Abstract:
Both fresh and dry mushrooms, Amanita muscaria, brave been examined chemically and physiologically. The mushrooms were subjected to elaborate fractionations with comparative pharmacological evaluation, to locate the hallucinogen 'pilzatropine' which has been elusive for over a century now. The hallucinogenic principles have been located definitively in a water soluble portion of the mushroom, freed from extraneous materials. Similar water concentrates from four successive batches of mushroom have been found to have LSD-like properties and devoid of muscarinic effects. Minute quantity of a potent crystalline substance, designated 'Z', m.p. I35-38°G (dec), C15H30015K4 (provisional) has been isolated from the water concentrate. Attempts are being made to characterize 'Z', with the micro quantity available. Some indolic components are being separated from mother liquors.

Constituents occur in very small amounts in the mushroom. Isolation procedures have been evolved to minute detail and for easy repetition to obtain constituents in sufficient quantity from large batches of mushroom.

Potassium gluconate and a fixed oil consisting of esters of oleic acid have been isolated for the first time from A. muscaria. Mannitol, fumaric acid and some minor constituents like crystalline substances, m.p. 172-74 °, m.p. I60-65 °, and a β-glycoside, m.p. 106 °C (dec) have been found in the mushroom.

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STUDIES IN THE HALLUCINOGENIC PRINCIPLES OF AMANITA MUSCARIA, L.

by

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A THESIS

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Laboratory, POONA-8, India. He was associated with Prof. R. C. Shah from February 1950 to July 1956 at the N. C. L.

His services were lent on deputation by the C.S.I.R. to the Aligarh Muslim University from the end of July 1956 for a period of three years to help the University in the development of researches on indigenous drugs, used in Unani Medicine. He stayed at the University till April 1958 as a Senior Research Fellow. On May 2, 1958 he sailed for U. S. A. for higher studies on leave of absence.

He has published some papers and taken some patents. These are listed under Appendix I. He was a member of the Institution of Chemists, India; Indian Science Congress Association; Northwest Scientific Association (U. S. A.). He has recently been awarded a grant by the Asia Foundation to defray the cost of membership in the American Chemical Society.

He married Srimathi. Shakuntala in September 1949. They have two daughters, Sumati (10) and Hema (4).

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ABSTRACT

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Constituents occur in very small amounts in the mushroom. Isolation procedures have been evolved to minute detail and for easy repetition to obtain constituents in sufficient quantity from large batches of mushroom.

Potassium gluconate and a fixed oil consisting of esters of oleic acid have been isolated for the first time from A. muscaria. Mannitol, fumaric acid and some minor constituents like crystalline substances, m.p. 172-74°C, m.p. 160-65°C, and a β-glycoside, m.p. 106°C (dec) have been found in the mushroom.

INTRODUCTION

Innumerable investigators, all over the world, botanists, chemists, explorers, mycologists, pathologists, pharmacologists, physiologists and practitioners of psycho-therapy have examined Amanita Muscaria, L., (Fr.) S. F. Gray[82], (L. ex Fr.) Quel.[88], from time to time, with different view points. As a result, an overwhelming mass of literature, at times mingled with seemingly contradictory observations, has accumulated around this mushroom. Naturally, one wonders why this particular mushroom, commonly known as fly agaric attracted so much attention.

Literature Review

Strange beauty is ascribed to Amanita muscaria: "crowned by a vivid red cap dotted with white spots, it was depicted by Walt Disney in the Dance of the Mushrooms with Tschaikowsky's Nutcracker Suite music in Fantasia."[25]

Before A. D. 1256, Albert Magnus[65] noted that the freshly cut mushroom placed in a dish of milk or water killed flies (Musca). The flies were said to have died by ingesting mushroom juice. Linnaeus[63] reported that the mushroom was advocated for killing bed bugs. However, the ability of the mushroom to kill flies has never been confirmed scientifically.

Wassons[85] have dwelt at length on the possible origin of the appellation 'fly' to the mushroom. They believe that the word 'fly' (or bug) was meant originally to describe the temporary demented condition produced by the fungus; derivation from the doubtful toxicity of of A. muscaria towards flies appears to be far fetched.

Explorers and travellers who toured Siberia early in the 18th century described vividly the use of A. muscaria by Siberian tribes in rituals and orgy. These reports [46,79,83,85] have been recounted in recent articles[61,63,82,88] (cf. Appendix 2 for a brief description). It has also been stated that the mushroom was responsible for the so-called Berserk fury of the Viking period. Fabing[25] has quoted references at length in his article "On Going Beserk: a Neurochemical Inquiry" in support of the hypothesis that the furious rage of the Berserks in the heyday of Viking culture, a thousand years ago, was brought about by A. muscaria. Tyler[82] contradicts the above view citing reference to review of literature on this topic by Thorsen[80].

A. Drew of the Department of Neurology at the University of Michigan has described the effects of A. muscaria on a middle-aged tavern keeper who ate the mushrooms accidently in October 1955. This private communication quoted by Fabing[25] is reproduced in Appendix 3. It must, however, be cautioned that in modern times there has not been any authentic case of definitive value to prove scientifically that hallucinations result from eating A. muscaria. Another point to be noted is that instances of death resulting from A. muscaria have been rare, and few.

On Muscarine

Studies in the isolation of the active principles of A. muscaria began nearly 150 years ago. All the investigations centered around mus-

Table I

Low yield of muscarine from *Amanita muscaria*

Authors	Wt. of Fresh Mushroom Processed	Yield
King, H.[50]	25.5 Kg	124 mg. muscarine aurichloride
Kogl, F. et al[51]	1250 Kg	137 mg. crystalline reineckate of muscarine.
Kogl, F. et al[53]	1250 Kg	540 mg. crude muscarine chlorite, admixed with choline chloride.*
Balenovic, K. et al[3]	1136 Kg	710 mg. crude muscarine chloride.*
Eugster, C. H.[20;21] and Waser	124 Kg	260 mg. pure muscarine chloride.

*These figures have been computed by me from the original articles. The authors referred to have not recorded their final yields.

carine, the main alkaloid. The isolation of muscarine calls for a lot of experience, patience and perseverance. The fresh mushrooms contain on an average 0.002% muscarine. About 500 Kg. of mushrooms will be required to isolate one gram of muscarine. Muscarine crystallizes only when relatively pure. During the purification and enrichment followed through biological evaluation, the loss in muscarine ranges about 30%. Table I gives an idea of the poor yeilds of muscarine obtained by four groups of representative investigators, from King[50] in 1922 to present day.

All these investigations were carried out in collaboration with pharmacologists. Biological evaluation, stepwise, of each fraction was found to be essential to locate the particular fraction rich in muscarine. Physiological properties of muscarine, like stopping an isolated frog's heart in diastole were used.

These bioassays were helpful in recording activity of various fractions from the mushroom in terms of muscarinic units and so forth. Yet the bioassays also caused a lot of confusion. _A. muscaria_ contains choline and acetyl choline besides muscarine. Bioassays could not easily distinguish between muscarine and acetylcholine. An isolation scheme based mainly on bioassay could not possibly lead to pure muscarine.

Kogl et al[53] realized this, quite late in their investigations. They reported, in 1957, the following activities on frog's heart.

Muscarine: 2.3×10^8 units/mg.

Acetyl choline: 10×10^8 units/mg.

Eugster and Waser[20] made use of bioassays, but at the same time developed specific partition chromatographic techniques to localise muscarine. Their investigations were extensive, processing 2600 Kg. of the mushrooms continually from 1953 onwards. Wilkinson[88] credited the above authors with success in obtaining the first pure muscarine chloride.

Balenovic et al[3] found that counter-current extraction was not suitable for the separation of reineckates of quarternary bases from A. muscaria. By switching to chromatography with ion-exchange resins and cellulose columns, Balenovic and Stefanac[4] could isolate muscarine pure.

Koehl et al[57] also obtained pure muscarine chloride by preliminary fractionations on resins and purification by chromatography. Eugster and Muller[22] used ion-exchange resins and column chromatography for the isolation of muscarine from Inocybe patouillardi (yield, 0.037%), Inocybe fastigiata (yield, 0.01%) and Inocybe umbrina (yield, 0.003%), during their search for better sources of muscarine.

The structure of muscarine baffled all the investigators, till very recent years. Structural studies resulted in varying degradation products in different laboratories. Functional group analyses and diagnostic reports were erroneous. Chemists considered the problem was intriguing because of stereo specificity of natural muscarine.

By partition chromatography, muscarine was at last obtained pure in milligram quantity but confirmed as a single entity in different laboratories. Immediately after, the structure of muscarine was elucidated, with the use of infrared spectroscopy and X-ray analysis. The various stereoisomers have been synthesized and evaluated pharmacologically.

All the above work leading to structure elucidation, synthesis and physiological properties of muscarine and related products has been the subject of three recent and excellent review articles with extensive bibliography:

1. The History and Chemistry of Muscarine. by S. Wilkinson,[88] Quart. Rev., XV (No. 2), 153-71 (1961). (87 main references, covering the period, 1811-1960).

2. Breve revue d'ensemble sur la chimie de la Muscarine, by C. H. Eugster,[23] (22 references from 1869-1960); Revue de Mycologie, XXIV (No. 5), 369-85 (1959)

3. The Story of Muscarine, by K. Bowden and G. A. Mogey[8]. J. Pharm. Pharmacol., 10, 145-56 (1958) (55 references from 1811-1957).

A chronological history of muscarine from 1811 to 1924 can be found in the first article of Kogl et al[51] in 1931 on A. muscaria.

A. muscaria interested not only the chemists for so long. It became an active source of experimentation for pharmacologists from 1869, with the work of Schmiedeberg and Koppe.[75] Dixon,[19] in 1907 was struck by the similarity of the effects of muscarine reproducing some of the responses, due to the vagus stimulation of the parasympathetic system. He advanced a hypothesis that the vagus nerve liberated a muscarine-like substance which acted as a chemical transmitter of nerve impulses. Dixon's hypothesis met with universal skepticism; he was discouraged from following this promising field of investigation. Dixon's hypothesis was proved to be substantially correct by Hunt and Taveau,[44] and by Dale[16,17,18] in their studies on acetyl choline. Muscarine became a foundation stone of modern pharmacology.

<u>Hallucinogenic factors</u>

Muscarine engulfed like an octopus the chemical and pharmacological study of *A. muscaria.* As a result the original impetus for research on this mushroom, to find out the active principles responsible for the psychotropic action was lost.

Schmiedeberg[76] became curious with the observation of Petri[69] in a dissertation in 1880 that there was a limiting factor in the dose of muscarine required to cause cardiac arrest. Petri mentioned that when the dose of muscarine exceeded the limit, the heart was accelerated instead of being stopped. Petri used in his experiments a commercial sample of muscarine prepared from *A. muscaria* by Gehe and Co. in Dresden. Schmiedeberg investigated an identical sample of commercial muscarine. He concluded that the product contained in addition to muscarine and choline, a significant quantity of a base, similar to atropine in antagonizing the activity of muscarine. Unfortunately, it was not ascertained if (1) this base was a new alkaloid which occurred in *A. muscaria* in some cases (2) this was an artifact formed from other basic compounds during the preparation of muscarine or (3) this was an accidental adulteration with one of the known alkaloids of Solanaceae.

Ten years later in 1891, Kobert[55,56] reported that fly agaric contained besides choline and muscarine, a new active principle, similar to the unknown base of Schmiedeberg. Kobert named this new compound "pilzatropine" or alternatively "muscaridine". He reported that pilzatropine dilated the constricted pupil, accelerated the slowed heart and was finally excreted unchanged in urine.

In 1903, Harmsen[35] could not find any atropine-like substance in the crude muscarine isolated by him from _A. muscaria_ collected in Bavaria, as determined by pharmacological tests on the frog heart and eye of the cat. Harmsen emphasized that his experiments should not rule out the possibility of pilzatropine occurring in the mushroom. He considered it as probable that developmental and environmental factors might influence the occurrence of pilzatropine in _A. muscaria_.

Honda[43], in 1911, isolated from _A. muscaria_ two bases, α- and β-myketosine in very small quantity, not sufficient to allow determination of their physical and chemical properties. Tests on frogs and rabbits indicated that only in relatively large doses, both α- and β- myketosine exhibited hypnosis. Honda refrained from speculating on the relationship of these compounds to pilzatropine.

In 1953, Wieland and Motzel[87] re-examined the fungus, _Amanita mappa_. They concluded that the base from _Amanita mappa_ which was previously named 'mappin' was identical with bufotenine. Here again was an example of coincidence in nomenclature. Mushrooms are also sometimes called toadstools. Bufotenine was first isolated from the secretion of the parotid gland of the common European toad, _Bufo vulgaris Laur._[70]

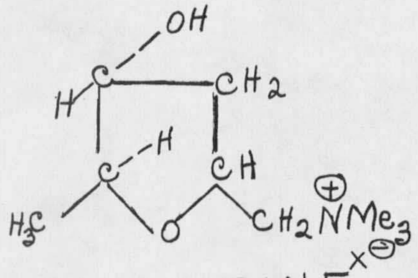
In the same communication Wieland and Motzel reported to have detected the presence of bufotenine in *Amanita pantherina* and in small quantities in *Amanita muscaria* by paper chromatography.

In 1955, Lewis[62] reported the isolation of ℓ-hyoscyamine from *Amanita pantherina* (yield, 1.8 mg/Kg) and from *Amanita muscaria* (yield, 1.1 mg/Kg). Lewis thought that Kobert's pilzatropine might have been ℓ-hyoscyamine. He cautioned that the usual administration of atropine for treatment of mushroom poisoning in general as inadvisable.

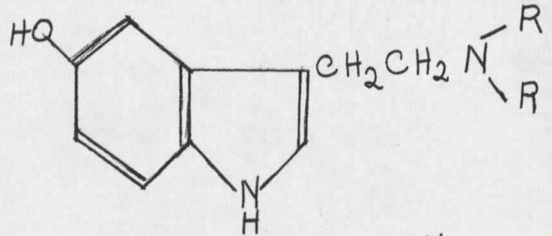
Curiously enough, just a year before the report of Lewis, in 1954, Kwasniewski,[59] examined 10 Kg. of fresh *A. muscaria.* The basic fraction did not dilate pupil of human eye. He concluded that no atropine-like substance was present in the mushroom investigated.

The presence of an atropine-like principle in the panther fungus, *Amanita pantherina* was reported by Inoko[45] towards the end of last century. Fiske[28] examined some poisonous plants of New Jersey, and reported that *A. muscaria* contained the alkaloids, muscarine, choline and atropine. Brady and Tyler[9] examined *Amanita pantherina* recently. They could not detect hyoscyamine or bufotenine by paper chromatography. But in *Panaeolus campanulatus,*[95] Tyler detected serotonin by paper chromatography.

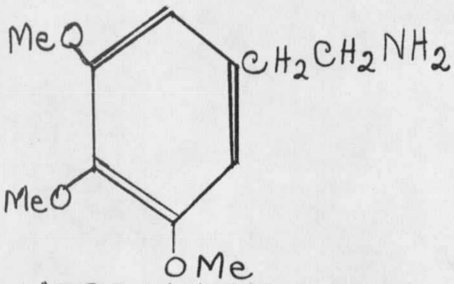
Eugster[23] has been working on *A. muscaria* persistently from 1953. He reports that though 2600 Kg. of the mushrooms were processed in his laboratories, hyoscyamine and bufotenine were not found. In his unpublished investigations with Muller,[23] he detected an indole with a spectrum



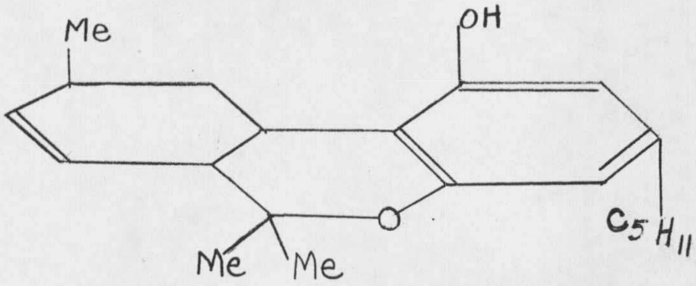
MUSCARINE



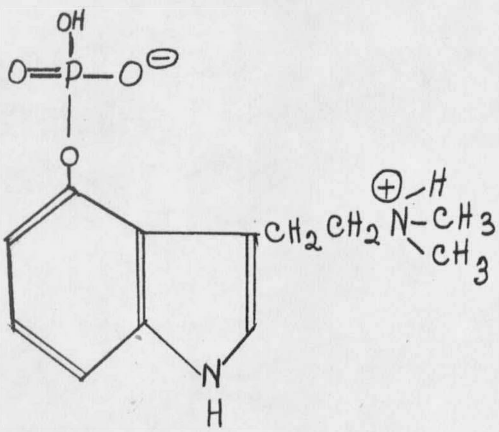
R=R=H, SEROTONIN
R=R=CH_3, BUFOTENIN



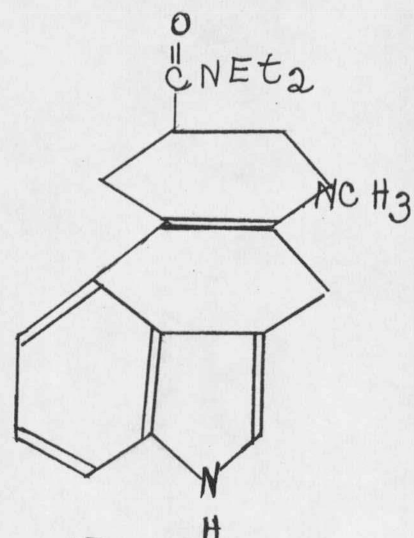
MESCALINE



TETRAHYDROCANNABINOL



PSILOCYBIN



L.SD - 25

Figure 35

Hallucinogens of vegetable origin

practically similar to that of 'psilocybin'.[41] However, the indole from A. muscaria which was suspected to be a 4-hydroxy derivative of indole was so labile that it could not be isolated. It appeared to have chemical properties different from 'Psilocybin'.

The research team headed by Dr. A. Hofmann at the Sandoz Laboratories, Basle, Switzerland have in recent years been screening intoxicating mushrooms of Mexican origin chemically and pharmacologically. They have been fortunate to have Prof. Roger Heim, an able mycologist as an active collaborator for the identification and collection of various species of Psilocybe, Stropharia, Conocybe, etc.[86] From Psilocybe mexicana, Heim, the above team isolated an active psychotropic principle, 'psilocybin'. Psilocybin has been proved to have the structure of phosphoric acid ester of 4-hydroxy-dimethyl tryptamine.[41] This has aroused wide interest adding to the speculation[24,26] that most of the known hallucinogenic materials of vegetable origin (Fig.35) excepting tetrahydrocannabinol are indolic. Pharmacological evaluation of various substituted indoles has also been carried out.[81]

It is of interest in this connection to refer to the 'ololiuqui' problem which has been the subject of research for some years in this laboratory by Cook and Keeland.[47] They isolated from the seeds of Rivea corymbosa, a glucoside to which they advanced a provisional structure on the basis of dehydrogenation studies on the aglycone and possible biogenetic relationships to other natural products. They found that the seeds contained minute quantity of alkaloids which could not be isolated.

Hofmann and Tscherter[42] using thin sheet chromatography have since then succeeded in isolating micro-quantities of three crystalline indole alkaloids identified as lysergic acid derivatives from the seeds of Rivea corymbosa (L.) Hallier Filius. They report that the same alkaloids were also found in Ipomoea tricolor, Cav.

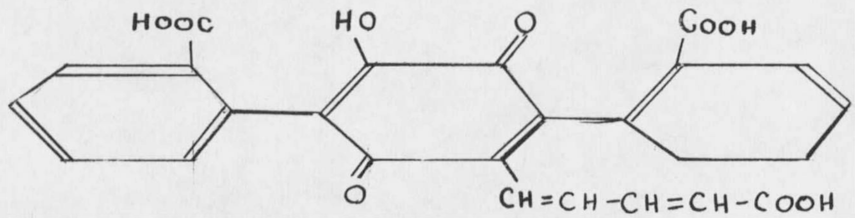
Just as the early erroneous formula of Kogl et al[51] for muscarine is quoted in many recent books on Medicinal chemistry, Biochemistry and Pharmacology, similarly statements have been made to the effect that muscarine is responsible for the psychotropic action of A. muscaria. Even Bowden and Mogey[8] in their recent painstaking review article, quote "the true base (muscarine) is almost certain to be a valuable heuristic tool - it has for instance already given clear confirmation[2] of the presence of atropine-like actions."

Eugster[23] has clarified this issue. He states that muscarine has no action on the central nervous impulses, while the intoxications of the mushroom are due to excitation of the CNS to a great extent.[37] Eugster[23] concludes nostalgically that the problem of muscarine has been solved but the original purpose of research on the hallucinogens of A. muscaria has been forgotten and remains unfulfilled. This conclusion is equally shared by Tyler[82] that "the final chapter of the story of the ambiguous alkaloid, pilzatropine, remains to be written."

Other Constituents of Amanita muscaria

A) Choline and acetyl choline have been encountered by many investigators. In their early work on the colouring matter of the mushrooms, Kogl and

Erxleben[52] isolated an orange yellow pigment, muscarufin, $C_{25}H_{16}O_9$, m.p. 275.5° (yield: 850 mg. from 500 Kg. of A. muscaria), and assigned to it the following structure:



Eugster[23] reports that tests carried out in his laboratory indicate that the red colour of A. muscaria is due to at least 4 compounds of different shades of colour. No one after Kogl has so far succeeded in isolating the colouring matter(s) in a crystalline state. Eugster[23] casts doubt on the terphenyl quinone structure of Kogl for muscarufin, on the basis of absorption spectra.

In a very recent preliminary communication, Kogl, Salemink and Schuller[54] reported the isolation of a new alkaloid, muscaridin (yield: 300 mg. of muscaridin aurichloride, m.p. 129-131°C (uncorrected) from 1035 Kg. of A. muscaria). Muscaridin is reported to be the quarternary trimethyl ammonium salt of 6-amino-2,3-dihydroxy-hexane:

$$CH_3-\overset{CH_3}{\underset{CH_3}{\overset{|}{\underset{|}{N^{\oplus}}}}}-CH_2-CH_2-CH_2-\overset{H}{\underset{OH}{\overset{|}{\underset{|}{C}}}}-\overset{H}{\underset{OH}{\overset{|}{\underset{|}{C}}}}-CH_3$$

$$Cl^-$$

B) Literature on other minor constituents in, and varied properties of A. muscaria collected through Chemical Abstracts from 1907 onwards by the author of this thesis:

Buschmann[12] found in A. muscaria, stored in methanol, one day after gathering, Xanthine and, in greater amount hypoxanthine.

Clark and Kantor[14] processed A. muscaria according to the method of Harmsen[35] for the isolation of muscarine and similar substances. The resulting waxy, semi-crystalline products were injected in lymph sacs of frogs. All the frogs died in a few minutes with the classical symptoms of muscarine intoxication.

Kung[58] reported that from extracts of A. muscaria, choline, putrescine, herzynine (histidine betaine) could be isolated.

Gerber[31] studied the coagulating properties in 86 species of Basidomycetes, on milk, faintly acidulated. He found that Amanita phalloides coagulated boiled or fresh milk at 40-55°; all other Amanitas required 2.5 hours for coagulation of milk.

Ford[30] studied the distribution of poisons in various Amanitas. He reported that A. muscaria contained muscarine, an agglutinin and a hemolysin. The agglutin was a glucoside, resistant to boiling for half an hour. For the isolation of the glucoside, the fungi were extracted with water and filtered through a berkefeld filter. To the filtrate evaporated to a small bulk, ethanol was added in excess. The resulting precipitate was freed from protein by uranyl acetate in alkaline solution. From the protein-free solution, the glucoside was precipitated by basic lead acetate. The precipitate was washed with water and decomposed by bicarbonate. The resulting dark brown filtrate possessed agglutinating powers. After hydrolysis with acid, it reduced Fehling's solution. It gave no colour reactions for pentoses. Ford reported that it was the first agglutinin to be isolated by chemical means; and the only glucoside known to have blood-agglutinating powers.

Radais and Sartory[73] studied immunity of rabbits toward mushrooms. Juice expressed from the caps of a large quantity of mushrooms, preserved with essence of mustard was injected intraperitoneally into rabbits. The minimum lethal dose was 8 c.c. Death occurred in 24 hours, preceded by gastrointestinal disturbance and paralysis. Continued small doses resulted in immunity, reaching a peak at the end of 4 months. But a suspension of injection for one month resulted in loss of resistance and the animals died from 8 c.c. doses.

Zellner[90] found that the substance he had isolated from A. muscaria earlier[89] was not pure ergosterol, but a mixture of two substances. The mixture yielded a new product, $C_{26}H_{40}O \cdot H_2O$, m. p. 159°, and a cerebroside, m. p. 133°. Among the minor constituents, King[50] obtained a phytosterol (yield: 89 mg. from 25.5 Kg. of A. muscaria), m. p. 158-59°C, whose analyses did not agree with $C_{26}H_{40}O$, but with the formula, $C_{26}H_{44}O$. King concluded that it must be ergosterol. He also obtained KCl, leucine, mannitol and fumaric acid. Ludgwig[64] noted the presence of leucine earlier in the mushroom. In an earlier communication, Zellner[89] mentioned that A. muscaria contained propionic and fumaric acids, mykose (trehalose) and other unidentified minor constituents.

Herrmann[40] has given data on the odour and other characteristics for identification of about 40 species of mushrooms. He reported that the odour of mushrooms was due to ethereal oils, resins, terpenes, etc. which need chemical investigation.

Bard and Zellner[5] examined the polysaccharides of various fungi including A. muscaria. Glucose (1.76%), mannitol (6.02%), mycetide (7.40%) and viscosin (5.43%) were found in A. muscaria. The last two components were colloidal and contained pentosans (0.86%) and methyl pentosans (0.62%). Lapicque and Nattan-Larrier[60] reported that the juice of A. muscaria acted in the same manner on nervous and on muscular excitability. Chretien and Leblois[13] studied the toxicity of various mushrooms to dogs. A. muscaria dried at 38° was found to be non-toxic.

Rabb and Renz[72] reported that A. muscaria causes no hemolysis while some other Amanita species do. Binet et al[6] reported that the extract of A. muscaria produced a marked contraction of the bronchioles of the perfused isolated dog lung. Atropine antagonized this action.

The toxic substance was found to be in the cap of the mushroom, especially in the cuticle and not in the footstalk. Similar results were obtained by the same authors[7] with experiments on guinea pig.

A. muscaria grown in Uruguay[29] was found to contain muscarine. Amanita muscarina, Pers., growing wild in Russia[49] was found to contain ascorbic acid: 11 mg. per 100g. of fresh mushroom and 4 mg. per 100g. of dry mushroom. Scandinavian workers detected dihydroxy glutamic acid in A. muscaria.[84]

DISCUSSION AND SUGGESTIONS FOR FURTHER RESEARCH

Important observations can be made as a result of experience gained in the present work and a review of literature on Amanita muscaria.

The ability of mushrooms to kill flies (or insects) or repel them needs to be verified.

The hallucinogenic principle(s) in the mushroom is/are not easily metabolised.

To somehow account for the ambiguous 'pilzatropine', investigators during the last hundred years and more looked for an atropine-type alkaloid, or an indole like bufotenin.

Climatic and environmental factors have been mooted to account for the variable presence/absence of pilzatropine.

Constituents are present in minute amounts in the mushroom. Definitive work demands processing of large quantities of mushroom, about 2000 Kg. at a time.

Constituents are labile. Routine techniques will not suffice for isolation. Specific dependable methods as have been evolved in this laboratory are necessary to follow each constituent.

Various pharmacological syndromes are present that are not always easily distinguishable. Biological data needs careful interpretation.

The present investigation on A. muscaria was undertaken with a single aim: to clarify the elusive 'pilzatropine' problem. Unlike other investigators, the search was not restricted to a mere hypothetical alkaloid. Investigations have been carried out in collaboration with an able team of pharmacologists. Both dry and fresh mushrooms collected by authentic agencies (Granger Research Laboratories, Washington, D. C., and Mycological Society, California) have been examined systematically. The various fractionation and isolation procedures are presented fully under four parts of the Experimental with illustrations. Each main fraction was subjected to pharmacological evaluation.

Biological data proved helpful, but no undue reliance was placed on them. Changes were made in isolation techniques progressively. Elaborate and elegant fractionations were carried out intuitively with 4 batches of mushroom. As a result, it has been established that the ultimate water soluble fraction of the mushroom freed from all possible lipids, proteins and other organic and inorganic constituents has LSD-like properties. This water concentrate has been cleanly separated from the antagonistic muscarine-like product.

An extremely hygroscopic but relatively pure crystalline substance has finally been isolated in a very small quantity (yield: 307 mg. from 40.5 KG mushrooms, Part IV of Experimental) from the potent water concentrate. The crystalline substance designated 'Z', m.p. 135-38°C (dec) has been found to have LSD-like effects. From the mother liquors and remaining water-concentrate freed from inorganic salts, like KCl, some indolic components are being separated through thin sheet chromatography. The work is laborious and time consuming. It is likely to involve well over 500 chromatographic plates and necessary zeal in developing specific techniques to locate each individual component in a pure state.

Whether 'pilzatropine' is concentrated in the above water concentrate or whether the hallucinogenic activity of the mushroom is due to a combined drug syndrome will have to be proved by ultimate human experiments. The point being stressed is this: there is a possibility that the psychic effects of the mushroom may not reside in one particular substance like the hypothetical 'pilzatropine'. This may account for the failure of all investigators in the past. For example, the water concentrate isolated, after so much struggle, with definite LSD-like effects may contain besides crystalline 'Z' a host of other substances. The presence in minute quantities of these other substances may accelerate or diminish psychic effects with variation in dosage of the water-concentrate. The hallucinogenic principles need not necessarily belong to the category of alkaloids, indoles or glucosides. They need not even be organic or organo-inorganic substances. Even an inorganic component like potassium thiocyanate,[93] if present, can contribute to the psychosis of the mushroom to some extent.

Curiously enough, the mushroom has been found to contain, in the present investigation, a number of potassium salts: KCl (considerable quantity); potassium gluconate (which has been isolated for the first time from A. muscaria); crystalline 'Z', $C_{15}H_{30}O_{15}K_4$ (provisional); A. M. 20 and A. M. 21, crystalline substances isolated from similar water-concentrates (Parts I and II of Experimental) with LSD-like effects.

Why does potassium occur so frequently? Has potassium any link with "sympathotonia", a term coined by our team of pharmacologists to express 'hyperpyrexia-mydriasis' drug syndrome, characteristic of LSD-like materials? Speculation will only add to the confusion of 'pilzatropine' problem. Experimentation alone can tell.

Experiments are underway to determine as much of the structure of crystalline 'Z' as possible by micromethods. With the micro-quantity available and hindered by its hygroscopic nature, 'Z' cannot possibly be worked through classical approach. Results of NMR spectra of 'Z' with interpretation are being eagerly awaited.

A. muscaria has not so far been reported to contain fixed oils. Considerable quantity of an oil consisting mainly of esters of oleic acid has been isolated and fractionated. Preliminary examination of the oil fractions has been carried out. The oils are being screened for insecticidal and insect repellant properties. Detailed examination of the oils will have to be carried out.

Various minor constituents have been encountered in the present investigations on A. muscaria. Mannitol and fumaric acid detected by investigators in the past were not apparently characterized well. In the present work these have been identified satisfactorily. Potassium gluconate is a new minor constituent isolated and characterized in the current work.

Investigations on A. muscaria have been extensive. The chances of obtaining other definitive constituents like the β-glucoside, m. p. 106°C (dec), (Chart 13, Part III) and the crystalline substances, m. p. 172-74°C (Chart 2, Part I) and m. p. 160-65°C (dec) (Chart 18, Part IV) in sufficient amounts will be meagre and difficult. Yet these possibilities should not be overlooked. The various fractions indicated in the experimental as reserve material will also have to be examined carefully.

Arrangements will have to be made to process at least about 2000 Kg of mushrooms. Crystalline 'Z' and various other constituents indicated so far can be isolated in sufficient amount for structural elucidation, if the procedures outlined are followed faithfully. The rest of the potent water concentrate should be worked up as indicated with the help of thin sheet chromatography. Comparative pharmacological evaluation may aid in locating other potent constituents which will have to be tackled ingenuously.

One point needs to be borne in mind. Literature indicates that only the Siberian tribes practiced the mushroom orgy with A. muscaria. It is possible that the mushrooms grown in that particular area alone may be hallucinogenic. One needs to verify by actual human consumption, with all precautions and proper scientific recording, the presence or absence of psychotropic activity in A. muscaria collected from various parts of the world. Studies in the variation, both qualitative and quantitative, of hallucinogenic principle(s) due to changes in climate and soil offer wide scope for research.

EXPERIMENTAL

The experimental data is presented under four parts for the sake of convenience, interspersed with flow-sheets (charts) and figures, often with cross references to maintain continuity and clarity. Negative results and fractionations etc. that ultimately are of no immediate value have been either deleted or at best merely indicated wherever deemed necessary.

No fraction, however insignificant it might have appeared from time to time, was ever discarded or belittled till the end, and was only set aside, on the basis of pharmacological data, for future work.

To prevent the formation of artefacts, high temperatures and undue exposure to air, strong acids and bases were avoided. As far as possible all the work was carried out at or near-about room temperature. An inert nitrogen atmosphere was maintained whenever considered expedient. That the experimental procedures did not produce chemical alteration of plant materials present in the fresh plant and that the test materials contained no bacterial pyrogens was confirmed by the pharmacologists by the "cleanness" of biological results they obtained.

All the melting points are corrected unless otherwise stated. Microanalyses were carried out by Berkeley Analytical Laboratory, University of California, Berkeley, California, and Geller Laboratories, Bardonia, New York. Solvents and chemicals of analytical reagent grade purity were used. Abbreviations like ether for diethyl ether and Skelly for Skellysolve 'F', a petroleum fraction with a boiling point range of 30-60° have been used in description.

PART I

EXAMINATION OF DRY MUSHROOM (255g)

Preliminary experiments

i) Alkaloidal constituents

a) Prollius fluid [ether (25), chloroform (8), ethanol (2.5) and ammonia (1)] extraction[39] of the powdered mushroom (20g) gave a basic fraction (40.8 mg., 0.204%) designated as A. M. 1 (code no. I.1) for biological evaluation. This fraction, however, did not give any appreciable precipitate with alkaloidal reagents (Mayer's, Dragendorff's).

b) To detect the presence of any alkaloidal complex, samples of dry mushroom were (A) extracted with acidified methanol and (B) mixed with sufficient aq. (5%) Na_2CO_3 to make a uniformly wetted pulp and then extracted with chloroform.

Examination of the above for liberated alkaloids gave no positive indication.

ii) Soxhlet extraction (Chart I)

The powdered drug (25g) was extracted in succession with Skelly, ether, chloroform and methanol in a soxhlet extractor (150 ml).

a) The residue from Skelly was a light brown oil with characteristic odour of the original dry mushroom (1.8051g). It was dissolved in ether (30 c.c.) and shaken out in a separating funnel twice with dilute HCl (2%, 10 c.c., 5 c.c.) and dilute acetic acid (2%, 10 c.c., 5 c.c.) and washed with water (5 c.c., 5 c.c.). The aqueous acidic solutions and washings did not contain any alkaloid.

AMANITA MUSCARIA

Dry powder - 25 g.

Extn. (soxhlet) with Skellysolve 'F'

- Skelly Ext.
  - Solvent removed
  - Residue
    Light Brown Oil (1.8051g)
    7.2204%
    - neutral frn.
      0.3390g
      A.M.7
      (T.7)
    - basic frn.
      negligible
    - acidic frn.
      (0.3230g)
      A.M.3
      (T.3)
- Marc
  - Soxhlet in succession with ether, $CHCl_3$ and CH_3OH

Ether	(0.2720)
$CHCL_3$	(0.2084)
CH_3OH	(6.4180)
Total	6.8984g. residue

  - neutral frn.
    (0.4970g)
    A.M. 7
    (T.7)
  - basic frn.
    (0.035g.)
    A.M. 2
    (T.2)
  - acidic frn.
    (0.0423g.)
    A.M. 4
    (T.4)

CHART I

The ethereal layers were extracted with dilute Na_2CO_3 (5%, 10 c.c., 10 c.c.) and washed with water. The carbonate and washings did not extract any significant amount of acidic constituents. The ethereal solution was then extracted with dilute KOH (2%, 10 c.c., 5 c.c.) and washed with water. The remaining ether solution was dried over anhydrous Na_2SO_4 and yielded on removal of solvent a neutral fraction A. M. 7 (code no. I.7 0.3390g).

The aqueous alkaline extract was cooled, acidified and extracted with ether. The ether layers were washed with water, dried over Na_2SO_4 and evaporated. The resulting acidic fraction (0.323g) was labeled A. M. 3 (code No. I.3) for biological tests.

b) From the ether extractive was obtained a yellowish brown semi-solid (0.2720g, 1.088%) which was almost insoluble in dilute H_2SO_4 and gave negative tests for alkaloids.

c) The residue from chloroform (0.2084g, 0.8336%) was dark brown with a characteristic odour. A small acid soluble fraction of the chloroform extract was found to contain no alkaloids.

d) The dark brown residue from methanol (6.418g, 25.672%) gave insignificant precipitates with alkaloidal reagents.

Microscopic manipulation by means of solvents and mixed solvents, of each residue separately, did not yield any crystalline product. Hence all of them were worked up together and separated into a neutral fraction A. M. 7 (I.7, 0.497g), basic fraction, A. M. 2 (I.2, 0.035g) and an acidic fraction, A. M. 4 (I.4, 0.0423g) for biological tests.

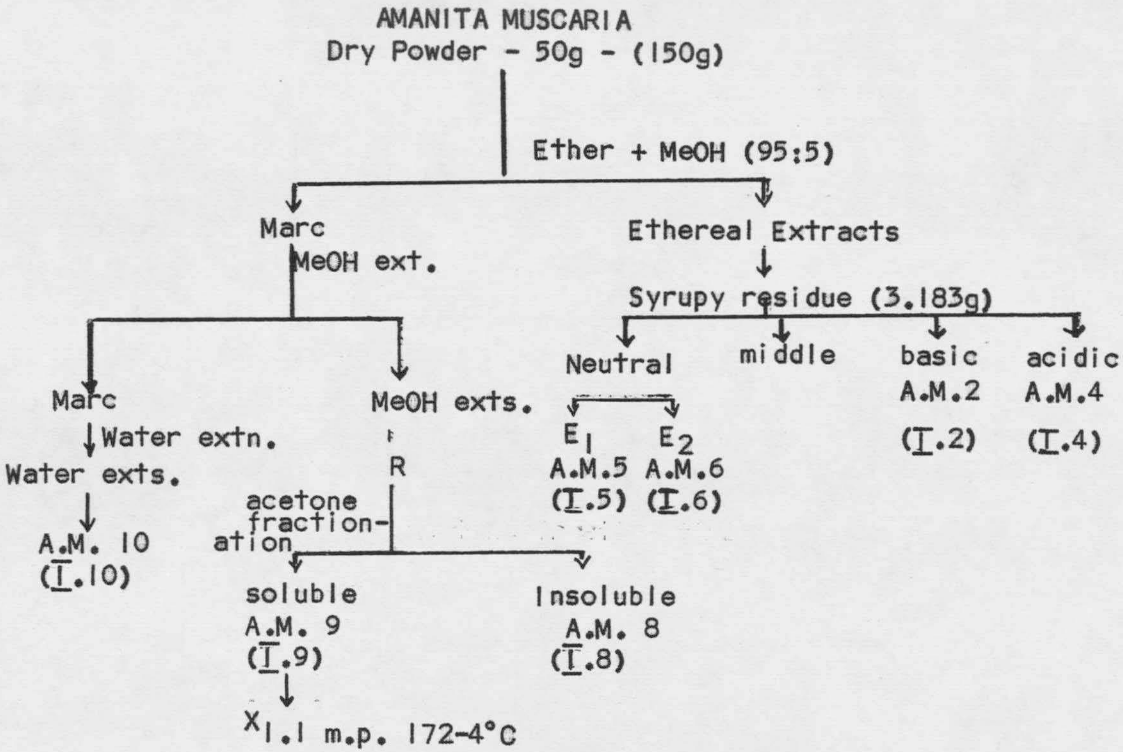

AMANITA MUSCARIA
Dry Powder - 50g - (150g)
Ether + MeOH (95:5)
Marc
MeOH ext.
Ethereal Extracts
Syrupy residue (3.183g)
Neutral
middle
basic
A.M.2
(I.2)
acidic
A.M.4
(I.4)
Marc
Water extn.
Water exts.
A.M. 10
(I.10)
MeOH exts.
R
acetone fraction-ation
soluble
A.M. 9
(I.9)
X I.I m.p. 172-4°C
Insoluble
A.M. 8
(I.8)
E1
A.M.5
(I.5)
E2
A.M.6
(I.6)

CHART 2

iii) <u>Extractions at room temperature</u> (Chart 2)

a) The powdered mushroom (50g) was macerated and percolated at room temperature with ether, containing 5% methanol, over a period of two weeks. On removal of solvent from the extracts, a thick syrup was obtained consisting mainly of neutral material with negligible amounts of acidic and basic fractions.

The neutral product on refrigeration yielded a pale yellow top fraction, A. M. 5 (T.5, 0.7074g) and a brown heavy wax (1.6573g) at the bottom. The wax on trituration with acetone in the cold yielded a light brown precipitate, A. M. 6 (T.6, 1.1813g) which was hygroscopic. In a capillary it changed in colour at 193°C, softened from 218°C and melted between 234-38°C (dec.).

The defatted mushroom was next extracted in lots with methanol in a Waring blendor. The filtrates on removal of solvent under reduced pressure yielded a thick syrup. This was triturated (magnetic stirrer) repeatedly with fresh acetone and centrifuged. The collective precipitates, A. M. 8 (T.8, 0.95g) were submitted for biological tests.

The acetone solution (supernatants) yielded a residue, A. M. 9. A major part of it (0.5258g) was sent for biological tests and the remainder worked up with solvents. Finally from methanol, colourless needles X_1, (62.8 mg), m.p. 172-74°C. were obtained.

Found: C, 39.42% and H, 7.64%; M. W. (?) - insoluble in camphor and
(Berkeley) no absorption in the u.v. (200-400 mμ)

The marc left after methanol extractions was then repeatedly extracted with distilled water (Waring blendor). The aqueous filtrates were concentrated under reduced pressure. The aqueous concentrate, A. M. 10 (12 c.c., T.10) had an intense fecal odour, was flourescent in in u.v. and gave a positive Keller's test (indicative of indoles).

Of the various fractions A. M. I to A. M. 10 (T.I to T.10) sent for biological evaluation, A. M. 10 (1/3 of the concentrate, 4 c.c. consumed for biological experiemtns) was reported to exhibit hallucinogenic activity in test animals. Attempts at isolation of crystalline products through various solvents (with the aid of microscope) like aq. methanol, acetone, dioxane etc. from A. M. 10 were not fruitful, even though some elusive crystalline materials could be observed under the microscope.

The concentrate was run through a cellulose column prepared with n-butanol and eluted with moist n-butanol. Dark coloured solutions were obtained first, followed by yellow eluants. The dark solution was run on a tightly packed cellulose column. This time it was held as the top zone and butanol elution yielded more of the yellow eluants. The dark zone was eluted with water.

The yellow butanol eluants were freed of solvent at room temperature (N_2). Descending paper chromatography of the residue with A) methanol (2), n-butanol (1), benzene (1) and water (1) and B) ethyl acetate (4), pyridine (2) and water (4) indicated the separation of at least two components flourescent in u.v.

At one stage, a micro quantity of crystalline product (u.v. maxima) λ 258 mμ, λ 198 mμ) was obtained.

The dark coloured solution was evaporated in vacuum and the residue in minimum quantity of water was shaken up with dioxane. The gummy mass which separated out was again taken up with water and subjected to repeated extraction with dioxane.

The total dioxane solutions were refrigerated overnight and centrifuged. A small dark brown viscous material separated out. From the clear golden yellow supernatants, solvent was removed. But crystallizations from various solvents were of no avail.

b) All the remainder of the dry powdered mushroom available for investigation (150 g.) was worked up as above and attention focused mainly on the ultimate water extract. The final aqueous solution, concentrated to about 120 c.c. (corresponding to A. M. 10) was absorbed on a tightly packed cellulose column, prepared with moist n-butanol. The column was carefully pushed out and cut into three zones: 1) top-black 2) middle-brown and 3) bottom-light brown.

Each zone was extracted in succession with n-butanol, dioxane and water.

The butanol, dioxane and water extractives of each zone were worked up separately. The residue from each fraction was subjected to crystallization by all known methods, but in vain.

Each of them was chromatographed on cellulose, chromosorb etc. Eluants were collected in fractions (arbitrarily, on the basis of visual colour and intensity of flourescence in u.v.) and worked up. The results were negative yielding no crystalline substances.

Finally, when all the above fractionations proved fruitless, they were all collected together, freed of solvents, dissolved in water, treated with silver carbonate and filtered. The filtrates were desilvered with H_2S, filtered and concentrated.[41] On refrigeration, a bitter semi-crystalline product was obtained. On initial crystallization from methanol and then thrice crystallized from water, a potassium salt, A. M. 20, $X_{I.20}$, long colourless needles (yield 606 mg) m. p. indefinite ranging between 200°-230°C (dec) was obtained and most of it (426 mg) subjected to biological tests.

PART II

EXAMINATION OF FRESH MUSHROOM

Earlier experiments indicated that the active principles were present mainly in the water soluble portion of the mushrooms. The fresh mushrooms contain on the average about 95% of water. Hence arrangements were made for the collection of fresh mushrooms which were asked to be dropped into cans of methanol immediately on picking.

The first shipment was received on Feb. 17, 1959 evening. The mushrooms strained through cheese-cloth folds (Chart 3) weighed 6.5 kg (dry wt.$\approx$488g). They were crushed in porcelain ware and the pulp was extracted with methanol at room temperature by maceration, percolation and shaking in lots (mechanical shaker). After six extractions of each lot with fresh solvent, the marc was pressed out of adhering solvent and similarly extracted with distilled water (5 extractions each batch).

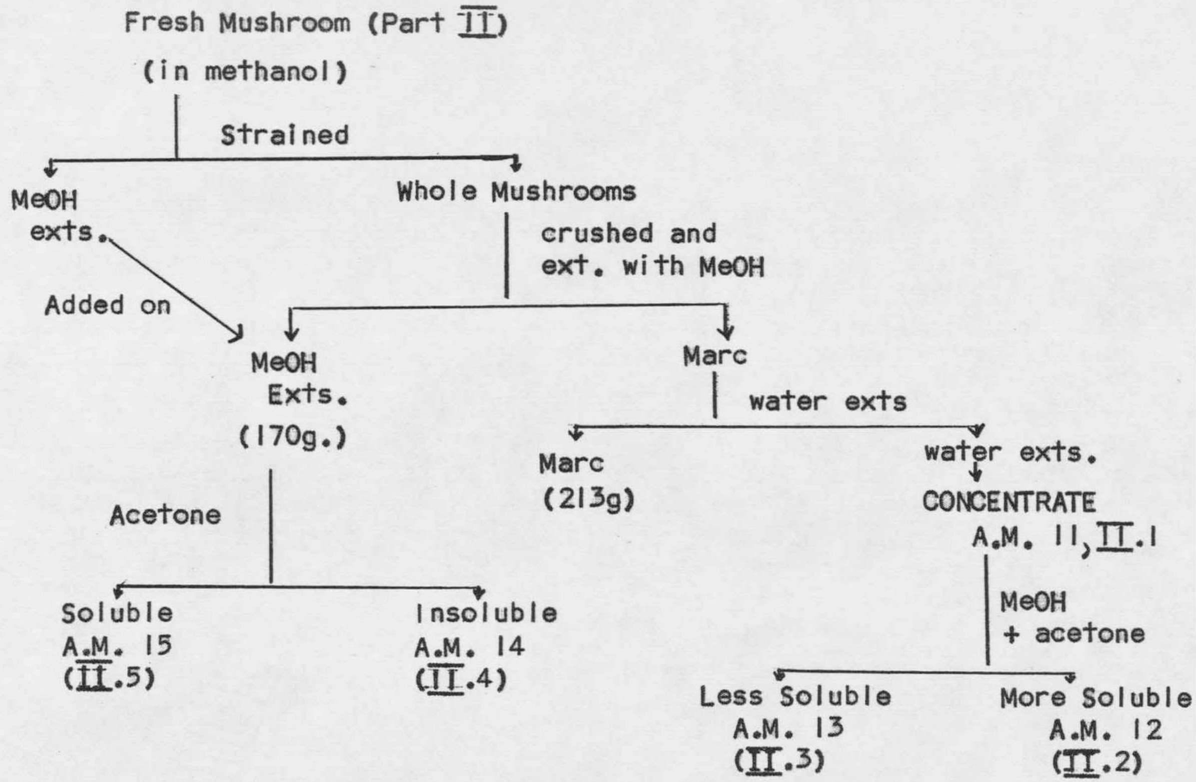

Fresh Mushroom (Part II)
(in methanol)
Strained
MeOH exts.
Whole Mushrooms
crushed and ext. with MeOH
Added on
MeOH Exts. (170g.)
Marc
water exts
Marc (213g)
water exts.
CONCENTRATE A.M. 11, II.1
Acetone
MeOH + acetone
Soluble A.M. 15 (II.5)
Insoluble A.M. 14 (II.4)
Less Soluble A.M. 13 (II.3)
More Soluble A.M. 12 (II.2)

CHART 3

The total methanol (aq) extractives were freed of solvent at room temperature. The dark brown residue (170 g) was triturated in batches repeatedly with acetone (magnetic stirrer). The acetone insoluble mass on drying in vacuum was a semi-solid material, A. M. 14 (II.4, 150 g) soluble in water (pH 5.0).

A brown, viscous oil, A. M. 15 (II.5), 18.95 g) miscible with water (pH 3.65) was obtained on removal of solvent from the acetone filtrates.

The water extracts of the marc were evaporated at room temperature. In spite of repeated filtrations, there was always some mushroom pulp carried through at each stage of extraction. Then by successive refrigeration and filtration all the "muck" (71 g) was removed. The aqueous concentrate, A. M. 11 (II.1, 34 g on dry weight basis, pH 4.0) corresponding to A. M. 10 (I.10) was treated with a mixture of methanol and acetone (2:1, 4 extractions, magnetic stirrer). The residue from the relatively more soluble fraction, A. M. 12 (II.2, 20.412 g) and also the residue obtained on removal of solvent in vacuum from the less soluble portion, A. M. 13 (II.3, 11.03 g, pH 3.9) along with A. M. 11 (II.1), A. M. 14 (II.4) and A. M. 15 (II.5) were sent for biological testing.

Examination of the various fractions

The residue from A. M. 13 was macerated with methanol (about a month, in the refrigerator, (Chart 4). The methanol insoluble portion was triturated exhaustively (magnetic stirrer) in succession with moist butanol and dioxane. The final insoluble residue was taken up with water,

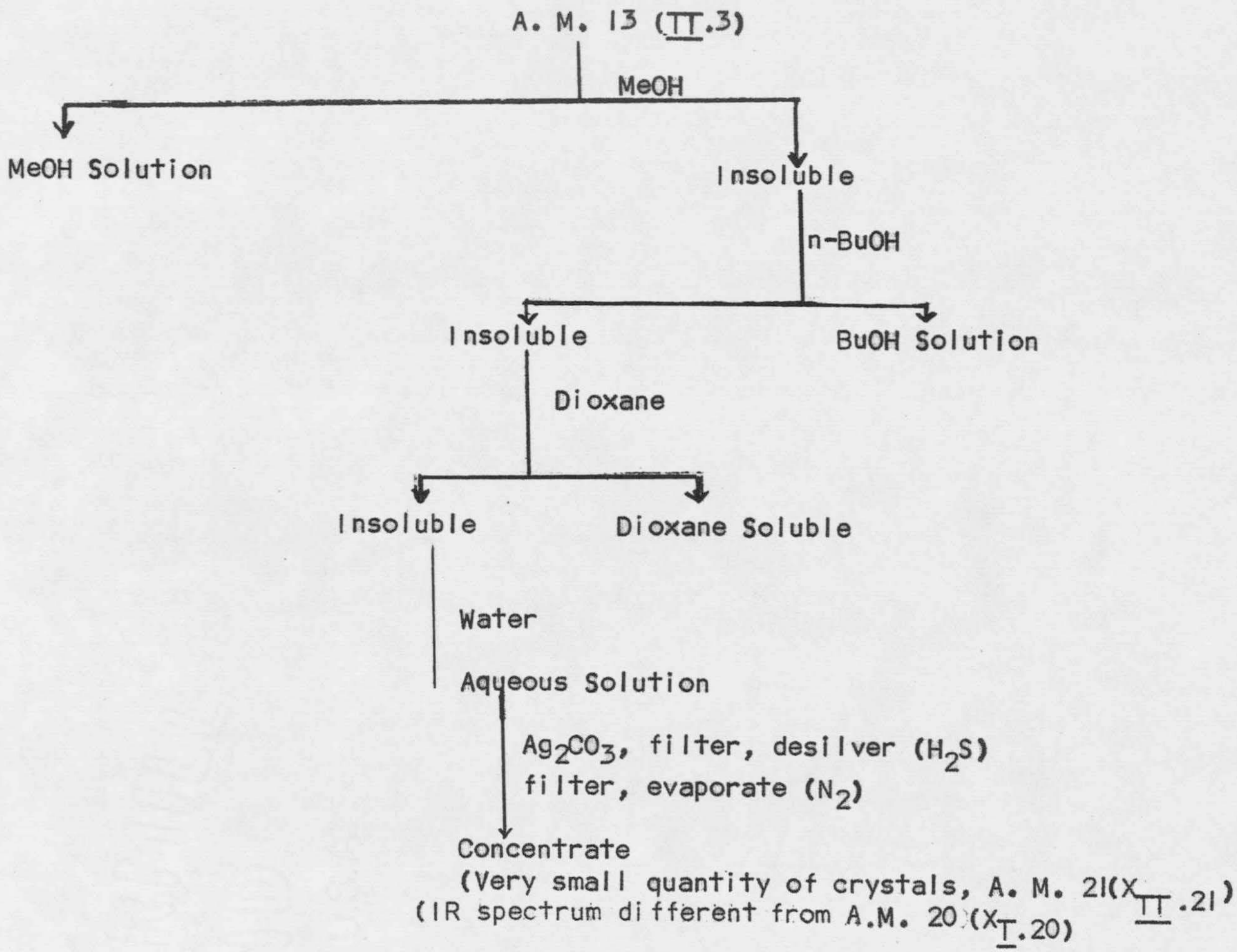

A. M. 13 (II.3)
MeOH
MeOH Solution
Insoluble
n-BuOH
Insoluble
BuOH Solution
Dioxane
Insoluble
Dioxane Soluble
Water
Aqueous Solution
Ag_2CO_3, filter, desilver (H_2S)
filter, evaporate (N_2)
Concentrate
(Very small quantity of crystals, A. M. 21(X_{II}.21)
(IR spectrum different from A.M. 20 (X_I.20)

CHART 4

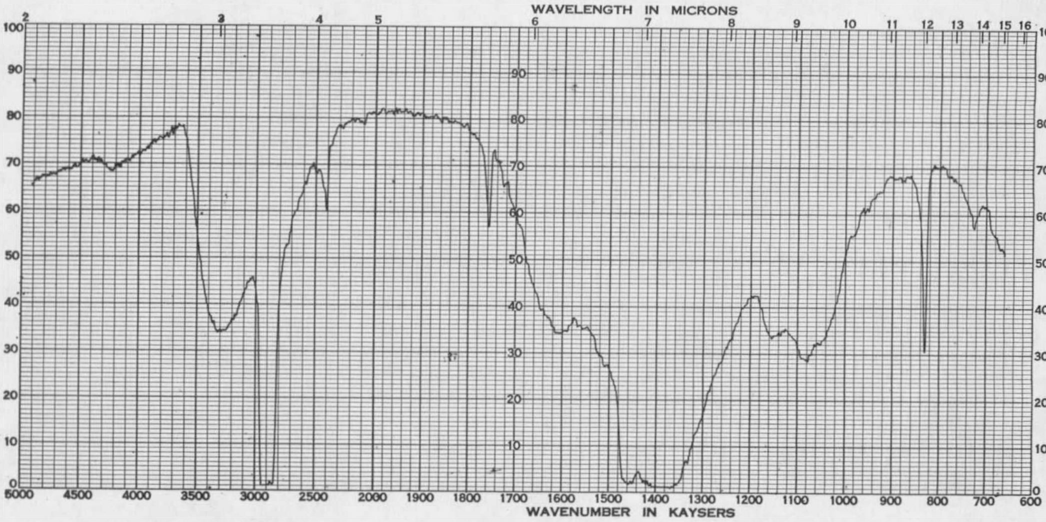


Figure 1

Infrared Spectrum (Nujol mull) of A. M. 20, $X_{\underline{I}}.20$

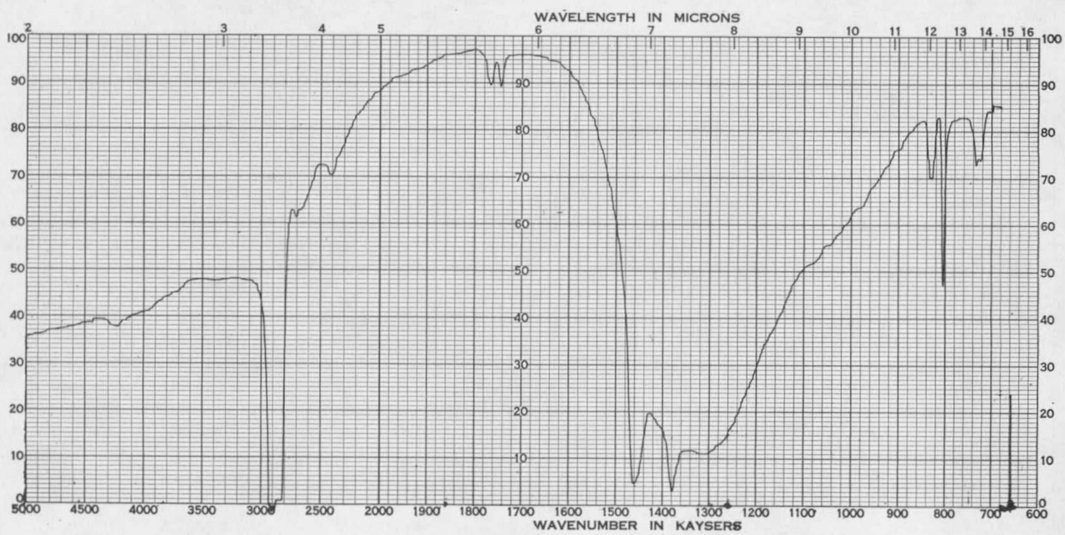


Figure 2

Infrared Spectrum (Nujol mull) of A. M. 21, $X_{\underline{II}}.21$

treated with silver carbonate,* filtered, and desilvered through H_2S. From the filtrates after patient working a small quantity of a crystalline product A. M. 21 ($X_{\underline{II}.21}$) was obtained which had infrared spectrum different from that of A. M. 20 ($X_{\underline{I}.20}$) isolated earlier from the sample of dry mushroom.

A. M. 12 (II.2) and A. M. 14 (II.4) were worked up as described for A. M. 13 (II.3). Each one of the corresponding fractions, soluble in methanol, n-butanol and dioxane was examined closely. Although traces of crystals (under microscope) could be discerned occasionally no crystalline product could be isolated.

A. M. 15 (II.5), dissolved in ether, was chromatographed on a column of alumina prepared with Skelly. Skelly eluants (fractions 1-5) did not contain any product. Elution with a mixture of ether and Skelly (9:1) (fractions 6-10) yielded an oil, A. M. 15 c/1 (II.5-01) consisting mainly of esters (from IR spectrum, Fig. 27). Elution with a mixture of ether (1) and methanol (3) (fractions 11-14) yielded a smaller quantity of another oil, A. M. 15 c/2 (II.5-02).

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* Silver carbonate was used for purification. Hofman[41] reported that silver carbonate aided in the isolation of 'psilocybin'.