Mushroom Ninhydrin-Positive Compounds

Amino Acids, Related Compounds, and Other Nitrogenous Substances Found in Cultivated Mushroom, *Agaricus campestris*

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A fraction, reactive to ninhydrin, was isolated from the edible, commercial mushroom, *Agaricus campestris*, by a novel isolation procedure. This isolate, when analyzed by an automatic amino acid analyzer, was found to contain amino acids, related compounds, and other nitrogenous substances heretofore undetected by previous investigators. The more important compounds definitely identified for the first time were α-aminoadipic acid, β-aminoisobutyric acid, canavanine, carnosine, creatinine, cystathionine, 2,4-diaminobutyric acid, homocystine, homoserine, hydroxylysine, kynurenine, and sarcosine. Other compounds were tentatively identified. These substances could be important from the nutritional and metabolic standpoint. Their presence may also contribute other physiological benefits to the consumer. Furthermore, the results of the present work shed new light on the complexity of the higher fungus.

The flavor-contributing properties of edible mushrooms have been known for a long time. As a departure from the investigation of mushrooms as a condiment, some workers (Anderson and Fellers, 1942; Mendel, 1898; Randoin, 1956) have studied the nutritional values of these higher fungi, thereby establishing them as a foodstuff having low calorific and good nutritional values.

Recently, one phase of mushroom study, which has attracted much attention, has been the separation and identification of the chemical constituents, particularly amino acids, present in the sporophores (fruiting bodies) of mushrooms, selected from different taxonomical families, such as *Agaricus campestris*, *Boletus edulis*, *Lactarius vellereus*, *Tricholoma georgi*, and many others. As a result, the amino acid constituents, both free (not derived from protein hydrolysis) and bound (protein-derived), have been investigated to some extent by Bano *et al.* (1964), Baglioni and Settimj (1927), Close (1960), Fitzpatrick *et al.* (1946), Hughes *et al.* (1958), Hughes and Rhodes (1959), Heinemann and Casimir (1961), and Inagaki (1934). Casimir and Trzcinski (1952) and Latche (1963) have extended this study by determining the distribution of the amino acids throughout the different parts (carpophore, pileus, and hymenium) of the mushroom sporophore, while Block *et al.* (1950) determined qualitatively the amino acids in the mycelium of *Agaricus blazei*. In these investigations, simple solvent extracts of the mushrooms were generally used. The methods of analysis (microbiological, animal feeding, paper chromatography, paper electrophoresis, and chemical tests) were laborious, time-consuming, and gave essentially qualitative and semiquantitative results.

Although much work has been performed in the past regarding the separation and detection of free and bound amino acids present in the extracts of fresh mushrooms from various species and genera of the family, Agaricaceae, few studies have been carried out in this area, particularly with respect to the free amino acids contained in *A. campestris*. Hughes *et al.* (1958), Hughes and Rhodes (1959), Heinemann and Casimir (1961), and Kissmeyer-Nielsen *et al.* (1966) have investigated extracts from this species (Table III).

At the present time, there is not sufficient knowledge regarding the presence of new and less common, free amino acids and other nitrogenous substances in the mushroom. Some of these compounds are present only in minute quantities. Conceivably, one reason for this situation has been the practice of studying solvent extracts derived from simple isolation procedures, as shown in Table III. To remove the less easily isolable substances from the mushroom, a new approach may be required—namely, the use of a more comprehensive isolation procedure, which would include steps for the efficient solvent fractionation and concentration of the compounds into a suitable concentrate or fraction. This fraction could then be assayed by a modern, high-resolution analytical instrument, such as an automatic amino acid analyzer, based on the standard biochemical method of Spackman, Stein, and Moore (1958). The introduction of the automatic and continuous ion-exchange chromatographic technique by these workers for the accurate separation, identification, and quantitative determination of amino acids and other ninhydrin-positive compounds in unknown mixtures has fulfilled the need for a method of analysis which does not possess the shortcomings of the earlier conventional methods. Kissmeyer-Nielsen *et al.* (1966) have recently used the Technicon AutoAnalyzer for assaying simple 2-propanol extracts of the edible mushroom, *A. bisporus*.

The present paper presents the results obtained by the use of a novel isolation procedure, which included features of both solvent fractionation and other methods of concentration of the compounds into a suitable fraction. This procedure was intended for the study of the less common, and heretofore undetected, amino acids and other nitrog-

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enous compounds present in the fresh, white, edible, and commercially cultivated mushroom, *Agaricus campestris* L. ex Fr. (Agaricaceae), sometimes referred to as *A. bisporus* or *Psalliota campestris*. The fraction obtained by this procedure was subsequently assayed by a Phoenix automatic amino acid analyzer.

EXPERIMENTAL

Material, Instrumentation, and Isolation Procedure. The fresh mushrooms (tight-button stage and harvested in July) were purchased from the Bay State Mushroom Co., Sudbury, Mass. These were grown under standard conditions required for commercial production and human consumption.

The analysis of the mushroom fraction was performed on a Model K-8000-C automatic recording amino acid analyzer (Phoenix Precision Instrument Co., Philadelphia, Pa.), a high speed analytical instrument based on the Moore-Stein dual column system, step elution technique. It utilizes the new Phoenix Spherix, high resolution ion-exchange resins for protein hydrolyzates in connection with high pressure columns for accelerated analyses. The method of analysis used with this instrument is essentially the one described by Spackman *et al.* (1958) as applied to physiological fluids, such as human urine (Stein, 1953) and blood plasma (Stein and Moore, 1954), except that the method now employed is on an accelerated basis. Specifications for the reagents, standard mixtures of amino acids, resins, column dimensions, buffers, and flow rates (buffer, ninhydrin, chart) for the acidic and neutral amino acids and for basic ones are given in the operational manual. The dual temperature system, 30° to 50° C. and 50° C., was used for both types of amino acids. The buffer solution (pH 3.25) was modified by the addition of 5% propanol.

Whenever possible, the concentration of a substance eluted from the columns was determined by the integration of the area under its peak on the effluent curve and the use of the prescribed calculations given in the manual. Planimetry of the area was resorted to when integration was unfeasible.

Evidence for the presence of an amino acid or related compound was based on the particular time of elution of its peak in the test chromatogram as compared with that of a model compound in a standard run, reference to elution chromatograms of mixtures of known compounds recorded in the literature, and/or comparison of its 440- to 570-mμ wavelength ratio with that found by previous workers for the same compound.

Isolation of Mushroom Fraction. The amino acid fraction was isolated in the following manner. The fresh mushrooms (3.2 kg.) were carefully and rapidly washed, freed of the small root sections, rinsed in distilled water, and dried in air at room temperature for about $2^1/_2$ hours. They were then weighed and ground in separate portions (400 grams) in absolute ethanol (3.3 liters) in a Waring Blendor at 10–15° C. for 5 minutes in a nitrogen atmosphere. The mixture was filtered (slight vacuum) and the moist solid was rehomogenized in 90% ethanol (1.5 liters) under the above conditions and filtered. The filtrates, ranging in color from pale yellow to light red, were stored separately under refrigeration (about 1° C.) for a day or two. Before removal of the solvent, the alcohol extracts were refiltered while cold to remove crystallized mannitol, and distilled (Büchi flask evaporator) in vacuo (50 to 55 mm. of Hg) at 35° to 40° C. to a semifluid mass which was finally dehydrated to constant weight by gradual reduction of pressure to, and kept at, about 4 mm. The colored residue (83.5 grams) was extracted seven times (400, 280, 5 × 200 ml.) with boiling petroleum ether (boiling range 30° to 60° C.) under anhydrous reflux conditions. The insoluble, caked solid was cautiously pulverized with the aid of absolute ethanol (150 ml.), the solvent removed by distillation, and the residue evacuated again to constant weight. The product (66.7 grams), a light brown powder, was shaken (10 × 200 ml.) with wet ether (ether saturated with water) at room temperature, the ether extracts were pooled (pale brown solution), the solvent removed, and the highly viscous residue was dried in vacuum to constant weight (743.6 mg.). The mixture was then stirred at room temperature with minimal methanol (24 ml.). The light red solution was filtered to remove any separated mannitol, diluted (2 × its volume) with methanol, and decolorized at room temperature by agitation with activated carbon (Darco, G-60). After filtration and removal (in vacuo at room temperature) of the solvent from the colorless filtrate, a pale straw-colored, viscous oil (291.3 mg.) was obtained. The oil was subsequently stirred with distilled water (30 ml.), the solution filtered from insoluble matter, the water removed (25–30° C., pressure, 15 mm. slowly reduced to 8 mm.), and the residue completely dehydrated under high vacuum. The procedure afforded 281.4 mg. of a viscous material which was finally agitated at room temperature with absolute ether (7 × 3.3 ml.). The insoluble fraction, after removal of the occluded solvent and reduction of the weight to a constant value, gave 181.7 mg. (0.006% of the initial weight of fresh mushrooms) of the desired amino acid isolate, as a slightly colored, highly viscous product. It was positive to ninhydrin and contained approximately 3.6% nitrogen. By contrast, the absolute ether–soluble fraction was ninhydrin-negative.

Individual solutions, prepared by dissolving this isolate (16.83 mg.) in buffer solution (1 ml.), were analyzed, in a manner similar to that used for physiological fluids, on the automatic amino acid analyzer (Tables I, II, and III).

RESULTS AND DISCUSSION

Tables I and II show 53 free, ninhydrin-positive compounds isolated and concentrated into one fraction by the novel procedure. The compounds in the fraction were then separated and recorded by the analyzer. Thirty-eight of these substances have been definitely identified. The amino acids and other substances detected for the first time in *A. campestris* were: sarcosine, homoserine, α-aminoadipic acid, cystathionine, homocystine, β-aminoisobutyric acid, hydroxylysine, 2,4-diaminobutyric acid, kynurenine, canavanine, creatinine, carnosine, ethanolamine, phosphoserine, phosphoethanolamine, *S*-methyl-L-cysteine sulfoxides and sulfone, methionine sulfoxides, γ-L-glutamyl-*S*-methylcysteine, L-lanthionine, *meso*-lanthionine, glucosamine, 3,4-dihydroxyphenylalanine (DOPA), galactosamine, 1-methylhistidine, and 3-methylhistidine. The presence of the last 12 substances in this series could not be definitely proved, and therefore their

Table I. Ninhydrin-Positive Compounds in the Mushroom Fraction

Acidic and neutral amino acids and related compounds arranged according to elution sequence from the columns

Compound	Amount Present, μmoles/100 Mg. of Fraction
Phosphoserine[a]	0.42
Phosphoethanolamine[a]	Trace[b]
Taurine	0.30
Urea	13.25
S-Methyl-L-cysteine sulfoxides and sulfone[a]	0.47
Methionine sulfoxides[a]	0.47
γ-L-Glutamyl-*S*-methyl-L-cysteine[a]	0.36
Aspartic acid	1.43
Threonine[c]	3.03
Serine	4.16
Asparagine + glutamine[d]	3.27
Sarcosine[e]	...[f]
Homoserine	0.18
L-Lanthionine[a]	Trace
meso-Lanthionine[a]	Trace
Glutamic acid	10.34
Proline	4.99
Unknown 1[g]	Trace
Unknown 2	Trace
Glycine	1.72
α-Alanine	17.53
α-Aminoadipic acid	1.66[h]
α-Aminobutyric acid	0.42[h]
Cystine	3.45
Valine[c]	3.45
Cystathionine	0.42
Methionine[a,c]	Trace
Isoleucine[c]	3.39
Leucine	5.05
Glucosamine[a]	Trace
3,4-Dihydroxyphenylalanine[a]	0.18
Tyrosine	0.36
Phenylalanine[c]	1.72
Homocystine	0.83[h]
β-Alanine[e]	...[f]
β-Aminoisobutyric acid	Trace

[a] Presence very possible.
[b] Less than 0.06 μmole/100 mg. of fraction for trace compounds.
[c] Nutritionally essential to man.
[d] Not separated by the amino acid analyzer.
[e] Appears as a shoulder in the chromatogram.
[f] Cannot be calculated.
[g] Not citrulline.
[h] Approximate value.

Table II. Ninhydrin-Positive Compounds in the Mushroom Fraction

Basic amino acids and related compounds arranged according to elution sequence from the columns

Compound	Amount Present, μmoles/100 Mg. of Fraction
Galactosamine[a]	Trace[b]
Hydroxylysine	0.59
γ-Aminobutyric acid	12.00
Ornithine	0.12[c]
2,4-Diaminobutyric acid	1.07[c]
Ethanolamine	0.71[c]
Ammonia	34.70
Lysine[d]	0.42[c]
Kynurenine[e]	...[f]
1-Methylhistidine[a]	Trace
Histidine[d]	0.06
3-Methylhistidine[a]	Trace
Canavanine	6.24
Tryptophan[d]	Trace
Creatinine	87.94[c]
Carnosine[g]	...[f]
Arginine[d]	0.53

[a] Presence very possible.
[b] Less than 0.06 μmole/100 mg. of fraction for trace compounds.
[c] Approximate value.
[d] Nutritionally essential to man.
[e] Tends to tail as a shoulder on lysine.
[f] Cannot be calculated.
[g] Appears as a shoulder in the chromatogram.

identifications were considered tentative. Two unidentified compounds were also present in the chromatograms. The tentative identification of 3,4-dihydroxyphenylalanine is supported by the findings of Hughes (1961). Phosphoethanolamine, methionine sulfoxides, α-aminoadipic acid, and ethanolamine have been reported by Craske and Reuter (1965) in *Boletus edulis*. Taurine and ammonia have been recently reported by Kissmeyer-Nielsen *et al.* (1966) in *A. bisporus*.

Of the 10 nutritionally essential amino acids present, tryptophan and methionine were detected in trace amounts. Presumably, the latter compound was either present originally in the form of its isomeric sulfoxides, or was converted to the sulfoxides during the isolation of the mushroom fraction.

Asparagine and glutamine failed to separate in both the 30° to 50° C. and 50° C. programs, a behavior also observed by Zacharius and Talley (1962). However, these two compounds are known to be present in *A. campestris* and taxonomically related mushrooms, having been detected by Hughes *et al.* (1958), Jadot *et al.* (1960), Casimir and Trzcinski (1952), and other investigators. It is assumed, therefore, that these substances were also present in the mushroom fraction.

Ornithine was present in a very small quantity. It appeared in the correct places in the chromatograms of both the 30° to 50° C. and 50° C. programs. By contrast, proline and urea were found in appreciable amounts. Some ornithine may have been converted to proline and urea in the mushrooms, since ornithine is known to be a metabolic precursor of proline and an important intermediate in the formation of urea (Krebs-Henseleit urea cycle). In past investigations by other workers, ornithine has not always been detected in *A. campestris* and related species. Paris *et al.* (1957) did not report the presence of this amino acid in their work, while Heinemann and Casimir (1961) listed it as doubtful in *A. campestris*. On the other hand, Hughes and Rhodes (1959) and Kissmeyer-Nielsen *et al.* (1966) have reported its presence in the same species of mushroom.

Some of the newly detected amino acids and related compounds, such as homoserine, homocystine, hydroxylysine, α-aminoadipic acid and phosphoserine, among others, may contribute to the nutritional value of the mushrooms. Vogel (1964) has found that α-aminoadipic acid takes part in the α-aminoadipic acid–lysine pathway in the biosynthesis of lysine (a nutritionally important amino acid) in the basidiomycetes. Recently, free homoserine has been found by Saarivirta and Virtanen (1965) in germinating peas, a highly nutritive foodstuff.

The presence of compounds exemplified by taurine, sarcosine, cystathionine, kynurenine, canavanine, crea-

Table III. Comparison of Present Results with Those of Previous Investigators

Comparison includes one different but taxonomically related mushroom

Ninhydrin-Positive Compounds	Hughes *et al.* (1958)[a]	Hughes and Rhodes (1959)[b]	Heinemann and Casimir (1961)[c]	Craske and Reuter (1965)[d]	Kissmeyer-Nielsen *et al.* (1966)[e]	Present Workers
Total amino acids and other compounds detected	27	30	21	37	21	53[f]
Compounds identified	23	25	20[h]	28	21[g]	38[f]
Compounds tentatively identified	0	0	1	4	0	13
Compounds unidentified	4	5	0	5	0	2

[a] *Agaricus campestris* extracted with 80% ethanol. Extracts analyzed by paper chromatography and chemical tests.
[b] Same as above. Rapid ion-exchange chromatography (gradient elution-pressure method) also used in analysis.
[c] *A. campestris* presumably extracted with ethanol. Analysis performed by paper chromatography and electrophoresis.
[d] Dehydrated *Boletus edulis* extracted with boiling water. Aqueous extracts fractionated by ion-exchange chromatography. Fractions assayed by paper chromatography and spectrophotometry.
[e] *A. bisporus* extracted with boiling 2-propanol. Extracts assayed by an automatic amino acid analyzer.
[f] Unseparated asparagine + glutamine considered as one substance (one peak). Presumably both compounds were present.
[g] Urea determined separately by a modified clinical method.
[h] Unseparated leucines considered as one substance (one spot).

tinine, and carnosine in edible mushrooms may have important physiological implications, other than the nutritional and metabolic aspect, unknown at the present time. In a consideration of these implications, it would be worthwhile to bear in mind known facts about some of these compounds—namely, that taurine is an important constituent of bile acids, that cystathionine is one of the major free amino acids in human brain, a deficiency of which would lead to homocystinuria (Perry *et al.*, 1966), and that creatinine is a normal constituent of human blood.

The tentative identity of γ-L-glutamyl-*S*-methyl-L-cysteine in the mushroom fraction receives some support from the fact that during the past six years, a number of interesting compounds, having the γ-glutamyl moiety in their structures, have been found in mushrooms. Levenberg (1961) isolated β-*N*-(γ-L-(+)-glutamyl)-*p*-hydroxymethylphenylhydrazine, trivially known as agaritine, from *A. bisporus*. Jadot *et al.* (1960) obtained *N*-γ-L(+)-glutamyl-*p*-hydroxyaniline from *A. hortensis*, and Casimir *et al.* (1960) found *N*-γ-glutamylethylamine (theamine) in *Xerocomus badius*.

Tables I and II also show that in the mushroom fraction, the most plentiful of the amino acids, listed in the order of decreasing amount, were α-alanine, γ-aminobutyric acid, glutamic acid, leucine, and proline. Of the related and other nitrogenous compounds, creatinine, urea, and canavanine were the most abundant.

Table III gives a comparison of the results obtained by the present authors with those of previous investigators covering approximately the last 10 years. It includes the results reported by Craske and Reuter (1965) for *Boletus edulis*, a wild, edible mushroom closely related to *A. campestris*, taxonomically.

In view of the interesting results obtained with the novel isolation procedure employed in the present study, it would seem worthwhile to consider briefly some other applications. For example, this method could be applied not only to other mushrooms of different species and genera, both edible and poisonous, but also to other types of complex biological mixtures. Presumably, new and/or previously undetected compounds might be revealed, especially if the fractions obtained were assayed by a modern analytical instrument such as the automatic amino acid analyzer. Valuable knowledge resulting from this research would help to elucidate the chemical composition of natural products from which fractions were derived.

ACKNOWLEDGMENT

The technical advice in the selection and procurement of the basidiomycetes rendered by Elwyn T. Reese of our Microbiology Group; the use of the amino acid analyzer, made available by Martin Kronman of our Biochemical Group; and the aid in the processing of the mushrooms given by Americo A. Deacutis are gratefully appreciated.

LITERATURE CITED

Anderson, E., Fellers, C. R., *Proc. Am. Soc. Hort. Sci.* **41,** 301 (1942).
Bano, Z., Ahmed, R., Shrivastava, H. C., *Indian J. Chem.* **2,** 380 (1964).
Baglioni, S., Settimj, L., *Atti Accad. Lincei* **6,** 377 (1927).
Block, S. S., Stearns, T. W., Stephens, R. L., McCandless, R. F. J., J. AGR. FOOD CHEM. **1,** 890 (1950).
Casimir, J., Jadot, J., Renard, M., *Biochim. Biophys. Acta* **39,** 462 (1960).
Casimir, J., Trzcinski, F., *Bull. Inst. Agron. Sta. Rech. Gembloux* **20,** 178 (1952).
Close, R., *Nature* **185,** 609 (1960).
Craske, J. D., Reuter, F. H., J. SCI. FOOD AGR. **16,** 243 (1965).
Fitzpatrick, W. G., Esselen, Jr., W. B., Weir, E., *J. Am. Dietet. Assoc.* **22,** 318 (1946).
Heinemann, P., Casimir, J., *Rev. Mycol.* **26,** 24 (1961).
Hughes, D. H., Ph.D. thesis, University of Delaware, Newark, Del., 1961.
Hughes, D. H., Lynch, D. L., Somers, G. F., J. AGR. FOOD CHEM. **6,** 850 (1958).
Hughes, D. H., Rhodes, Jr., Y. E., *Mushroom Sci.* **4,** 176 (1959).
Inagaki, B., *J. Pharm Soc. Japan* **54,** 824 (1934).
Jadot, J., Casimir, J., Renard, M., *Biochim. Biophys. Acta* **43,** 322 (1960).
Kissmeyer-Nielsen, E., McClenden, J. H., Woodmansee, C. W., J. AGR. FOOD CHEM. **14,** 633 (1966).
Latche, J. C., *Compt. Rend.* **257,** 2145 (1963).
Levenberg, B., *J. Am. Chem. Soc.* **83,** 503 (1961).
Mendel, J. B., *Am. J. Physiol.* **1,** 225 (1898).
Paris, R., Durand, M., Bonnet, J. L., *Ann. Pharm. Franc.* **15,** 677 (1957).
Perry, T. L., Hansen, S., Bar, H-P., MacDougall, L., *Science* **152,** 776 (1966).
Randoin, L., *Mushroom Sci.* **3,** 59 (1956)
Saarivirta, M., Virtanen, A. I., *Acta Chem. Scand.* **19,** 1008 (1965).
Spackman, H., Stein, W. H., Moore, S., *Anal. Chem.* **30,** 1190 (1958).
Stein, W. H., *J. Biol. Chem.* **201,** 45 (1953).
Stein, W. H., Moore, S. *J. Biol. Chem.* **211,** 915 (1954).
Vogel, H. G., *Am. Naturalist* **98,** 435 (1964).
Zacharius, R. M., Talley, E. A., *Anal. Chem.* **34,** 1551 (1962).

Received for review March 27, 1967. Accepted August 14, 1967.