhimurium* in the absence or presence of a metabolising system. Usually, for historical reasons, microsomes from livers of aroclor-treated rats have been used as metabolising systems in these assays, but hepatic activation systems from mice and hamster have also been used with similar results (Papaparaskeva et al., 1991). These hepatic activation systems contain little γ -glutamyl transpeptidase activity to efficiently remove the glutamyl moiety from agaritine.

In all cases, except one, the *Salmonella* strains used in the tests have been designed to detect reverse mutations from histidine auxotrophy (his^-) to histidine prototrophy (his^+). The strains designated TA97, TA98, TA1537, TA1538 and TA2637 are particularly suitable to detect frame-shift mutations, whereas strains TA100 and TA1535 are good at detecting base-pair substitutions. Strains TA102, TA104 and TA1530 detects various types of mutations. Since the end-point is histidine-prototrophy (his^+), tests with all these strains are vulnerable to impurities with histidine in the test solution.

Fifteen out of the 20 mutagenicity studies in *S. typhimurium* have been performed with mushroom extracts (Table 13). Of these 15 studies, twelve have found extracts to have a weak mutagenic activity (approximately corresponding to a doubling of the spontaneous mutation frequency). Three studies were unable to detect any mutagenic activity in *Salmonella* strains TA98 and TA100 (De Flora et al., 1979; Morales et al., 1990b; Uejima et al., 1986). One of the negative studies were performed on canned *Agaricus bisporus* which are known to contain only low levels of agaritine (Morales et al., 1990b).

Of the twelve positive studies in the *Salmonella* assay, five used water extracts and six alcohol extracts of the mushroom. Extracts of *Agaricus bisporus* are expected to contain histidine since around 50 % of the amino acids in the mushroom occurs in free form. Measurements of the amino acid contents of ethanol extracts of fruiting bodies have revealed 5.38 μmol histidine per g fruiting body in one study (Oka et al., 1981), 2.46 μmol histidine per g dry material in another (Abe et al., 1980), and 16 mg histidine per kg fresh weight in a third (Minamide et al., 1980). The amount of free histidine was reduced during storage of mushrooms at room temperature, but slightly increased during storage at 1°C (Minamide et al., 1980). Thus, since mushroom extracts may contain quite high concentrations of histidine it is important to control for this artefact-creating

Table 13. Mutagenicity of extracts of *Agaricus bisporus* in various strains of *Salmonella typhimurium* in the absence or presence of a metabolising system (S9) obtained from the liver of aroclor 1254-treated rats (if not otherwise stated).

Solvent for extract	Strain TA97 -S9 +S9		Strain TA98 -S9 +S9		Strain TA100 -S9 +S9		Strain TA102 -S9 +S9		Strain TA104 -S9 +S9		Strain TA1535 -S9 +S9		Strain TA1537 -S9 +S9		Strain TA1538 -S9 +S9		Strain TA2637 -S9 +S9		Strain TA1530 -S9 +S9		Strain TM677 -S9 +S9		Reference
water (a)				(+)		(+)												(+)					Sterner et al., 1982
water (a, b, c)			-	-	(+)	(+)					(+)	-	-	-	-	-							von Wright et al., 1982
water (a, d)			(+)	(+)	-	(+)																	Morales et al., 1990a
water (a, e)			-	-	-	-																	Morales et al., 1990b
water (f)											+	+	+	+									Toth et al., 1992
water (f, g)											+	+	+	+									
water	-	-	(+)	(+)	(+)	(+)																	Andersson et al., 1998
water (a, h)			-	-																			Kellman and Berstein, 1978
ethanol (a)			-	(+)												-							
acetone (a)			-	-		-							-										
ether (b)			-	-		-																	
DMSO (a, h)			-	+		(+)							+		+								
methanol (a)				-		-																	De Flora et al., 1979
ethanol	(+)	(+)	(+)	(+)	(+)	(+)							(+)	(+)									Pool-Zobel et al., 1990
ethanol (a)	(+)	(+)	-	(+)	(+)	(+)	+	(+)	+	+	-	(+)	-	-	-	-			(+)	(+)			Papaparaskeva et al.,1991
ethanol (a, i)			(+)	(+)					(+)	(+)													
ethanol (a, j, k)									+	+													
ethanol (a)	(+)		(+)		(+)		(+)		+		(+)		-		-								Papaparaskeva-Petrides et al., 1993
ethanol (a, f)			-	-	-	-																	Ueijma et al., 1986
ethanol (l)																					(+)	-	Grüter et al., 1991
ethanol (m)									+	+											(+)	-	Walton et al., 1997
ethanol (a, g, m)									+	+											(+)	-	Walton et al., 1998
ethanol (a, n, m)									+	+													

- = no mutagenic activity; (+) = borderline mutagenic activity - less than doubling in mutation frequency but statistically significant; + = mutagenic activity; (a) no control for histidine; (b) mushrooms stored frozen at -20°C before analysis; (c) metabolising system from phenobarbital-induced mouse liver; (d) metabolising system from phenobarbital-induced rat liver; (e) extract from canned mushrooms; (f) preincubation assay instead of plate incorporation assay; (g) extract from dry-baked mushrooms; (h) boiled; (i) metabolising system from mouse liver; (j) mushrooms with various agaritine contents; (k) metabolising system from aroclor-induced hamster liver; (l) mushrooms purchased on a market were put in plastic bags sealed under vacuum and frozen - subsequently extract was prepared from freeze dried material; (m) metabolising system from homogenised rat kidney; (n) extract from freeze-dried mushrooms

factor when testing such extracts for mutagenic activity. Although higher histidine concentrations are found in water extracts than in alcohol extracts, Grüter and co-workers (1991) showed that the histidine level in ethanol extracts were high enough to more than double the spontaneous frequency of revertant colonies. The same conclusion has been drawn by Andersson et al. (1998) and suggested by Pool-Zobel and co-workers (1990). Of the twelve positive studies eight had not controlled for the histidine content of the extract (Kellman and Berstein, 1978; Sterner et al., 1982; von Wright et al., 1982; Morales et al., 1990a; Papaparaskeva et al., 1991; Papaparaskeva-Petrides et al., 1993; Walton et al., 1997a; Walton et al., 1998), and in four cases the detected mutagenic activity could very well be explained solely as an artefact due to histidine (Kellman et al., 1978; Sterner et al., 1982; von Wright et al., 1982; Morales et al., 1990a). Thus, there are a number of studies with *Salmonella typhimurium* in which the positive response of *Agaricus bisporus* extracts can not easily be explained as artefacts resulting from impurities of histidine in the mushroom extract.

Nearly all studies with mushroom extracts have used freshly prepared extracts, and these have seldom induced mutation frequencies higher than three times the control. Unexpectedly, the study that used the oldest extract (approximately 24 h old) and that accordingly had the highest risk that agaritine could be degraded, was the study that observed the strongest mutagenic activity: extracts from raw mushrooms increased the mutation frequency over the background approximately 19 times in strain TA1535 in the absence of a metabolising system and approximately five times in strain TA1537 (Toth et al., 1992). Even larger increases were observed in the presence of a metabolising cytosolic system from aroclor-induced rat liver.

A mutagenic activity comparable to that of extracts from fresh *A. bisporus* was found in extracts from dry-baked (10 min 225°C) mushrooms (Toth et al., 1992). Amino acid analysis showed the extracts from unbaked mushrooms to contain 0.54 mg histidine per ml, while the equivalent value of the baked mushroom was 0.37. According to the authors, the addition of these amounts of free histidine is insignificant in the Ames assay (Toth et al., 1992).

The observation that extracts from dry-baked (10 min 225°C) mushrooms harbours a mutagenic activity of similar magnitude as extracts from fresh *A. bisporus* has recently been confirmed by Walton et al. (1998). A more prolonged baking at a lower temperature, that is at 100°C for 4 hr, suppressed the mutagenic response of extracts. However, since these investigators evaporated the filtrate extract down to about 10% of its original volume, they not only concentrated the presumed mutagenic activity but also the co-extracted histidine, increasing the likelihood that the observed direct mutagenicity were an artefact due to the presence of histidine (Grüter et al., 1991). Inclusion of an activation system comprising hepatic cytosol from Aroclor 1254-induced rats clearly induced a mutagenic response of extracts from raw as well as dry-baked (10 min at 225°C and 4 hr at 100°C) mushrooms (Walton et al., 1998). Also extracts from freeze-dried mushrooms were reported to contain mutagenic activity but again the observed activity might be an artefact due to the presence of histidine.

Cooking *Agaricus bisporus* before preparation of mushroom extracts reduced the mutagenic activity of extracts more efficiently than dry-baking - boiling a water extract for 10 minutes reduced the volume of the extract by less than 10 % but the mutagenic activity by 50 % (von Wright et al., 1982).

Extracts of course contain a magnitude of different compounds. Their number and their quantity being dependent on the solvent used to prepare the extract. The contribution of agaritine to the mutagenic activity of ethanol extracts from *Agaricus bisporus* has been questioned by some investigators (Papaparaskeva et al., 1991; Papaparaskeva-Petrides et al., 1993). They have observed a lower mutagenic activity of agaritine than of mushroom extracts containing equivalent amounts of this compound. Furthermore, and in contrast to most other investigators, they have obtained a potentiation of the mutagenicity of mushroom extracts in the presence of rat metabolising systems (hepatic cytosolic fractions). However, they found no effect of the metabolising system on the mutagenicity of agaritine. The cytosol-induced increase in mutagenic activity was impaired by dicoumarol and menadione, an excellent substrate and a potent inhibitor, respectively, of the cytosolic enzyme DT-diaphorase. DT-Diaphorase is largely linked with the deactivation of quinones. The most susceptible *Salmonella* strain to the mutagenicity of mushroom extracts in this study was TA104, which is a strain particularly sensitive to reactive oxygen-generating species such as hydroperoxides and quinones. In agreement with the indication that the extract might contain oxidising compounds, the investigators found the mutagenicity of mushroom extracts to be reduced when the incubation mixture was supplemented with superoxide reductase, catalase, reduced glutathione or DMSO. To explain these results the investigators suggested that some of the mushroom phenol quinones are activated to genotoxic species by endogenous tyrosinase during the extraction procedure (Papaparaskeva-Petrides et al., 1993).

One explanation to these deviating observations might be that the mushroom extract contain chitin/chitosan that may chelate metal ions having a hydrogen peroxide (H_2O_2)-generating ability (Chung et al., 1998). If this is the case, a part of the mutagenic activity of mushroom extracts could very well be due to reactive oxygen species.

Morales et al. (1990a) reported a slightly higher mutagenic activity in aqueous extracts prepared from previously frozen mushrooms (maintained at $-20^{\circ}C$ for 3 months) than in extracts prepared from fresh mushroom. This observation may suggest that chemical changes during freezing affect the structure and activity of some of the many compounds present in the mushroom flesh. Since freezing and thawing the cultivated mushroom have been reported to reduce the level of agaritine, also the observation of Morales et al. (1990a) could be interpreted as other compounds than the mushroom phenylhydrazines being responsible for a part of the mutagenic activity of mushroom extracts.

Grüter and co-workers (1991) are the only investigators that have used a *Salmonella* strain (TM677) which detects forward mutations and not reverse mutations to histidine prototrophy, to determine a mutagenic activity in *Agaricus bisporus* extracts. This strain detects mutations to 8-azaguanine resistance and is not sensitive to the presence of histidine in the tested extract. Studies with this strain revealed that extracts of fresh mushrooms contained low but significant mutagenic activity in the absence of a metabolising system. No mutagenic activity was observed in the presence of a metabolising system or in extracts from canned mushrooms.

It should be mentioned that also extracts of *A. silvaticus* and *A. arvensis*, both growing in the Nordic countries and picked by mushroom hunters, have been reported to contain a mutagenic activity (Table 14). Extracts from another popular species among mushroom hunters, *A. campestris*, were void of such an activity. Neither of these studies have

Table 14. Mutagenicity of extracts of *Agaricus* species growing in the nature in various strains of *Salmonella typhimurium* in the absence or presence of a metabolising system (S9) obtained from the liver of aroclor 1254-treated rats (if not otherwise stated).

Solvent for extract	Strain TA97 -S9 +S9		Strain TA98 -S9 +S9		Strain TA100 -S9 +S9		Strain TA102 -S9 +S9		Strain TA104 -S9 +S9		Strain TA1535 -S9 +S9		Strain TA1537 -S9 +S9		Strain TA1538 -S9 +S9		Strain TA2637 -S9 +S9		Strain TA1530 -S9 +S9		Strain TM677 -S9 +S9		Reference
ethanol (a) ethylacetate (a) <i>A. silvaticus</i>				(+) -		+												(+) +					Sterner et al., 1982
ethanol (a) ethylacetate (a) <i>A. arvensis</i>				(+) -		+												- -					Sterner et al., 1982
water (a, b) <i>A. campestris</i>			-	-	-	-																	Morales et al., 1990a

(a) no control for histidine; (b) metabolizing system from phenobarbital-induced rat liver.

controlled for the presence of histidine in the extracts (Sterner et al., 1982; Morales et al., 1990a).

Perhaps the strongest indication that mushroom extracts might contain mutagenic activity comes from studies with purified compounds. When these were tested in the *Salmonella* mutagenicity assay, all hydrazine derivatives that have been shown to occur in *Agaricus bisporus* have been identified as mutagenic in at least one strain of *S. typhimurium* (Table 15). In general, the mutagenic activity of these compounds were not influenced, or at least not increased, by a metabolising system prepared from livers of aroclor 1254-exposed rats. Furthermore, strains TA97 and TA1537 were most sensitive to these compounds, indicating a frameshift activity. 4-(Carboxy)-phenylhydrazine (CPH), a suggested precursor to agaritine, was tested at doses over 1 mg per plate but had the lowest mutagenic activity. Perhaps due to the high concentrations, CPH was very toxic in the absence of a metabolising system. Agaritine (100-2000 µg/plate) was weakly mutagenic in most *Salmonella* strains, whereas the 4-(hydroxymethyl)benzene-diazonium ion (HMBD; 5-135 µg/plate) was the most mutagenic compound of the *Agaricus*-hydrazines tested. When the bacteria were incubated with agaritine in the presence of γ -glutamyl transferase from bovine kidney, a pronounced increase in the mutagenic activity was obtained (Friederich et al., 1986; Walton et al., 1997a). A doubling in the mutation frequency induced by agaritine was also obtained in the presence of a kidney homogenate (Walton et al., 1997a). The increased mutagenic efficiency of agaritine in the presence of γ -glutamyl transferase (purified or in a kidney homogenate) could possibly be a result of a continuous production of HMBD when the glutamyl moiety is enzymatically removed from agaritine. Incorporation of an incubation system consisting of rat hepatic microsomes fortified with an NADPH-generating system further potentiated the mutagenic response of agaritine elicited in the presence of rat kidney homogenate (Walton et al., 1997a). The liver homogenate had no effect in the absence of the kidney homogenate, presumably because it is devoid of significant γ -glutamyl transpeptidase activity.

These observations after co-incubation of *Salmonella* bacteria with agaritine and γ -glutamyl transferase contradict observations made in studies where bacteria were incubated with mushroom extracts and either γ -glutamyl transferase or a kidney homogenate containing this enzyme activity. In these later studies neither γ -glutamyl transferase, nor a kidney homogenate had any influence on the mutagenic activity of mushroom extracts (Pool-Zobel et al., 1990; Papaparaskева et al., 1991; Walton et al., 1997a).

An increased mutagenic activity of agaritine in *S. typhimurium* (TA1537) was not only observed in the presence of γ -glutamyl transferase, but also after incubating the agaritine-exposed bacteria at 25°C for 16 h in the presence of alkaline pH (pH 8.2 and 9.0), as compared to the directly determined agaritine mutagenicity at neutral and slightly acidic pH - pH 5 and 7 (Friedrich et al., 1986).

Besides agaritine, a weak mutagenic activity was also induced by the unstable 4-(hydroxymethyl)phenylhydrazine (HMPD), which have not yet been identified in *Agaricus bisporus*, and its synthetically stabilised form N'-acetyl-4-(hydroxymethyl)-phenylhydrazine (AHMPH; 100-1000 µg/plate), Table 10 (Friederich et al., 1986; Rogan et al., 1982).

Table 15. Comparative mutagenicity of the hydrazine derivatives occurring in *Agaricus bisporus* in various strains of *Salmonella typhimurium* in the absence or presence of a metabolising system (S9) obtained from the liver of aroclor 1254-treated rats.

Tested hydrazine derivatives were β -N-(γ -L(+)-glutamyl)-4-hydroxymethylphenylhydrazine (agaritine), 4-(carboxy)phenylhydrazine (CPH), 4-(hydroxymethyl)phenylhydrazine (HMPH), N'-acetyl-4-(hydroxymethyl)phenylhydrazine (AHMPH) and 4-(hydroxymethyl)benzenediazonium ion (HMBD).

Compound tested	Strain TA97 -S9 +S9	Strain TA98 -S9 +S9	Strain TA100 -S9 +S9	Strain TA102 -S9 +S9	Strain TA104 -S9 +S9	Strain TA1535 -S9 +S9	Strain TA1537 -S9 +S9	Strain TA1538 -S9 +S9	Strain TA2637 -S9 +S9	Type of assay	Reference
Agaritine (a)		-	-	(+)	(+)					spot test	De Flora et al., 1979
Agaritine (a)						(+)	(+)	+	(+)	preincubation	Rogan et al., 1982
Agaritine (a)	(+)	-	-		(+)	(+)	(+)	-		plate incorporation	Papaparaskeva-Petrides et al., 1993
Agaritine (b)		-	-	-	-				(+)	plate incorporation	Sterner et al., 1982
Agaritine (c)		(+)	(+)	(+)	(+)					preincubation	Ueijma et al., 1986
Agaritine (d)	+	-	-	-		-	(+)	-		preincubation	Friederich et al., 1986
Agaritine (i)					(+)					plate incorporation	Walton et al., 1997b
Agaritine (d) + γ -GT	+	(+)	+	+		-	+	+		preincubation	Friederich et al., 1986
CPH								-	-	preincubation	Malca-Mor and Stark, 1982
CPH	+	-	-	-		-	-	-		preincubation	Friederich et al., 1986
CPH	+*	+	-*	(+)	-*	(+)				plate incorporation	Andersson et al., 1998
CPH					(+)					plate incorporation	Walton et al., 1997b
HMPH (e)		-	(+)				+	(+)		preincubation	Friederich et al., 1986
AHMPH (f)						-	-	+	-	preincubation	Rogan et al., 1982
AHMPH (j)					(+)					plate incorporation	Walton et al., 1997b
HMBD (g)						(+)*	+			preincubation	Rogan et al., 1982
HMBD (h)	+	+	+	+		-	+	+		preincubation	Friederich et al., 1986
HMBD				+						plate incorporation	Lawson et al., 1995
HMBD (i)					+					plate incorporation	Walton et al., 1997b

- = no mutagenic activity; (+) = borderline mutagenic activity - less than doubling in mutation frequency but statistically significant; + = mutagenic activity (a) agaritine synthesised by Toth and colleagues (>98 % pure); (b) agaritine purified from mushrooms; (c) origin of agaritine unknown; (d) isolated from *A. bisporus*, 76.5 % pure; (e) gift from Hoechst; (f) AHMPH synthesised by Toth and colleagues (>98 % pure); (g) HMBD synthesised by Toth and colleagues (>98 % pure); (h) synthesized; (i) agaritine synthesised by Walton and colleagues (91 % pure); (j) AHMPH synthesised by Walton and colleagues (97 % pure); * pronounced cytotoxicity

Urine collected from rats and mice during a single dose balance study with agaritine, and which contained agaritine metabolites, was assayed for mutagenicity in the Ames test using *Salmonella typhimurium* strains TA98, TA100 and TA104, with and without metabolic activation. No increase in mutation frequency above background was detected in any sample (Walker et al., 1997).

Only one short-term study have used other bacteria than *Salmonella*, and this study was negative. In the bioluminescence test 4-(carboxy)phenylhydrazine was unable to induce any reverse mutations of *Photobacterium leiognathi* SD18 from dark variant to luminescent state (Levi et al., 1986).

It should be stressed that the observations reported in chapter 3 on differential stability of agaritine solutions under various conditions of storage may have a bearing on the interpretation of the mutagenicity data of agaritine reported above, and possibly also on the interpretation of the data obtained with mushroom extracts. Generally, investigators have not stated under which conditions and for how long agaritine solutions and mushroom-extracts have been stored. Thus, it is possible that in some studies the concentration of agaritine present during treatment, if present at all, is substantially less than the concentrations reported.

8.4. Tests in cultured mammalian cells

Cancer cells often become resistant to cytostatic drugs. One mechanism whereby resistance may develop is through amplification of genes important for cell proliferation. When tested at concentrations up to 25 µl per incubation mixture, ethanol- and ethylacetate-extracts of *A. bisporus* did not induce adeno-associated virus amplification in Syrian hamster embryo cells *in vitro* (Pool-Zobel et al., 1990). At higher concentrations the solvent (a 1:1 mixture of ethanol and DMSO) were toxic to the cells.

Similarly, no DNA single-strand breaks were observed in rat hepatocytes exposed for 1 h *in vitro* to extracts (ethanol/ethylacetate) derived from cooked or raw *A. bisporus* (Pool-Zobel et al., 1990). Neither was any genotoxic effect detected in cells cultured in the presence of mushroom extracts for longer periods of time (8-24 h).

In an investigation reported only in the form of an unpublished abstract, test substances in the form of fresh or canned *A. bisporus* were tested for their ability to induce gene mutations (6-thioguanine resistance) and sister chromatid exchanges in Chinese hamster ovary cells (Santa Maria and Caballo, 1989). No mutations were induced by canned mushrooms. Fresh mushrooms were not tested in this assay. Both fresh and canned mushrooms stimulated the induction of sister chromatid exchanges. However, it is questionable whether the increase in sister chromatid exchange frequency observed in this study is of any biological significance. Furthermore, canned mushrooms were more efficient than fresh mushrooms to induce sister chromatid exchanges.

In a study exploring the possibility to use cell culture techniques to determine toxicity and antitoxicity of test compounds, water- and methanol-extracts of *A. bisporus* were found to be moderately or weakly active in three assays (inhibition of protein synthesis, MMT conversion to formazan and Kenacid blue staining), and without effect in a fourth (release of lactate dehydrogenase into the culture media) (Phillips, 1996).

Very few short-term studies with pure phenylhydrazines or the 4-(hydroxymethyl)-benzenediazonium ion have been performed in cultured mammalian cells. In one study,

the 4-(hydroxymethyl)benzenediazonium ion was found to induce gene mutations (thioguanine resistance) in V79 cells (a lung fibroblast cell line derived from the Chinese hamster) in a concentration-dependent manner (the concentrations varied between 10 and 100 μ M) (Lawson et al., 1995). In another study, only reported in the form of a short abstract, agaritine, 4-(hydroxymethyl)benzenediazonium tetrafluoroborate and N'-acetyl-4-(hydroxymethyl)phenylhydrazine did not induce any mutations to ouabain resistance in V79 cells in the absence or presence of metabolically competent liver cells (Kuszynski et al., 1981). Since the concentrations of the various hydrazine derivatives used in these experiments were not stated, it is possible that the contradictory results obtained with the diazonium ion in the studies of Lawson et al. (1995) and Kuszynski et al. (1981) either is due to the slightly different experimental conditions of the two studies or to different concentrations of the diazonium ion being used by the two groups of investigators.

The hepatocyte/DNA repair test, which measures unscheduled DNA synthesis, is known to be sensitive to various classes of DNA-reactive carcinogens, and is regarded as a suitable short-term test for *in vitro* detection of chemical carcinogens. The test combines intact cell metabolism with the broadest capability for xenobiotic biotransformation (isolated primary hepatocytes), together with a biochemical endpoint for genotoxicity. Whereas agaritine (10-1000 μ M) neither elicited unscheduled DNA synthesis in rat hepatocytes nor in mouse hepatocytes, N'-acetyl-4-(hydroxymethyl)phenylhydrazine was positive in both types of hepatocytes (Mori et al., 1988). However, the magnitude of the positive response was low compared to that of other types of carcinogenic chemicals.

8.5. In vivo tests

Recently, Big Blue transgenic mice were used to study the mutagenic activity of *A. bisporus in vivo* (Shepard et al., 1995). These mice (a strain derived from C57Bl6 mice) bear the bacterial lacI gene, incorporated into a lambda bacteriophage shuttle vector, stably inserted into the DNA of every cell. Following dietary exposure to fresh mushrooms, dried mushrooms and mushroom extracts enriched in agaritine (30 %, w/w), the shuttle vector was specifically isolated from genomic DNA of forestomach, liver, lung, kidney and glandular stomach tissue, and the marker gene expressed in *E. coli* bacteria. Material from forestomach, liver and lung were selected for analysis because they were target organs in the fresh mushroom carcinogenicity study (Toth and Erickson, 1986). Kidney was examined because the tissue level of γ -glutamyl transferase, the first enzyme in the postulated bioactivation pathway of agaritine, is highest in this organ. The glandular stomach was examined in some studies for comparison with forestomach results. Mutations in the lacI gene of the five tissues were detected with a colour test. On the basis of previous work with other compounds correlating carcinogenic potency with results of the lacI in the transgenic mouse, a 15-week exposure period was predicted to produce a significant increase in mutation frequency in target organs, if the tumours observed in Toth's study (Toth and Erickson, 1986) were indeed due to a genotoxic substance(s).

An obvious weakness of the study was the low number of animals used, only 2 mice per treatment. Dietary administration of crude agaritine (75 % pure) in the feed, resulting in an exposure of 120 mg/kg body weight/day, increased the mutation frequency by

100 % in the kidney and by 50 % in the forestomach. The agaritine-feed did not influence the mutation frequency in the liver and lungs, whereas a significant decrease in mutation was observed in the glandular stomach. The possible antimutagenic effect of agaritine in the glandular stomach was not pursued further. Mice receiving fresh or dried mushrooms in the diet (30 and 80 mg agaritine/kg body weight/day, respectively) showed no increase in mutant frequency in any of the target organs.

It is not easy to compare the carcinogenic potency of different carcinogens in experimental systems. One method used in studies on transgenic rodents is based on the TD₅₀ doses (the daily dose rate that produced a 50 % increase in tumours over controls after lifelong exposure). When the increased mutation frequency induced by feeding transgenic mice 120 mg agaritine/kg body weight/day were compared with the mutation frequency induced by TD₅₀ doses of various organ-specific carcinogens in mice, the TD₅₀ for agaritine was estimated to approximately 250 mg/kg body weight/day, putting agaritine into the class of weak genotoxic carcinogens (Shepard et al., 1995). It should be emphasised that doses above 100 mg agaritine/kg body weight/day were precluded in a 2-year feeding study due to toxicity (Toth et al., 1981a), see section 9.2.2.1. The improvement in sensitivity offered by the transgenic methods in comparison with traditional carcinogenicity studies lies in the fact that a gene, rather than the animal itself, is the unit of observation, and that mutations in the target gene can accumulate over the whole treatment period (Shepard et al., 1995). However, a gene mutation is not equivalent to the induction of a tumour.

Pool-Zobel and co-workers (1990) have tested mushroom extracts in two other *in vivo* assays. In one of these they investigated the induction of DNA single-strand breaks in liver cells of male Sprague Dawley rats at 1h after an oral exposure to ethanol extracts of fresh *A. bisporus* purchased at the local market. This endpoint could be viewed as a secondary effect to primary DNA damage. No single-strand DNA breaks were detected in this experiment. In the other study male C57BL/JC mice (5 animals per group) were dosed with the same type of extracts (corresponding to 13.25 g mushroom/kg body weight) and killed 24 hours later. The bone marrow was prepared and the incidence of micronucleated polychromatic erythrocytes determined. The extract had no influence on the frequency of micronuclei in bone marrow cells of. The latter experiment had two weaknesses which makes it difficult to interpret the results. Firstly, the extract was an ethanol solution, whereas the control solution was distilled water. Secondly, only one (and a very early) fixation time was used, which increases the likelihood of missing a potential effect.

However, in agreement with the German study, investigators at the National Food Administration i Sweden found water extracts of *A. bisporus* (corresponding to approximately 14.5 mg agaritine/kg body weight) intraperitoneally injected into 6 weeks old NMRI male mice not to induce micronuclei in polychromatic bone marrow erythrocytes (Andersson et al., 1998). In the same study 200 mg/kg body weight 4-(carboxy)phenylhydrazine, dissolved in DMSO, had no influence on the frequency of micronucleated polychromatic erythrocytes, although there was an indication of an increased micronucleus incidence in this experiment. It was concluded that the potential of 4-(carboxy)phenylhydrazine to induce micronuclei *in vivo* should be investigated further.

Japanese investigators have studied the influence of the antioxidant status of the mouse feed on the efficiency of micronuclei induction in peripheral reticulocytes of male ICR

mice intraperitoneally injected with the 4-(hydroxymethyl)benzene-diazonium ion, HMBD (Kato et al., 1995). The antioxidant status of the feed was regulated by its vitamin E content. The vitamin E level in the blood of mice fed vitamin E-deficient diet for a week was significantly lower than that in mice fed a diet adequate in vitamin E. Independently on the type of mouse feed given to the animals (10 animals per group), a single administration of 90 mg HMBD/kg body weight resulted in a significant induction of micronuclei in peripheral reticulocytes, particularly at 48 h after injection of the test-compound (Kato et al., 1995; Hiramoto et al., 1995a). At this HMBD concentration, feeding a vitamin E deficient diet resulted in a significantly ($p < 0.05$) enhanced micronucleus frequency as compared to the frequencies observed in animals fed vitamin E adequate diet. These studies indicate that the HMBD ion gives rise to chromosome damage and that vitamin E deficiency leads to a reduction in protective ability against chromosome damage.

8.6. Antimutagenic effects of *A. bisporus*

Agaricus bisporus does not only contain mutagens, but also antimutagens. For example, there is one report claiming that ethanol extracts of *A. bisporus* and *A. abruptibulvus* inhibits the mutagenesis of 2-nitrofluorene in strain TM677 of *Salmonella typhimurium* (Grüter et al., 1990). To what extent these antimutagenic compounds in mushroom extracts masks an effect of mutagenic compounds is not known.

8.7. Conclusions on effects in short-term tests

The 4-(hydroxymethyl)benzenediazonium ion (HMBD) is a reactive compound believed to be produced from agaritine in the mushroom or when agaritine is metabolised by γ -glutamyl transpeptidase in mammals. The HMBD is metabolically converted to two types of radical species, aryl diazenyl radicals and aryl radicals, that may react with carbon atoms of deoxyribose units in DNA, and nitrogen atoms and the C-8 position of purines to create specific types of DNA damage (see, Figure 9). DNA damage has been observed in various tissues of mice orally exposed to HMBD. Also *in vitro* studies have revealed that HMBD may induce DNA damage in deoxyribonucleotides, isolated calf thymus DNA, and cultured mammalian cells.

Also agaritine and/or its metabolites have been shown to bind covalently to liver, kidneys and stomach DNA of mice exposed *in vivo*. The binding was five times higher in males than in females. The highest specific activity was observed in the stomach, the first site of contact between agaritine and the tissues.

In vitro studies have been performed with the bacterium *Salmonella typhimurium* and with cultured mammalian cells. The bacterial tests (Ames assay) have revealed a weak mutagenic activity of water- and alcohol-extracts of *A. bisporus*. Available information indicate that the mutagenic activity could be due to the phenylhydrazines (4-(carboxy)phenylhydrazine, agaritine, 4-(hydroxymethyl)phenylhydrazine) and the 4-(hydroxymethyl)benzenediazonium ion, and/or to other hitherto unidentified compounds occurring in the mushroom. The latter compounds could possibly be quinone derivatives or reactive oxygen species.

The interpretation that mushroom hydrazines may partly be responsible for the genotoxic effect of mushroom extracts is strengthened by the observation that purified

mushroom hydrazines, including agaritine and the 4-(hydroxymethyl)benzenediazonium ion, give rise to mutations in the *Salmonella* assay. It should also be noted that the mutagenic activity of agaritine was increased upon removal of the glutamyl moiety by γ -glutamyl transpeptidase.

The few *in vitro* studies that have been performed with cultured mammalian cells are generally of poor quality and can not be used independently to establish whether extracts of *A. bisporus* or the purified mushroom hydrazines are mutagenic.

The *in vivo* studies on mice have produced contradictory results. Whereas extracts of *A. bisporus* had no influence on the frequency of micronuclei in bone marrow cells of male mice in two separate experiments, other *in vivo* studies on mice revealed that agaritine induced gene mutations and the 4-(hydroxymethyl)benzenediazonium ion micronuclei in peripheral reticulocytes. In addition, one study indicated that intraperitoneally injected 4-(carboxy)phenylhydrazine might induce micronuclei in polychromatic bone marrow erythrocytes, but this possibility has to be investigated further.

If HMBD is continuously produced from agaritine in the cultivated mushroom or by the internal metabolism in humans consuming *Agaricus bisporus*, any of the lesions formed by the reaction of HMBD with DNA might be genotoxic, and possibly stimulate cancer induction.

9. Effects on experimental animals

9.1. Acute and subchronic toxicity of mushroom hydrazines and HMBD

The first toxicity study with the phenylhydrazines and the 4-(hydroxymethyl)-benzenediazonium ion (HMBD) occurring in *Agaricus bisporus* dates back to 1924. In these early studies Bodansky compared the severity of anemia and liver damage induced by hydrazine with that induced by various hydrazine derivatives in the dog and the rabbit. Unlike hydrazine and phenylhydrazine, 4-(carboxy)phenylhydrazine had marginal effects on both endpoints. No serious liver damage as determined by histological examination and measurement of blood sugar levels could be detected in a female dog injected twice, two days apart, with 0.3 g 4-(carboxy)phenylhydrazine. Also a subsequent administration of 2.2 g of this compound did not produce any detectable effects (Bodansky, 1924a). The weight of the dog was not given, but the total dose would be around 280 mg/kg body weight for a dog weighing 10 kg. Similarly, four subcutaneous injections with 0.025, 0.1, 0.3 and 1.0 g 4-(carboxy)phenylhydrazine in a rabbit (total dose 790 mg/kg body weight) only resulted in a marginal reduction in red blood cell count and blood hemoglobin level (Bodansky, 1924b). The author suggested that 4-(carboxy)phenylhydrazine is easily detoxified in the animal body.

When it some years later was observed that compounds with hydrazine groups may hinder the formation of tuberculous lesions, Minami (1933) studied the effect of exposing experimental animals to 4-(carboxy)phenylhydrazine and more than ten other hydrazine derivatives regarding the degree of anemia produced by the treatment and what influence it had on the oxygen levels in blood. In these studies male guinea pigs (350 g) were subcutaneously injected every second day, for two weeks, with aqueous solutions of 4-(carboxy)phenylhydrazine, the cumulative dose for the two dose groups being around 410 and 820 mg per animal, respectively. The body weight of animals injected with both doses increased during the experiment, but the magnitude of the increase was higher for those that received the lower dose. No toxicity was registered by visual inspection. No splenic enlargement was found at biopsy, neither was anemia found in other organs, although such autopsy findings were recorded in animals exposed to other hydrazine derivatives. The exposure had no influence on the number of blood corpuscles, red blood cells and amount of hemoglobin. No control animals were used in this study. Instead, the values registered in the animals before the beginning of the treatment was used as control values. The conclusion was that 4-(carboxy)phenylhydrazine neither induced anemia, nor any other observable toxicity in male guinea pigs. When it comes to predicting the risk for humans consuming *A. bisporus*, the relevance of these toxicity studies that administered the test compounds by subcutaneous injection is unknown.

However, toxicity studies (28 days long) have also been performed in connection with carcinogenicity studies, mainly to determine the maximum tolerated dose of a chemical, which could then be given for life. In these tests, four parameters for toxicity; survival

rates, body weights, chemical consumption levels (in drinking water or feed), and histological changes, were used to assess toxicity (Toth et al., 1975). Table 16 summarises the toxicity data obtained in such dose-setting experiments. In the studies with subcutaneously injected agaritine (isolated from *A. bisporus*) the highest doses were 200 and 400 mg/kg body weight. Mice treated with these doses clinically showed excitation, uncoordinated runs, convulsions, paralysed lower backs and ultimately died. Histopathological findings included vacuolated cells in the liver and kidney tubules, edema and desquamation of bladder epithelium and hyperemia and haemorrhages of the lungs (Toth and Sornson, 1984). The highest dose of N²-[γ-L(+)-glutamyl]-4-carboxyphenylhydrazine tested in the dose-setting experiment, 2 400 mg/kg body weight, was non-toxic in Swiss mice when given as a single intragastric instillation. A toxic dose level can, therefore, not be given for this compound. In addition to the observations from dose-setting experiments reported for Swiss mice in Table 16, Pilegaard and co-workers (1997) have found 1 550 mg 4-(carboxy)phenylhydrazine/kg drinking water to be toxic in A/J mice (a mouse strain easily developing lung tumours).

Table 16. Toxic doses of various phenylhydrazines and the 4-(hydroxymethyl)-benzenediazonium ions occurring in *A. bisporus*, as determined by a set of criteria in Swiss albino mice (Toth et al., 1975).

Compound	Administration	Toxic dose level	Reference
4-(carboxy)phenylhydrazine	In drinking water for 35 days	2.5 g/l	McManus et al., 1987
Agaritine	A single intragastric instillation	625 mg/l; single administration	Toth et al., 1981a
Agaritine	Subcutaneous injection	200 mg/g body weight for females; 100 mg/g body weight for males; 28 days	Toth and Sornson, 1984
4-(hydroxymethyl)-benzenediazonium ion, sulfate form	A single subcutaneous injection	100 µg/g body weight	Toth, 1987
4-(hydroxymethyl)-benzenediazonium ion, tetrafluoroborate form	A single subcutaneous injection	50 µg/g body weight	Toth et al, 1981b
4-(hydroxymethyl)-benzenediazonium ion, tetrafluoroborate form	A single intragastric instillation	450 µg/g body weight	Toth et al, 1982

9.2. Long-term carcinogenicity studies.

The observation that chemotherapeutic agents containing a nitrogen-nitrogen bond induce tumours in experimental animals, has stimulated a systematic search for tumourigenic hydrazine derivatives. This search did not focus only on the synthetic hydrazine derivatives used in industry, agriculture, and medicine, but also included hydrazine derivatives occurring naturally in tobacco, "edible" mushrooms and bay leaves. Today alkyl-, aryl-, and acyl-substituted hydrazines have been identified as tumour-inducing substances in many different types of tissue in mice, hamsters and rats.

In the middle of the seventies Toth and his co-workers at the Eppley Institute for Research on Cancer, University of Nebraska Medical Center, initiated a concentrated effort to reveal the possible significance of hydrazines in the causation of dietary cancer (Toth, 1975). Toth and his co-workers have concentrated on the substances present in the False Morel (*Gyromitra esculenta*) and the cultivated mushroom (*Agaricus bisporus*).

The compounds of interest in *Agaricus bisporus* are agaritine, its presumed precursors 4-(carboxy)phenylhydrazine and β -N-(γ -L(+)-glutamyl)4-(carboxy)phenylhydrazine, as well as the presumed degradation products 4-(hydroxymethyl)phenylhydrazine and the 4-(hydroxymethyl)benzenediazonium ion.

Table 17A and 17B shows the experimental results obtained when these compounds, and the fresh and dry-baked cultivated mushroom have been tested for their ability to induce tumours in mice: the tumour types that were found in statistically increased frequencies (indicated by *, ** or ***) or that were most common are given, as well as the number of tumour-bearing animals and the tumour incidences. In many cases one animal carried more than one tumour, but the multiplicity of tumours is not given in the table. If an animal were found to have both benign and malignant tumours, it is only counted in the group of animals bearing malignant tumours. The malignant tumours are identified by italics in Fig. 17A and 17B. In addition to the 13 published long-term studies on mice (all of which are presented in Table 17A and 17B), two rather poor studies with oral administration of cooked and dried cultivated mushroom in rats have been published.

In the standard design of the carcinogenicity studies performed by Toth and co-workers at the Eppley Institute for Research on Cancer, the tested compound was dissolved in the drinking water at concentrations that had been determined from toxicity studies performed prior to the chronic experiment. The solution was prepared trice weekly and protected from light, and the total consumption of drinking water measured at the same intervals during the treatment period. Each concentration tested was given for the life-span of 50 males and 50 females randomly bred Swiss albino mice that were 6 weeks old at the beginning of the experiment. Thus, in contrast to many other long-term carcinogenicity studies, the animals were not killed after a definite treatment period or at a definite percentage of survivors in the experiment. If not otherwise stated, the control group was made up of the same number of males and females as the exposed groups, and the animals were of the same age at the beginning of the experiment as the animals in the exposed groups. However, in a substantial number of experiments the control group was not started at the same time as the treated groups. A control group with a particular route of administration and a fixed number of animals were used as control group for all long-term studies that were started with the same type of dosing regimen

as long as at least 50% of the original number of animals in the control group was still living. When 50% of the animals in the control group had died a new control group was started and all studies that were started from this point in time would have the new group as the control group. As a consequence, the time span between starting the control group and the treated groups could be as long as around 1.5 years (Gry and Andersson, 1998).

The treated and the control animals were carefully checked and weighed at weekly intervals, and gross pathologic changes recorded. The animals were either allowed to live until they died by themselves, or were killed with ether when found in poor condition. Complete necropsy and histological examinations were performed on all animals. Statistical analysis on tumour induction was carried out by using Fisher's exact test for 2x2 tables (Armitage, 1971) on the sum of animals with benign and malignant tumours. In the tables, the symbols *, **, and ***, represents the statistical significance, e.g. the probability that chance alone had produced a difference in tumour incidence between treated and control groups larger than 0.05, 0.01 and 0.001, respectively. Usually, no statistical analysis was carried out on animals with specific benign (normal letters in Table 17A and 17B) and malignant (italics in Table 17A and 17B) tumours.

Though the essential information is included in Tables 17A and 17B, the following text will summarise the findings in the separate experiments for the interested reader. The general experimental design in studies by Toth and co-workers will not be reported again when the respective studies are discussed. The reader is referred to the original paper to get information on the histological characterisation of the neoplastic alterations.

9.2.1. Studies on the cultivated mushroom (*Agaricus bisporus*).

In 1986, Toth and Erickson published the results of a study in which groups of 50 female and 50 male Swiss albino mice were fed uncooked *Agaricus bisporus ad libitum* for 3 days, followed by semisynthetic diet for 4 days each week for their life span. Control animals were fed the semisynthetic diet. The study lasted until all mice had died or had been killed with ether when found in poor condition; that is, between 150 and 160 weeks after the start of the experiment.

The estimated average daily mushroom consumption was 11 g per mouse for both sexes, which seems rather high. The authors emphasised that the figure takes into account the fact that, on the average, 48% of the fresh mushroom is dehydrated in 24 hours at room temperature. Since no specific information was given, this intake presumably was an average over the entire experimental period. The mushrooms used were purchased twice weekly locally. There was no indication that the contents of agaritine or other phenylhydrazine derivatives were measured in the mushrooms during the study.

The treatment had no substantial effect on the survival rate as compared with that of the untreated controls. However, the average weekly weight curves of the animals given mushroom supplementation were substantially lower throughout the study than that of the untreated animals. The dose of *A. bisporus* given to the animals in the chronic experiment had been selected from an initial toxicity study in which mice were fed the mushroom *ad libitum* for 4.5, 4, 3.5, 3 and 2.5 days each week followed by semisynthetic diet for the rest of the week for a total of 70 days. The feeding did not result in a weight gain for all dose groups in this toxicity study. Because the body weight of mice

treated with *A. bisporus* for 3 days increased steadily, with the exception of an initial short decrease, this dose regimen was selected for the chronic experiment.

As shown in Table 17A, feeding of uncooked mushrooms to mice induced a statistically significant increase in the total number of tumours of bone and forestomach in both sexes and of lung tumours in the males (Toth and Erickson, 1986). An increase in tumours of the liver was suggestive but not statistically significant ($p < 0.07$ for the females and $p < 0.06$ for the males).

Recently, Toth and co-workers have presented data from two carcinogenicity studies with dry-baked cultivated mushrooms. The *A. bisporus* mushroom is mainly eaten in baked form (pizza) in the United States. The baking was done at 220-230°C for 10 minutes. In one of the studies the baked *A. bisporus* mushroom was fed to Swiss mice (50 females, 50 males) for 3 days followed by a semisynthetic diet for 4 days each week for life. The mushroom consumption was 14.7 g per day per female and 13.0 g per day per male when the age of the mice were 57-60 weeks. The same number of animals in the untreated control group were fed the semisynthetic diet.

Though the survival were good in both groups (information on weight gain of animals missing), mice that had received the interrupted lifelong oral administration of baked *A. bisporus* feed developed statistically increased incidences of tumours in the forestomach, glandular stomach, duodenum and ovary (Table 17A). In the females, the incidences were 20, 12, 14 and 12 %, while in males they were 16, 20 and 4% in the relevant tissues (the last two non-significant). In the untreated control only an ovarian tumour (incidence 2%) was detected (Toth et al., 1997a).

In another experiment with baked *A. bisporus* mice were fed the mushroom for 12 hours each day for 5 days each week for life (Toth et al., 1997b). After each feeding cycle, the animals receive a well-balanced semisynthetic diet for 12 hours each day for 5 days, plus the remaining 2 days each week. Consumption data were generated during one month when the mice were 57-60 weeks old. The females consumed 4.8 g baked *A. bisporus* per day and the males 4.2 g per day. In this experiment with baked *A. bisporus*, as well as in the experiment reported above (Toth et al., 1997a), treatment did not influence the survival negatively. Information on weight gain of the animals is missing. The tumour incidences in lungs, blood vessels, cecum, and colon were increased in the group fed the baked mushroom as compared to the incidence in the untreated control group. However, none of the increases were statistically significant (Table 17A).

Toth and co-workers (1998) have reported that Swiss mice fed for life with a semisynthetic diet mixed with 10%, 5% or 2.5% lyophilised *A. bisporus* powder had similar survival rates as animals fed a semisynthetic diet non-substituted with lyophilised mushrooms. However, no information on weight gain of the animals was given. The mice given the mushroom-containing feed developed tumours in the lungs, forestomach, glandular stomach, and ovaries in certain exposure groups. Some of the tumour incidences in mushroom-fed mice were found to be significantly increased (10%-dose: females-ovaries 22%, males-lungs 60%; 5%-dose: females-glandular stomach 12% and ovaries 8%; 2.5%-dose: males and females-forestomach 10% and 10%, respectively), although no dose-response relationship was established (Table 17A). According to some estimates, less than 10% of the *A. bisporus* is consumed in lyophilised form in the United States.

Table 17A. Tumourigenicity of the cultivated mushroom (*Agaricus bisporus*) in randomly bred Swiss albino mice.

Compound tested	Route of administration	Dose	Exposure	Effect of treatment					Reference
				treated animals males (50/group)	females (50/group)		control animals males (100/group)	females (100/group)	
<i>Agaricus bisporus</i> uncooked	p.o.	3 days per week	lifelong	31 (62%)** 18 13 6 (12%) 6 14 (28%)** 11 3 8 (16%)** 5 3	20 (40%) 9 11 4 (8%) 4 19 (38%)* 16 3 8 (16%)** 6 2	lung adenomas adenocarcinomas liver benign hepatomas forestomach squamous cell papillomas squamous cell carcinomas bone osteomas osteosarcomas	17 (38%) 8 6 9 7 1 (2%) 0 (0%) 1 2 (4%) 0 0 0 (0%) 0 0	13 (26%) 6 7 0 (0%) 0 0 (0%) 0 0 0 (0%) 0 0	Toth and Erickson, 1986
<i>Agaricus bisporus</i> dry-baked	p.o.	3 days per week	lifelong	8 (16%)* 7 1 0 10 (20%)* 0 10 2 (4%) 2	10 (20%)* 9 0 1 6 (12%)* 1 5 7 (14%)* 7 6 (12%)* 5 1	forestomach squamous cell papillomas leiomyocarcomas squamous cell carcinomas glandular stomach polypoid adenomas adenocarcinomas duodenum adenocarcinomas ovaries adenomas adenocarcinomas	0 (0%) 0 0 0 0 (0%) 0 0 0 (0%) 0 0 0 0 1 (2%) 0 1	0 (0%) 0 0 0 0 (0%) 0 0 0 (0%) 0 0 0 0 1 (2%) 0 1	Toth et al., 1997a

Table 17A cont. Tumourigenicity of the cultivated mushroom (*Agaricus bisporus*) in randomly bred Swiss albino mice.

Compound tested	Route of administration	Dose	Exposure	Effect of treatment					Reference
				treated animals		control animals			
				males (50/group)	females (50/group)	males (100/group)	females (100/group)		
<i>Agaricus bisporus</i> lyophilized	p.o.	10% of feed	lifelong	30 (60%)*	13 (26%)	lung	19 (38%)	14 (28%)	Toth et al., 1998
				20	12	adenomas	11	9	
				10	1	adenocarcinomas	8	5	
				3 (6%)	0 (0%)	forestomach	0 (0%)	0 (0%)	
				2	0	squamous cell papillomas	0	0	
				1	0	squamous cell carcinomas	0	0	
		5% of feed	lifelong	1 (2%)	1 (2%)	glandular stomach	0 (0%)	0 (0%)	
				1	1	adenocarcinomas	0	0	
					11 (22%)**	ovaries		1 (2%)	
					3	adenomas		0	
					8	adenocarcinomas		1	
				25 (50%)*	19 (38%)	lung	19 (38%)	14 (28%)	
	p.o.	2.5% in feed	lifelong	21	13	adenomas	11	9	
				4	6	adenocarcinomas	8	5	
				2 (4%)	1 (2%)	forestomach	0 (0%)	0 (0%)	
				2	1	squamous cell carcinomas	0	0	
				3 (6%)	6 (12%)*	glandular stomach	0 (0%)	0 (0%)	
				1	1	adenomas	0	0	
				2	5	adenocarcinomas	0	0	
					4 (8%)	ovaries		1 (2%)	
					2	adenomas		0	
					2	adenocarcinomas		1	
				23 (46%)*	17 (34%)	lung	19 (38%)	14 (28%)	
				16	12	adenomas	11	9	
				7	5	adenocarcinomas	8	5	
				5 (10%)*	5 (10%)*	forestomach	0 (0%)	0 (0%)	
				3	5	squamous cell papillomas	0	0	
				2	0	squamous cell carcinomas	0	0	
				2 (4%)	3 (6%)	glandular stomach	0 (0%)	0 (0%)	
				2	3	adenocarcinomas	0	0	
					1 (2%)	ovaries		1 (2%)	
					1	adenocarcinomas		1	

Table 17A cont. Tumourigenicity of the cultivated mushroom (*Agaricus bisporus*) in randomly bred Swiss albino mice.

Compound tested	Route of administration	Dose	Exposure	Effect of treatment					Reference
				treated animals males females (50/group) (50/group)		control animals males females (100/group)(100/group)			
<i>Agaricus bisporus</i> dry-baked	p.o.	12 h a day for 5 days per week	lifelong	26 (52%)	17 (34%)	lung	19 (38%)	14 (28%)	Toth et al., 1997b
				17	8	adenomas	11	9	
				9	9	adenocarcinomas	8	5	
				4 (8%)	12 (24%)	blood vessels	3 (6%)	7 (14%)	
				2	3	hemangiomas	2	5	
				2	12	hemangiosarcomas	1	2	
				9 (18%)	6 (12%)	cecum	4 (8%)	4 (8%)	
				9	6	polypoid adenomas	?	?	
				3 (6%)	1 (2%)	colon	0 (0%)	0 (0%)	
				2	1	polypoid adenomas	0	0	
				1	0	adenocarcinoma	0	0	

In addition to these more or less criticised long-term studies on mice, two rather poor long-term studies on rats have been published. The first of these were intended to compare the wholesomeness of irradiated *Agaricus bisporus* with that of non-irradiated *Agaricus bisporus*, but data from the control groups in this experiment gives some information on the effect of consuming cooked *Agaricus bisporus* (van Logten et al., 1971). In this study 4 groups of 10 female and 10 male Wistar rats, weanlings of treated parents or controls, were fed on a standard diet (control group), on a diet containing 25% cooked non-irradiated mushrooms (a second control group) or on a diet containing 25% cooked mushrooms previously irradiated with γ -rays (350 krad) or with electrons (500 krad). The mushroom diet was prepared by cooking washed mushrooms (1600 g) for 2.5 minutes in a domestic pressure cooker, after which the mushrooms were homogenised in the cooking liquid, made up to 2 litre with tap water, mixed with the normal standard diet, pelletised and oven-dried (60°C). Food and drinking water were given *ad libitum*.

The study lasted for up to 112 weeks, at which time over 50 % of the animals were still alive. There was no difference in survival between experimental and control groups. The food intake and the average daily intake of mushrooms were not recorded, and the phenylhydrazine content in the feed not determined. The method of producing the feed should have reduced the phenylhydrazine level substantially in comparison to the level that would have been expected in a feed produced in a similar way from uncooked cultivated mushroom. No evidence was found that a feed containing 25% irradiated or 25 % non-irradiated mushrooms had any effect on the growth of the rats. Neither did the treatment induce tumours.

The value of this study when it comes to evaluating the effect of *A. bisporus* consumption is questionable. The authors had originally planned the experiment as a reproduction study, but as the animals had already been on the mushroom diet for 1 year when the breeding was finished they were kept for another year. The female rats were used for breeding five litters during the study and the parents of the treated group had been treated as well. Comparison of the results of the first 90-day test with those of the second, carried out with animals of the F₃ generation, indicates that the feeding of irradiated mushrooms during four generations was without effect (van Logten et al., 1971). The experimental groups (treated as well as control groups) consisted of only 10 male and 10 female rats, and the diets in the respective groups were not isocaloric.

The second long-term study on rats were performed with twenty 50-day-old female Sprague-Dawley rats (from Charles River) that for 500 days (1.37 years) - until the termination of the experiment - were fed a pelletised diet containing 30 % *A. bisporus* (Matsumoto et al., 1991). The diet was prepared by mixing milled cultivated mushroom that had been dried at room temperature with a powdered basal diet. Twenty-four control animals were feed the basal diet only. Water and feed were given *ad libitum*. Although all animals survived until the end of the experiment, the average body weight gain of rats receiving cultivated mushroom in the diet were not as pronounced from week 30 and onwards as that of the control rats. The difference in body weight between the two groups 40 weeks after the start of the experiment were statistically significant. In the 20 rats fed the *A. bisporus* diet, mammary tumours developed in 6 rats, thymoma adenoma in 1 rat, adrenal adenomas in 3 rats and pituitary adenoma in 1 rat. In the control group of 24 rats the corresponding tumour numbers were 12, 0, 4 and 1, respectively. Thus, no significant difference was observed in the incidence of these tumours

between the *A. bisporus* fed group and the control group. The study was performed with only one sex (female rats), was based on too few animals, and was too short to make it possible to evaluate *A. bisporus* for carcinogenicity. Furthermore, the investigators did not determine the influence of the drying procedure of the prepared feed on the agaritine level in the consumed product.

In addition to the described long-term carcinogenicity studies, Danish investigators have studied whether *A. bisporus* is able to induce lung adenomas in A/J mouse, a short-term carcinogenicity model for lung tumours (Pilegaard et al., 1997). Only female mice were used in the study and the exposure period was 26 weeks. And only 15 animals were used per treatment group. The mice were fed lyophilised *A. bisporus* in the semisynthetic diet; 11% and 22% of the diet was replaced by freeze-dried mushrooms. The intake of the mushroom diets were equivalent to an intake of 92 or 166 mg agaritine per kg body weight per day. The exposure did not influence the frequency of lung adenomas in this study. The specificity of the assay is good, but false-negative results are not uncommon because of weak sensitivity (Griesemer, 1991).

It is well known that certain lectins derived from beans belonging to genus *Phaseolus* are toxic to animals and human beings fed with either raw bean or the purified lectin. The bean lectins impair protein digestion and cause inadequate absorption of nutrients, particularly amino acids, thus resulting in protein malnutrition and, occasionally, death. The fact that also *A. bisporus* contain lectins may influence the interpretation of the carcinogenicity studies where *A. bisporus* has been fed to experimental animals. In a study aiming to explore the possible mechanism of lectin-induced malabsorption, Ortiz and coworkers (1992) fed young Sprague-Dawley rats with a rat chow containing 25-75% dried *A. bisporus* mushroom or 100 mg of purified *A. bisporus* lectin for 5 days. Animals fed mushroom concentrations of $\geq 50\%$ or purified lectin showed a significant decrease in body weight and protein digestibility. Their small intestine showed a flattened mucosa with fused villi as well as alterations from the columnar to the cuboidal type of absorptive surface cells. Some rats were fed with a 75% supplemented diet consisting of *A. bisporus* fruitbodies heated at 100°C for 1 h before they were incorporated into the chow. No histological lesions were found in the small intestine of these animals. Unfortunately, it is not known to what extent the lectins occurring in *A. bisporus* influence the outcome of carcinogenicity studies with uncooked mushrooms. It is clear, however, that the control animals have not been exposed to these compounds.

9.2.2. Studies on phenylhydrazines in the mushroom and related compounds

9.2.2.1. Agaritine.

Toth and colleagues have performed two studies with agaritine. In the first of these studies, Swiss mice received chemically synthesised agaritine (purity 98%) as a 0.0625% solution in the drinking water for their lifetime (Toth et al., 1981a). This dose was, however, too toxic for the males and an additional group of 50 males receiving a 0.0313% agaritine-solution in the drinking water was started separately. In this study, the untreated control group was made up of 100 animals of each sex, and these were 5 weeks of age at the beginning of the study.

The study lasted 120-130 weeks (until all animals had died or were killed with ether when moribund). The average daily intake of agaritine in the groups receiving a 0.0625% solution was 3.15 mg for females and 3.94 mg for males. The average daily

intake of agaritine for males receiving the lower dose agaritine in drinking water (0.0313% solution) was 2.68 mg per male. Agaritine at the 0.0625% level substantially reduced the survival rates in males compared to the survival rates in untreated control males, whereas this dose when given to the females and the lower dose level when given to the males did not effect the survival rates.

As shown in Table 17B, none of the exposures to agaritine in drinking water resulted in a detectable carcinogenic effect. However during the course of the study, a substantial number of animals developed convulsions and eventually died of the seizures.

In the second long-term carcinogenicity study with agaritine on mice, the compound was isolated from commercially purchased cultivated mushroom and administered as subcutaneous injections (Toth and Sornson, 1984). One of the two agaritine-exposed groups received five subcutaneous injections of 100 µg agaritine (purity 98%) in 0.01 ml physiological saline per gram body weight at weekly intervals. The other exposed group of 50 Swiss mice of each sex received a single subcutaneous injection of 100 µg (females) or 50 µg (males) agaritine in 0.01 ml physiological saline per gram body weight. Also in this experiment, the untreated control group of 100 female and 100 male Swiss mice were observed from 5 weeks of age. This experiment apparently used the same control group as the experiment which had administered agaritine orally in drinking water (Toth et al., 1981a). Thus, the animals in the control group were neither injected with the solute, nor kept concurrently with the treated animals. The study lasted 140-150 weeks (until all mice had died or had been killed when found in poor condition). Toth and Sornson (1984) concluded that subcutaneous injection with agaritine at the two dose levels tested, neither had an effect on the survival rates, nor on the cancer incidences as compared with that of the untreated control (Table 17B).

The observations reported in chapter 3 on the differential stability of agaritine solutions under various conditions of storage may be important for the interpretation of the carcinogenicity studies with agaritine. In the first study with agaritine reported above (Toth et al., 1981), agaritine was dissolved in drinking water and the animals consumed the solution *ad libitum*. The solution was prepared three times weekly, and the total consumption of water containing agaritine was measured at the same intervals during the treatment period. The investigators reported that the drinking solution was contained in brown bottles because of the possible light sensitivity of the chemical. When an agaritine solution (625 mg/l) for the chronic experiment was analysed by HPLC chromatography after standing for 48 hours at room temperature, it was found to contain 90% of the original compound unchanged. However, since bubbles of air will pass through the solution every time the mice drinks from the bottle, the agaritine solution in the carcinogenicity study will continuously be supplied fresh oxygen that may contribute to the degradation of agaritine. Thus, it is possible that the mice in this study have been exposed to much lower agaritine levels than were reported.

The agaritine used in the other carcinogenicity study with this compound was isolated from the commercial cultivated mushroom, *A. bisporus*. Neither was the purity of the isolate stated, nor the time period given for how long the agaritine solutions (in physiological saline) were left standing before being injected into the mice. Because this information is lacking it is difficult to estimate whether there is a possibility that the agaritine exposure could be less than stated also in this experiment.

Table 17B. Tumourigenicity of the phenylhydrazines and related compounds occurring in the mushroom in randomly bred Swiss albino mice.

Compound tested	Route of administration	Dose	Exposure	Effect of treatment					Reference
				treated animals		control animals			
				males (50/group)	females (50/group)	males (100/group)	females (100/group)		
Agaritrine	p.o.	0.0625 %	lifelong	6 (12%)	13 (26%)	lungs	19 (19%)	29 (29%)	Toth et al., 1981a
				4	9	adenomas	10	15	
				2	4	adenocarcinomas	9	14	
				2 (4%)	2 (4%)	blood vessels	5 (5%)	7 (7%)	
				0	0	angiomas	2	4	
				2	2	angiosarcomas	3	3	
				1 (2%)	6 (12%)	Malignant lymphomas	10 (10%)	15 (15%)	
	p.o.	0.03125% (males only)	lifelong	8 (16%)		lungs	19 (19%)		
				7		adenomas	10		
				1		adenocarcinomas	9		
				5 (10%)		blood vessels	5 (5%)		
				2		angiomas	2		
				3		angiosarcomas	3		
				1 (2%)		Malignant lymphomas	10 (10%)		

Table 17B cont. Tumourigenicity of the phenylhydrazines and related compounds occurring in the mushroom in randomly bred Swiss albino mice.

Compound tested	Route of administration	Dose	Exposure	Effect of treatment					Reference
				treated animals males (50/group)	females (50/group)		control animals males (100/group)	females (100/group)	
Agaritine	s.c.	100 mg/kg	5 weekly	12 (24%) 7 5 3 (6%) 1 2 3 (6%)	13 (26%) 8 5 9 (18%) 7 2 10 (20%)	lungs adenomas <i>adenocarcinomas</i> blood vessels angiomas <i>angiosarcomas</i> Malignant lymphomas	19 (19%) 10 9 5 (5%) 2 3 10 (10%)	29 (29%) 15 14 7 (7%) 4 3 15(15%)	Toth and Sornson, 1984
	s.c.	100 mg/kg to females, 50 mg/kg to males	single	9 (18%) 4 5 1 (2%) 1 0 3 (6%)	12 (24%) 9 3 5 (10%) 3 2 14 (28%)	lungs adenomas <i>adenocarcinomas</i> blood vessels angiomas <i>angiosarcomas</i> Malignant lymphomas	19 (19%) 10 9 5 (5%) 2 3 10 (10%)	29 (29%) 15 14 7 (7%) 4 3 15(15%)	
N'-acetyl-4-(hydroxy-methyl)phenylhydrazine	p.o.	0.0625 %	lifelong	24 (48%)*** 16 8 15 (30%)*** 7 8	17 (34%)** 10 7 16 (32%)*** 9 7	lungs adenomas <i>adenocarcinomas</i> blood vessels angiomas <i>angiosarcomas</i>	22 (22%) ? ? 5 (5%) ? ?	15 (15%) ? ? 8 (8%) ? ?	Toth et al., 1978
N'-acetyl-4-(hydroxy-methyl)phenylhydrazine	s.c.	500 mg/kg	26 weekly	2 (4%) 1 1	0 (0%) 0 0	soft tissue tumours <i>fibrosarcomas</i> <i>angiosarcomas</i>	2 (4%) 2 0	0 (0%) 0 0	Toth and Nagel, 1981
4-(hydroxy-methyl)benzenediazonium tetra-fluoroborate	p.o.	400 mg/kg	single	16 (32%)*** 9 7	15 (30%)*** 3 12	glandular stomach polypoid adenomas <i>adenocarcinomas</i>	0 (0%) 0 0	0 (0%) 0 0	Toth et al., 1982

Table 17B cont. Tumourigenicity of the phenylhydrazines and related compounds occurring in the mushroom in randomly bred Swiss albino mice.

Compound tested	Route of administration	Dose	Exposure	Effect of treatment					Reference
				treated animals males (50/group)	females (50/group)		control animals males (100/group)	females (100/group)	
4-(hydroxy-methyl)benzenediazonium tetrafluoroborate	s.c.	50 mg/kg	26 weekly	9 (18%)* 0 5 2 2 3 (6%) 2 1	11 (22%)* 0 10 0 1 9 (18%)* 4 5	subcutaneous tissue fibroma fibrosarcomas rhabdomyosarcomas angiosarcomas skin squamous cell papillomas squamous cell carcinomas	3 (6%) 1 2 0 0 0 (0%) 0 0	3 (6%) 0 3 0 0 0 (0%) 0 0	Toth, 1987
4-(hydroxy-methyl)benzenediazonium sulfate	s.c.	50 mg/kg	26 weekly	20 (40%)* 19 0 1 2 (4%) 0 2	16 (32%)* 13 2 1 7 (14%)* 2 5	subcutaneous tissue fibrosarcomas rhabdomyosarcomas angiosarcomas skin squamous cell papillomas squamous cell carcinomas	2 (4%) 2 0 0 0 (0%) 0 0	0 (0%) 0 0 0 1 (2%) 1 0	Toth, 1987
4-(carboxy)-phenylhydrazine	p.o.	0.125%	lifelong	21 (42%)* 6 15	7 (14%)* 3 4	aorta and large arteries leiomyomas leiomyosarcomas	2 (4%) 0 2	0 (0%) 0 0	McManus et al., 1987
N ² -[γ-L(+)-glutamyl]-4-(carboxy)-phenylhydrazine	p.o.	1.4 g/kg	52 weekly	13 (26%)* 0 13 0	1 (2%) 0 0 1	subcutaneous tissue fibroma fibrosarcomas myxosarcomas	0 (0%) 0 0 0	3 (6%) 1 2 0	Toth, 1986a

9.2.2.2. *N'*-acetyl-4-(hydroxymethyl)phenylhydrazine

The American investigators at University of Nebraska Medical Centre have performed two studies with *N'*-acetyl-4-(hydroxymethyl)phenylhydrazine, a compound that does not occur in the cultivated mushroom. In *A. bisporus* agaritine is believed to be enzymatically cleaved to L-glutamate and 4-(hydroxymethyl)-phenylhydrazine. Since 4-(hydroxymethyl)phenylhydrazine is a relatively unstable compound the investigators attached an acetyl group to it to render the compound feasible for biological experiments.

In the first study with *N'*-acetyl-4 (hydroxymethyl)phenylhydrazine (purity >97%), the compound was given as a 0.0625% solution in drinking water for the lifetime of Swiss albino mice (Toth et al., 1978). The average daily intake of *N'*-acetyl-4-(hydroxymethyl)phenylhydrazine was 6.4 mg for female mice and 6.6 mg for male mice. The experiment lasted for up to 120-130 weeks. The control group of 100 mice of each sex was 5 weeks at the beginning of the study.

There was no significant difference in survival between the *N'*-acetyl-4 (hydroxymethyl)phenylhydrazine-treated mice and the mice fed the control diet. As shown in Table 17B the exposure gave rise to statistically increased frequencies of lung tumours and blood vessel tumours in both sexes. The authors did not indicate the number of benign and malignant tumours for the control group.

In the second study with *N'*-acetyl-4-(hydroxymethyl)phenylhydrazine Swiss albino mice received 26 separate injections of 500 µg of the compound in 0.01 ml physiological saline per gram body weight at weekly intervals (Toth and Nagel, 1981). The control animals received 26 subcutaneous injections 0.01 ml physiological saline per gram body weight at weekly intervals. The experiment lasted for 120-130 weeks. The survival rate and tumour incidence among the treated animals were not different from that in control animals (Table 17B).

9.2.2.3. 4-(Hydroxymethyl)benzenediazonium ions

Two forms of the 4-(hydroxymethyl)benzenediazonium ion have been tested in long-term carcinogenicity studies - the tetrafluoroborate and the sulfate form. Both compounds were chemically synthesised by the investigators. The purity of the 4-(hydroxymethyl)benzenediazonium tetrafluoroborate was 95% and that of the 4-(hydroxymethyl)benzenediazonium sulfate 98%.

The 4-(hydroxymethyl)benzenediazonium tetrafluoroborate was tested both after oral application and after subcutaneous injections. In one study with the 4-(hydroxymethyl)benzenediazonium tetrafluoroborate, the compound was given as a single intragastric dose by gavage at a concentration of 400 µg in 0.01 ml 0.9% NaCl solution per gram body weight, which was close to the maximum tolerated dose (Toth et al., 1982). The control groups were inadequately characterised in this study. As a solvent-instilled control, 50 mice of each sex, received 41 weekly intragastric doses of 100 µg sodium tetrafluoroborate in 0.01 ml NaCl solution per gram body weight. A third group consisted of untreated mice. The study lasted for 120-130 weeks. Only data on the 4-(hydroxymethyl)benzenediazonium tetrafluoroborate-treated group was presented.

The investigators claim that the 4-(hydroxymethyl)benzenediazonium tetrafluoroborate-treatment had no significant effect on the survival when compared with the survival of the solvent-injected control animals or the untreated controls. This seems to be

a rash statement, since none of the 50 exposed males survived longer than 80-90 weeks. Whereas the 4-(hydroxymethyl)benzenediazonium tetrafluoroborate-treatment induced glandular stomach tumours, no such tumours were found in the untreated controls (Table 17B). Toth et al. (1982) mentioned that some of the adenocarcinomas probably started to grow as polypoid adenomas whereas others developed into malignancy without the transitory benign stage.

When Swiss albino mice were given 26 subcutaneous injections at weekly intervals with 50 µg 4-(hydroxymethyl)benzenediazonium tetrafluoroborate in 0.01 ml 0.9% sodium chloride solution per gram body weight, the treatment shortened the survival when compared with the solvent-injected controls (Toth et al., 1981b). The control received 26 subcutaneous weekly injections with sodium tetrafluoroborate (the dose of tetrafluoroborate corresponded to the dose given to the treated group). Whereas all treated animals had died within 70-80 weeks after the beginning of the treatment, some of the control animal lived for up to 110-120 weeks. As shown in Table 17B, the subcutaneous injections with 4-(hydroxymethyl)benzenediazonium tetrafluoroborate induced a statistically significant increase in tumours of the subcutis and skin of female Swiss mice.

In the study with the sulfate form of the 4-(hydroxymethyl)benzenediazonium ion, Swiss albino mice received 26 subcutaneous weekly injections at 50 µg in 0.01 ml 0.9% NaCl solution per gram body weight (Toth, 1987). The control animals received the same number of subcutaneous injections with 31 µg sodium sulfate in 0.01 ml 0.9% NaCl solution per gram body weight. As was the case after treatment with the tetrafluoroborate of the 4-(hydroxymethyl)benzenediazonium ion, treatment with the sulfate form of the compound resulted in shortened survival in comparison with the control group. Furthermore, the treatment resulted in statistically increased frequencies of tumours of cubcutis (both sexes) and skin (only females) - Table 17B.

9.2.2.4. 4-(Carboxy)phenylhydrazine

Commercially available 4-(carboxy)phenylhydrazine hydrochloride (p-hydrazinobenzoic acid; purity >97%) was administered to Swiss albino mice at a dose of 0.125% in drinking water for lifetime; that is for 120-130 weeks for the treated mice and 150-160 weeks for the control animals (McManus et al., 1987). The average daily intake of 4-(carboxy)phenylhydrazine per animal was 4.7 mg for females and 5.1 mg for males.

Treated mice showed an increased mortality rate. Particularly at the early stages of the study, a substantial numbers of mice died suddenly with ruptured aortae (32% of females and 50% of males). As shown in Table 17B the treatment also induced a statistically significant increase in smooth muscle cell tumours of the aorta and the large arteries.

Two major histopathological changes observed could contribute to the explanation of both the ruptures and the tumours. First the aortic walls had undergone a diffuse intimal and inner medial fibrinoid necrosis, accompanied by widespread medial elastinolysis. Secondly, cells from the media proliferated.

Already twenty years earlier, Roe and his coworkers (1967) had tested 4-(carboxy)phenylhydrazine in Swiss mice. Only 25 female mice were used in this experiment, and only one dose level was tested. Virgin animals were treated 5 days each

week for 40 weeks with 1 mg 4-(carboxy)phenylhydrazine in 0.2 ml distilled water by gavage. 85 untreated female Swiss mice served as controls.

The authors studied the incidence of lung tumours in treated and control animals 40-60 weeks after treatment. At 40-50 weeks after treatment, one out of the 7 exposed mice still living had developed lung tumours, whereas the corresponding incidence among the control animals were 2 out of 37. At 50-60 weeks after the beginning of the treatment one out of 5 survivors had developed lung tumours. In the controls 6 out of 42 survivors had developed lung tumours. The authors concluded that 4-(carboxy)phenylhydrazine was without apparent carcinogenic effect in the lung.

In addition to the carcinogenicity studies with 4-(carboxy)phenylhydrazine described above, Danish investigators have studied whether supplementation of this compound (1000 ppm) to the drinking water for 26 weeks could give rise to lung adenomas in female A/J mouse, a short-term carcinogenicity model for lung tumours (Pilegaard et al., 1997). The average intake of 4-(carboxy)phenylhydrazine in this study was 106 mg/kg body weight/day. No lung adenomas were induced. However, the data was based on only 15 animals and the exposure to 4-(carboxy)phenylhydrazine was lower in this experiment than in the positive long-term carcinogenicity study of McManus et al. (1987).

9.2.2.5. β -N-(γ -L-(+)-Glutamyl)-4-(carboxy)phenylhydrazine

Swiss mice received chemically synthesised β -N-(γ -L (+)-glutamyl)-4-(carboxy)phenylhydrazine (purity: 95%) as 52 weekly intragastric installations of 1.4 mg in 0.01 physiological saline per gram body weight (Toth, 1986). The experiment lasted for 150-160 weeks (until all mice had died or were killed when found in poor condition).

The treatment had no substantial effect on the survival time. Although β -N-(γ -L-(+)-glutamyl)-4 (carboxy)phenylhydrazine was administered by the oral route, the treated males developed a statistically significant increase in subcutaneous tumours which were located on the shoulder areas and backs of animals. The number of tumour bearing animals is shown in table 17B. Toth (1986) proposed that some of the chemicals might have been regurgitated after the application and might have been rubbed and dispersed on the skin of the animal. If the subcutaneous tumours of the males are induced by regurgitation, then the authors information on the toxicity by oral administration is sparse. Instead of giving the control animals water (the solute) weekly by gavage, they were given water *ad libitum* in ordinary drinking bottles.

9.2.2.6. Other phenylhydrazines

A compound closely related to the agaritine degradation product 4-(hydroxymethyl)-benzenediazonium ion has been identified in *A. xanthodermus*, a wild nonedible relative to *A. bisporus* (Dornberger et al., 1986). This compound was the 4-(hydroxy)-benzenediazonium ion (Figure 10A). When the 4-(hydroxy)benzenediazonium sulfate was subcutaneously injected 36 times at weekly intervals in Swiss mice at 2 μ g per gram body weight, tumours were induced in the subcutis (Toth et al., 1989a). In parallel experiments two injections with 10 μ g 4-(hydroxy)benzenediazonium sulfate per gram body weight, one week apart, had no tumourigenic activity.

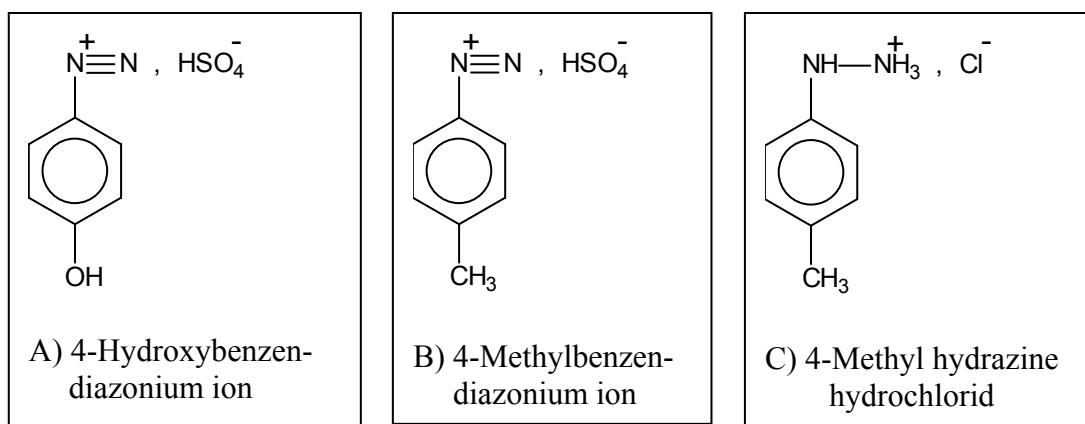


Fig. 10. One phenylhydrazine and two benzenediazonium ions that do not occur in *A. bisporus* but have been tested for carcinogenicity in Swiss albino mice; (A) 4-hydroxybenzenediazonium sulfate; (B) 4-(methyl)benzenediazonium sulfate; (C) 4-methylphenylhydrazine hydrochloride.

The third benzenediazonium ion that has been shown to induce tumours in Swiss mice is the 4-(methyl)benzenediazonium ion (Figure 10B). This compound has never been identified in any *Agaricus* species, but it has been speculated that it could be formed from the 4-(hydroxymethyl)benzenediazonium ion. When 4-(methyl)benzenediazonium sulfate was administered by subcutaneous injection at weekly intervals 10 or 16 times at 50 µg per gram body weight, tumours of the subcutaneous tissue and skin were induced (Toth et al., 1989b).

Another compound speculated to be produced from 4-hydroxymethylphenylhydrazine (the direct degradation product of agaritine) is 4-methylphenylhydrazine (Figure 10C). When this compound in hydrochloride form was administered to Swiss mice by subcutaneous injection at 140 µg per gram body weight weekly for 10 weeks, or as 7 weekly intragastric instillations of 250 µg per gram body weight, tumours were induced in the exposed animals. The subcutaneous injection resulted in lung tumours, whereas the intragastric treatment, in addition to lung tumours in the female, induced tumours in the blood vessels in both sexes (Toth et al., 1977). In subsequent studies 4-(methyl)phenylhydrazine unexpectedly induced soft-tissue tumours at injection sites of Swiss mice administered the hydrochloride form of the compound by 26 weekly subcutaneous injections (Toth and Nagel, 1981). The reason behind the different types of tumours found in these two studies is not known.

9.3. Non-conventional carcinogenicity studies

In one study, the activity of synthesised agaritine and methanol extracts from *A. bisporus* were tested for their ability to induce tumours in mouse bladder epithelium implanted with these compounds in paraffin wax (Hashida et al., 1990). The observed rate of mouse urinary bladder carcinoma was 30.8% in animals exposed to methanol extracts of *A. bisporus*. Although the agaritine content of shiitake extracts was more than 200 times lower than the agaritine content of *A. bisporus*-extract, the tumour rate was 23.5 % in animals administered extracts from fresh shiitake (*Lentinus edodes*). The tumour

rates in animals exposed to extracts of dry shiitake and synthesised agaritine was 9.8 and 50%, respectively. The tumour incidence in the negative control was 5.4%. A statistically significant increase in the tumour incidence, at the $p < 0.01$ level, were observed for animals exposed to agaritine and methanol extracts of fresh *A. bisporus*.

9.4. Antineoplastic effects of phenylhydrazines

Today 96 hydrazine derivatives and related compounds have been studied for carcinogenic action in long-term experiments in laboratory animals and 82 have given a positive response (Toth, 2000). In addition, case-control and cohort studies of one common hydrazine in industrial settings and four hydrazines used in medicine indicate that they play a role in the development of certain types of human cancer (Toth, 1994). However, hydrazines and hydrazine-containing natural products have also been reported to have antineoplastic activity (Toth, 1996, 2000).

In one of these studies 5-6 weeks old male C3H and CDF₁ mice were implanted with MM-46 carcinoma cell or IMC carcinoma cells and given a diet including 10, 20 or 30% powdered *A. bisporus* (Nanba and Koruda, 1988). On the 30th day after tumour transplantation, the experiment was terminated. The tumour inhibition ratio in the 10, 20 and 30% mushroom-fed groups were 59, 67 and 76% in C3H mice and 33, 45 and 55% in the CDF₁ mice. The experiment also included studies on phagocytic activity of macrophages, and measurement of superoxide anion production and activities of lymphokine-activated killer cells and cytotoxic T cells. It was concluded that a mushroom-supplemented diet suppressed the growth of tumours.

As estrogens have a role in breast cancer development, and estrogen production by aromatase/estrogen synthetase in breast cancer plays a dominant role in tumour proliferation, it is interesting to note that extracts of *Agaricus bisporus* inhibits the aromatase activity dose dependently *in vitro* (Grube et al., 2001). It is not known whether these observations have any bearing on the *in vivo* situation.

9.5. Reproduction, embryotoxicity and teratogenicity

There are only three reports on embryotoxicity and teratogenicity of phenylhydrazines in *A. bisporus*.

In an old report describing the teratogenic effects of semicarbazide hydrochloride injected (2-3 mg) into 4-6 days old embryonated White Leghorn eggs, the authors state that similar effects to semicarbazide is produced by 4-(carboxy)phenylhydrazine (Neuman et al., 1956). The effect is characterised by production of lower beak and skeletal malformations (bent tarsometatarsal and tibiotarsal bones).

An embryotoxicity and teratogenicity study with water extracts of *A. bisporus* have been performed in rats within this Nordic project. In this study pregnant Sprague-Dawley rats were administered a freshly prepared water extract of the mushroom by gastric intubation daily on days 6-15 of pregnancy and killed on day 19 of gestation (Andersson et al., 1994). The extracts were prepared from freeze-dried *A. bisporus*, containing 340 mg agaritine per kg mushroom, and the amounts administered corresponded to a human intake of 3 kg mushrooms per day. When treated and control dams were examined for embryotoxicity (preimplantation loss, number of resorptions) and fetuses

for teratogenicity (external and skeletal malformations), using standard teratological techniques, no significant differences could be detected between the groups. Neither could any influence on the body weights of the mothers and the fetuses be detected.

In an investigation on the effect of gamma irradiation on the storage characteristics and nutritional quality of mushrooms, dried and ground unirradiated, organically grown *A. bisporus* were incorporated in feed and given to pregnant albino CF1 mice (2-5 days before term) as a control exposure when exploring the effects of irradiated mushrooms on female animals and their offspring (Campbell et al., 1968). The agaritine content of the mouse feed was not determined and the feeding period of the pregnant dams was too late for any teratogenic effects to be expected in the offspring. No exceptional findings were noted in this experiment, but very little information was given on the results.

9.6. Biological effects of *A. bisporus*

In this section some studies on physiological effects related to *A. bisporus* consumption are reported. Some of the physiological effects are adverse, others of a protective nature. It should be kept in mind that it generally is unknown which compounds in the mushroom are responsible for the observed effects. There is no direct reason to suspect the phenylhydrazines to be responsible for the effects.

Although *Agaricus bisporus* is not generally recognised as having medicinal value (Chang, 1996), some investigators have explored the cultivated mushroom also in this respect. Consumption of various types of mushroom have been shown to lower the plasma cholesterol concentration. The mechanism for this effect is not known. Recently, Dutch investigators studied the hypocholesterolemic properties of *A. bisporus* by feeding groups of female rats for 6 weeks with diets where parts of the corn starch and dextrose of the commercial diet had been exchanged for a small quantity of cholesterol and in total 2.5, 5 or 10 % mushroom (Beynen et al., 1996). Inclusion of *A. bisporus* in the rat diet had no effect on the plasma and liver cholesterol concentrations of the rats.

The cultivated mushroom has been used in folklore medicine for treatment of diabetes in northern Europe, although scientific evidence for its efficiency have been lacking. To explore a possible hypoglycaemic effect of mushroom consumption, normal and streptozotocin diabetic adult male lean mice (a colony breed at Aston University, UK) were fed a pelleted diet containing 6.25 % dried fruit bodies of *A. bisporus* during 28 days (Swanston-Flatt et al., 1989). The glucose homeostasis was not affected in non-diabetic mice, but in mice with induced streptozotocin diabetes (reduction in hyperphagia, polydipsia and body weight loss) hyperglycaemia was reduced already during the first week after induction of the disease. The percentage glycated haemoglobin two weeks after streptozotocin administration was also reduced and the initial reduction in plasma insulin and pancreatic insulin counteracted. Thus, the mushroom improved insulin-induced hypoglycaemia in streptozotocin diabetic mice.

Since consumption of *A. bisporus* reduced the initial insulinopenic effect of streptozotocin and countered the depletion of pancreatic insulin, the mechanism for the amelioration of diabetes by this mushroom may be connected to a protective effect against islet B-cell destruction. Extracts of the cultivated mushroom have been reported to affect islet B-cells by stimulating calcium uptake and insulin release (Ahmad et al., 1984a). This has been attributed to a lectine activity in the mushroom (Ahmad et al., 1984b; Ewart et al., 1975).

In addition to the phenylhydrazines shown in Figures 1 and 2, *A. bisporus* contains another nitrogen-containing compound that also containing a glutamyl moiety. That compound is γ -L-glutaminy-4-hydroxybenzene which is believed to be used in a tyrosinase-catalyzed metabolic pathway that produces inhibitors ostensibly responsible for the induction of dormancy in this species (Weaver et al., 1971a). As a first step γ -L-glutaminy-4-hydroxybenzene is hydrolysed to γ -L-glutaminy-3,4-dihydroxybenzene, which is oxidised to γ -L-glutaminy-3,4-benzoquinone, both reactions being catalysed by tyrosinase (Weaver et al., 1971b). The quinone compound undergoes a base-catalysed non-enzymatic reaction to yield 2-hydroxy-4-iminoquinone. Both quinones may inhibit sensitive enzymes, for example enzymes involved in energy production and DNA synthesis, and give rise to cytostatic and cytotoxic activity. Studies with isolated enzymes and permeabilised L1210 lymphocytic leukemia cells have revealed that the mechanism by which DNA synthesis is affected by γ -L-glutaminy-4-hydroxybenzene is by an inhibition of ribonucleotide reductase and not DNA polymerase (FitzGerald et al., 1984). The inhibition occurs in the range at which γ -L-glutaminy-4-hydroxybenzene is found in gill tissue, close to 1 g/kg tissue. Histological evidence of severe kidney and pancreas damage (necrosis) was observed in neonatal C57Bl/6J mice injected with γ -L-glutaminy-3,4-dihydroxybenzene, but not in mice injected with N²-methyl- γ -L-glutaminy-3,4-dihydroxybenzene (Boekelheide et al., 1980). The LD₅₀ value for the active compound was 100 to 200 mg/kg. Since γ -L-glutaminy-3,4-dihydroxybenzene, but not the methylated derivative, was an excellent substrate for γ -glutaminy-transpeptidase, it was believed that the transpeptidase produces glutamic acid and 4-aminocatechol from γ -L-glutaminy-3,4-dihydroxybenzene, and that air oxidises 4-aminocatechol to the ultimate cytotoxic compound 2-hydroxy-4-iminoquinone. The toxic effect was registered in the two tissues having the highest γ -glutaminy-transpeptidase activity.

From *Agaricus campestris* a similar precursor for γ -L-glutaminy-3,4-benzoquinone has been found in the form of the amino acid agaridoxin, 3,4-dihydroxy-(γ -L-glutamyl)-anilide (Szent-Gyorgyi et al., 1976).

9.7. Conclusion on effects on experimental animals

Only a few and poorly performed studies have dealt with the *subchronic toxicity* of mushroom hydrazines. The only observation of importance coming from these studies is the comparatively low toxicity of 4-(carboxy)phenylhydrazine in dogs, rabbit and guinea pigs.

Since also the few published studies on *embryotoxicity and teratogenicity* are of poor quality, very little information can be extracted from these reports of reproductive toxicity of phenylhydrazines in *A. bisporus*, water extracts of the mushroom and dried and ground mushroom. Whereas orally supplied water extracts of *A. bisporus* had no teratogenic activity in Sprague Dawley rats, high concentrations of 4-(carboxy)phenylhydrazine injected into eggs, much higher concentrations than man can ever be exposed to through consumption of the cultivated mushroom, produced lower beak and skeletal malformations in embryonated White Leghorn chickens.

None of the 13 long-term *carcinogenicity* studies with *A. bisporus* or the phenylhydrazines in the mushroom in mice, or the 2 long-term carcinogenicity studies with *A. bis-*

porus in rats have been performed according to approved standard protocols for carcinogenicity studies. All cancers studies on mice have been performed in a single laboratory and with only one strain of mice, the outbred Swiss albino mice. Factors that did not follow standard procedures and that might have had profound influence on the end results were that mice were exposed to the tested compounds from the sixth week of age until they died or were found in poor condition instead of for a predetermined length of time, and that the control group were usually not started at the same time as the treated groups.

Of the mushroom hydrazines, 4-(carboxy)phenylhydrazine, N^2 -[γ -L(+)-glutamyl]-4-(carboxy)phenylhydrazine and 4-(hydroxymethyl)benzediazonium ion (as tetrafluoroborate and sulfate) were identified as carcinogens at high doses in Swiss albino mice. Furthermore, a stabilised derivative of 4-(hydroxymethyl)phenyl-hydrazine, N' -acetyl-4-(hydroxymethyl)phenylhydrazine, was carcinogenic in the same system. On the other hand agaritine was found not to be carcinogenic when administered by the oral and subcutaneous route. Since agaritine was administered in drinking water changed only three times a week and the concentration of agaritine in the drinking water was not analysed, it is possible that the compound was partly degraded in the drinking water.

However, tumours were induced in several tissues when fresh or dry-baked *A. bisporus* were fed to mice *ad libitum* for 3 days, followed by semisynthetic diet for 4 days each week for life. Whereas the fresh mushroom induced tumours in the lung, forestomach and bone, the dry-baked mushroom induced tumours in the forestomach, glandular stomach, duodenum and ovaries. Since the average weekly weight curves of the animals given fresh *A. bisporus* were substantially lower than that of untreated animals it has been speculated that the carcinogenic response of the treatment was induced by the treatment protocol and not the cultivated mushroom. Although feeding mice with dry-baked mushrooms for 12 hours a day for 5 days each week for life and a well-balanced semisynthetic diet for the rest of the time (periodic alterations in nutrient levels) increased the number of tumours in some tissues, none of the increases in tumour frequency were statistically significant. It is impossible to determine whether the lower carcinogenicity registered in this study was due to a lower intake of mushrooms (and phenylhydrazines) or a more balanced feeding protocol. Both studies in which rats were fed *A. bisporus* were of poor quality. Thus, if *A. bisporus per se* is carcinogenic in experimental animals, its activity should be low.

10. Human implications

There are two aspects of human intoxication after consumption of *A. bisporus* or its wild-growing relatives. One possibility, although unlikely, is that the cultivated mushroom itself could give rise to acute intoxications of the consumer. The second and more likely possibility is that consumers of what is believed to be the cultivated mushroom or edible wild-growing *Agaricus* species might be exposed to toxic look-alike contaminating mushrooms. The risk for such incidents is of course highest when eating the wild-growing *Agaricus* species.

Consumers are exposed to wild mushrooms from many sources. They can harvest wild mushrooms for their own personal use, buy mushrooms from businesses that collect and sell mushrooms to the public and retail distributors, or order dishes that contain wild mushrooms in restaurants. Wild mushrooms from commercial sources may be contaminated by toxic look-alikes, which are collected with edible mushrooms through either ignorance or negligence. Mushroom pickers are often paid by the weight for their wild harvest, a practice that discourages selective harvesting and culling for the elimination of undesirable species and that encourages fraud. Field drying the wild harvest before sale to cooperative processing and packaging operations makes effective culling more difficult because the appearance of the dried products has changed (Gecan and Cichowicz, 1993). Consequences of poisoning due to contamination with toxic look-alikes range from unpleasant gastrointestinal disturbances to major organ failure and death.

The risk for intoxications occurring after consumption of products sold on the market has been studied in the United States. Ten Food and Drug Administration Districts each were directed to collect 30 samples of canned and dried imported mushrooms during one year (October 1988-October 1989), and 20 samples of canned, dried, and fresh imported and domestic mushrooms for 1 year (October 1989-October 1990) (Gecan and Cichowicz, 1993). Among the 344 mushroom samples collected and analyzed during the 2-year survey, only 3 samples were supposed to be *Agaricus* species (Meadow Mushroom; *Agaricus campestris*). The toxic look-alikes to *Agaricus* species are mainly Death Angel (*Amanita bisporigera*), Spring Amanita (*Amanita verna*), Destroying Angel (*Amanita virosa*), and Death Cap (*Amanita phalloides*). None of these toxic look-alikes were found in the 3 *Agaricus*-samples.

11. Previous estimations of risk

In 1983 the International Agency for Research on Cancer evaluated agaritine for carcinogenicity based on data available up until then (see chapter 9). No evidence was found for the carcinogenicity of agaritine in experimental animals. However, there was limited evidence of the metabolites of agaritine being carcinogenic in experimental animals. In the absence of epidemiological data, no evaluation of the carcinogenicity of agaritine to humans could be made. No carcinogenicity study of the cultivated mushroom *per se* was available at the time of the IARC evaluation.

Schlatter (1986) approached the task of performing a risk assessment of phenylhydrazines in *A. bisporus* in several ways. One approach was a rough linear extrapolation of the cancer risk from mouse to man using as experimental data the carcinogenicity study of Toth and Erickson (1986) in which mice were fed *A. bisporus* 3 days a week for their life-span. In that study the intake of mushrooms lead to a tumour incidence of 0.4. Assuming agaritine and/or its metabolites to be responsible for the carcinogenic effect and the mushroom consumption to correspond to an intake of 50 mg agaritine/kg body weight/day, as well as the metabolism of Swiss mice being comparable to that of man, Schlatter (1986) calculated that a human cancer risk of $1/10^6$ would correspond to a daily intake of approximately 0.1 µg agaritine/kg body weight/day (corresponding to about 6 µg daily for a human). Another approach by Schlatter (1986) to attempt a risk assessment of agaritine and its hydrolysis products was drawn upon the DNA binding and *in vivo* mutagenesis studies of Shepard et al. (1986). In this study the DNA binding of the 4-(hydroxymethyl)benzenediazonium ion (HMBD), the anticipated ultimate carcinogen arising from agaritine metabolism, was measured in mice orally supplied with this compound. The HMBD bound weakly to stomach DNA, but made very few DNA adducts to liver DNA. To be able to compare the quantity of DNA adducts produced by HMBD with that produced by well known animal carcinogens the author calculated the Covalent Binding Index, CBI (Lutz, 1979). The HMBD had a CBI value of 13 in the stomach and below one in the liver. For comparison, the strong carcinogen nitrosodimethylamine has a CBI value of 6000 and aflatoxin B₁ a value of 12000, both measured in rat liver (Lutz, 1979). Since there was no DNA binding data available for agaritine, but this mushroom compound according to other investigators (Friedrich et al., 1986) was 10-100 times less mutagenic than HMBD in bacterial test systems, a CBI value of 0.3 for all tissues was estimated and assumed for agaritine. By making a number of somewhat speculative assumptions related to DNA adducts and to which risks are tolerable and which are not, Schlatter (1986) compared the CBI values and concluded that an exposure to about 200 µg agaritine/person/day or 0.5 g fresh *A. bisporus*/person/day would be tolerable. This exposure corresponds to one mushroom meal every three to four months.

In 1993 Toth and Gannett attempted an assessment of the carcinogenic potency of *Agaricus bisporus* based on their long-term carcinogenicity studies in the Swiss mouse with the mushroom itself, the phenylhydrazines and benzenediazonium ion occurring in the mushroom, and N'-acetyl-4-(hydroxymethyl)-phenylhydrazine. The latter compound does not occur in the mushroom. The N-acetylation of 4-(hydroxymethyl)phenyl-

hydrazine (the unstable substance formed by hydrolysis of agaritine) was made in order to stabilise the molecule. Although treatment with agaritine itself resulted in no detectable carcinogenic action in Swiss mice, treatments with the other test compounds and the raw mushroom (*A. bisporus*) induced tumours in various tissues of the animals. By taking into account the daily or weekly dose levels of individual phenylhydrazines or the mushroom *per se*, as well as the length of the exposure period, the following total amounts were calculated to induce a significant increase of tumours in Swiss mice (25 g mouse, average life span: 70 weeks): 10 mg 4-(hydroxymethyl)benzenediazonium sulfate, 1 800 mg N²-[γ-L(+)-glutamyl]-4-carboxyphenylhydrazine, 2 400 mg 4-(carboxy)phenylhydrazine and 23 kg *A. bisporus*. Assuming that the susceptibility of the Swiss mouse to the carcinogenic stimulus is similar to humans, and that an average person has a weight of 70 kg and a life span of 50 years, Toth and Gannett (1993) calculated that the following amounts of these compounds would be required to induce tumours in man: 28 g 4-(hydroxymethyl)benzenediazonium sulfate (corresponding to 2.6 kg mushroom/day for 50 years, with mushrooms containing 0.6 mg/kg of the compound), 5.04 g N²-[γ-L(+)-glutamyl]-4-carboxyphenylhydrazine (corresponding to 6.7 kg mushroom/day for 50 years, with mushrooms containing 42 mg/kg of the compound), 6.72 g 4-(carboxy)phenylhydrazine (corresponding to 36 kg mushroom/day for 50 years, with mushrooms containing 10 mg/kg of the compound) and 350g *A. bisporus* per day for 50 years. The authors estimated that the phenylhydrazines studied only accounted for a fraction of the carcinogenicity demonstrated for raw *Agaricus bisporus*. It should be stressed that no dose-response studies were performed with the phenylhydrazines of the mushroom or with the mushroom itself. At the single dose used the most sensitive tumour type was induced in approximately one third of the exposed animals. Therefore, it is very likely that the induction of tumours could be demonstrated at lower dose levels as well. The calculated exposure level that would give rise to tumours in man is related to that high tumour incidence.

More recently, Swiss investigators updated their risk assessment of agaritine in the cultivated mushroom, now basing it on *in vivo* mutagenicity studies in transgenic Big Blue mice (Shepard et al., 1995). Their studies revealed that the mutation frequencies were increased by 100% in the kidney and by 50% in the forestomach of mice fed during 105 days a powdered chow containing a mushroom extract with 30% agaritine (120 mg agaritine/kg body weight/day). Based on the assumption that fresh, cooked and canned mushroom on average contain 150 mg agaritine/kg mushroom and that the per capita consumption of the cultivated mushroom is 4 g a day for a Swiss consumer, the investigators estimated a daily agaritine exposure of 0.6 mg/person. This would correspond to approximately 10 µg/kg body weight for a person weighing 60 kg. Using the TD₅₀ value (see section on mutagenicity) of agaritine derived from the *in vivo* mutagenicity data in Big Blue mice, a rough estimate of the cancer risk at the calculated human exposure level of agaritine was obtained by a linear extrapolation. The lifetime cancer risk from an average daily *A. bisporus* consumption was estimated to 20x10⁻⁶, based solely on the genotoxic properties of the mushroom. Substances in the mushroom that might positively or negatively influence the carcinogenicity through non-genotoxic mechanisms are not detected in this *in vivo* assay and have not been included in the analysis (Shepard et al., 1995). This estimate of cancer risk is based on mutagenicity data from a very low number of animals.

12. Present nordic risk assessment

Due to the high content of biologically active phenylhydrazines in *Agaricus bisporus*, coupled with the popularity of this mushroom in the European kitchen, several attempts have been made to estimate the potential risk related to consumption of *Agaricus bisporus* in man as discussed in the previous chapter.

In 1991 the Nordic Working Group on Food Toxicology and Risk Evaluation (NNT) expressed the opinion that published toxicological studies give rise to serious concern as to a possible human health risk from consumption of the cultivated mushroom. As no epidemiological data were available and the long term carcinogenicity studies with the mushroom and its hydrazine constituents had an inadequate standard or apparently were contradictory, it was not possible to evaluate the human risk at that time (Gry and Pilegaard, 1991).

Since then several experimental studies have become available that could contribute to the *present risk assessment*:

12.1. Hazard identification.

Agaritine (β -N-[γ -L-(+)-glutamyl]-4-hydroxymethylphenylhydrazine) was identified in *A. bisporus* already in the early 1960s. The level of agaritine found in fresh mushrooms is between 80 and 1 700 mg/kg, with average values between 200 and 400 mg/kg. In addition to agaritine, the cultivated mushroom also contains small quantities of 4-(carboxy)phenylhydrazine (10-11 mg/kg fresh weight), β -N-[γ -L-(+)-glutamyl]-4-(carboxy)phenylhydrazine (42 mg/kg fresh weight) and 4-(hydroxymethyl)benzene diazonium ions (0.6-4 mg/kg fresh weight). In addition to a limited number of natural hydrazine derivatives, the industry has produced a number of synthetic compounds for industrial use. Since most of the synthetic hydrazines have significant biological activity, and many are toxic and/or carcinogenic, Toth and co-workers undertook the work to assess the possible carcinogenic activity of the phenylhydrazines and related compounds in *A. bisporus*. Their studies indicated that most of the phenylhydrazines and related compounds in the mushroom are carcinogenic in Swiss albino mice. The only compound that was tested negative was agaritine, a finding that significantly muddled the interpretation of the carcinogenicity data. The suspicion that the phenylhydrazines and the benzenediazonium ion in the mushroom are carcinogenic has subsequently been strengthened by biochemical and mutagenicity studies. Furthermore, the cultivated mushroom *per se* has been shown to possess mutagenic and carcinogenic activity.

12.2. Hazard characterization.

Absorption and metabolism. Studies in experimental animals have shown that agaritine, and/or its degradation products, are to a significant degree and rapidly absorbed from the gastro-intestinal tract. Blood levels peak within a few hours, but no unchanged agaritine is detected. No major qualitative differences in the pharmacokinetic behaviour of agaritine in the rat and mouse have been observed, and, furthermore, the metabolic profile of this compound seems to be the same in both species. None of the metabolites have been identified. Nonetheless, the enzyme γ -glutamyl transpeptidase, present in various tissues of the body, is believed to be the enzyme responsible for cleaving agaritine into L-glutamic acid and 4-hydroxymethylphenylhydrazine. The latter compound is subsequently transformed to HMBD. It is the HMBD that is believed to produce reactive radical species able to damage macromolecules.

Subchronic toxicity. Very little is known about the subchronic toxicity of mushroom phenylhydrazines. The only information of importance is the rather low toxicity found for 4-(carboxy)phenylhydrazine in dogs, rabbits and guinea pigs.

Teratogenicity. There are no indications that *A. bisporus* or the phenylhydrazines occurring in the mushroom are embryotoxic or teratogenic in mammals at biologically relevant doses. However, it should be stressed that the design of the few studies available did not follow modern guidelines for these types of study. It should also be stressed that very few toxicity studies other than carcinogenicity studies have been performed with the phenylhydrazines in the mushroom.

DNA-binding and mutagenicity in vitro and in vivo. Much more interest has been focused on whether the phenylhydrazines may induce cancer or not. A low frequency of DNA damage has been observed in various tissues of experimental animals exposed orally to agaritine and HMBD, an observation indicating that these compounds may be genotoxic carcinogens. This interpretation is supported by the observation that mushroom extracts, as well as agaritine and the other phenylhydrazines occurring in *A. bisporus*, induce a low frequency of mutations in bacterial test systems *in vitro*. Furthermore, *in vivo* studies on mice have revealed that agaritine induces gene mutations in transgenic animals and that the 4-(hydroxymethyl)benzediazonium ion induces micronuclei in peripheral lymphocytes.

Carcinogenicity. Several controversial carcinogenicity studies with *A. bisporus* and its phenylhydrazine constituents have shown carcinogenic effects in Swiss mice; the fraction of treated animals with the highest increase of a specific tumour compared to the controls is shown in Table 18. Thus, in three out of four long-term carcinogenicity studies tumours were induced in various tissues of mice fed raw, baked or freeze-dried *A. bisporus*. In the fourth study, with baked mushrooms but with another (more balanced) feeding schedule, the increase in tumour incidence was not significant. No tumours were induced in two long-term studies in rats, but in these rather inadequate studies the number of animals were too low to detect a relatively small increase in tumour incidence. Furthermore, the processing (pre-treatment) of the mushrooms (ordinary drying and milling, or washing, combined with pressure cooking, homogenising and mixing with tap water) might well have resulted in a significant degradation of the potentially active phenylhydrazines in these studies. Three of the four phenylhydrazines and related compounds known to occur in *A. bisporus* - CPH, GCPH and HMBD - were carcino-

genic at high doses when administered orally (by gavage or in drinking water) and also when

Table 18. Fraction of treated animals with the most pronounced increase of a specific tumour.

Parameter	Material or compound tested in the cancer study (reference)					
	Fresh raw mushroom (Toth and Ericson, 1986)	Dry-baked mushroom (Toth et al., 1997a)	Freeze-dried mushroom (Toth et al., 1998)	CPH (McManus et al., 1987)	GCPH (Toth, 1986a)	HMBD (Toth et al., 1982)
Daily exposure per mouse	4.7 g	5.57 g	1.25 g*	1250 mg/l drinking water for life	1400 mg/kg body weight/1 day weekly for 52 weeks	Single dose 400 mg/kg body weight
Fraction of tumour-bearing animals with the highest increase of a specific tumour (type of tumour)	38% (fore-stomach in males)	20% (fore-stomach in females, glandular stomach in males)	22% (lung in males)	38% (aorta and large arteries in males)	26% (subcutaneous tissue in males)	32% (glandular stomach in males)

* 1.25 g freeze-dried mushroom approximately corresponds to 12.5 g raw mushroom

injected subcutaneously in Swiss mice. The fourth - and major - phenylhydrazine, agaritine, was found not to be carcinogenic when given in drinking water or by subcutaneous injection - but no precautions were taken in these studies to protect against a possible oxidative degradation of agaritine in water. Agaritine has recently been shown to be vulnerable to such degradation in aqueous solution (Hajkova et al., submitted).

It should be stressed that dose-response effects were only investigated in one of the carcinogenicity studies. In this study no significant dose-response effect could be demonstrated. Otherwise, the studies only contained two groups, one control group and one exposed group, with 50 animals of each sex in each group. It should also be noted that the tumour profiles induced by the various mushroom phenylhydrazines and by the mushroom *per se* differ.

12.3. Exposure characterization.

The estimated annual per capita intake of the cultivated mushroom (*Agaricus bisporus*) varies between the Nordic countries - being 0.6 kg in Finland having the lowest intake among Nordic countries and 2.4 kg in Sweden with the highest intake. Also the habit of using fresh mushrooms for dietary purposes varies between countries. The market share of fresh mushrooms was between 33 and 70%. The rest being preserved mushrooms, mainly sold in cans.

Since the per capita intake is an estimation of average intake and does not take into consideration the fact that mushrooms usually are consumed only by a part of the population, the per capita consumption is too low to express the consumption among mushroom eating consumers. Danish data, based on a limited consumer study, indicate that only around 50% of the population consume the cultivated mushroom, and that 5% of the population consume five times the median intake and 0.1% thirty times the median intake.

The potentially toxic compounds in the cultivated mushroom are the phenylhydrazines. The most abundant compound being β -N-[γ -L-(+)-glutamyl]-4-hydroxymethylphenylhydrazine (agaritine) which usually occurs in quantities between 200 and 400 mg/kg in the fresh mushroom. Sometimes levels up to 1700 mg/kg have been found. An anticipated metabolite of agaritine, the 4-hydroxymethylbenzenediazonium ion (HMBD), is believed to be the most potent phenylhydrazine but it occurs at low levels, between 0.6 and 4 mg/kg fresh weight. The other phenylhydrazine derivatives in the mushroom are 4-(carboxy)phenylhydrazine (CPH, 10-11 mg/kg fresh weight) and γ -glytamyl-4-(carboxy)phenylhydrazine (GCPH, 16-42 mg/kg fresh weight).

The data on the influence of food processing on the amount of phenylhydrazines in the consumed mushroom are scanty. Frozen and freeze-dried mushrooms seems to contain similar agaritine levels as fresh mushrooms, whereas air-dried, cooked and fried mushrooms contain reduced levels, but to what extent is not well known. Canned mushrooms only contain up to 10% of the agaritine level in fresh mushrooms.

Taken together, these data allows an estimation of the per capita exposure to agaritine in the Nordic countries. Agaritine was used as a representative of the phenylhydrazines in the mushroom. The estimated exposure in the Nordic countries is somewhere between 48 and 788 mg agaritine/year, corresponding to a daily exposure of between 2.1 and 36 μ g/kg body weight. The exposure being influenced by the quantity of mushrooms consumed (nationality), the amount of agaritine in the fresh mushroom (200-400 mg/kg) and the preference of using fresh instead of processed mushrooms (33-70%). The effect of household processing of the mushroom on the agaritine content was otherwise not taken into consideration. A single mushroom dish with 75 g fresh *A. bisporus* would result in an exposure to 15-33 mg agaritine, that is, 250-550 μ g/kg body weight.

12.4. Risk characterization.

Six carcinogenicity studies in mice with oral application (by gavage, in drinking water or in the diet) were considered in combination with an estimated human intake to *A. bisporus* in the Nordic countries for the sake of performing a quantitative risk assessment of the cultivated mushroom. Of these studies, three were long-term studies with *A. bisporus per se*, and three were long-term studies with the phenylhydrazines and related compounds occurring in the mushroom. The calculation of the human lifetime cancer risk at average intake of *Agaricus bisporus* (or the corresponding amounts of the phenylhydrazines in the mushroom) in the Nordic countries are presented in Table 19. The calculations were done by linear extrapolation from high to low dosages using one-dose carcinogenicity studies in Swiss mice (assuming man and mouse having identical metabolism and sensitivity). The exercise was based on a number of additional assumptions: the weight of a mouse on average being 25 g and that of man 60 kg; the average life span of a mouse being 70 weeks; there is no reduction in weight during dry-baking

of mushrooms for 10 minutes; freeze-dried mushrooms weighing 10% of raw mushrooms; and the influence of processing on phenylhydrazine content of mushrooms not being taken into consideration.

Table 19. Estimated life-time human cancer risk from the intake of *Agaricus bisporus* in the Nordic countries (average of intakes in Denmark, Iceland, Norway and Sweden). (Finnish figures within brackets.)

Parameter	Material or compound tested in the cancer study					
	Fresh raw mushroom (Toth and Erickson, 1986)	Dry-baked mushroom (Toth et al., 1997a)	Freeze-dried mushroom (Toth et al., 1998)	CPH (Mc-Manus et al., 1987)	GCPH (Toth, 1986a)	HMBD (Toth et al., 1982)
Daily exposure per mouse	4.7 g	5.57 g (males)	1.25 g*	1250 mg/l drinking water for life	1400 mg/kg body weight/1 day weekly for 52 weeks	Single dose 400 mg/kg body weight
Daily exposure per kg body weight (mouse)	189 g	229 g	50 g	204 mg	149 mg	0.82 mg
Daily human intake of mushroom per kg body weight	0.1 g (0.025 g)	0.1 g (0.025 g)	0.1 g (0.025 g)	0.1 g (0.025 g) (containing 10-11 mg CPH/kg)	0.1 g (0.025 g) (containing 16-42 mg CCPH/kg)	0.1 g (0.025 g) (containing 0.6-4 mg HMBD/kg)
Human cancer risk due to life-long exposure	193 x 10 ⁻⁶ (52x10 ⁻⁶)	86 x 10 ⁻⁶ (23x10 ⁻⁶)	211 x 10 ⁻⁶ (56x10 ⁻⁶)	1.8-2.0 x 10 ⁻⁶ (0.5x10 ⁻⁶)	2.7-7.0 x 10 ⁻⁶ (0.7-1.9 x10 ⁻⁶)	23-150 x 10 ⁻⁶ (6-40x10 ⁻⁶)

* 1.25 g freeze-dried mushroom approximately corresponds to 12.5 g raw mushroom

Thus, with all the uncertainties mentioned above in mind the calculations result in an estimated lifetime human cancer risk for an “average“ Nordic consumer of fresh (or freeze-dried) *A. bisporus* up to 200 x 10⁻⁶. In reality an average consumer will consume a mixture of fresh and processed mushrooms. Some types of processing (i.e. canning) will reduce the phenylhydrazine content of the mushroom substantially, whereas other types of processing seem to have a much less pronounced effect. However, the influence of standard cooking procedures on the phenylhydrazine content of the cultivated mushroom is not very well studied. Of the phenylhydrazines and related compounds investigated in carcinogenicity studies and occurring in the mushroom, HMBD had the highest carcinogenic potency. This finding agrees with what is expected based on the anticipated mechanism of action.

The human exposure estimation (per capita intake) is based on production volume information which suffers from a number of shortcomings. In Denmark, for example, around half of the adult population claim not to consume the cultivated mushroom, 5% eat more than five times the median intake and 0.1% thirty times more than the median intake. Non-consumers of the cultivated mushroom would of course not have an additional cancer risk from the phenylhydrazines in the mushroom, whereas sub-populations of “high” consumers would have correspondingly higher cancer risks. It should be noticed that the Finnish population only consumes one quarter of what is consumed of the cultivated mushroom in the other four Nordic countries. The estimated lifetime cancer risk for the average Finnish consumer should accordingly be lower than in the other Nordic countries.

Clearly, the carcinogenic potential of *A. bisporus* and the possible contribution of agaritine are difficult to assess in the face of contradictory experimental data, which may be due to the different exposure protocols employed in the carcinogenicity studies. In contrast to *A. bisporus*, its major phenylhydrazine, agaritine, did not induce tumours when administered in the drinking water to mice. Therefore, no risk assessment has been made for this compound. However, it may be anticipated that due to the study design, agaritine could have been significantly degraded in the drinking water.

It must be underpinned that the long-term carcinogenicity studies on which the risk assessment has been made, might be criticised for several reasons. Only a single mouse strain, the Swiss mouse, has been used in the studies. In contrast to most mouse strains, which are inbred, the Swiss mouse is a randomly bred strain. This results in a heterogeneity in sensitivity between various animals. The studies have often used improper controls – either a historical control group, or a control group that was not started at the same time as the exposed group. In stead of carrying out the carcinogenicity studies in the normal way of exposing the animals for a predetermined time period (usually 2 years), the studies were continued until the mice died or were found in poor condition. Standard carcinogenicity studies have three dose groups but these studies have only used a single dose group. Some of the studies have been criticised for resulting in an unbalanced diet. In general a number of biochemical and toxicological endpoints are studied besides the main end-point (tumours) in order to be able to interpret the data. Such studies have rarely been reported in the carcinogenicity studies with the cultivated mushroom and its phenylhydrazines.

Conclusion: The carcinogenic effect of three of the four phenylhydrazines (4-(carboxy)phenylhydrazine (CPH), 4-(hydroxymethyl)benzenediazonium ion (HMDB), β -N-(γ -L-glutamyl)-4-carboxyphenylhydrazine (GCPH)) or related compounds occurring in the “Cultivated Mushroom” (*Agaricus bisporus*), as well as the fresh, baked and freeze-dried mushroom *per se* in mice is taken as the critical effect.

The carcinogenicity has been investigated only in an out-bred mice strain, the Swiss albino mice; not in two animal species as usually required for carcinogenicity testing.

Various *in vitro* and *in vivo* studies have shown that *A. bisporus* extracts contain mutagenic activity, and that the mushroom constituents agaritine and HMDB are mutagenic. Except for the latter compound (HMDB), the mutagenic activity is very weak. These findings suggest that consumption of *A. bisporus* might impose a risk for the consumer. The magnitude of the risk is difficult to estimate. However, using data obtained in the mouse studies and extrapolating to mean human consumption in the

Nordic countries by the use of a linear model, indicate that in the worst case life-time risk might be in the order of $50\text{-}200 \times 10^{-6}$. For most consumers the actual risk is probably lower than this estimate both because of the conservative risk model used and because processing (e.g. boiling, frying, microwave heating or drying) will reduce the agaritine content in *A. bisporus* by up to 50% or even more. Further, it is anticipated that the content of the other phenylhydrazine derivatives also will be reduced during processing.

13. Recommendations

In order to perform a more well founded risk assessment of the phenylhydrazines in *Agaricus bisporus* the present report has revealed that more studies are required particularly in relation to the following subjects:

Carcinogenicity study

- A carcinogenicity study of fresh *Agaricus bisporus* with an appropriate feeding protocol using several doses on rats should be performed.

Other toxicity studies

- The metabolites/degradation products of *Agaricus bisporus* phenylhydrazines occurring in blood of consumers of the mushroom should be identified.
- The role of γ -glutamyl transferase *in vitro* and *in vivo* should be further studied.
- The DNA-binding of agaritine should be investigated further after appropriate conditions of treatment.
- The mutagenic activity of agaritine, 4-(carboxy)phenylhydrazine, β -N-(γ -L(+)-glutamyl)-4-(carboxy)phenylhydrazine and 4-(hydroxymethyl)benzenediazonium ion in mammalian cells *in vitro* should be investigated.

Chemical studies

- The occurrence of 4-(carboxy)phenylhydrazine, β -N-(γ -L(+)-glutamyl)-4-(carboxy)phenylhydrazine and 4-(hydroxymethyl)benzenediazonium ion in *Agaricus bisporus* should be confirmed.
- The content of agaritine and other phenylhydrazines in other (wild and cultivated) *Agaricus* species should be studied.
- The influence of storage and household processing conditions on the levels of 4-(carboxy)phenylhydrazine, β -N-(γ -L(+)-glutamyl)-4-(carboxy)phenylhydrazine and 4-(hydroxymethyl)benzenediazonium ion in *Agaricus bisporus* should be investigated.
- The degradation products of agaritine and other phenylhydrazines formed in *Agaricus bisporus* under household conditions should be identified.

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