



Studies in the hallucinogenic principles of *Amanita muscaria*, L.
by A V Subbaratnam

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in Chemistry at Montana State College
Montana State University
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Abstract:

Both fresh and dry mushrooms, *Amanita muscaria*, have been examined chemically and physiologically. The mushrooms were subjected to elaborate fractionations with comparative pharmacological evaluation, to locate the hallucinogen 'psilocybin' 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. 135-38°C (dec), C₁₅H₃₀O₁₅K₄ (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. 160-65 °, and a β-glycoside, m.p. 106 °C (dec) have been found in the mushroom.

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A. V. SUBBARATNAM

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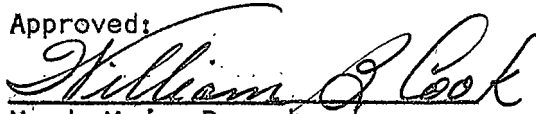
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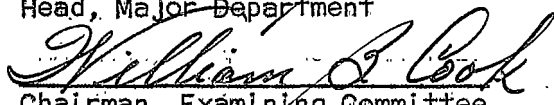
Doctor of Philosophy in Chemistry

at

Montana State College

Approved:


Head, Major Department


Chairman, Examining Committee


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Bozeman, Montana
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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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Finally, I dedicate this thesis in fond memory of three of my best friends, who inspired me with confidence during their short lives and died tragically young: (late) Prof. A. N. Ratnagiriswaran; (late) Dr. K. Ramachandran and (late) Dr. S. Rajagopalan.

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ABSTRACT

Both fresh and dry mushrooms, Amanita muscaria, have been examined chemically and physiologically. The mushrooms were subjected to elaborate fractionations with comparative pharmacological evaluation, to locate the hallucinogen 'pilzotropine' 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. 135-38°C (dec), $C_{15}H_{30}O_{15}K_4$ (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°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. Gray⁸², (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 Tchaikowsky's Nutcracker Suite music in Fantasia."²⁵

Before A. D. 1256, Albert Magnus⁶⁵ 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. Linnaeus⁶³ reported that the mushroom was advocated for killing bed bugs. However, the ability of the mushroom to kill flies has never been confirmed scientifically.

Wassons⁸⁵ 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. Fabing²⁵ has quoted references at length in his article "On Going Berserk: 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. Tyler⁸² contradicts the above view citing reference to review of literature on this topic by Thorsen⁸⁰.

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 accidentally in October 1955. This private communication quoted by Fabing²⁵ 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

<u>Authors</u>	<u>Wt. of Fresh Mushroom Processed</u>	<u>Yield</u>
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 yields of muscarine obtained by four groups of representative investigators, from King⁵⁰ 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 al⁵³ realized this, quite late in their investigations. They reported, in 1957, the following activities on frog's heart.

Muscarine: $\sim 2.3 \times 10^8$ units/mg.

Acetyl choline: $\sim 10 \times 10^8$ units/mg.

Eugster and Waser²⁰ 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. Wilkinson⁸⁸ credited the above authors with success in obtaining the first pure muscarine chloride.

Balenovic et al³ found that counter-current extraction was not suitable for the separation of reineckates of quaternary bases from A. muscaria. By switching to chromatography with ion-exchange resins and cellulose columns, Balenovic and Stefanac⁴ could isolate muscarine pure.

Kuehl et al⁵⁷ also obtained pure muscarine chloride by preliminary fractionations on resins and purification by chromatography. Eugster and Muller²² used ion-exchange resins and column chromatography for the isolation of muscarine from Inocybe patouillardii (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. Moge⁸. 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 al⁵¹ 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.

Hallucinogenic factors

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.

Schmiedeberg⁷⁶ became curious with the observation of Petri⁶⁹ 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 "pilzatrophine" or alternatively "muscaridine". He reported that pilzatrophine dilated the constricted pupil, accelerated the slowed heart and was finally excreted unchanged in urine.

In 1903, Harmsen³⁵ 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 pilzatrophine occurring in the mushroom. He considered it as probable that developmental and environmental factors might influence the occurrence of pilzatrophine in A. muscaria.

Honda⁴³, 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 pilzatrophine.

In 1953, Wieland and Motzel⁸⁷ 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, Lewis⁶² 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 pilzatrope 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, Kwasniewski⁵⁹ 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 Inoko⁴⁵ towards the end of last century. Fiske²⁸ examined some poisonous plants of New Jersey, and reported that A. muscaria contained the alkaloids, muscarine, choline and atropine. Brady and Tyler⁹ 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.

Eugster²³ 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

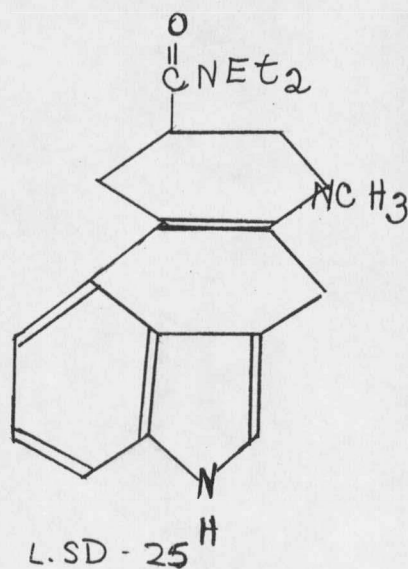
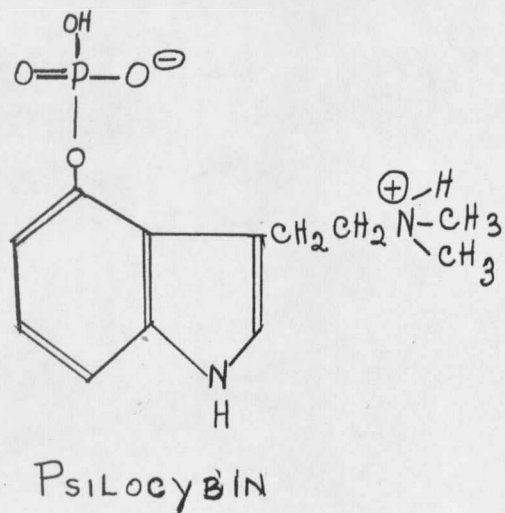
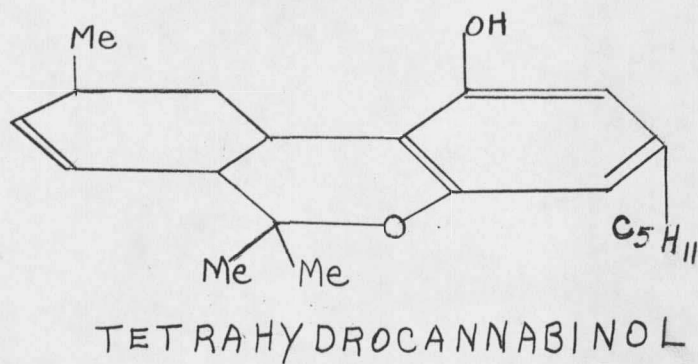
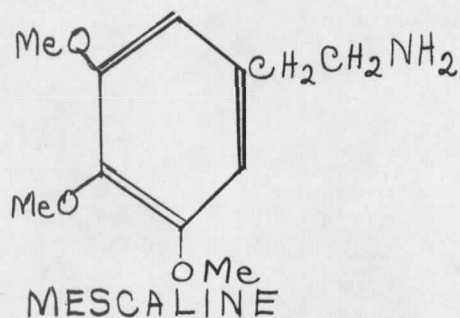
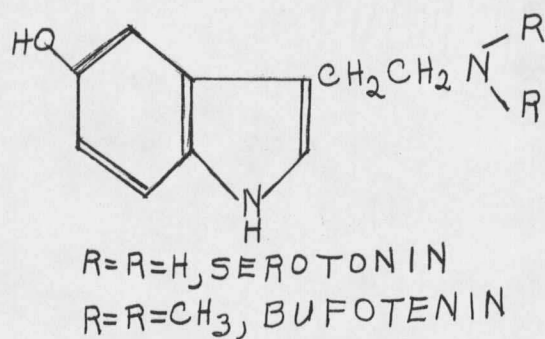
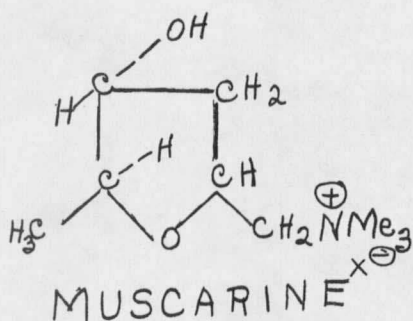


Figure 35

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 Tschertter⁴² 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 Filiius. They report that the same alkaloids were also found in Ipomoea tricolor, Cav.

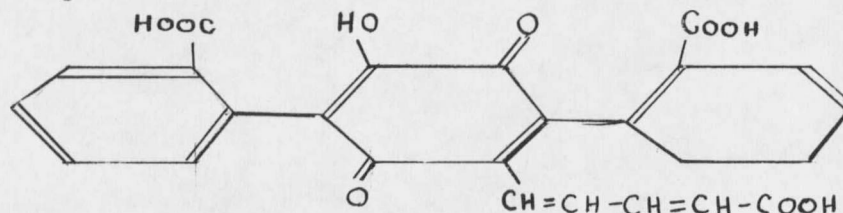
Just as the early erroneous formula of Kogl et al⁵¹ for muscarine is quoted in many recent books on Medicinal chemistry, Biochemistry and Pharmacology, similarly statements have been made to the effect that muscarine is responsible for the psychotropic action of A. muscaria. Even Bowden and Mogey⁸ in 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 confirmation² of the presence of atropine-like actions."

Eugster²³ 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 Eugster²³ 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 Tyler⁸² that "the final chapter of the story of the ambiguous alkaloid, pilzatropane, remains to be written."

Other Constituents of Amanita muscaria

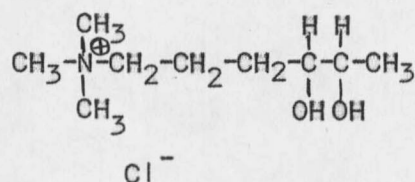
A) Choline and acetyl choline have been encountered by many investigators. In their early work on the colouring matter of the mushrooms, Kogl and

Erxleben⁵² 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:



Eugster²³ 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. Eugster²³ casts doubt on the terphenyl quinone structure of Kogl for muscarufin, on the basis of absorption spectra.

In a very recent preliminary communication, Kogl, Saleminck and Schuller⁵⁴ reported the isolation of a new alkaloid, muscaridin (yield: 300 mg. of muscaridin aurichloride, m.p. $129-131^\circ C$ (uncorrected) from 1035 Kg. of A. muscaria). Muscaridin is reported to be the quarternary trimethyl ammonium salt of 6-amino-2,3-dihydroxy-hexane:



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:

Buschmann¹² found in A. muscaria, stored in methanol, one day after gathering, Xanthine and, in greater amount hypoxanthine.

Clark and Kantor¹⁴ processed A. muscaria according to the method of Harmsen³⁵ 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.

Kung⁵⁸ reported that from extracts of A. muscaria, choline, putrescine, herzynine (histidine betaine) could be isolated.

Gerber³¹ studied the coagulating properties in 86 species of Basidiomycetes, 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.

Ford³⁰ studied the distribution of poisons in various Amanitas. He reported that A. muscaria contained muscarine, an agglutinin and a hemolysin. The agglutinin 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 Sartory⁷³ 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.

Zellner⁹⁰ found that the substance he had isolated from A. muscaria earlier⁸⁹ 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, King⁵⁰ obtained a phytosterol (yield: 89 mg. from 25.5 Kg. of A. muscaria), m. p. $158-59^\circ C$, 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. Ludwig⁶⁴ noted the presence of leucine earlier in the mushroom. In an earlier communication, Zellner⁸⁹ mentioned that A. muscaria contained propionic and fumaric acids, mykose (trehalose) and other unidentified minor constituents.

Herrmann⁴⁰ 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 Zellner⁵ 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-Larrier⁶⁰ reported that the juice of A. muscaria acted in the same manner on nervous and on muscular excitability. Chretien and Leblois¹³ studied the toxicity of various mushrooms to dogs. A. muscaria dried at 38° was found to be non-toxic.

Rabb and Renz⁷² reported that A. muscaria causes no hemolysis while some other Amanita species do. Binet et al⁶ 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 authors⁷ with experiments on guinea pig.

A. muscaria grown in Uruguay²⁹ was found to contain muscarine. Amanita muscarina, Pers., growing wild in Russia⁴⁹ 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 'pilzatrophine', 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 pilzatrophine.

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 'pilzatrophine' 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°0 (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 'pilzotropine' 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 ingeniously.

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 "clean-ness" 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)]³⁹ extraction of the powdered mushroom (20g) gave a basic fraction (40.8 mgr, 0.204%) designated as A. M. I (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.

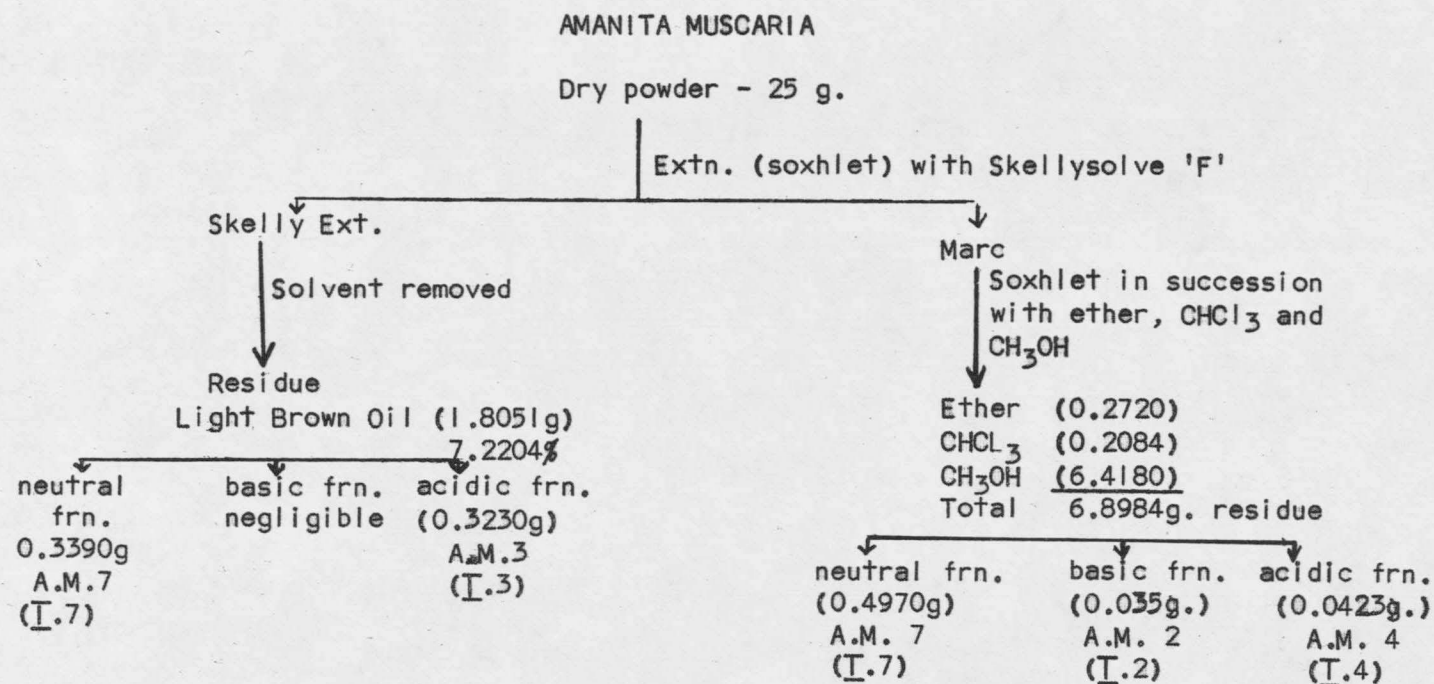


CHART I

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.

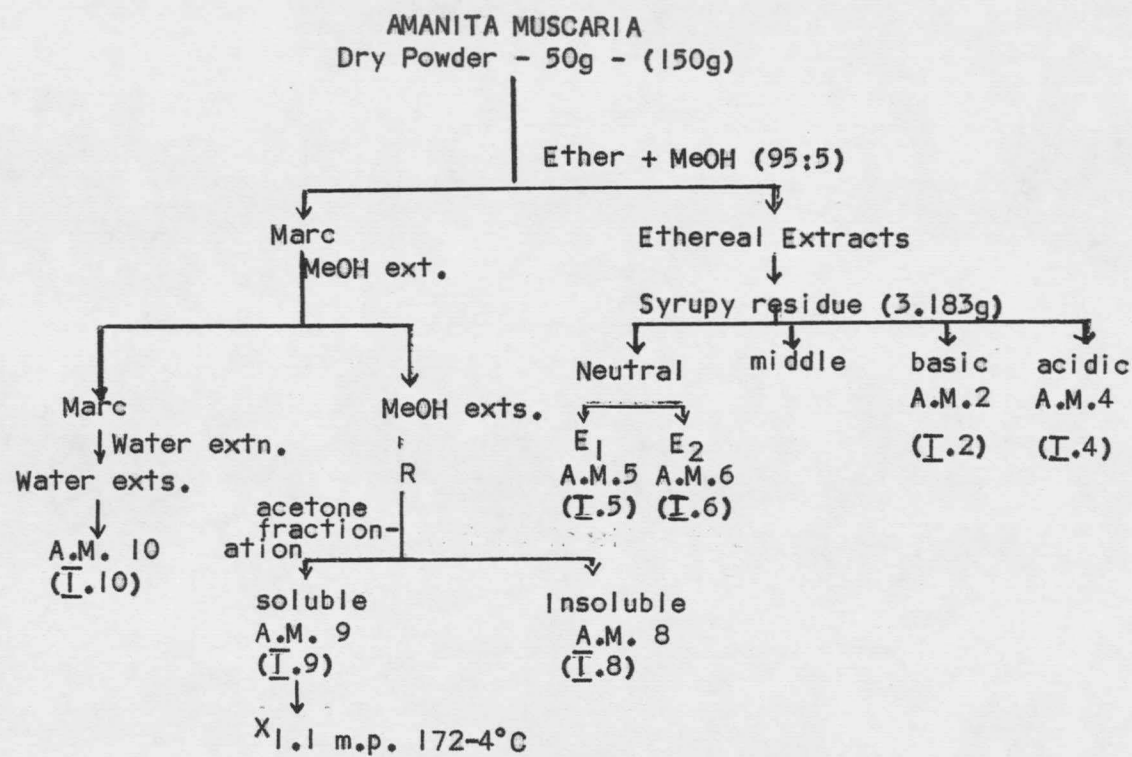


CHART 2

iii) Extractions at room temperature (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 (I.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 (I.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 blender. 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 (I.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 XI, (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 blender). The aqueous filtrates were concentrated under reduced pressure. The aqueous concentrate, A. M. 10 (12 c.c., I.10) had an intense fecal odour, was fluorescent in u.v. and gave a positive Keller's test (indicative of indoles).

Of the various fractions A. M. 1 to A. M. 10 (I.1 to I.10) sent for biological evaluation, A. M. 10 (1/3 of the concentrate, 4 c.c. consumed for biological experiments) 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 fluorescent in u.v.

At one stage, a micro quantity of crystalline product (u.v. maxima) λ 258 m μ , λ 198 m μ) 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 fluorescence 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, $X_{1.20}$, long colourless needles (yield 606 mg) m. p. indefinite ranging between 200° - $230^{\circ}C$ (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).

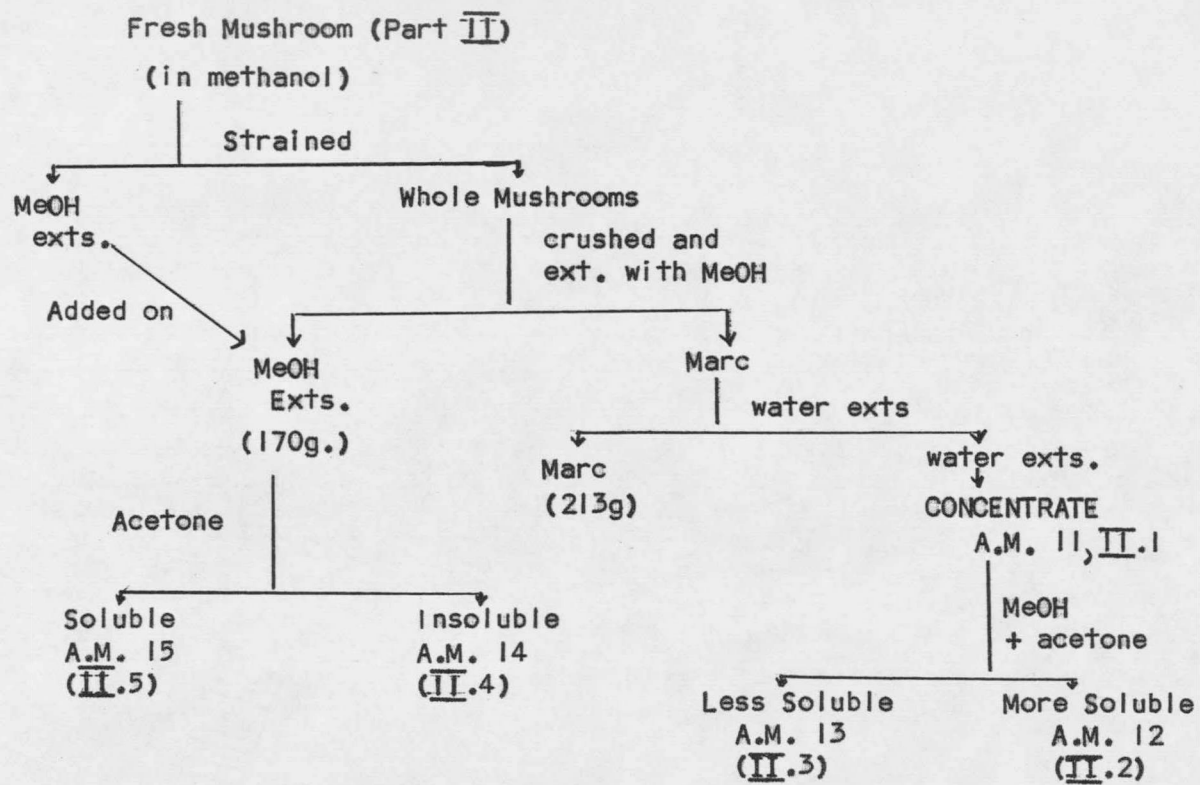


CHART 3

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,

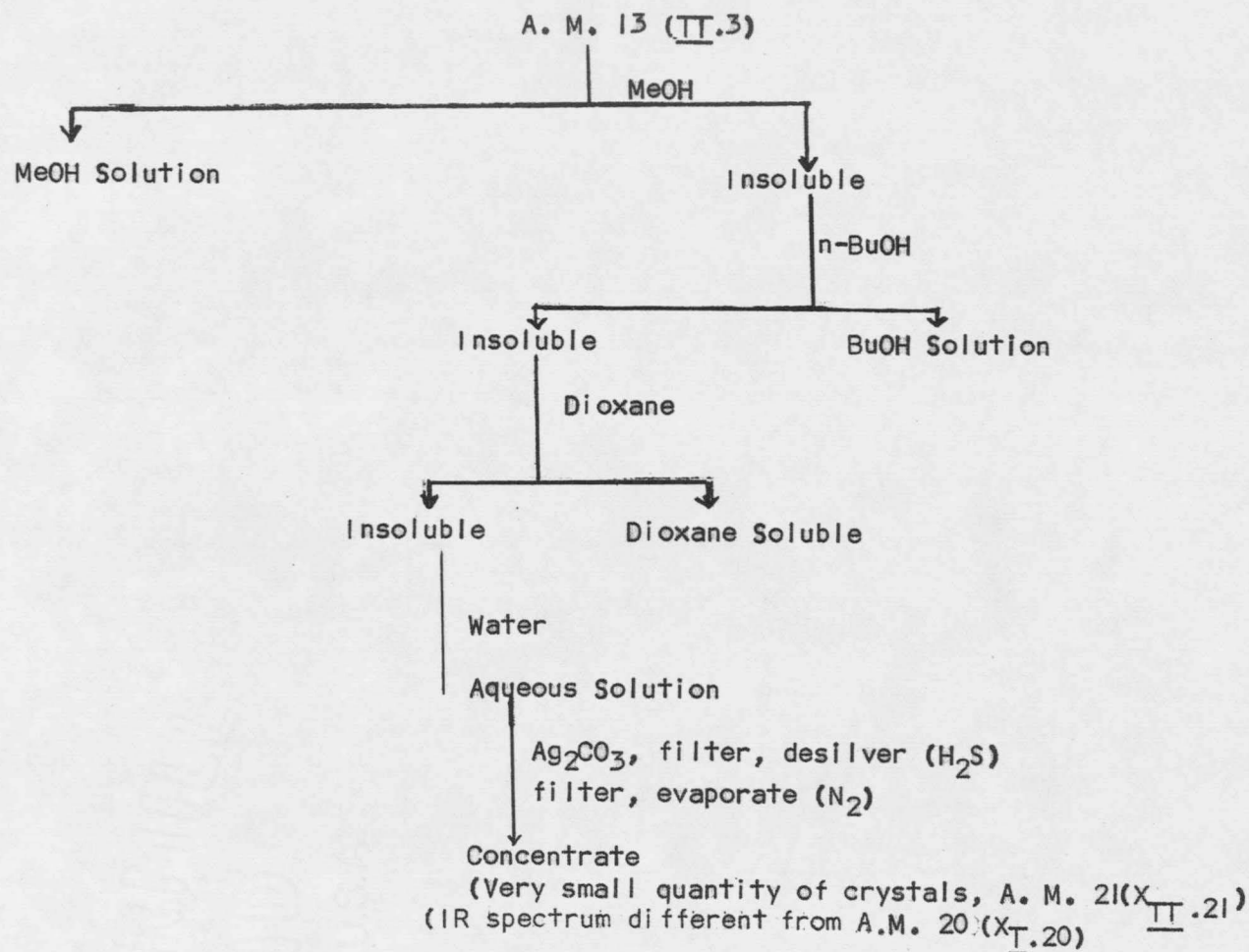


CHART 4

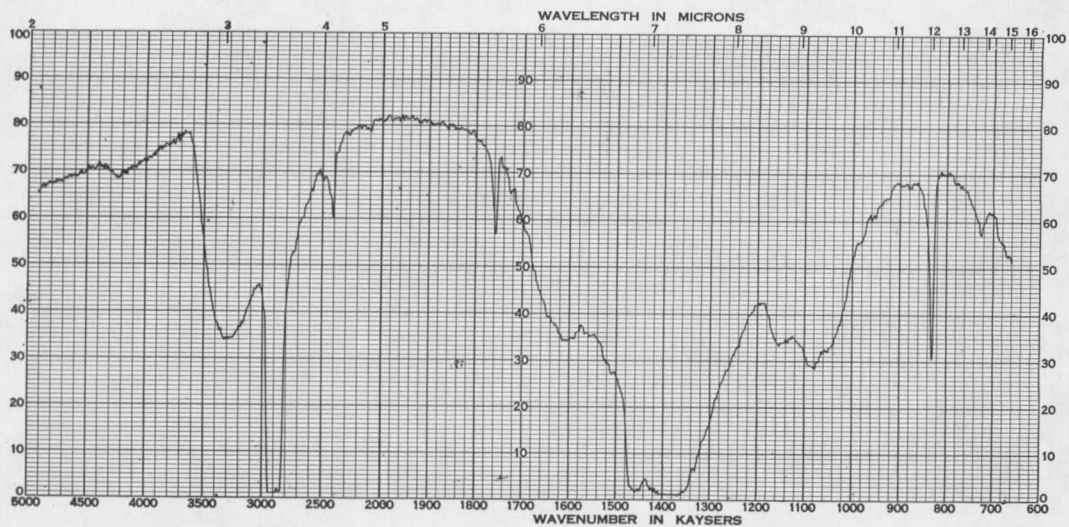


Figure 1

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

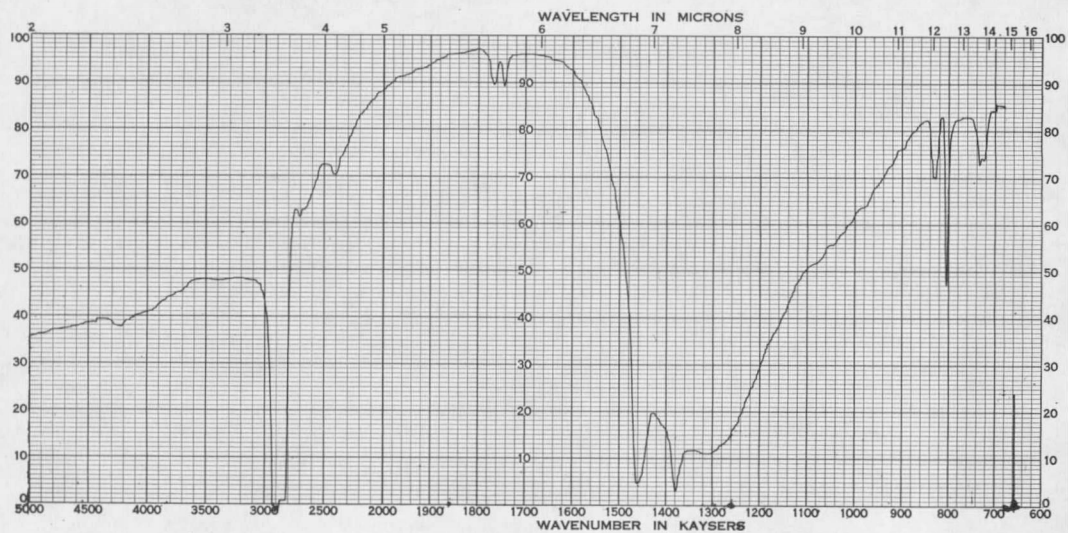


Figure 2

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

treated with silver carbonate,* filtered, and desilvered through H₂S.

From the filtrates after patient working a small quantity of a crystalline product A. M. 21 (X_{II.21}) was obtained which had infrared spectrum different from that of A. M. 20 (X_{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).

* Silver carbonate was used for purification. Hofman⁴¹ reported that silver carbonate aided in the isolation of 'psilocybin'.

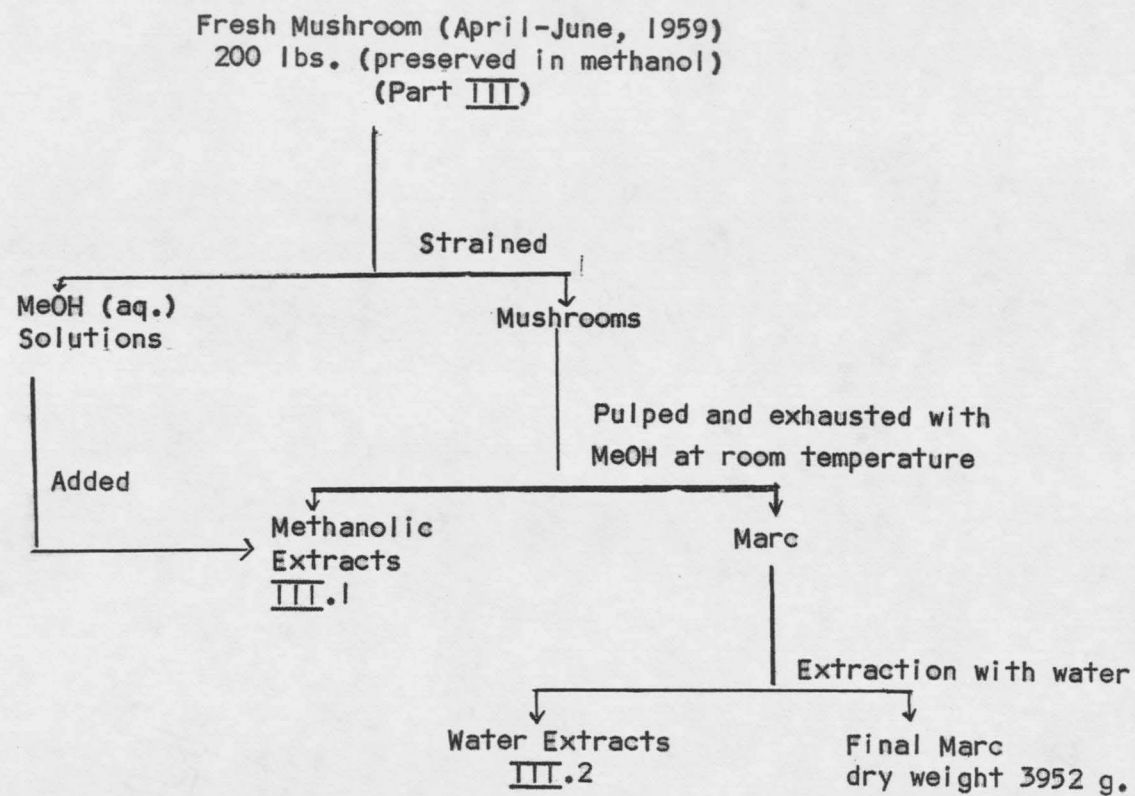


CHART 5

PART III

FRESH MUSHROOMS - 200 lbs.

Early in March 1959 a shipment of 8 cans (25 lbs. of mushroom in each) of fresh mushroom preserved in methanol was received. Impurities like mud, dirt and straw in the cans were separated. The aqueous methanolic dialysate (about 15 gallons) was filtered through cheese-cloth (Chart 5) and then filter-paper. The mushrooms were ground (in the stainless steel meat grinder of Animal Husbandry Department, Campus) on March 24, 1959 to a fine pulp. The pulpy mass was extracted with methanol at room temperature by maceration and percolation during a period of three months (April-June 1959) in improvised conical-shaped stainless steel percolators. The extracted mushrooms were pressed, filtered (basket centrifuge) and then extracted exhaustively with distilled water.

A.) Methanolic Extracts

Solvent was removed from the methanolic extracts (about 85 gallons) under reduced pressure at room temperature, yielding a thick syrup (1675 g). A number of preliminary experiments were carried out with the concentrate. Following is a typical fractionation carried out with a portion of the concentrate, (Chart 6), in these preliminary trials.

i) The concentrate (80 g) was mixed with crushed ice and repeatedly extracted with ether. The concentrate from ether solution was freed of a small quantity of gummy mass by judicious addition of Skelly. The filtrates were chromatographed on a column of alumina. Elution with a mixture of Skelly and ether, increasing the ether content of the mixture gradually, resulted in the separation of three oil fractions,

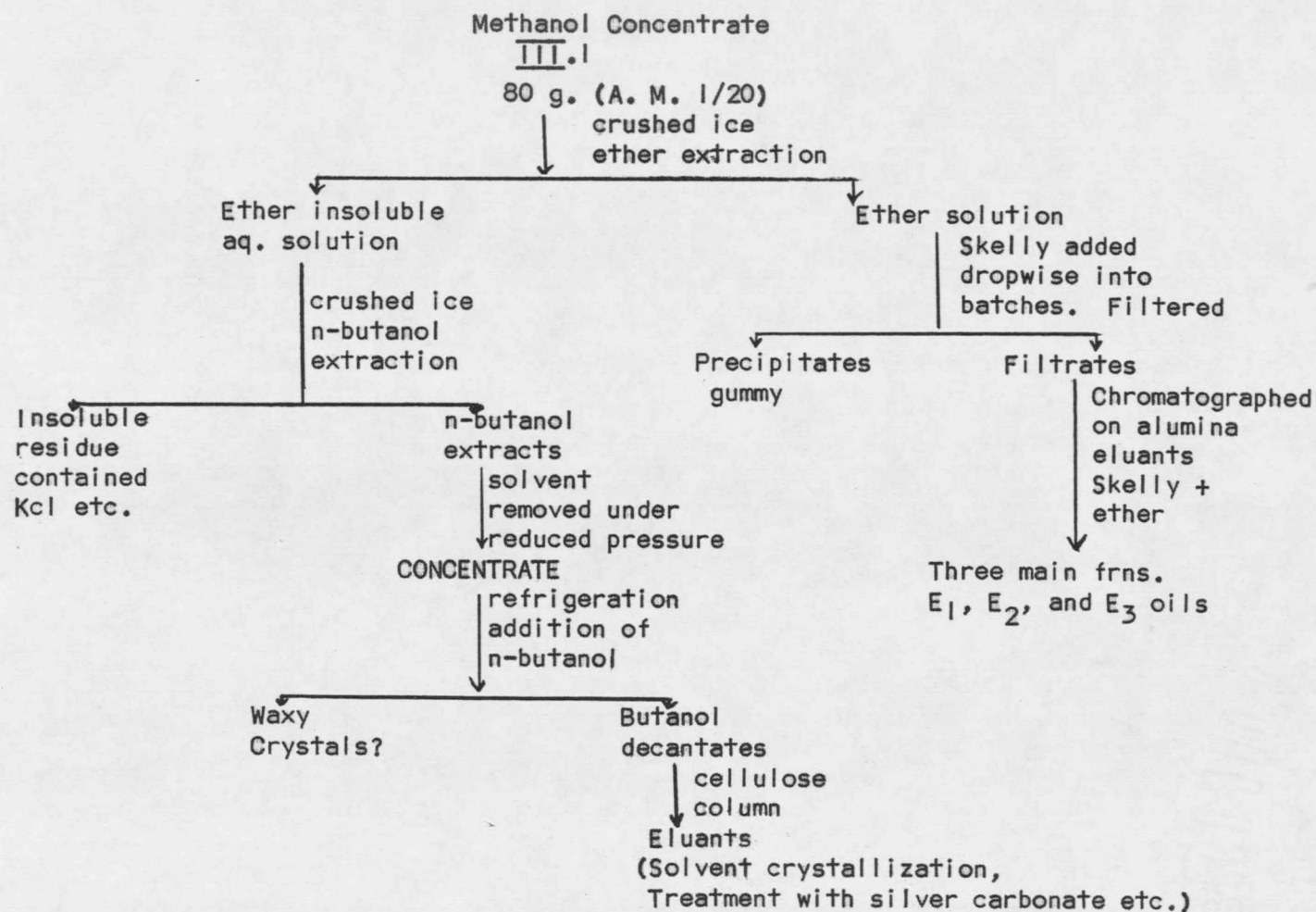


CHART 6

Total Concentrate
(1590g.), III.1, A.M. 19/20

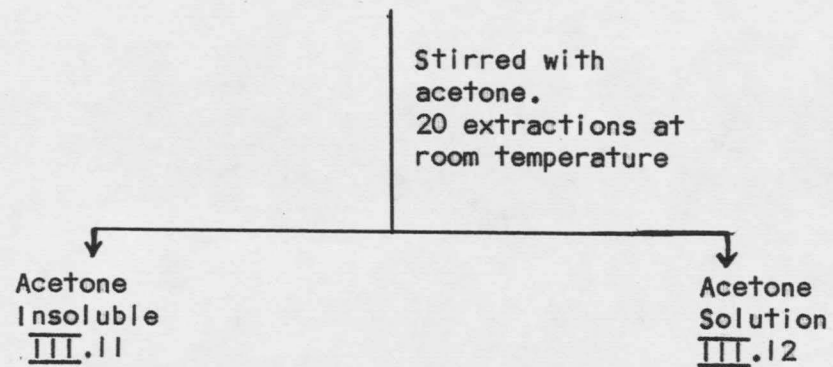


CHART 7a

(A. M. 1/20 E₁, E₂ and E₃).

The ether insoluble fraction from the original methanolic concentrate was mixed with additional crushed ice and extracted with n-butanol. The butanol insoluble residue was found to contain inorganic salts (KCl etc.)

The butanol extract was concentrated at reduced pressure. Experiments with various solvent systems were tried with the concentrate. No crystalline material was isolated. Refrigeration for considerable time and treatment with increasing quantities of n-butanol resulted in the separation of a waxy material. This material, semi-crystalline under the microscope, yielded no definite product. The butanol decantates were chromatographed on cellulose, and the various eluant (n-butanol) fractions examined. Crystallization could not be induced through solvents. Treatment with silver carbonate, desilvering and further work failed to yield a pure substance.

ii) The final scheme of fractionation of the methanolic concentrate is outlined under Chart 7, and following Charts.

The concentrate (1590g) was stirred in batches with acetone continuously (about 20 extractions of each, with fresh solvent each time, using magnetic stirrer), thus separating it into two main fractions: that soluble in acetone and that insoluble in acetone.

a) The acetone soluble fraction (III.12) on removal of solvent was obtained as a thick concentrate (730 g) soluble freely in ether and less in Skelly. It was partitioned in lots, between equal amounts of Skelly and 70% methanol (aq).

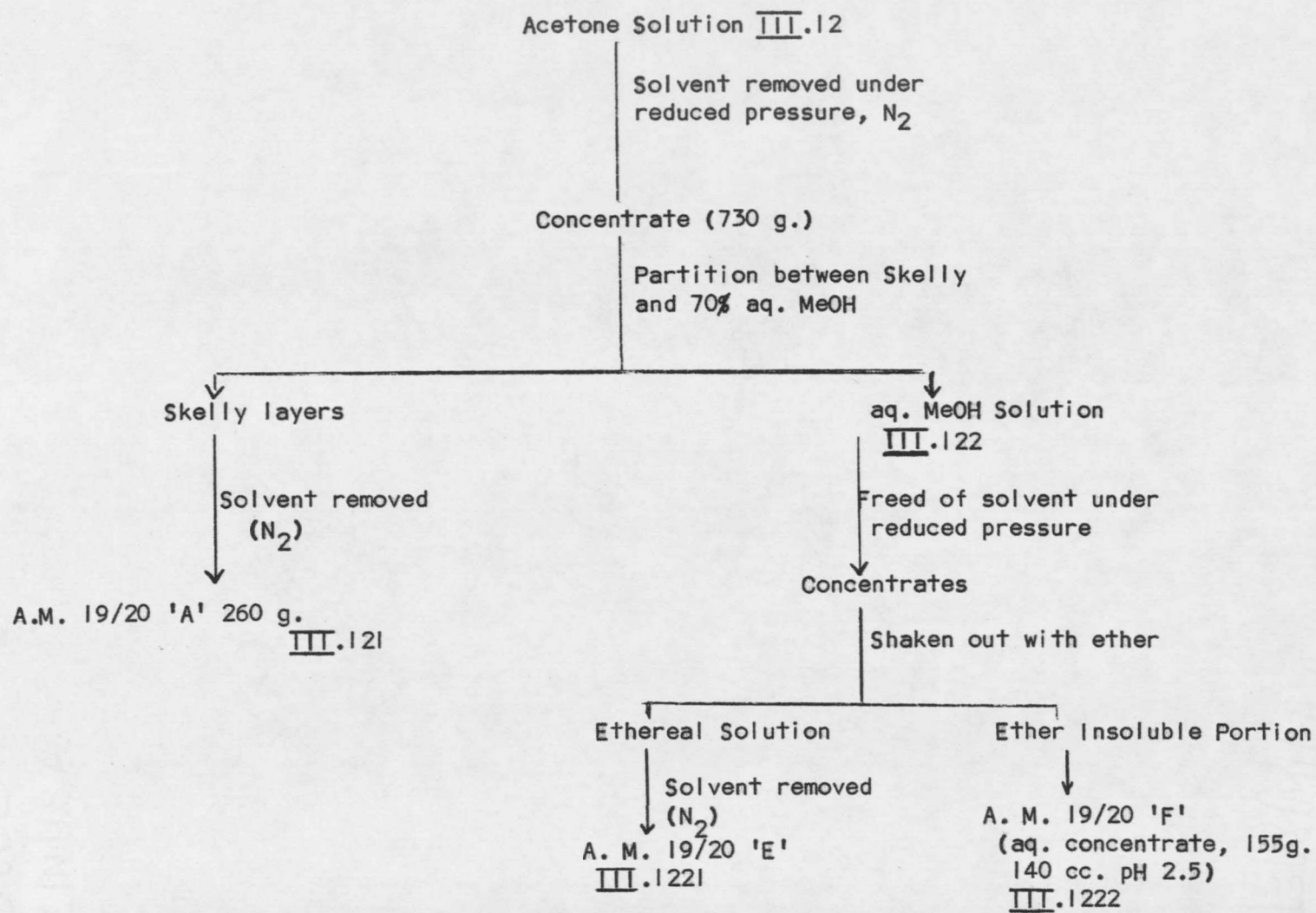


CHART 7

A. M. 19/20 'A' (240g.) III.121

Alumina Chromatography
(23 x 5.5 cm.)

A. M. 19/20 A/1, III.1211
Light yellowish oil (58g.)
(skelly eluants)
Sap. equivalent = 235.1
Neut. equivalent of acids
(on hydrolysis) = 298.4

A. M. 19/20 A/2, III.1212
Thick, brownish oil (124g)
(skelly eluants)
Sap. equivalent = 245.8
Neut. equivalent of acids
(on hydrolysis) = 290.3

IR spectra of original 'A', Fraction 1,
acids from 1 and Methyl esters of acids;
Fraction 2, acids from 2 and Methyl
esters of acids.

Gas Chromatogram of Methyl esters of
acids from 1 and 2.

CHART 8

The Skelly solution on removal of solvent (N_2) yielded an oil 'A' (III.121, 260 g). It was chromatographed on a column of alumina (23×5.5 cm) prepared in Skelly. The first eluants with Skelly (1500 c.c.) yielded, on removal of solvent (Chart 8), a light yellowish oil (58 g, III.1211, A. M. 19/20 A/1). Subsequent Skelly eluants (3 liters) yielded a viscous brown oil (124 g, III.1212, A. M. 19/20 A/2). Further eluants (fractions 3-16) with Skelly, mixture of Skelly and ether (with gradual increase of ether), ether, mixture of ether and methanol and ultimately methanol and water did not give any definite product.

The original oil ('A', III.121) and the two oil fractions (A. M. 19/20 A/1 = III.1211 and A. M. 19/20 A/2 = III.1212) obtained on chromatographic separation were all submitted for biological tests. At one stage, the pharmacologists reported that fraction III.1212 was potent, hallucinogenically while the others, III.121 and III.1211 exhibited insignificant effects.

The oils were examined as it was felt that there might be a lipid soluble hallucinogen with the fatty oils acting as carriers. No such product could be isolated through the usual methods of solvent fractionation. The saponification equivalents of the oils⁷⁷ (weight of fat saponifiable by one gram - equivalent of KOH) were very close, as well as the neutralization equivalents⁶⁷ of their fatty acids.



Figure 3

Infrared Spectrum (capillary film) of A. M. 19/20 A/1, III.1211

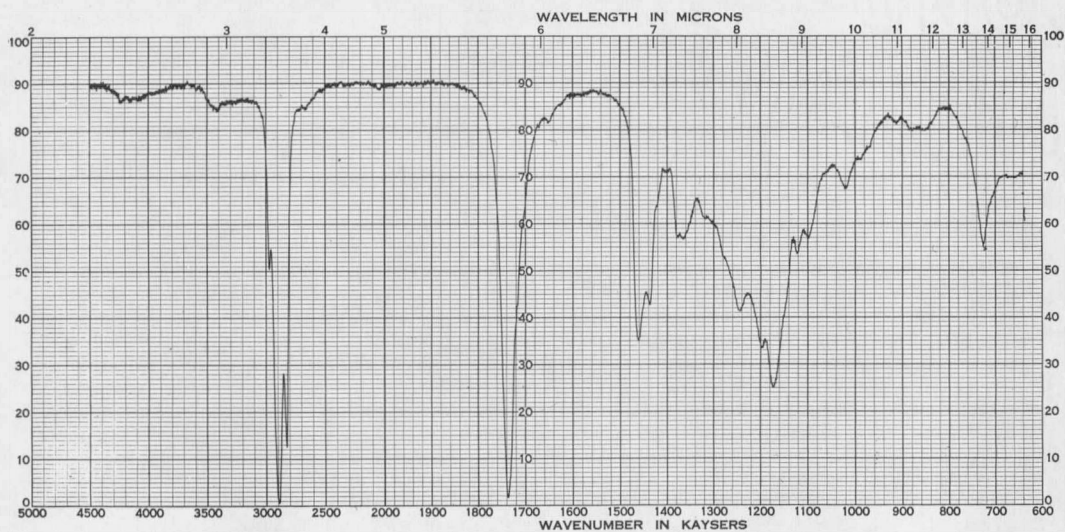


Figure 4

Infrared Spectrum (capillary film) of A. M. 19/20 A/2, III.1212

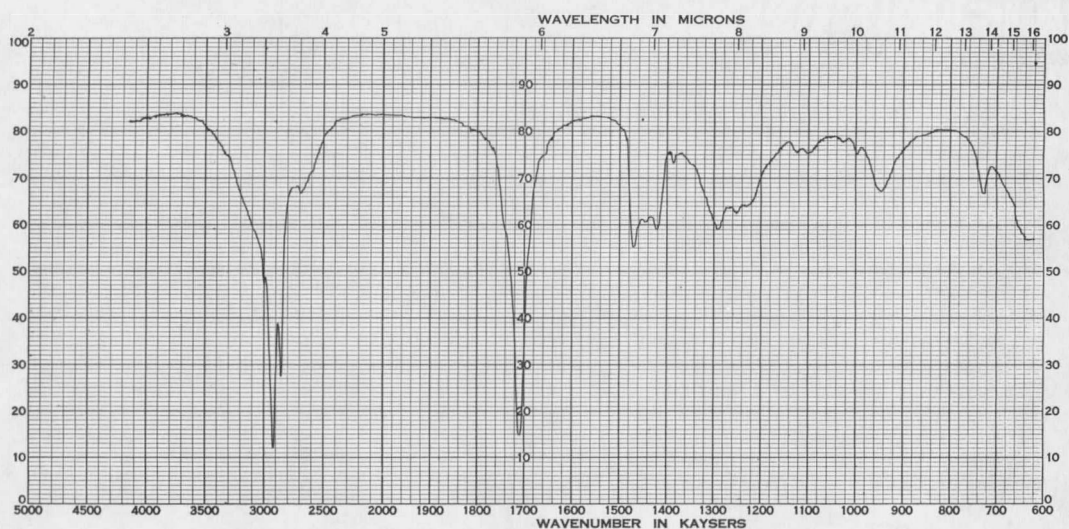


Figure 5

Infrared Spectrum (capillary film) of acids from
A. M. 19/20, A/1 (III.1211)

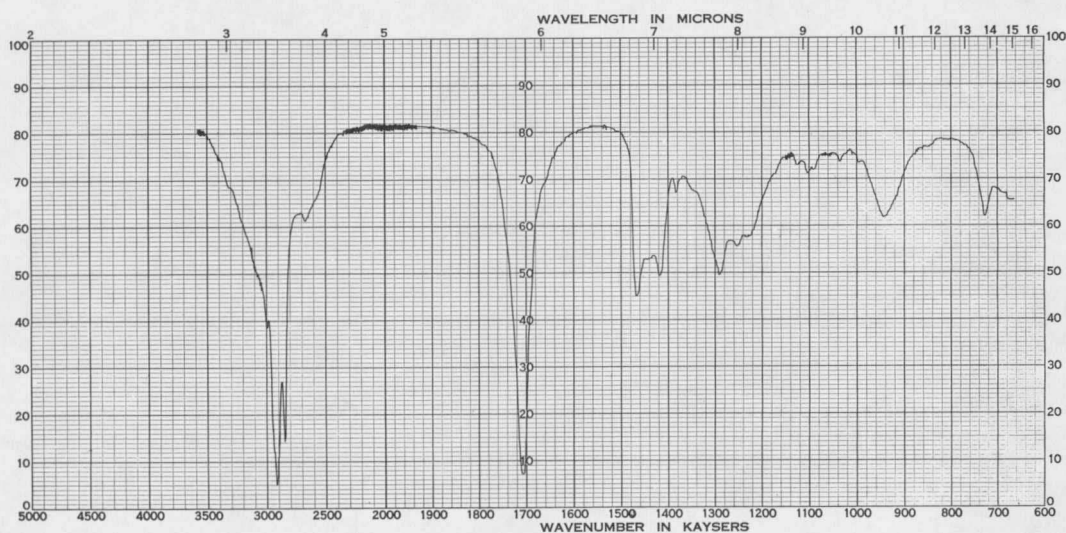


Figure 6

Infrared Spectrum (capillary film) of acids from
A. M. 19/20, A/2 (III.1212)

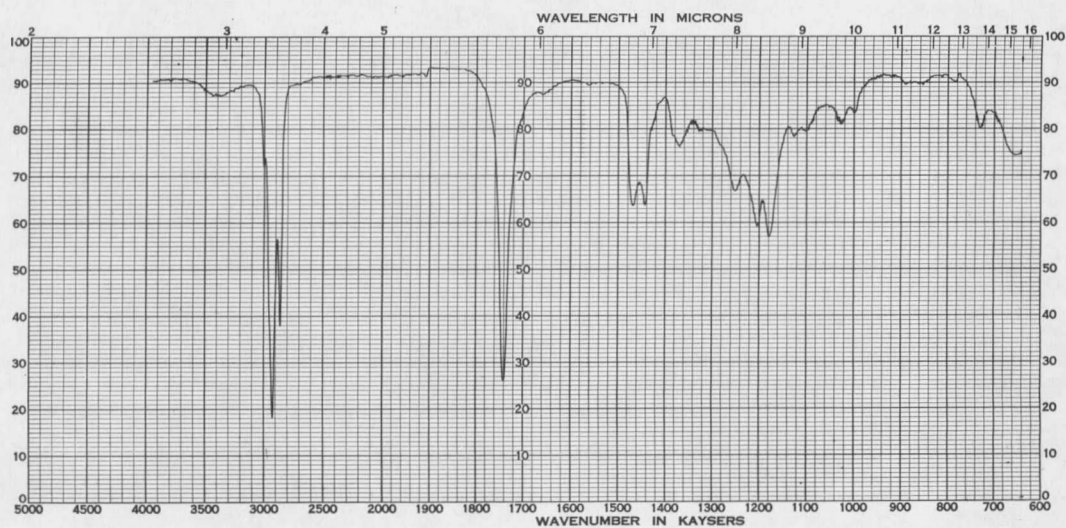


Figure 7

Infrared Spectrum (capillary film) of Methyl Esters
of Acids from A. M. 19/20 A/1 (III.1211)

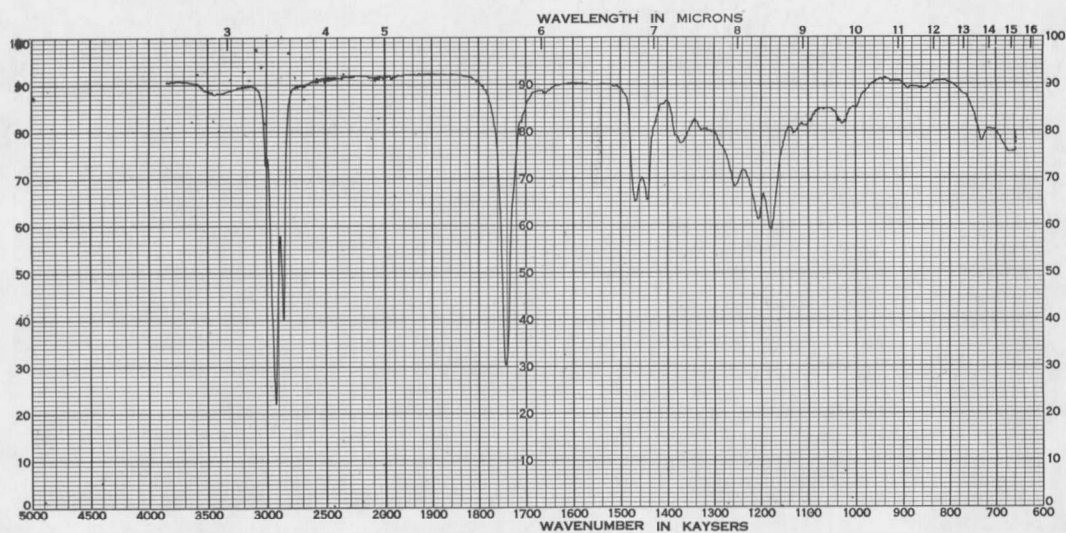


Figure 8

Infrared Spectrum (capillary film) of Methyl Esters
of Acids from A. M. 19/20 A/2 (III.1212)

<u>Fraction</u>	<u>Sapn. equivalent</u>	<u>Neutralization Equivalent</u>
<u>III</u> .1211	235.1	298.4
<u>III</u> .1212	245.8	290.3

For the determination of saponification equivalent,⁷⁷ weighed quantity of oil was refluxed on steam-bath with an excess of standard KOH in ethylene glycol. The amount of alkali consumed was estimated by back-titration with standard acid. A blank was run under the same conditions.

For collecting the fatty acids, oil was saponified with KOH (in methanol or ethylene glycol) and the saponified product freed of neutral fraction by extraction with Skelly, ether and ethyl acetate and then acidified with cold dilute HCl. The liberated acids were taken up in ether, carefully rinsed with water, dried over anhydrous sodium sulphate and evaporated under nitrogen. The acids were dried over paraffin shavings in H₂SO₄ in vacuum desiccator for IR spectra, determination of neutralization equivalents⁶⁷ and for the preparation of methyl esters using ethereal diazomethane. No serious attempts were made to isolate the neutral fraction, suspected to be glycerol.

The infra red spectra of the oils, the acids and their esters from both the fractions III.1211 and III.1212 are shown in Fig. 3-8. Gas chromatograms of the methyl esters were identical. The indication was that from both fractions they were principally esters of oleic acids. Small quantities of other acids saturated and unsaturated appeared to be present.

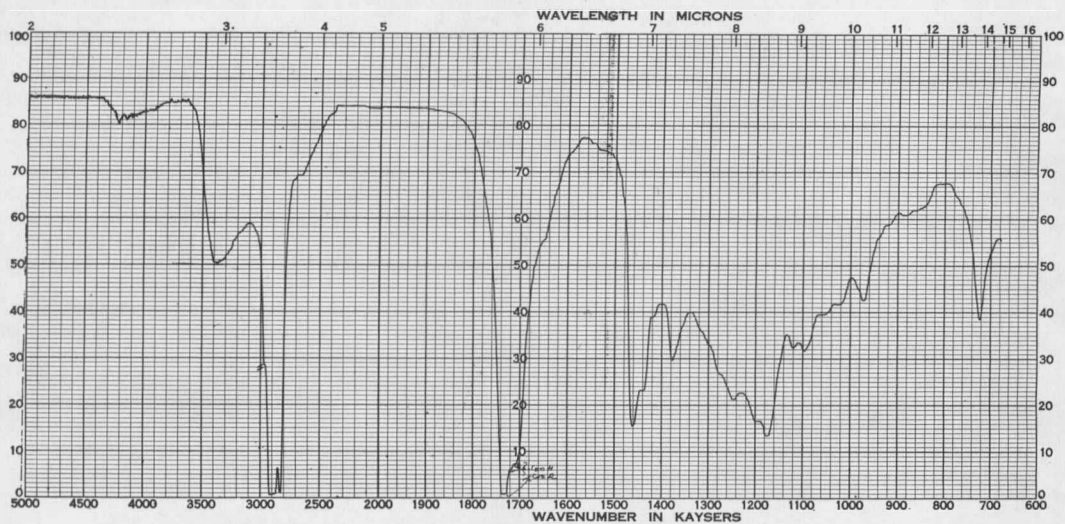


Figure 27

Infrared Spectrum (capillary film) of A. M. 15 C/I, II.5-01

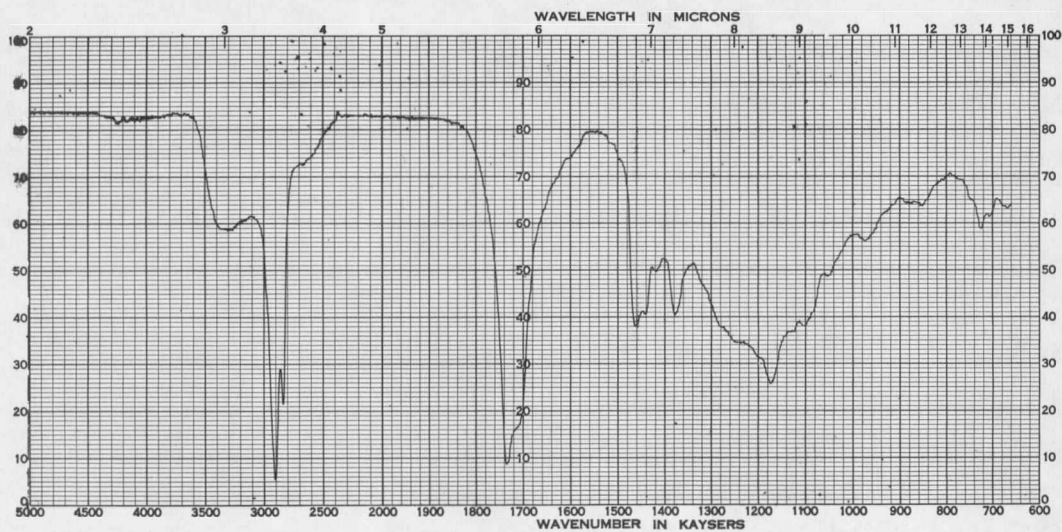


Figure 28

Infrared Spectrum (capillary film) of A. M. 1/20 E₁

The IR spectra of oils obtained similarly from earlier samples of mushroom viz. A. M. 15c/1 (II.5-01), A. M. 15c/2 (II.5-02) and A. M. 1/20, E₁E₂ and E₃ were very similar to those of III.1211 and III.1212.

b) The 70% methanol extracts (III.122) were concentrated under reduced pressure. The concentrate was shaken out repeatedly with diethyl ether to remove any oil (which being very sparingly soluble in Skelly might have escaped into the methanol layer during partition). On removal of ether, an oil 'E' (III.1221) was obtained which was suspended in a large amount of Skelly and chromatographed on alumina (3.5 x 29 cm) (Chart 9).

<u>Eluant</u>	<u>Remarks</u>
1. Skelly	No residue
2. Skelly (90) Ether (10)	No residue
3. Skelly (70) Ether (30) 400 c.c.	Oil
4. Skelly (50) Ether (50) 1 liter	Semi-crystalline residue
5. Skelly (20) Ether (80) 700 c.c.	Little residue
6. Skelly (10) Ether (90) 500 c.c.	Little residue
7. Ether 300 c.c.	Little residue
8. Ether (90) MeOH (10) 200 c.c.	Little residue
9. Ether (80) MeOH (20) 200 c.c.	Little residue
10. Ether (60) MeOH (40) 200 c.c.	Little residue
11. Ether (40) MeOH (60) 600 c.c.	Very little residue
12. Ether (20) MeOH (80) 200 c.c.	No residue.

A. M. 19/20 'E', III.1221

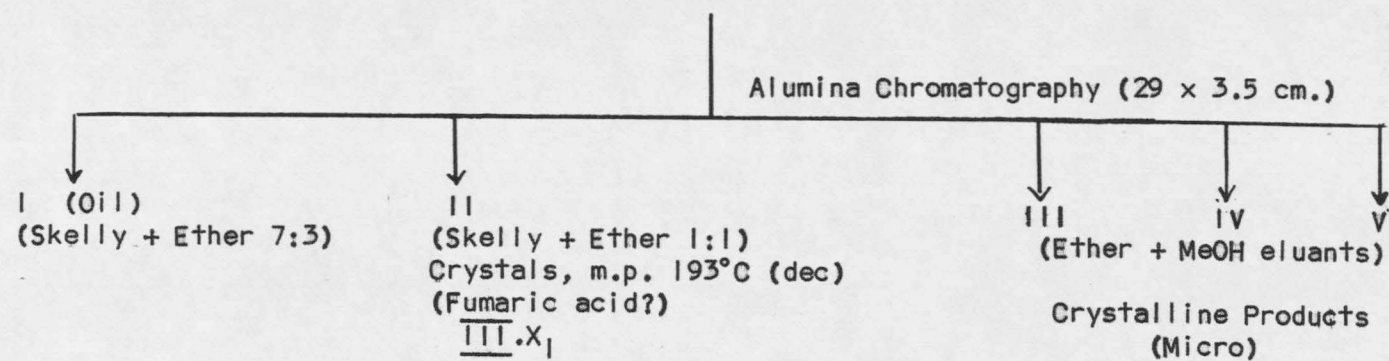


CHART 9

- | | |
|-----------------------------------|---------------------|
| 13. Ether (10) MeOH (90) 400 c.c. | Very little residue |
| 14. Methanol 1 liter | Very little residue |
| 15. Water | Little residue |

Solvent was removed from chromatographic fraction number 4, Skelly (50) and ether (50). The residue on refrigeration yielded a small quantity of a crystalline product (100 mg). On crystallization from water it came out as needles, m. p. 196-97°C (dec.). Capillary introduced at 100°C; melting point depended on rate of heating. About half of the material (44 mg), III.X, was used for biological tests. It was soluble in water and methanol and very sparingly so in ether. Sodium fusion indicated the absence of nitrogen. It gave negative tests with Folin's,⁴⁷ Keller's,^{41,86} and ninhydrin reagents. An aqueous solution was acidic (pH 2.8-3.0) and decolorized bromine-water and KMnO_4 .

The neutralization equivalent of the product, carried out as below was found to be 57.22.

The substance (22.8 mg) was taken up in warm, neutral, 50% ethanol in a stoppered flask, cooled and titrated against standard NaOH (37.80 c.c. of 0.01054 'N' alkali was consumed) using phenolphthalein indicator.⁶⁷

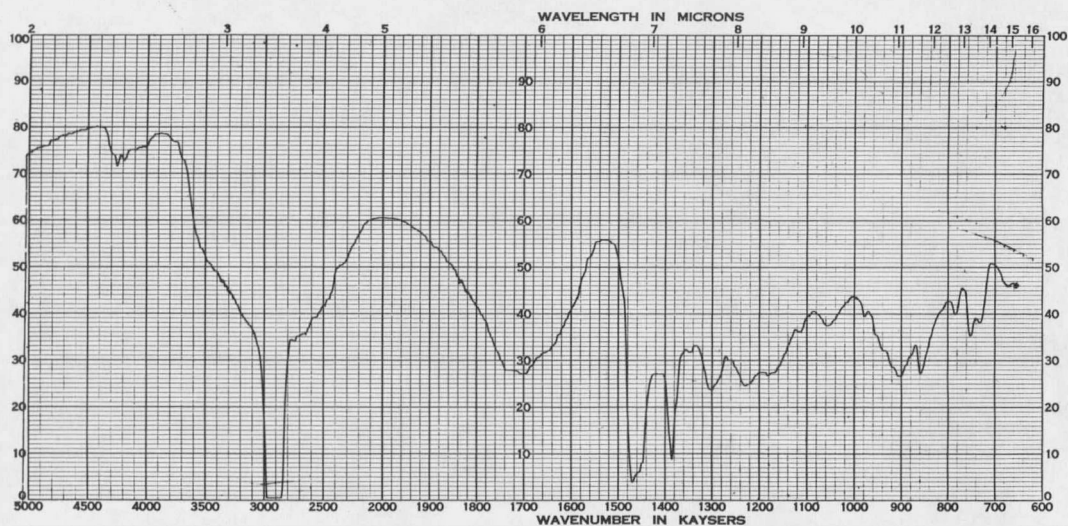


Figure 9

Infrared Spectrum (Nujol mull) of trans-Aconitic Acid

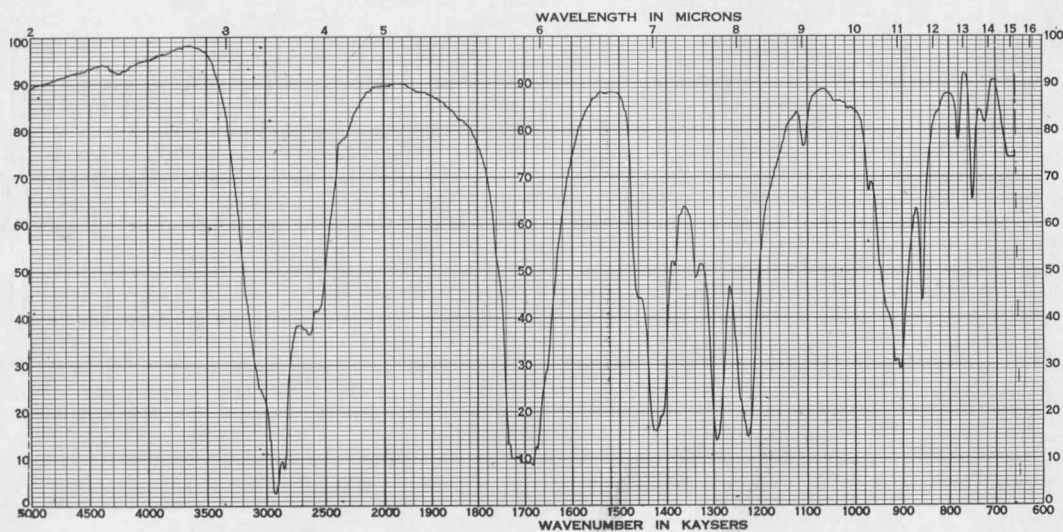


Figure 10

Infrared Spectrum (Nujol mull) of cis-Aconitic Acid

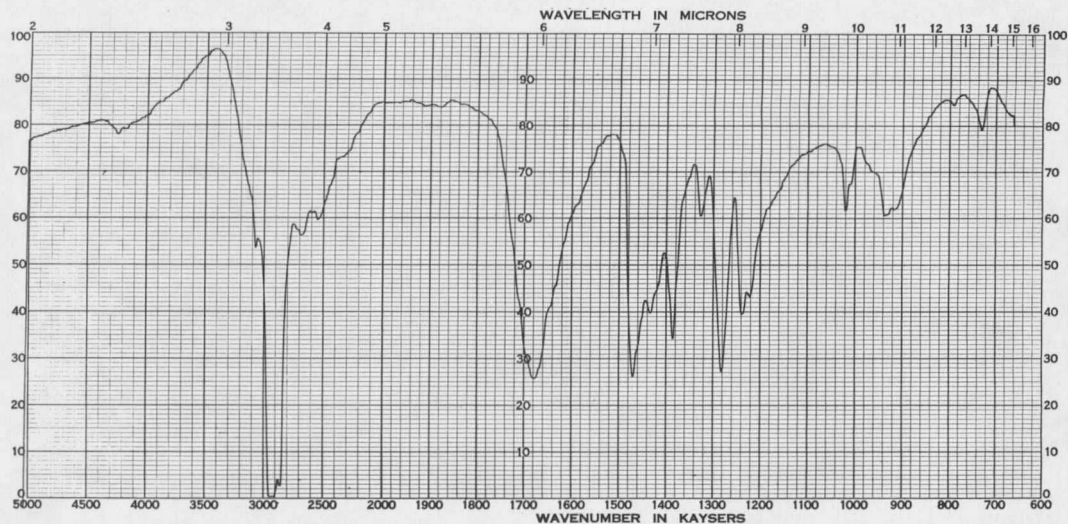


Figure 11

Infrared Spectrum (Nujol mull) of Fumaric Acid

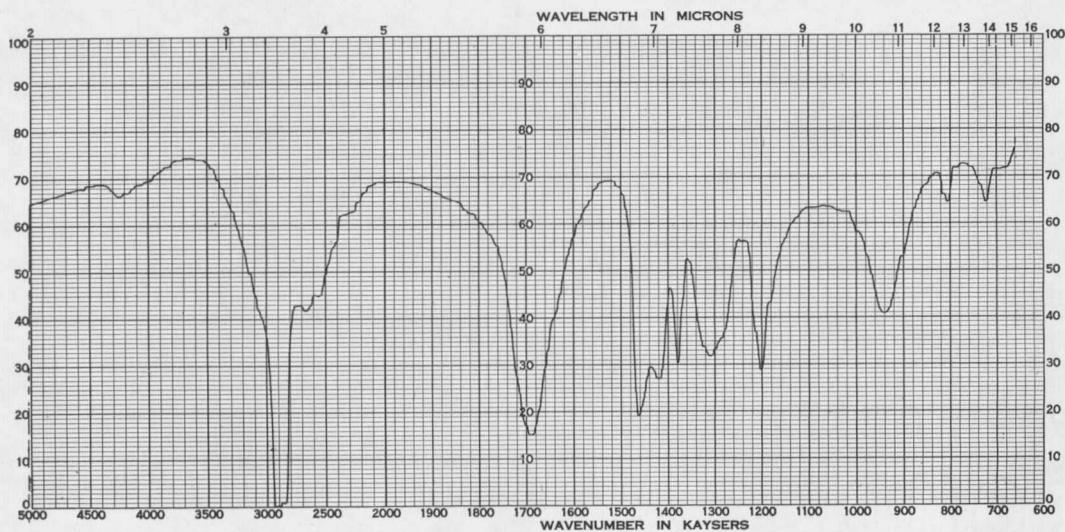


Figure 12

Infrared Spectrum (Nujol mull) of III.X₁, m.p. 196-97°C (dec)

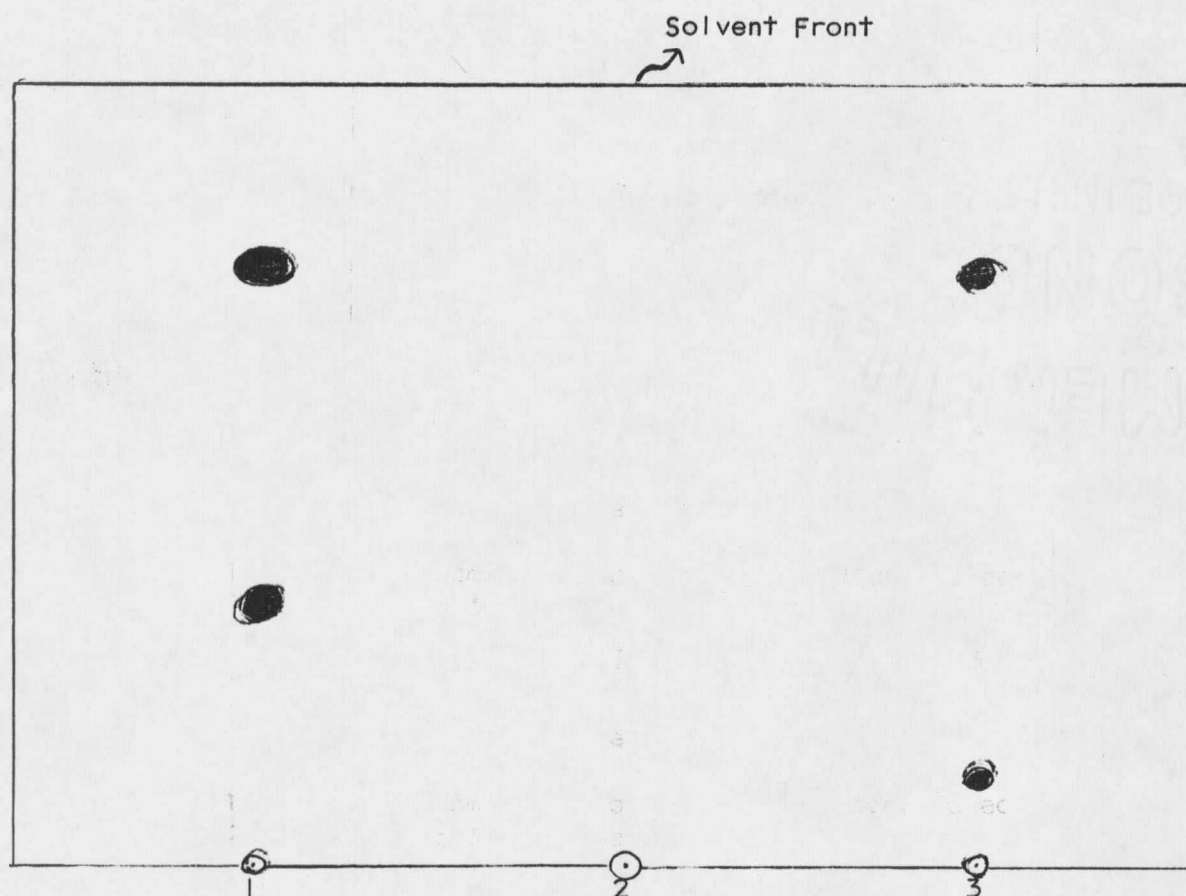


Figure 31

Thin sheet chromatogram of: 1) trans-aconitic acid
2) III.X₁ and 3) cis-aconitic acid

The melting point, 196-97°C (dec.), unsaturation, and neutralization equivalent, 57.22 (N. E. may be such as 1, 1/2 or 1/3 of molecular weight) indicated that it could be trans-aconitic acid,³⁴ m.p. 191°, 194-95°C (N. E. = 58.03, M. W. = 174.11). However, infrared spectra of samples of trans-aconitic acid (General Bio-chemicals, Ohio) and cis-aconitic acid (Nutritional Biochemicals Corporation, Ohio) differed considerably from the spectrum of the mushroom product, III.X₁, m. p. 196-97°C (dec). (Fig. 9-12).

Ascending thin sheet chromatography⁷⁸ confirmed the non-identity. Aqueous solutions of trans-aconitic acid, III.X₁ and cis-aconitic acid were spotted parallel on a silica sheet. The silica sheet was kept for three hours in a glass chamber containing 200 ml of the solvent system: Ethyl acetate (50), Pyridine (30) and water (20). The sheet was taken out, and allowed to dry at room temperature. It was then sprayed with a freshly mixed reagent:⁹⁴ Pyridine (7) and acetic anhydride (3). The sprayed sheet was dried in a current of hot air. Both cis- and trans-aconitic acids exhibited brown spots (on a white background) while the crystalline product III.X₁ did not.

Fumaric acid has been reported to be present in Amanita muscaria by earlier investigators, though apparently not characterized.^{50,89} The melting point of fumaric acid has been variedly reported: 200°C⁷⁷; 287° (293-5)°C, sublims at 200°C, b.p. 290°C³⁴; 300-2° (286-7, 282-4)°C In a sealed tube, sublims above 200° in open vessels³⁶; 287° (closed capillary, rapid heating), sublims at 200°C.⁶⁶ Fumaric acid being dicarboxylic (C₄H₄O₄, M. W. 116.07) has the same neutralization equivalent,

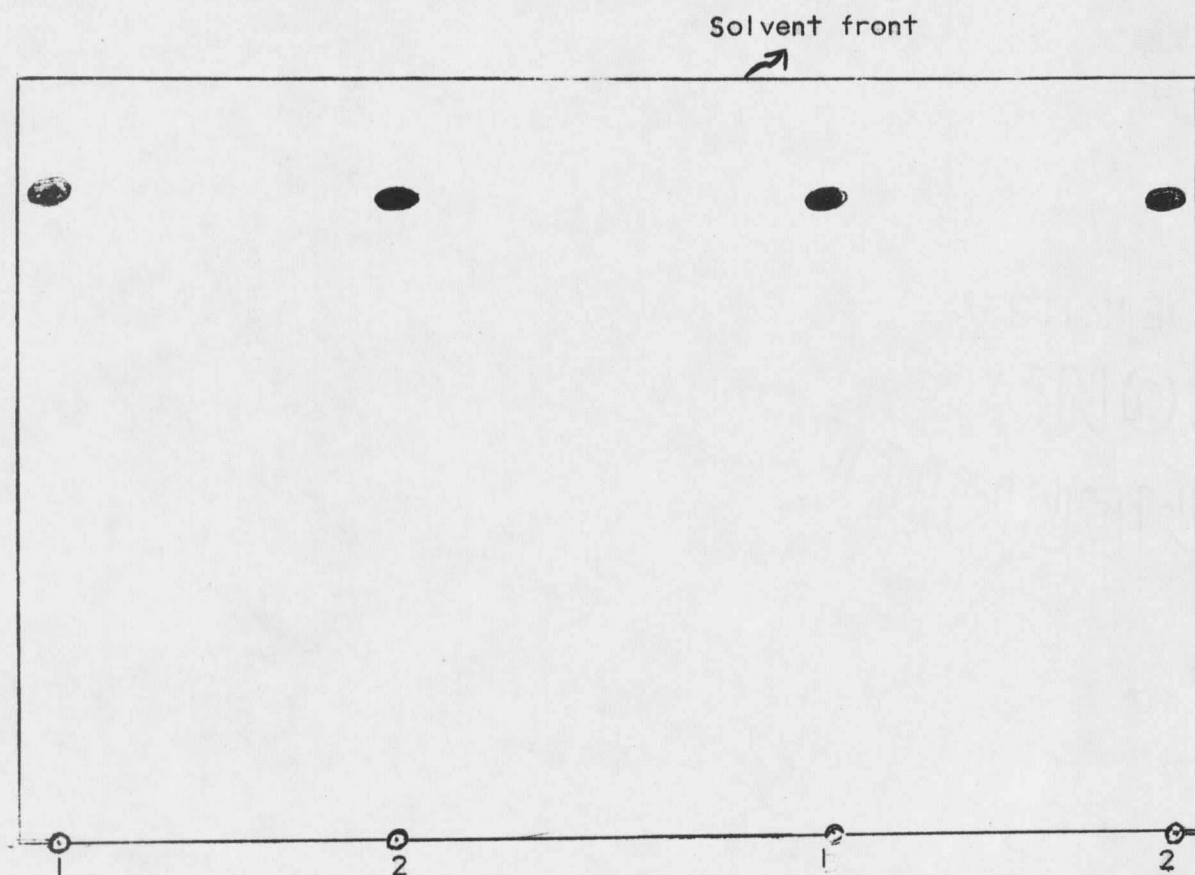


Figure 32

Thin sheet chromatogram of: 1) III.X and 2) fumaric acid

58.03 as aconitic acid ($C_6H_6O_6$, M. W. 174.11), a tricarboxylic acid.

Thin sheet chromatography of III.X₁ parallel with a sample of fumaric acid (Nutritional Biochemicals Corporation, Ohio) indicated identity. A silica sheet with samples spotted was developed for 1 hour and 40 minutes in the solvent system: n-butanol (10), formic acid (2) and water (15). Bromophenol blue¹⁰ (0.04% in 95% ethanol and a few drops of dilute NaOH for adjusting to a definite blue colour, pH 6.7) was used as spraying reagent. Identical yellow spots on a blue background were obtained. (Fig. 32).

The crystalline product III.X₁ was readily soluble in water. Fumaric acid is only sparingly soluble in water, 0.7 g/100 ml at 25°C and 9.8 g/100 ml at 100°C.³⁴ Even in a closed capillary, III.X₁ melted at 195°C in parallel experiments with fumaric acid. Maleic acid, m. p. 130.5°C is easily soluble in water, 78.8 g/100 ml at 25°C and 392.6 g/100 ml at 97.5°C.³⁴

Samples of fumaric acid, III.X₁, maleic acid prepared by gentle warming with water of maleic anhydride (Eastman Kodak) and also an old sample of maleic acid (Eastman Kodak) were chromatographed on a silica sheet, for two hours, in the same solvent system: n-butanol (10), formic acid (2) and water (15). Bromophenol spray¹⁰ gave identical yellow spots for fumaric acid and III.X₁, while the yellow spots of maleic acids were lower. The old sample of maleic acid gave a tiny yellow zone at the same level of fumaric acid indicating the presence of some fumaric acid in it. (Fig. 33). The chromatogram of aconitic acids (Fig 31) gave identical

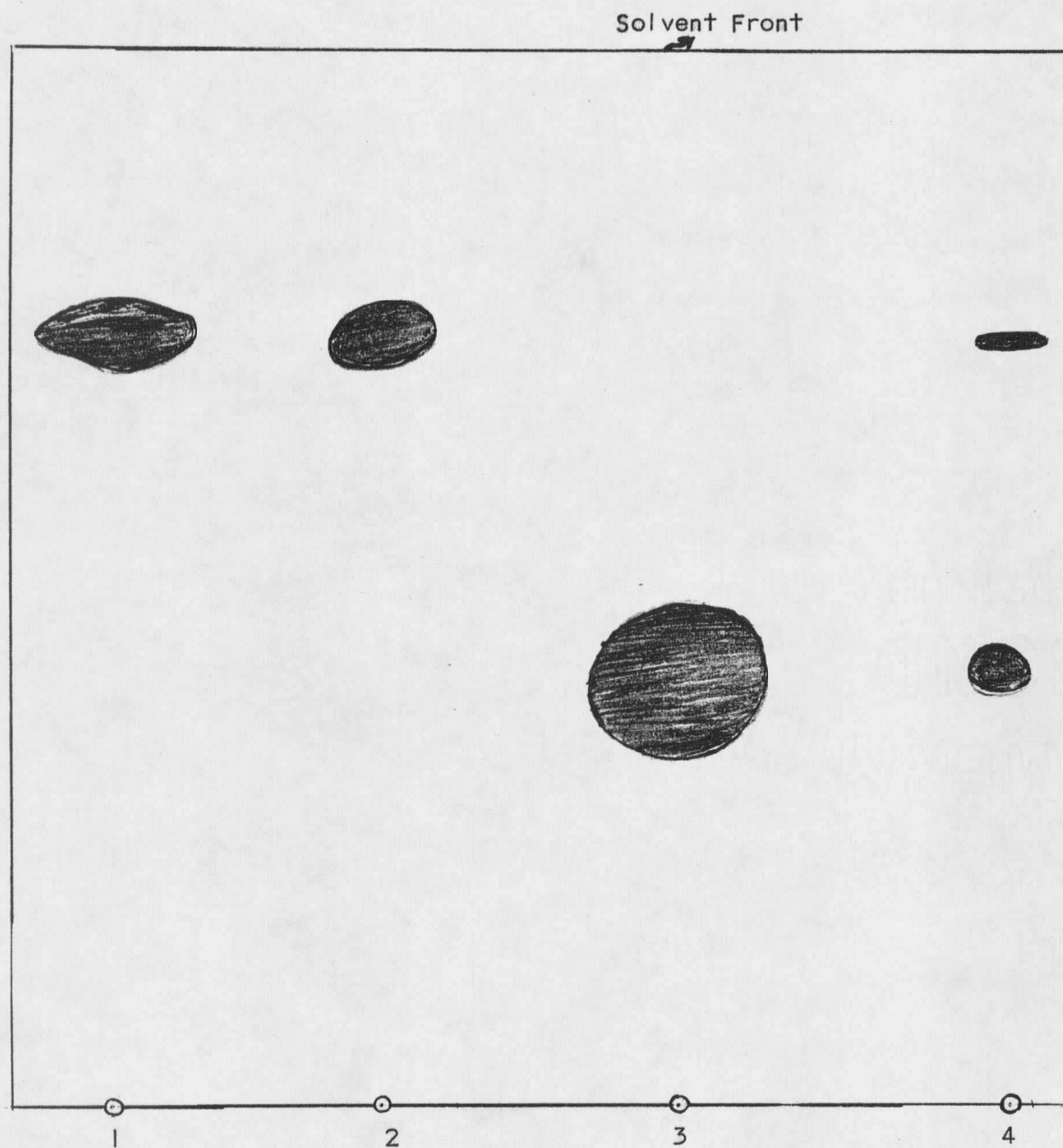


Figure 33

Thin sheet chromatogram of: 1) Fumaric acid;
2) III.X_1 ; 3) Maleic acid from maleic anhydride;
4) Maleic acid

brown spots for cis- and trans-isomers. Of course, in the trans-acid there was a lower brown spot indicating the presence of some other constituent or impurity.

The infra red spectra of fumaric acid and III.X₁ were very close. However, it appears that while III.X₁ consists mainly of fumaric acid, there is some impurity present as further evinced by elemental analysis below:

Found: C, 41.65%; H, 4.64%; M. W. 136
(Geller)

Fumaric acid (C₄H₄O₄, M. W. 116.07) requires: C, 41.39%;
Aconitic acid (C₆H₆O₆, M. W. 174.11) H, 3.47%

Pharmacologists reported that III.X₁ produced only hyperpyrexia and no other objective symptoms, like mydriasis. No more material is available for further purification and preparation of derivatives like methyl esters.

Micro quantity of crystalline products separated from the other chromatographic fractions, but further work on these was shelved.

The ether insoluble fraction of the concentrate from 70% methanol (Chart 7). 'F' (III.1222) was taken up with water (140 c.c., 155 g, pH 2.5) and refrigerated. No crystalline product could be isolated through the usual methods. It was passed through a DEAE (diethyl-aminoethyl) cellulose column (Chart 10) and eluted with n-butanol, arbitrarily collecting various fractions. None of them yielded directly any crystalline substance. The total concentrates were then taken up with absolute ethanol and fractionally precipitated with ether. Gummy insoluble materials which

A. M. 19/20 'F' (III.1222)

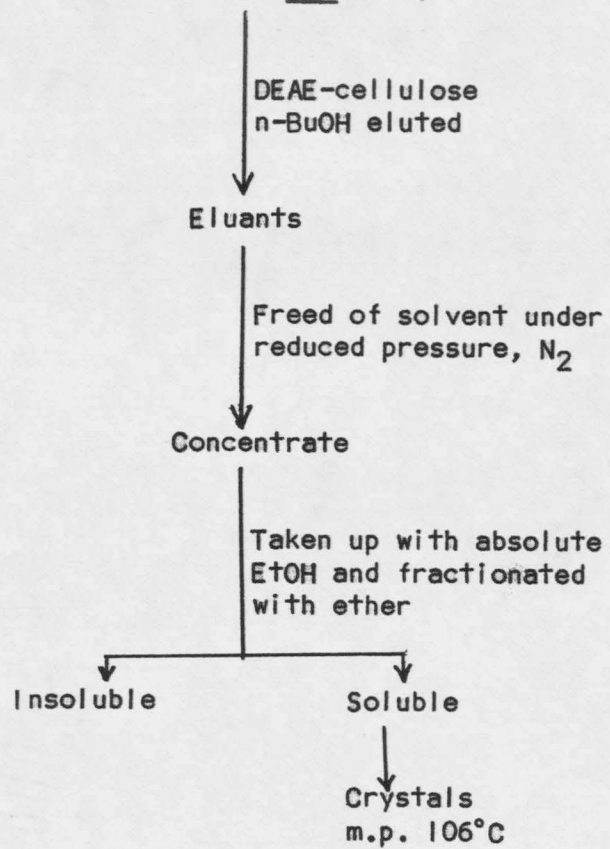


CHART 10



Figure 13

Infrared Spectrum (capillary film) of C_A (chart 13)

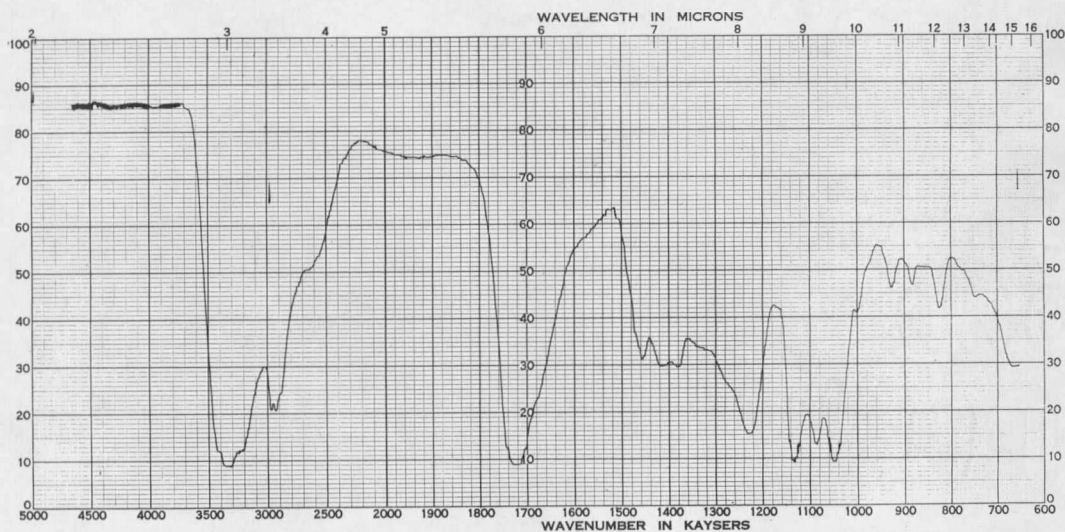


Figure 14

Infrared Spectrum (capillary film) of
ether-soluble fraction of 'F' (III.1222)

separated did not yield any crystalline substance on further manipulation.

The soluble fraction was found to have an infra red spectrum (capillary film) identical with the ether soluble fraction C_A (Chart 13) from the butanol concentrate of the total acetone insoluble fraction (III.11) separated from the main methanol concentrate of the mushrooms. (Charts 7, 11, 12 and 13). Pharmacological reports indicated similar muscarinic activity concentrated in both fractions 'F' (III.1222) and 'C' (III.111).

By persistent working a very small quantity of a crystalline product, m. p. 106°C (dec) was collected from the alcohol-ether soluble portion identical with the same product obtained from C_A (Chart 13) described later on.

Examination of the acetone-insoluble fraction III.11 (Charts 7 & 11)

This was extracted exhaustively with n-butanol in batches (mechanical shaker). The butanol insoluble pitchy brown semi-solid yielded a fraction soluble in absolute methanol. The concentrate 'D' (III.112, 250g) was soluble in water (pH 4.7) and consisted mostly of inorganic material, KCl being predominant. Pharmacologists reported that this fraction did not appear to be hallucinogenic. It was set aside.

From the butanol solutions, solvent was removed under reduced pressure (N₂) and the concentrate 'C' (III.111, 160.5 g, pH 3.4) on refrigeration over a period of two months yielded a semi-crystalline deposit which was filtered (Chart 12). The crystalline mass was washed (to remove the adhering colouring matter and impurities) with methanol

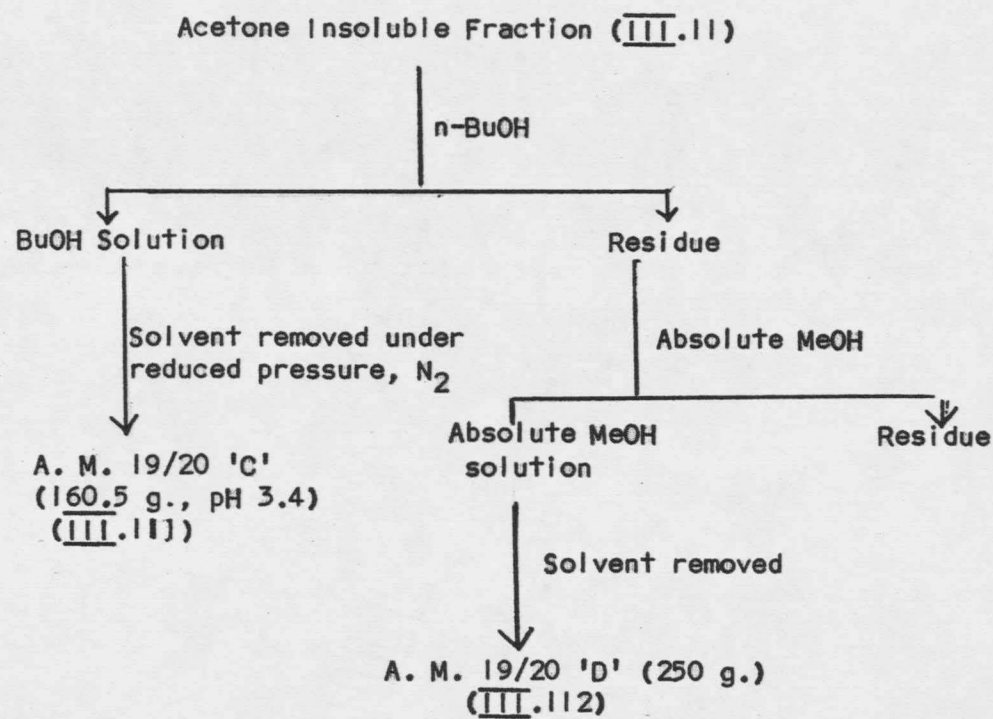


CHART 11

A. M. 19/20 'C' (160.5 g., pH 3.4)
(III.III)

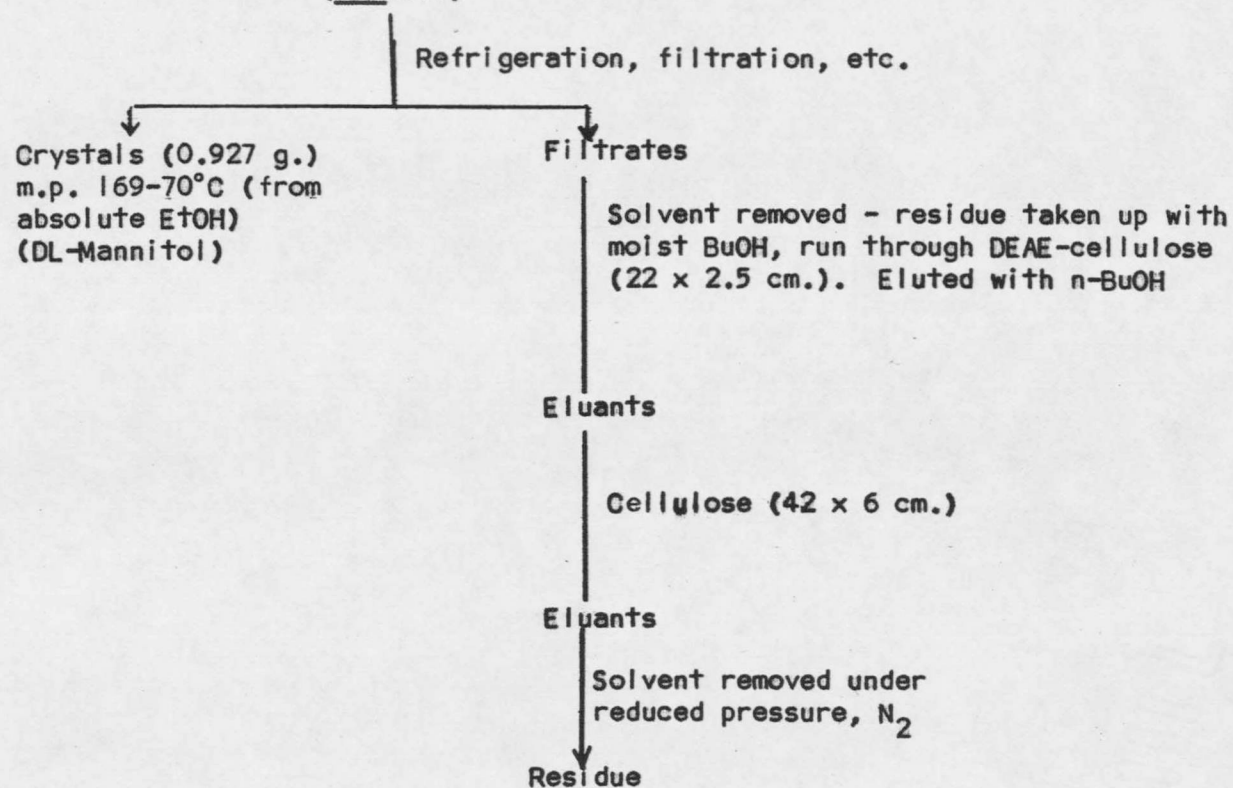


CHART 12

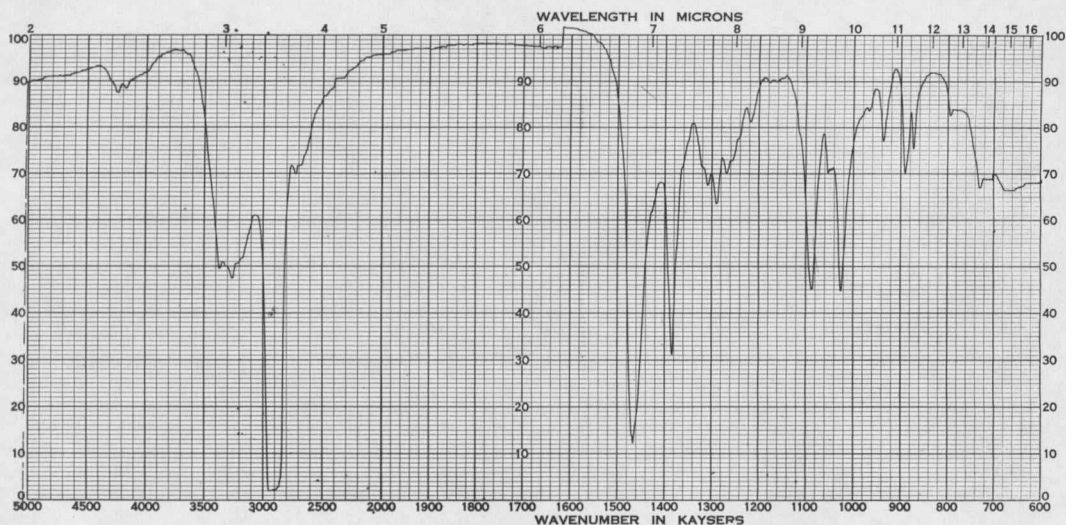


Figure 15

Infrared Spectrum (Nujol mull) of Mannitol

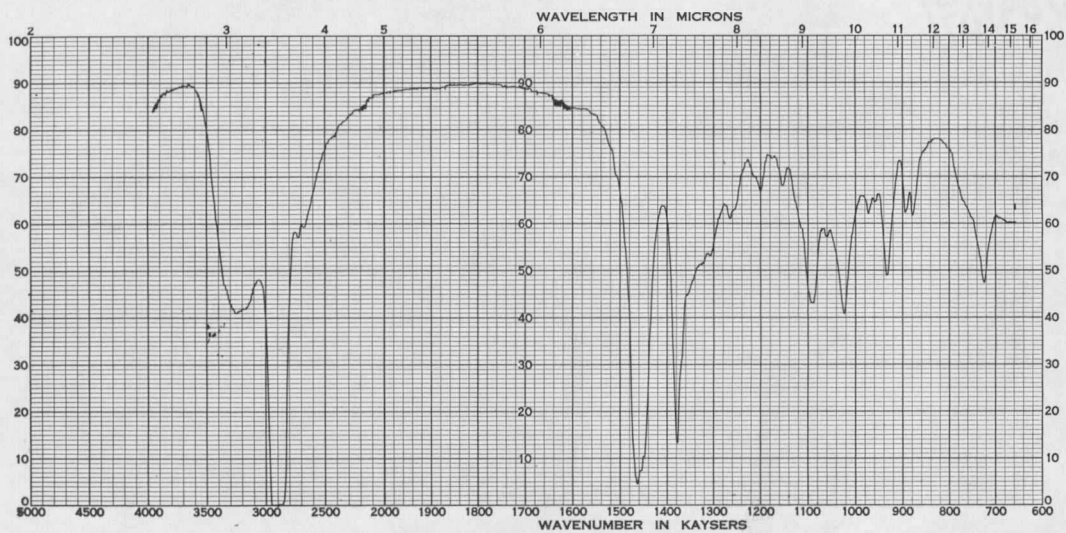


Figure 16

Infrared Spectrum (Nujol mull) of Mannitol from mushroom

and fractionally crystallized from hot absolute ethanol, when it came out as colourless needles, m. p. 169-70°C (yield, 0.927 g). It gave negative tests with ninhydrin, Folin's, Molisch,⁷¹ Keller's and Liebermann reagents. It gave a positive test with periodic acid indicating a vicinal hydroxyls. It did not show any depression in m.p. on admixture with a sample of mannitol, m. p. 166-67°C. From elemental analysis and melting point it could reasonably be identified to be DL-Mannitol⁷¹ (or possibly a mixture of D-Mannitol, m. p. 166° and DL-Mannitol, m. p. 170°). The infrared spectra of the substance and D-mannitol sample were almost identical (Fig. 15-16). There were minor differences in the finger print region.

Thin sheet chromatography⁷⁸ confirmed the identity with mannitol. Aqueous solutions of the crystalline product parallel with a sample of mannitol (Eastman Kodak) were spotted on a silica sheet. The silica sheet was kept for 55 minutes in a glass chamber containing the solvent system: Ethyl acetate (50), Pyridine (30) and Water (20). The silica sheet was taken out and dried at room temperature. It was then sprayed with 50% H₂SO₄ and dried in oven. Identical dark zones were observed with both the products (Fig. 36).

Found: C, 39.10%; H, 7.56%
(Geller)

Mannitol requires: C, 39.56%; H, 7.74%
(C₆H₁₄O₆, M. W. 182.17)

Thin sheet chromatography is faster than the currently known paper chromatographic methods, for the identification of carbohydrates. Both the techniques involve equally the same length of time in the search for

-80-

Solvent Front

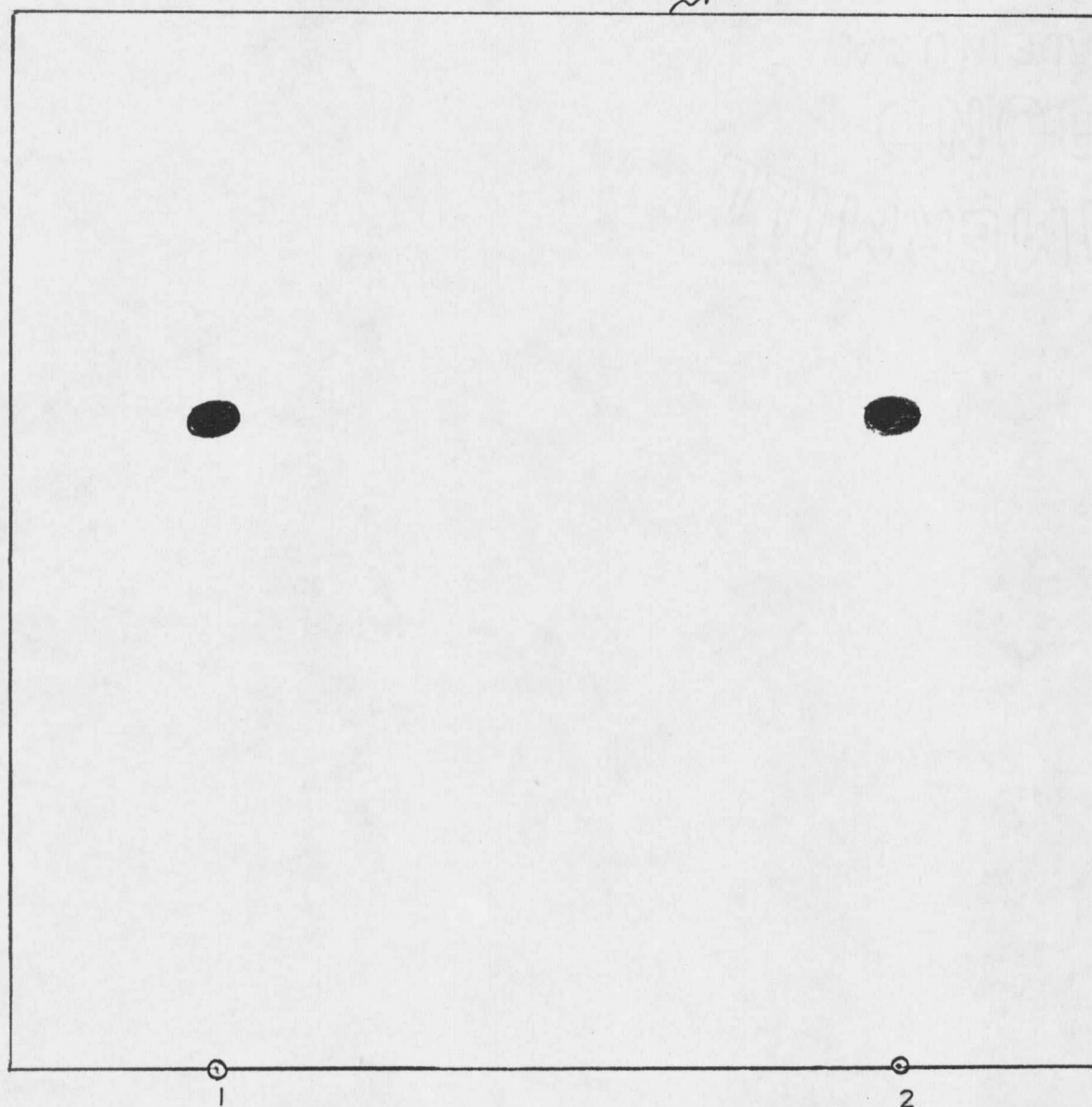


Figure 36

Thin sheet chromatogram of:

- 1) Mannitol (sample from Eastman Kodak)
- 2) Mannitol isolated from A. muscaria

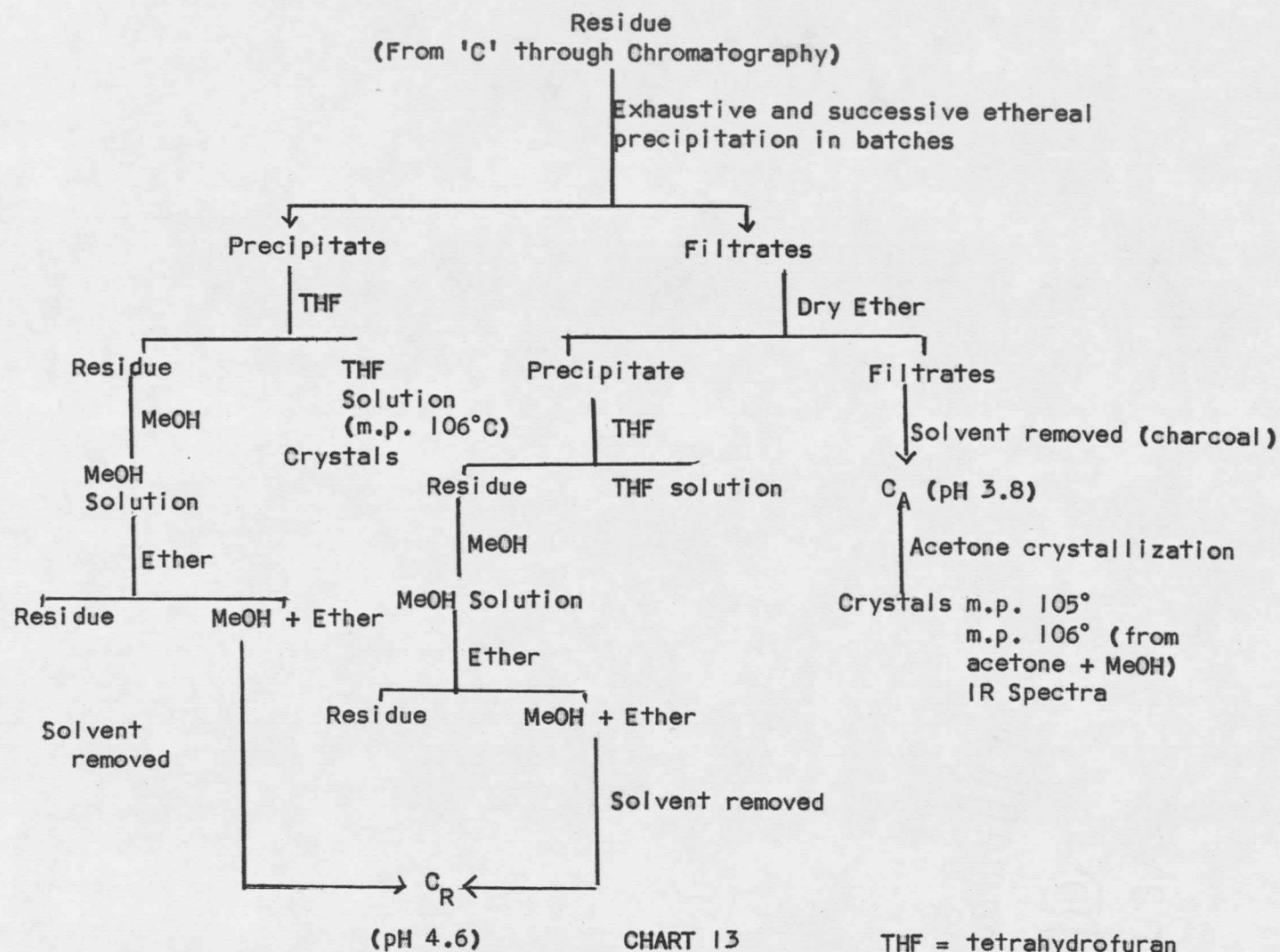
suitable solvent systems and spray-reagents for identification.

Paper chromatography was carried out earlier to identify the mannitol isolated from A. muscaria with a known sample of mannitol.

From 10 to 14 hours were needed for development using the solvent system: n-butanol (6), pyridine (4) and water (3). Ammoniacal silver nitrate was used as a spraying reagent.^{91, 92} If the sprayed paper is dried fast in a current of hot air, the background burns dark brown, making it difficult to see clearly the resulting identical chocolate coloured spots of mannitol. This difficulty can be avoided by allowing the paper, after spraying with ammoniacal silver nitrate, to dry first at room temperature for a few hours and then exposing for a few minutes to hot air.

The filtrates left after removal of mannitol were freed of solvent, taken up in minimum quantity of moist butanol and passed through a DEAE (diethylaminoethyl) cellulose column (22 x 2.5 cm). The butanol eluants were then passed through a cellulose column (42 x 6 cm) and eluted again with n-butanol. Various arbitrarily collected fractions of eluant were subjected to a number of preliminary experiments in the hope of isolating some crystalline substance but without success. Then the total residue was subjected to elaborate fractionations as indicated under Chart 13.

The infra red spectrum of C_A, the ether soluble fraction in the above scheme (Fig. 13) was found to be identical with that of the ether soluble portion of 'F' (III.1222) (Fig. 14) as were the pharmacological reports of muscarinic activity concentrated in these two fractions, as



already noted. The maximum amount of the crystalline product m. p. 106°C (dec) was obtained from C_A .

Characterization of the crystalline product m. p. 106°C (dec).

The total crude crystallizate (0.5285g) was crystallized from a mixture of methanol and acetone and finally from hot absolute ethanol. On very slow crystallization during a period of nearly three months, colourless needles (yield, 292 mg), m. p. 106°C (dec) were obtained. Sodium fusion indicated the absence of nitrogen and halogen. Tests with Molisch and Red Tetrazolium²⁷ for reducing sugars were negative. Test with periodic acid was positive.

No residue was left after ignition and emission spectrum indicated absence of inorganic contaminants.

After acid hydrolysis (20 mg. refluxed with 5 c.c. of 5% HCl on steam bath for 2.5 hours) it gave a positive test with Red Tetrazolium. Besides, the time taken to develop the pink colour²⁷ when tested with Red Tetrazolium along with known sugars indicated the likelihood of glucose being released on hydrolysis.

The hydrolysate was neutralized with Na_2CO_3 , concentrated and chromatographed on Whatman paper parallel with standard glucose. The solvent system consisted of Pyridine (4), n-butanol (6) and water (3). Spraying with CD-1³³ indicated identity with glucose. There was a little streaking due to the NaCl present. The hydrolysate was desalted by passing through M. B. 3 resin (mixed bed) and then chromatographed as above. Glucose and no other sugar was observed to be present when sprayed with CD-1 (Fig. 34).

Solvent Front

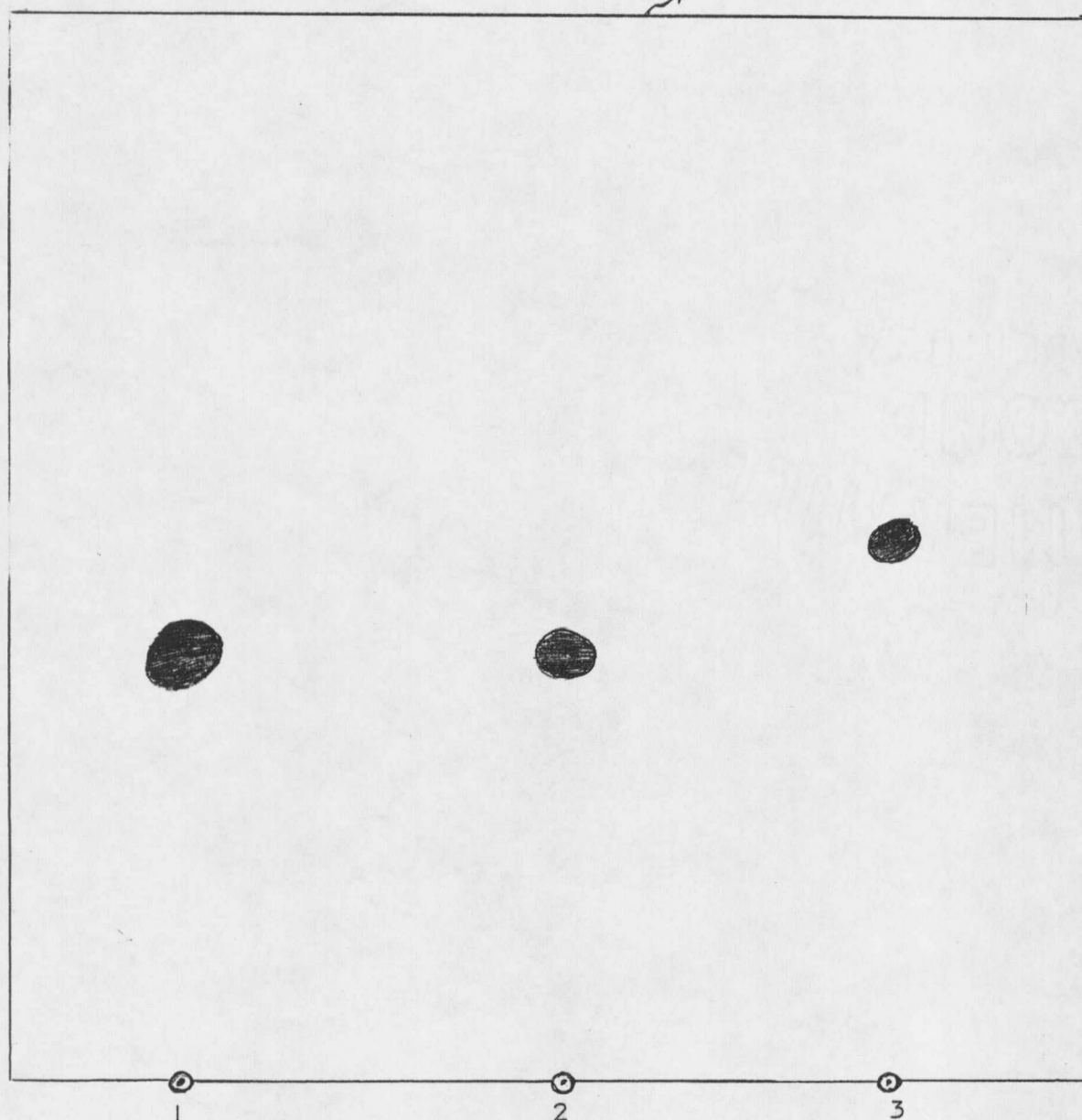


Figure 34

Paper chromatogram of:

- 1) Glucose sample;
- 2) acid hydrolysate of β -glucoside, m.p. 106°C (dec) from A. muscaria
- 3) Xylose sample

In another experiment, the crystalline product was incubated (42°C, 6 hours) with emulsin and chromatographed, parallel with standard glucose. Spraying with CD-1 indicated identical R_f value and no other components. This indicated that the crystalline substance m.p. 106°C could be a B-glucoside. Acetylation of the glucoside with acetic anhydride and pyridine was attempted twice but no crystalline acetyl derivative could be collected. No material was left for the isolation and identification of the aglycone. The substance had a slight tendency to sublime in vacuum at 100°C (1 mm pressure) but in an open capillary it decomposed only above its melting point.

Found:

(Berkeley) C, 40.73%; H, 6.80%; M. W. 350

(Geller) C, 44.08%; H, 7.51%; Loss in weight 6.95%

The difference in analytical values, for the same substance, is probably due to varying degree of hydration of sample analyzed in the above two laboratories. All the available evidence indicates that the substance is a glucoside. This can be settled only after the nature of the aglycone is known. In the meanwhile one may feel that the substance may be a disaccharide monohydrate, $C_{12}H_{22} \cdot H_2O$ (M. W. 360) will analyze for C, 40.00% and H, 6.60%, in close agreement with analytical values obtained from Berkeley Analytical Laboratory for the substance, glucoside, m.p. 106°C isolated from A. muscaria.

Trehalose, $C_{12}H_{22}O_{11} \cdot 2H_2O$, m. p. 96.5-97.5°C is the closest known disaccharide with non-reducing end. Trehalose is also reported to be found at times in young mushrooms. However, the infrared spectra of a sample of trehalose and the crystalline substance, m. p. 106°C (dec) from the mushroom differ considerably (Fig. 17, 18) in spite of unequal intensity of absorption spectra.

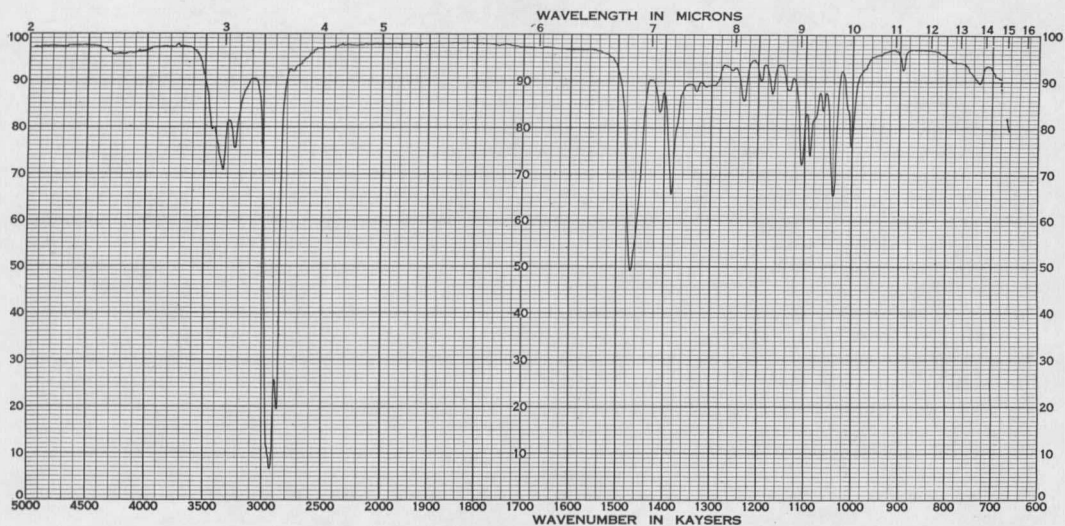


Figure 17

Infrared Spectrum (Nujol mull) of β -glucoside,
m. p. 106°C (dec) from mushroom

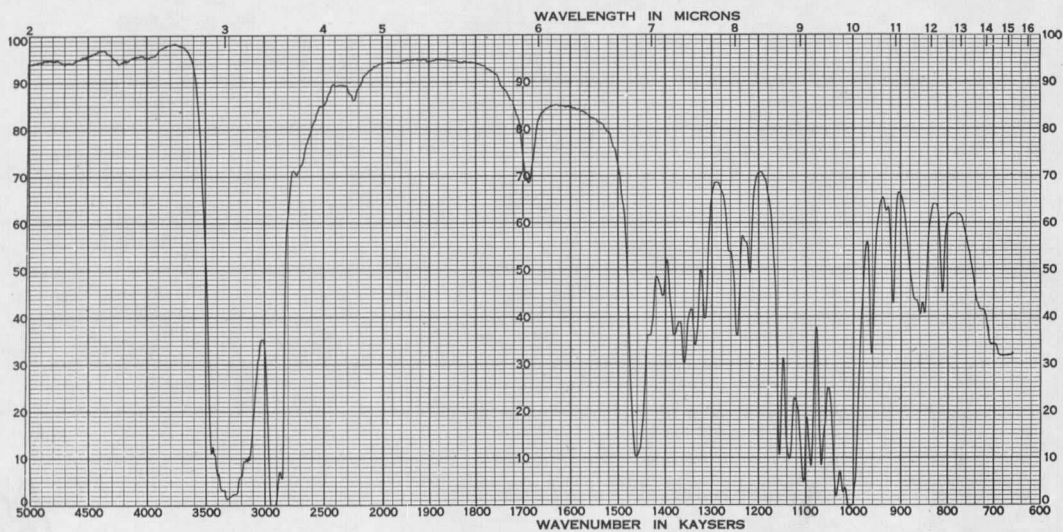


Figure 18

Infrared Spectrum (Nujol mull) of Trehalose

B. Water Extracts

The pressed marc (left after extraction with methanol) was extracted with distilled water at room temperature by maceration, percolation, shaking in batches (mechanical shaker), filtration in a basket centrifuge etc. This was a very tedious process during a period of two months (July-August 1959). The filtrates were concentrated at room temperature (air-blowing, vacuum distillation etc).

The concentrate (fecal odour) was chromatographed on a cellulose column (42 x 6 cm) (Chart 14) and eluted with moist butanol. The butanol eluants on removal of solvent under reduced pressure yielded a golden yellow syrup (1.5 g), containing micro-crystalline material, fluorescent in u.v. and giving a positive test with Keller's reagent for indoles. With small quantities of the concentrate at a time, various micro experiments were tried (chromatography, solvent fractionation, etc), but the major part was reserved for future work. Attention was focused on the next lot of mushrooms, described under Part IV.

After butanol elution, the column was washed with distilled water, filtered (celite) and concentrated. It was found to contain a good deal of inorganic components.

The final dry marc, after water extraction, weighed 3952 g.

PART IV

As a result of experience gained in working up the initial batches of fresh mushrooms it was considered desirable to process the mushrooms more efficiently in a pharmaceutical pilot plant and obtain the concentrates for investigation. Accordingly, six cans of fresh mushroom in

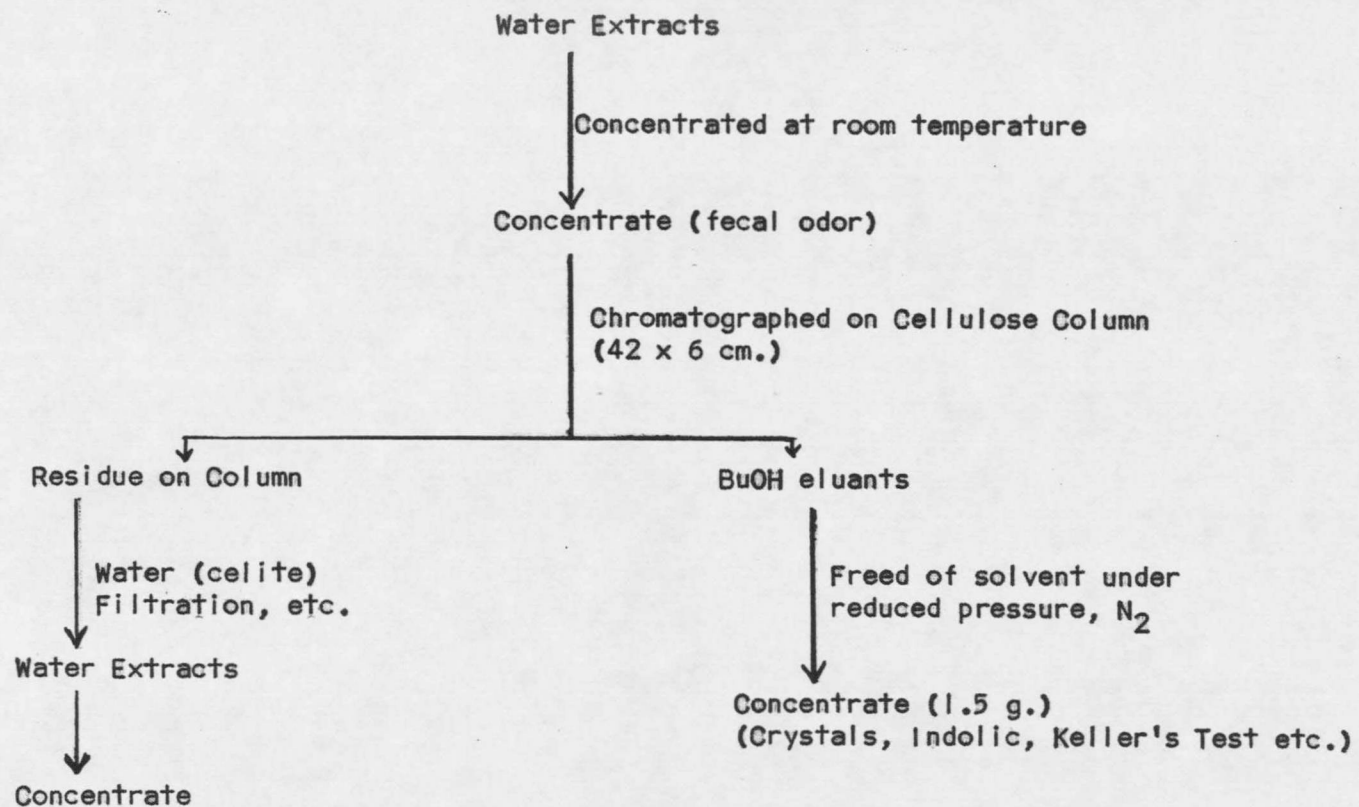


CHART 14

methanol received in May 1960 were processed at the Abbott Laboratories, North Chicago, Illinois, in June 1960 under the direct supervision of Dr. Cook. The extractions were based upon those adopted earlier.

One significant change was that the initial aqueous methanolic extracts were collected separately and worked up, distinguishing them from subsequent dry (absolute) methanolic extractives.

The mushrooms and methanol together weighed 98.6 Kg. Solvent was drained through cloth (58.1 Kg) and the whole mushrooms (40.5 Kg) were pulped in a meat grinder (Chart 15). Filtration through a stone-ware filter under vacuum was not satisfactory. Only four gallons of filtrate was collected by this method. Then, the pulpy mushrooms were run in batches in a basket centrifuge (4 Kg. at a time till the weight was reduced to about 1.2 Kg). About 20-25 gallons of filtrates was collected by this method.

The total aqueous methanolic extracts (pH 6.00, containing approximately 60% water) were freed of solvent below 25°C under reduced pressure in a L. T. V. (low temperature vacuum) still. The concentrate 'A' (IV.1) was obtained as a thick, dark brown syrup (3628 g, pH 5.1).

The residual pulp (11.4 Kg) was air-dried at 35°C overnight to 5.9 Kg (corresponding to 3.604 Kg dry weight) and was extracted with absolute methanol (99.6%) in batches (Chart 16). Each batch was extracted with about 12 gallons of methanol by the following successive operations using lightning stirrer for circulation of solvent:

Fresh Mushroom (Part IV)
 (Preserved in MeOH; received in May, 1960
 Extractions at Abbot Laboratories in June, 1960)

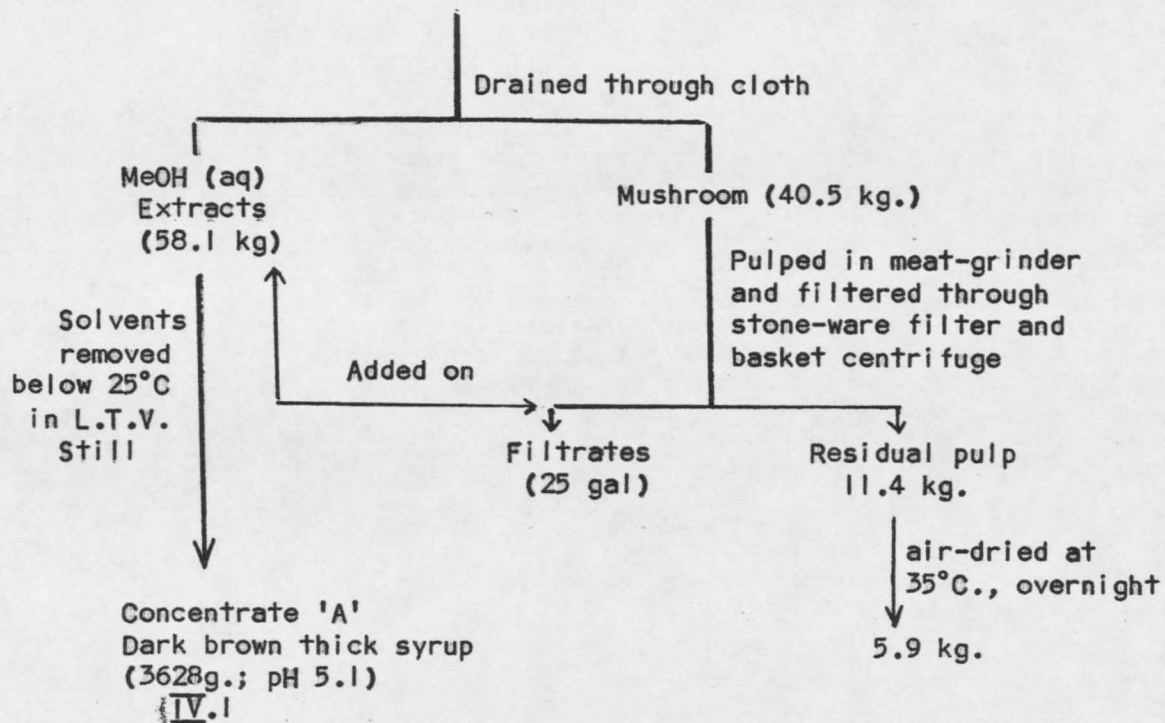


CHART 15

Extraction with
MeOH (99.6%) in batches
12 gallons each time
stirring
circulating
standing with MeOH, etc.
filter



1. Stirring for half an hour, standing for 1/2 hour and filtering. (Filtrates contained $\approx 4.7\%$ water)
2. Stirring for 1 hour, circulating for 2 hours and filtering (water content of filtrates $\approx 1.4\%$)
3. Circulating for 2 hours and filtering (water content $\approx 0.6\%$) (Chart 16).

The extracted mushroom pulp was centrifuged when about 3 liters of filtrates were collected. The marc weighed 4.5 Kg (dry weight 2.656 Kg). It was extracted with deionized water by stirring for about an hour at a time and filtering. The filtrates were concentrated in the L. T. V. still. The concentrate (4.2 Kg) was processed in this laboratory. It was found to contain mushroom pulp and was therefore refiltered. This filtrate weighed 3288 g. corresponding to 33 g. dry weight, 'C', IV.3.

The combined absolute methanolic extracts on distillation in L. T. V. still below 25°C gave a light brown syrup 'B' (IV.2, 2947 g, pH 7.5). On keeping for a few days in the refrigerator, it deposited a waxy mass embedded with crystalline substance and was worked up as outlined under Chart 17.

Characterization of the crystalline product

The total crystalline product, m. p. 183°C , recrystallized from aqueous methanol etc., (illustrated in Chart 17) was finally recrystallized by taking up in minimum quantity of water, adding absolute ethanol dropwise to faint turbidity and setting aside. Thus repeated fractional crystallizations yielded a total of 606.6 mg., colourless needles, m. p. 186°C (dec) (First crop of crystals, 0.4120g; Second crop, 0.1926g and

Concentrate 'B' IV.2

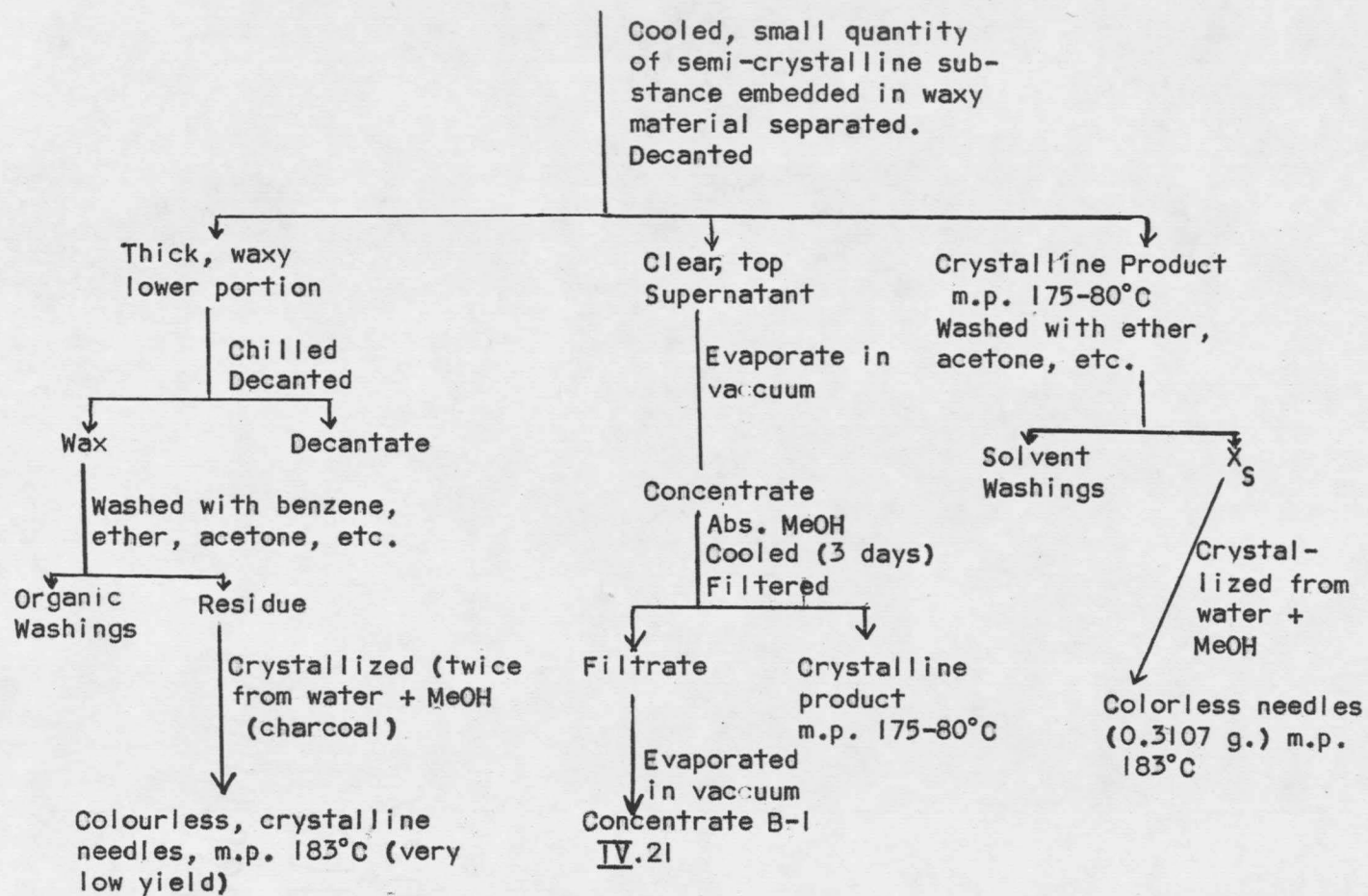


CHART 17

the last crop about 2 mg).

Aqueous solution of the crystals was nearly neutral (pH 7.00 with close-range pH paper). Fusion with sodium indicated absence of nitrogen and halogen. Tests with Molisch, Folin's, FeCl_3 , Keller's and Liebermann's reagents were negative. It gave a positive test with periodic acid but acetylation was not successful. It gave negative tests with Red Tetrazolium both before and after treatment with acid. Paper chromatography of acid hydrolysate parallel with known sugars was not definitive.

When heated on a platinum spatula, it burned like sugars. The flame was characteristic of potassium salts. The small amount of residue on the spatula gave a basic aqueous solution (pH 10.00). Emission spectrum indicated the presence of only potassium in moderate concentration.

From elemental analyses, m. p. etc., the substance was suspected to be potassium gluconate. Hence a sample of potassium gluconate was prepared from glucono delta lactone (Nutritional Biochemicals Corporation, Ohio) and their infrared spectra appeared to be very similar (Fig. 19, 20).

Found: C, 30.33%; H, 4.81%

(Berkeley)

(Geller) C, 30.70%; H, 4.91%; K, 16.59%; O, 47.72%; M. W. 107,

C, 30.99%; H, 4.57%; K, 16.54%; O, 47.97%; M. W. 107

Potassium Gluconate, $\text{C}_6\text{H}_{11}\text{O}_7\text{K}$ (M.W. 234) requires:

C, 30.77%; H, 4.70%; K, 16.66%; O, 47.86%

The preparation of a pure salt of gluconic acid presents considerable problems¹. The infrared spectra of samples of potassium gluconate obtained from Chas. Pfizer (99% pure) and Matheson Coleman & Bell (98%)

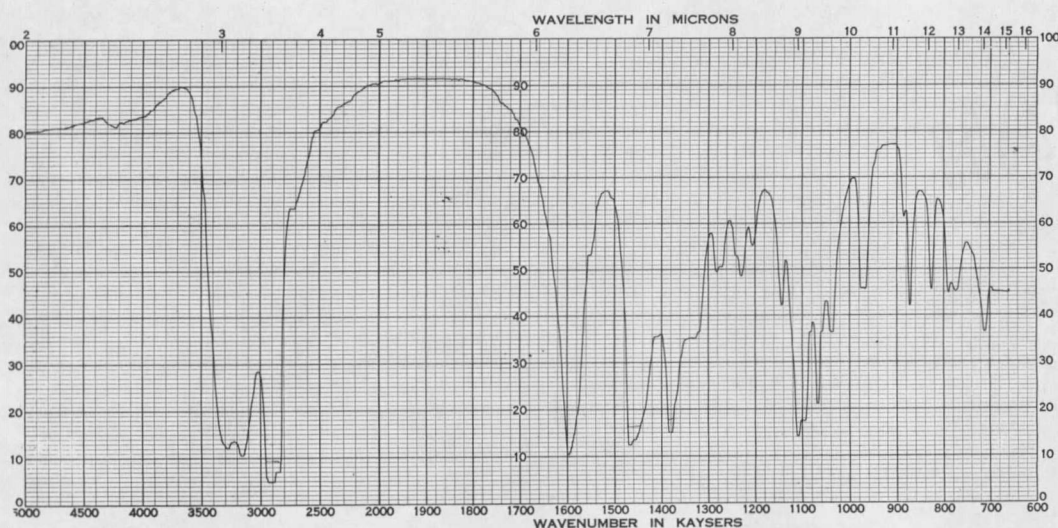


Figure 19

Infrared Spectrum (Nujol mull) of potassium gluconate from mushroom



Figure 20

Infrared Spectrum (Nujol mull) of potassium gluconate prepared from glucono-delta-lactone

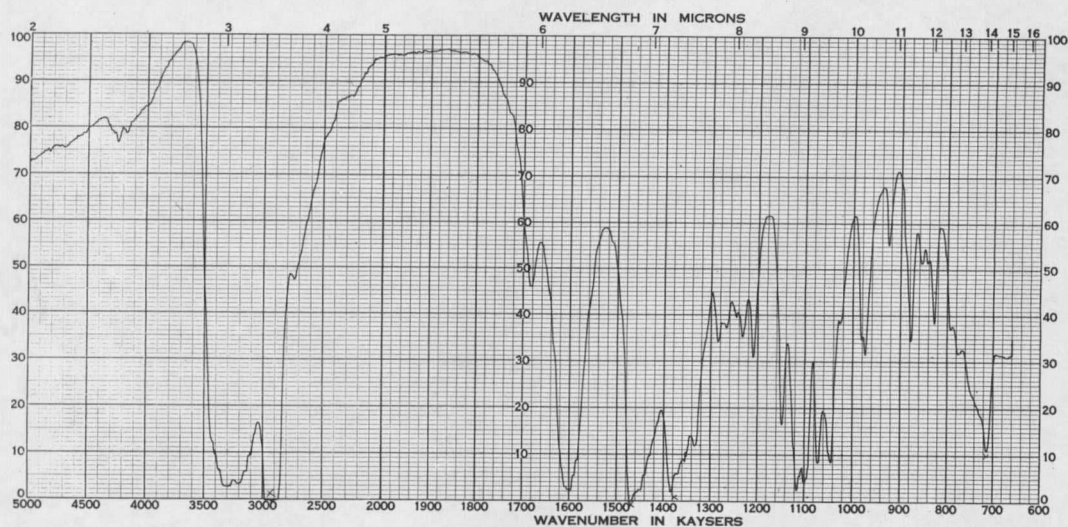


Figure 21

Infrared Spectrum (Nujol mull) of
potassium gluconate (Chas. Pfizer)

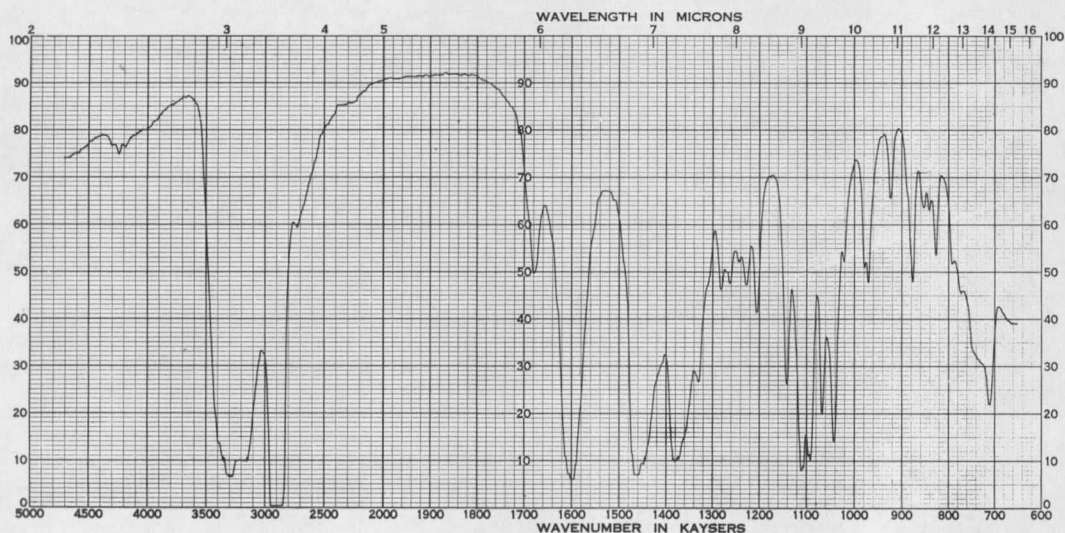


Figure 22

Infrared Spectrum (Nujol mull) of
potassium gluconate (Matheson, Coleman & Bell)

are shown in Fig. 21, 22. A thorough search of literature revealed that the melting point of potassium gluconate has been recorded by only one investigator,⁶⁸ in India, as 185°, Keenan and Weisberg⁴⁸ measured optical properties of some salts of gluconic acid for identification. Their data for potassium gluconate agrees well for the substance isolated from the mushroom.

1. Irregular grains and rods.
2. The indices of refraction: $n_{\alpha} = 1.523$, $n_{\beta} = 1.548$
 $n_{\gamma} = 1.565$, all ± 0.003 .
3. Extinction parallel

For microscopical identification, the crystalline material from the mushroom was immersed in a liquid with index 1.524. The crystals consisted of irregular grains and rods. A single crystal was located. On rotating the stage, the extinction was parallel with the disappearance of crystal boundary. On another slide the substance was immersed in a liquid with index 1.564. Rotating the stage, a full circle, caused no disappearance of the crystal boundary.

Identity with potassium gluconate was confirmed further by thin sheet chromatography on silica sheet. Aqueous solutions of samples of potassium gluconate (Chas. Pfizer and Matheson, Coleman & Bell) were spotted parallel with a solution of the crystalline product from the mushroom. The silica sheet was kept for two hours in a glass chamber containing the solvent system: n-butanol (10), formic acid (2) and water (15). The sheet was taken out and dried at room temperature. On

-98-

Solvent Front

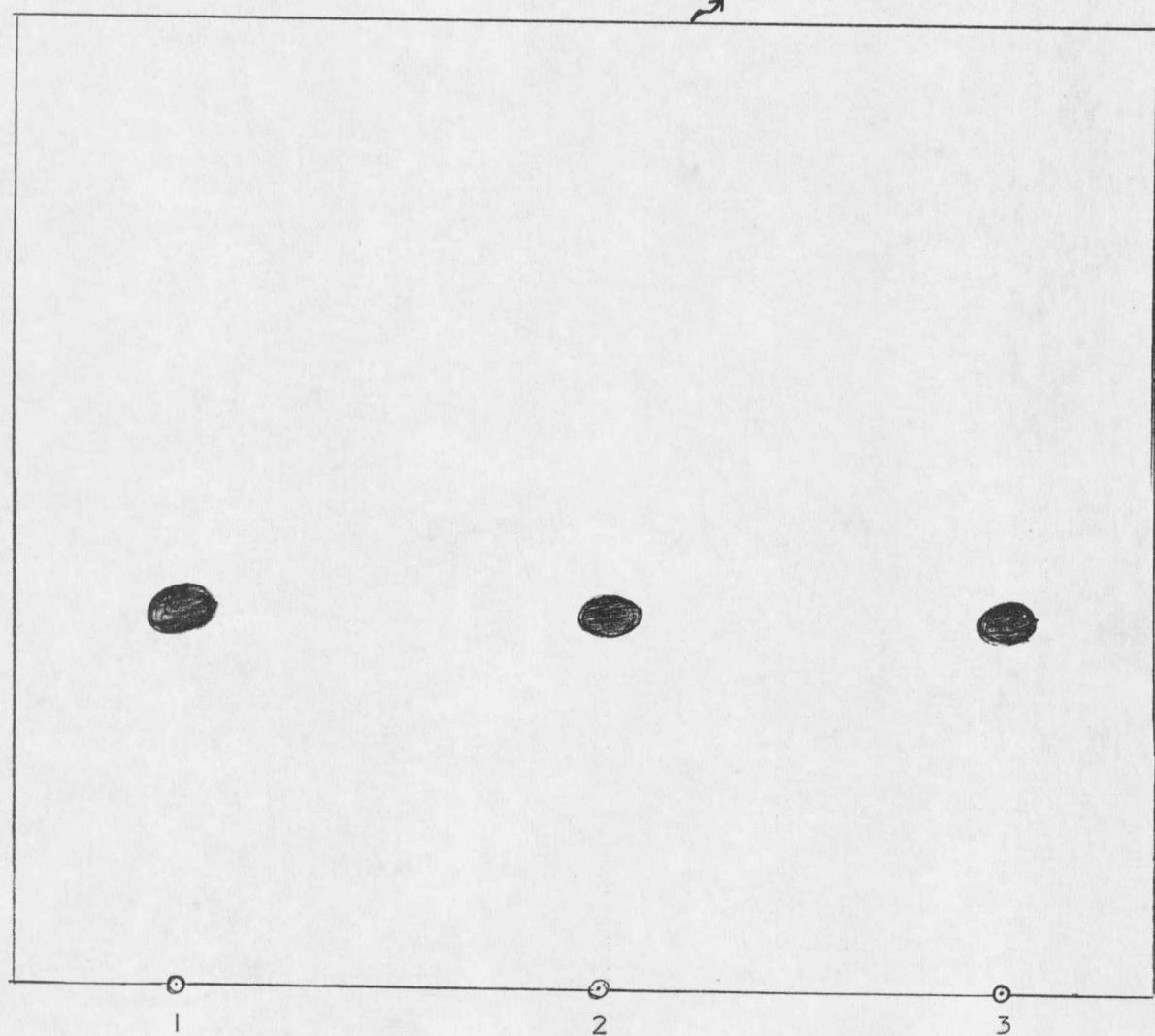


Figure 37

Thin sheet chromatogram of: Potassium gluconate
1) Sample from Matheson; 2) Isolated from
A. muscaria; 3) Sample from Chas. Pfizer

spraying with 50% H_2SO_4 and drying in oven, identical dark zones, on a white background, were exhibited by all the three substances (Fig. 37).

The concentrate B-I (IV.21) from the main decantate (from the absolute methanolic extract, 'B' = IV.2) after removal of the above crystalline product was fractionated through extraction with ether (containing a little Skelly to aid in clarification) into an insoluble fraction, B-R (IV.211) and an ethereal solution (IV.212) as outlined in Chart 18.

From the ethereal solution on removal of solvent was obtained an oil, corresponding to the oil 'A', III.121 (Charts 7, 8), obtained in earlier work. It was taken up in Skelly and chromatographed on alumina (30 x 5.5 cm). (Chart 18)

<u>Eluant</u>	<u>Remarks</u>
1. Skelly 1 liter	25.983g - yellow oil (Ab. A. <u>III</u> = <u>IV.2121</u>)
2. Skelly 1.5 liters	54.6224g - reddish brown oil (Ab. A. <u>IV</u> = <u>IV.2122</u>)
3. Skelly 1 liter	12.6726g - brown oil (Ab. A. <u>V</u> = <u>IV.2123</u>)
4. Sk (80) Ether (20) 400 c.c.	Very little residue
5. Sk (60) Ether (40) 1 liter	Very little residue
6. Sk (20) Ether (80) 1 liter	A little more residue
7. Ether, 4 liters	Very little residue
8. Ether (60) MeOH (40) 500 c.c.	Very little residue
9. Ether (20) MeOH (80) 500 c.c.	Very little residue
10. MeOH, 4 liters	Small quantity of thin film

The infrared spectra of the oil fractions were identical with

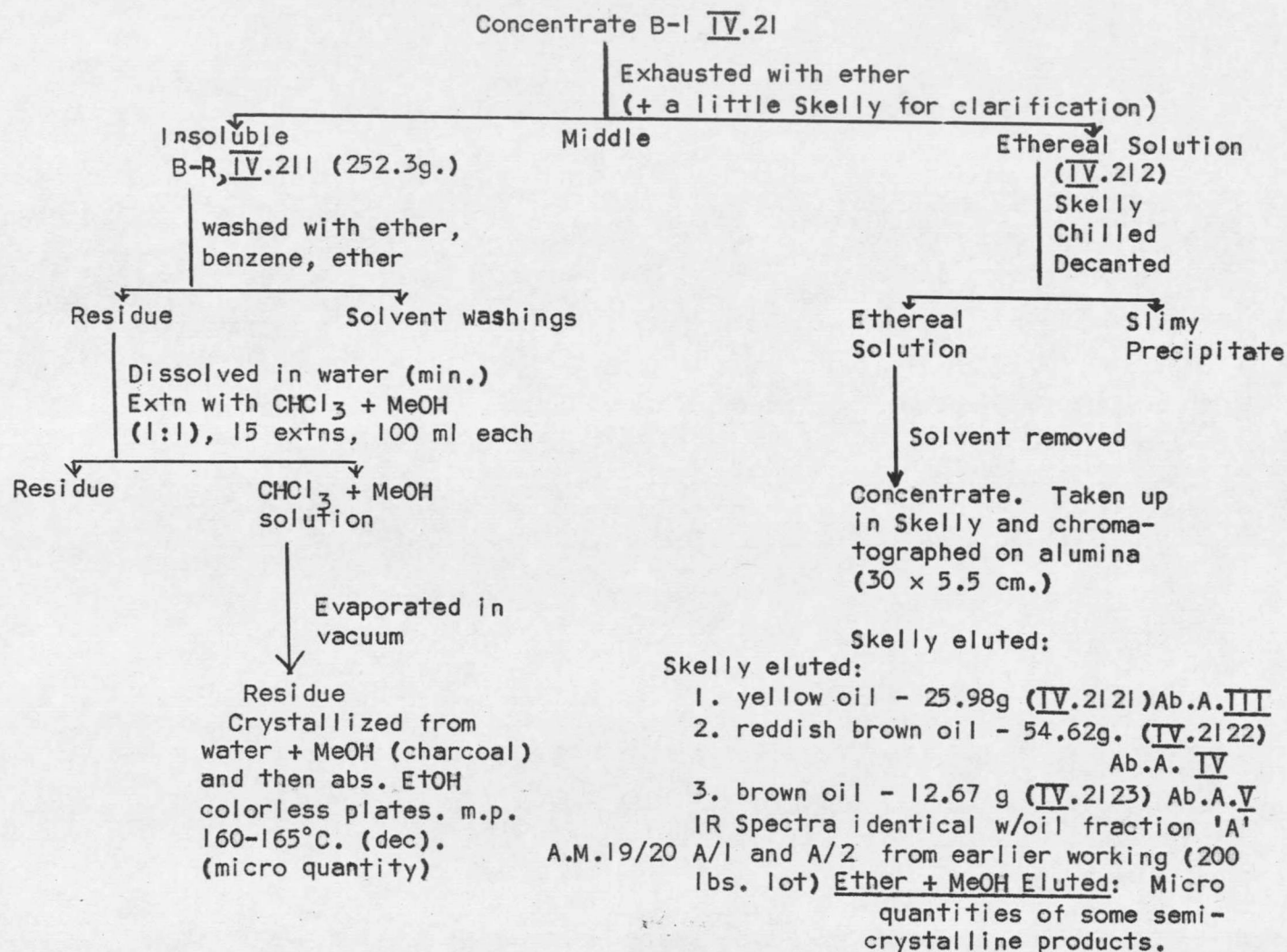


CHART 18

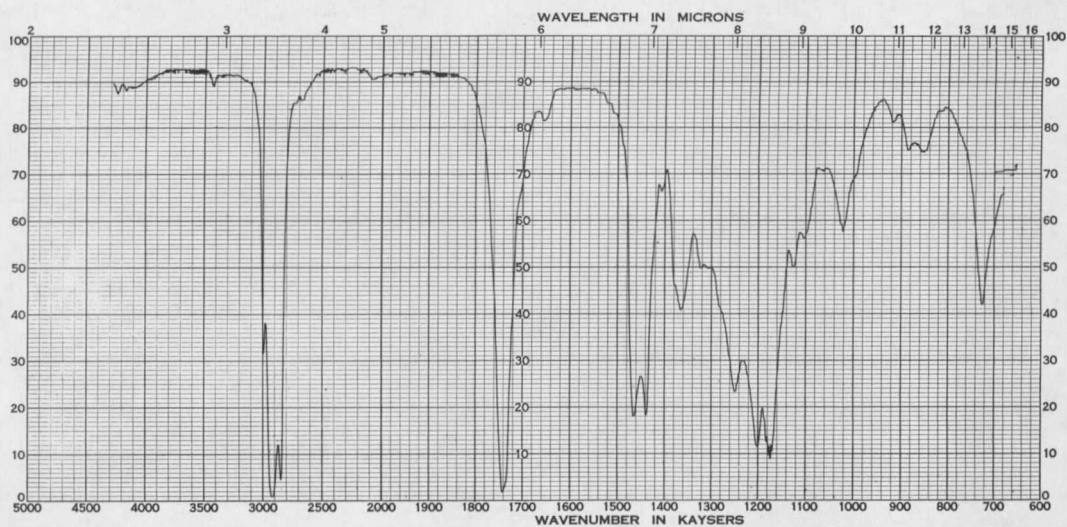


Figure 23

Infrared Spectrum (capillary film) of oil, Ab. A. III, IV.2121

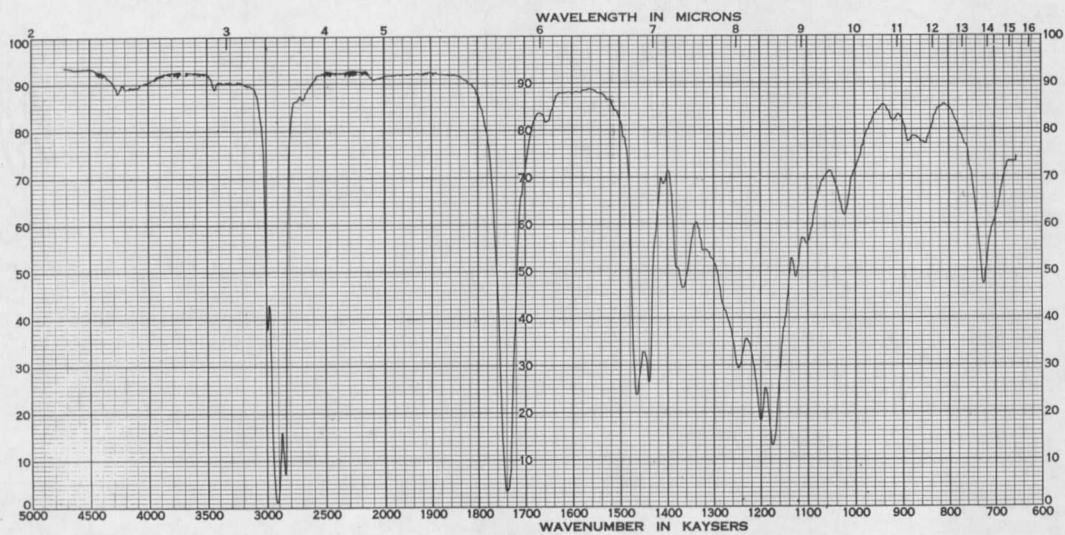


Figure 24

Infrared Spectrum (capillary film) of oil, Ab. A. IV, IV.2122

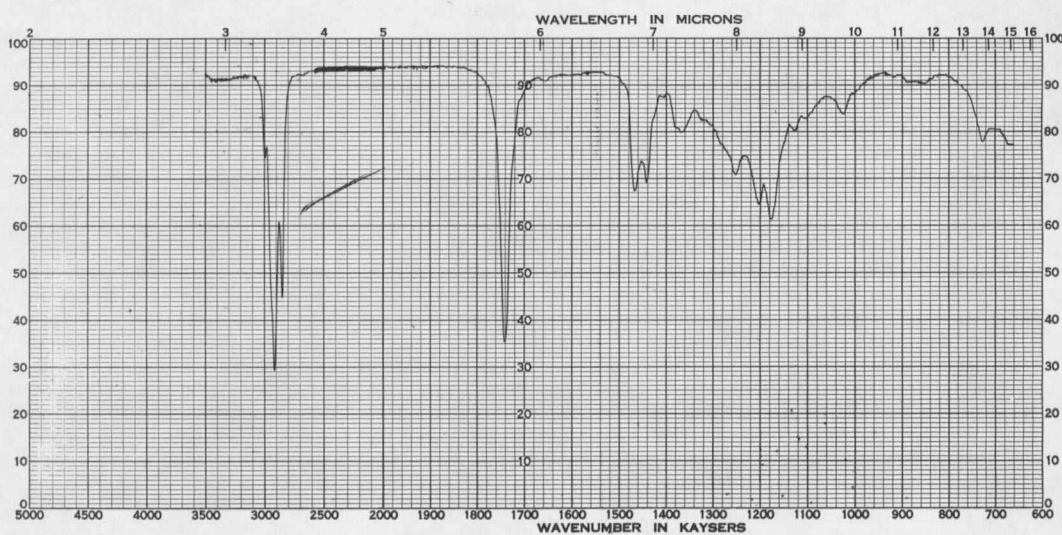


Figure 25

Infrared Spectrum (capillary film) of oil, Ab. A. V, IV.2123

A. M. 19/20, 'A' A/1, and A/2 obtained earlier from fresh mushroom, 200 lbs. (Chart 8). Pharmacological evaluation of these oils indicated them to be devoid of any noticeable hallucinogenic properties.

Experiments are underway to determine whether these oils have any insecticidal and/or insect repellant properties.

The ether insoluble fraction B-R (IV.211) was also reported to have no hallucinogenic activity. By fractionations, as illustrated in Chart 18, a micro-quantity of crystalline product, m. p. 160-65°C (dec) was separated but further experiments on this fraction were postponed.

Examination of aqueous methanolic concentrate (IV.1)

As the hallucinogenic principles were mainly concentrated in the water soluble extractive of the mushrooms, the mushrooms were so processed (at the Abbott Laboratories) as already explained in the beginning of Part IV to collect the initial aqueous methanolic concentrate (60% water content) separately and examine it. Unfortunately, pharmacological reports on this fraction was far from encouraging. It was worked up as illustrated under Chart 19 and the various fractions reserved for future exploration. Attention was mainly focused on the final water extractive from the 'marc'.

Aqueous concentrate from the marc 'C' IV.3

The aqueous concentrate (3288 g. corresponding to only 33 g. of total solids) was again the most active hallucinogenic fraction in this whole working.

Innumerable experiments were carried out with this particular

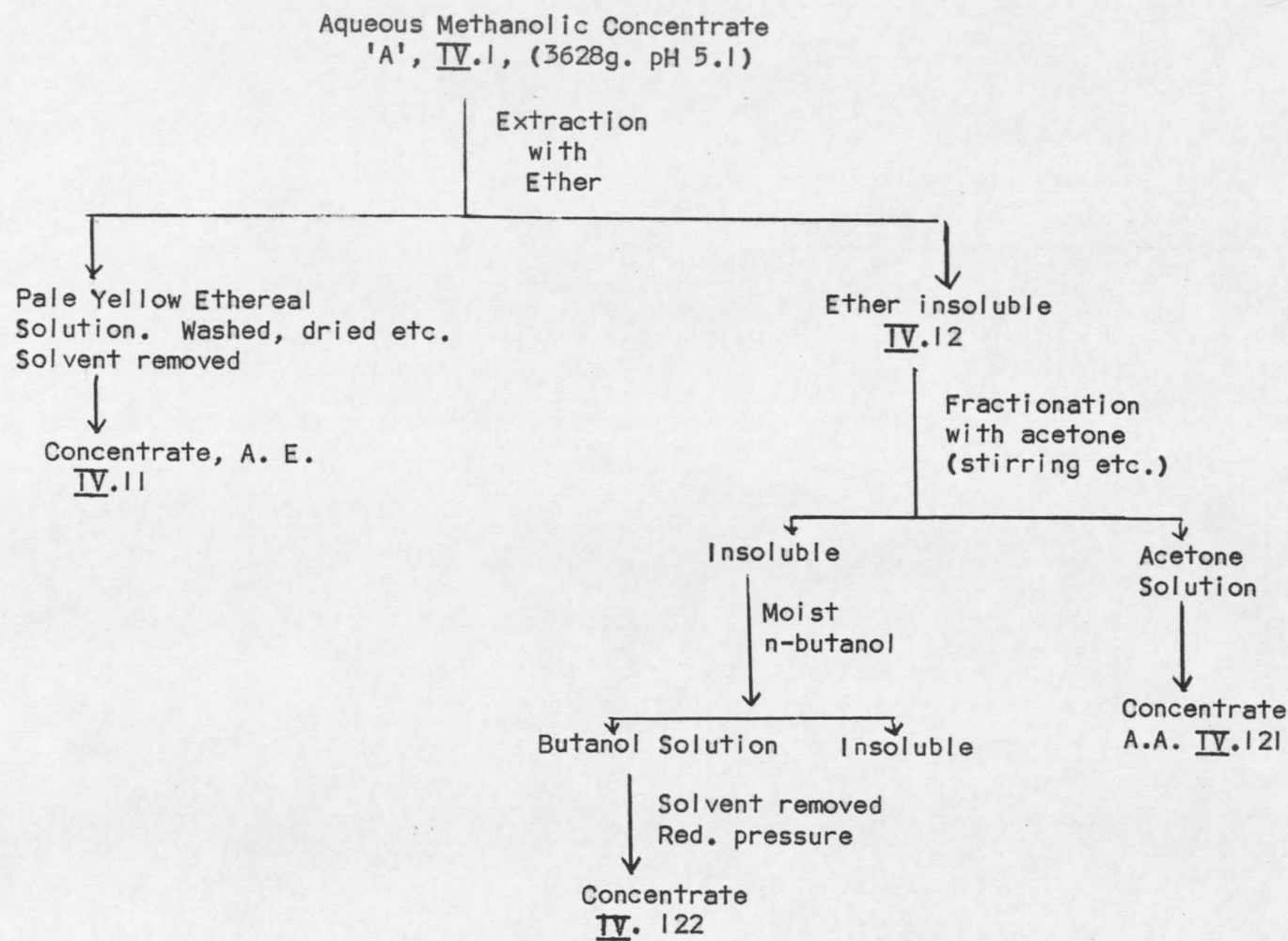


CHART 19

fraction. Various solvent systems were tried with chromatographic screening (column, paper, thin-sheet etc.) Finally the following procedure was found to be best suited and adopted. (Chart 20). Most of the water was removed by evaporation at room temperature under nitrogen atmosphere in batches. The syrupy concentrate was refrigerated for a few days when an inorganic deposit consisting mostly of KCl settled down. The decantate was divided into small portions and then added to an array of flasks containing absolute ethanol. Alcoholic filtrate from each on microscopic manipulation with solvents indicated presence of crystalline material, pale yellow in colour with characteristic fecal odour, flourescent in u. v. and generally responding to Keller's test for indoles.

The alcoholic filtrates, collected together (pH 6.0) were evaporated at room temperature (Rinco). The residue (about 2.5 g) was induced to crystallize by successive solution in minimum quantity of methanol, and treatment with a judicious mixture of ethyl acetate and acetone. The entire quantity of crystalline material (246.8 mg) obtained thus (over a period of two months) was almost colourless, excepting for a faint yellowish tinge. It melted indefinitely between 130-35°C (dec) (capillary introduced at 100°C). On heating over a platinum spatula it burned with a smoky flame. Its aqueous solution (pH 6.1-6.2) had a tingling taste and was flourescent in u.v. Absorption maxima at $\lambda 260\text{m}\mu$ and $\lambda 310\text{m}\mu$ was recorded for the substance. Test for nitrogen on sodium fusion was very doubtful, but it gave positive tests with ninhydrin and Keller's.

Thin sheet chromatography (silica plates) with various solvent systems, (along with samples of psilocybin, psilocin, etc.) indicated the separation of two to three components fluorescent in u.v.

After repeated crystallization from absolute methanol, colourless crystals (104.8 mg) were obtained (weakly fluorescent in u.v. and not showing any other component on thin sheet chromatography). Unfortunately, about 1/3 of the pure crystalline product (34.7 mg) sent for biological evaluation was lost.

Through persistent crystallization of mother liquors and also subjecting the various precipitates from absolute ethanol obtained earlier to the same treatment (i.e., dissolving in minimum quantity of water, adding in batches to absolute ethanol in a series of containers, and working up the ethanolic filtrates after removal of solvent with methanol, ethyl acetate and acetone), three more crops of the same crystalline material (total of 202.6 mg) were collected and the various mother liquors etc. carefully preserved in a refrigerator.

For biological tests, 12 mg dissolved in 1 c.c. of water were sent (in a sealed ampoule) again. As expected it was reported to be active and devoid of muscarinic effects.

The final pure crystalline product (total yield: 307.4 mg) was extremely hygroscopic. It did not exhibit any sharp m. p. and melted between 135-38°C (dec) (m.p. recorded after repeated trials, capillary being introduced at 100°C and examined under microscope immediately after melting showing no residue). On ignition over a platinum spatula,

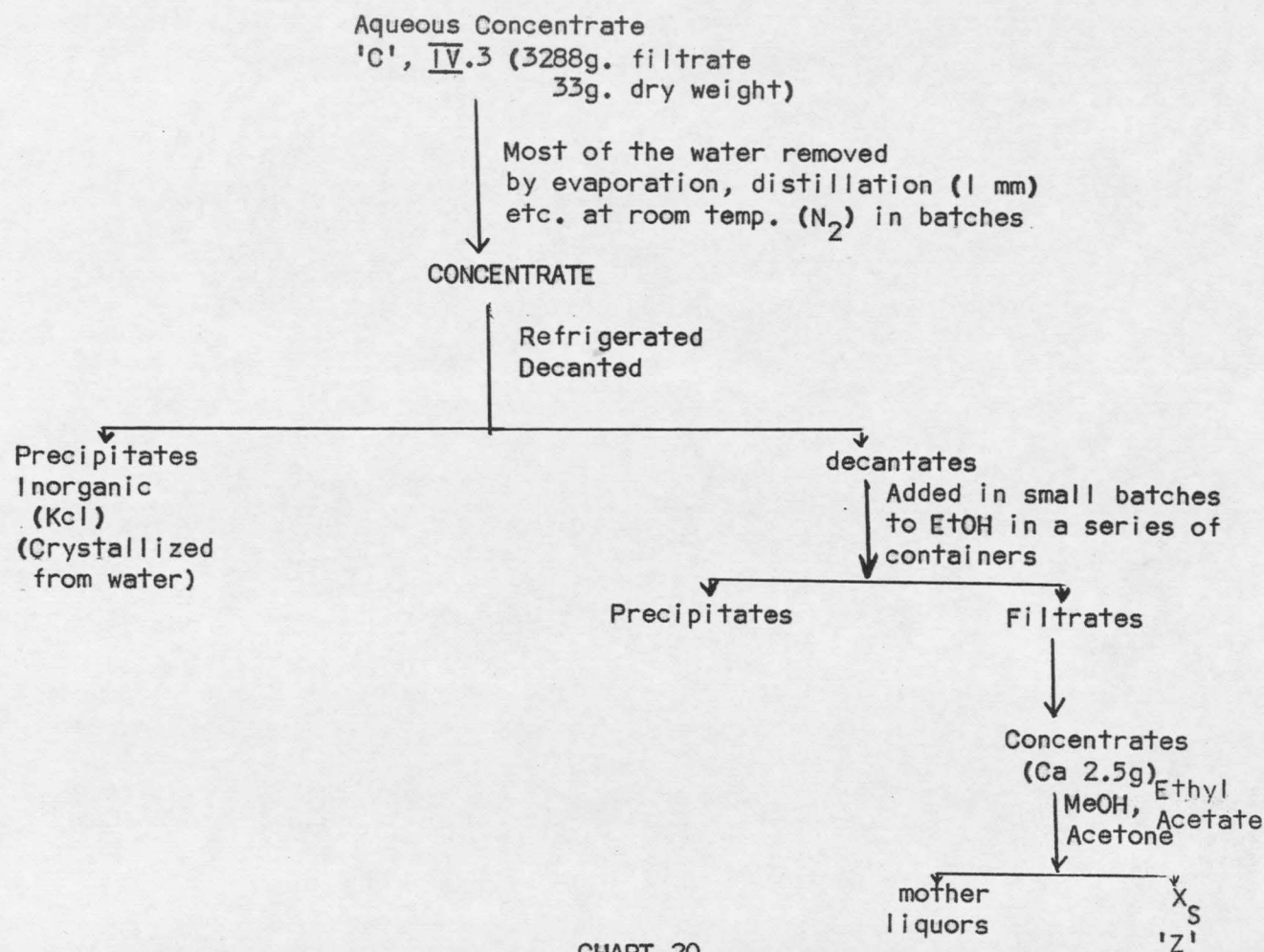


CHART 20

it burned like an organic substance but gave a purple flame characteristic of potassium. The residue left was very little whose aqueous solution was basic. Emission spectrum indicated only the presence of potassium.

Found: C, 30.17%; H, 4.83%; K, 25.23% and M. W. 615; (N, absent)
(Geller)

$C_{15}H_{30}O_{15}K_4$

(M.W. 606.79)

requires: C, 29.7%; H, 4.98%; K, 25.77%

The crystalline substance, designated as 'Z', does not appear to contain any sugar moiety in the molecule. Tests with Molisch and Red Tetrazolium were negative both before and after treatment with acid and emulsin. These experiments were carried out parallel with the β -glucoside, m. p. 106°C (dec) isolated from the mushroom.

The infrared spectra (Nujolmull and flourolube) of the crystalline substance are shown in Fig. 29-30. A sample of the substance (23.4mg) has been sent to the Varian Associates, Palo Alto, California for NMR spectrum and interpretation.

The final mother liquors preserved in the refrigerator, have again been subjected to intensive chromatographic fractionations. Attempts are being made to isolate any further crystalline products, probably indolic in nature.

Column chromatography was not successful. Thin sheet chromatography on silica sheet, for one hour, in the solvent system: Ethyl acetate (50), Pyridine (30) and water (20) resulted in the separation of three distinct zones all showing bluish green fluorescence in u. v.

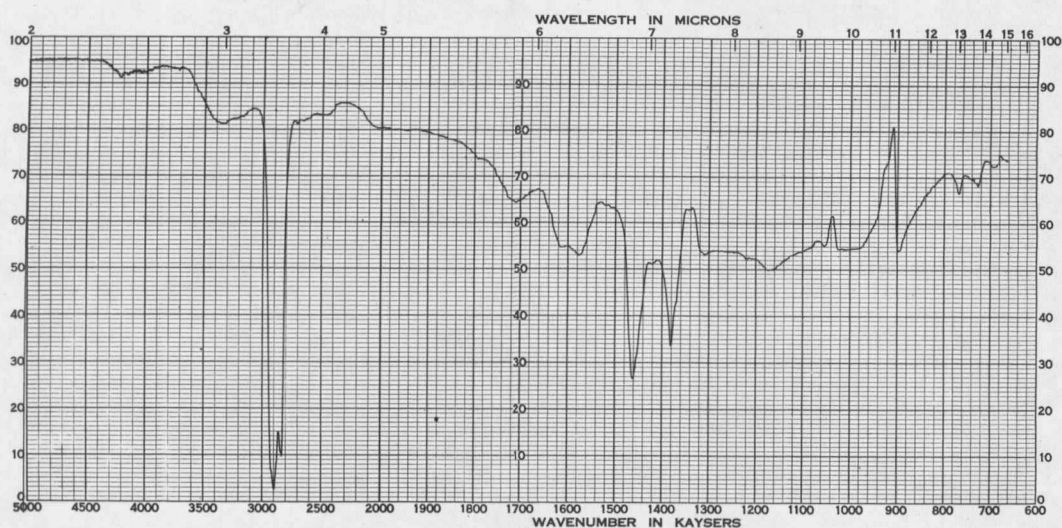


Figure 29

Infrared Spectrum (Nujol mull) of crystalline product, 'Z',
m. p. 135-38°C (dec) from water concentrate

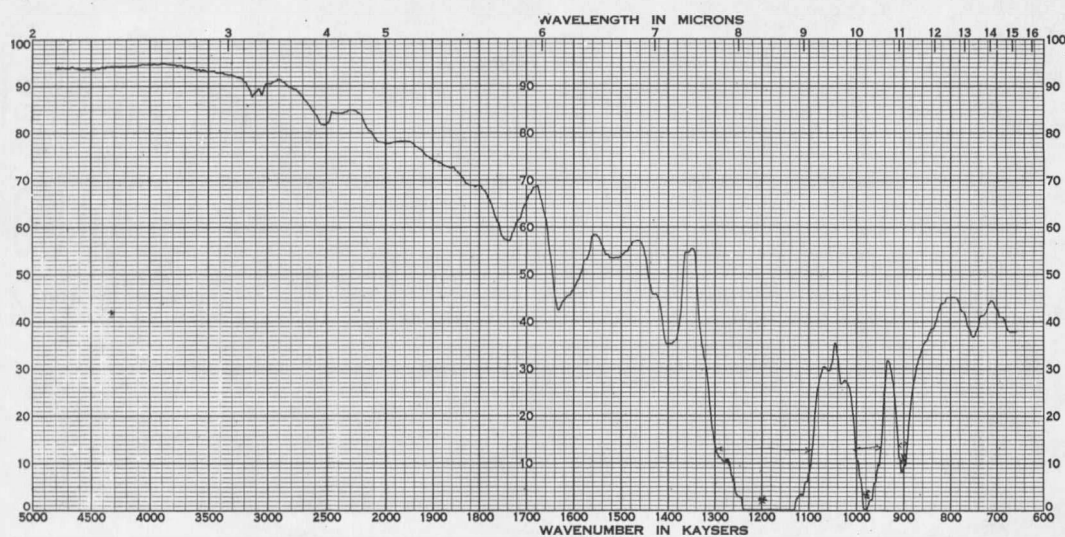


Figure 30

Infrared Spectrum (Flourolube) of crystalline product 'Z',
m. p. 135-38° (dec) from water concentrate

Hence, the total mother liquors are being worked up by this technique. This may involve more than 500 plates. Each zone will have to be eluted separately. Concentrate from each should again be sent for biological tests, to find out whether any of these have LSD-like properties.

The eluants from lowest zone on rechromatography on alumina sheet in the same solvent system gives further separation. Besides the zones exhibiting bluish green fluorescence in u. v., there is a distinct yellow component.

Working on the above lines, combined with fractional crystallization may ultimately lead to some positive results.

ADDENDA

a) 'Z' was sent to 'Berkeley' for elemental analysis to check the values obtained earlier from 'Geller'.

Found: C, 30.3%; H, 4.6%
(Berkeley)

Equivalent Weight (from potentiometric titration curve) = 218.

Apparently three free carboxyl groups (COOH) are present in 'Z' besides four COOK.

b) N. M. R. data from Varian Associates, Palo Alto, California, on 'Z':- 6 labile hydrogens, 8 methyl groups and one methyl keto group in the molecule.

c) Preliminary experiments indicate that the oil fractions isolated from A. muscaria are devoid of oral toxicity towards insects.

PHARMACOLOGICAL DATA

At the outset, it was planned to submit each fraction, systematically, for biological evaluation. Biological screening has been of assistance in the present investigation in the separation of a potent water soluble fraction. This fraction has been found by our team of pharmacologists to have LSD-like properties and free from muscarinic effects.

Dr. Edward Pelican (Acting Head, Department of Pharmacology and Experimental Therapeutics, Boston University College of Medicine) and his associates carried out the biological assays. Concise data from their unpublished results and private communications are presented along with relevant information from standard treatises on Pharmacology,^{11,32} for coherence.

Human experiments were evidently ruled out at these stages of investigation. Some reasonable biological criteria had, perforce, to be used, with tests carried out on dumb animals. Hence the primary interest of the pharmacologists was to measure the relative abilities of various materials, isolated by us from the mushroom, to produce effects in animals similar to those observed by the administration of LSD and muscarine respectively.

LSD (Lysergic acid diethyl amide) is used in psychiatric therapy and research because of its unique central actions.³² In extremely minute doses (less than 0.5 microgram per Kg. orally) it is reported to produce highly specific psychic alterations. Most impressive among these

are disturbances of visual perception. Visual hallucinations are vividly coloured and rapidly change.

Pelican et al have termed the drug syndrome characteristic of LSD in animal species, like rabbits, as "Sympathotonia". This means production of hyperpyrexia (fever) and mydriasis (pupillary dilatation). Contrarily, "Parasympathotonic" effects, like miosis (pupillary constriction), excessive salivation, excessive lacrimation are associated with muscarine-like products.

The term "muscarinic effects" is widely used in physiology and pharmacology while describing the role of endogenous acetyl choline in neuro-effector transmission of parasympathetic impulses. Muscarine was once thought to be the substance which transmitted parasympathetic impulses.

"While the sympathetic system is known to be active in all parts of the body during conditions of stress, fight and fear, the parasympathetic system is active in a resting person, e. g. asleep after a meal. Impulses passing down the vagus nerve (1) slow the pulse (2) quicken the movements of the intestines (3) increase the secretions into the alimentary tract and (4) constrict the bronchioles so that breathing may be noisy. The effects of acetyl choline are similar to those just described, but it has other effects as well. Dale¹⁶⁻¹⁸ showed that it acted in part like muscarine and in part like nicotine. The muscarinic actions are the same as the parasympathetic actions. The muscarinic actions of acetyl choline are on the smooth muscle while the nicotinic action is on the striated muscle.

Properties of acetyl choline

1. (a) A small dose causes a fall in blood pressure due to vasodilatation.
- (b) A larger dose causes vasodilatation and slows the heart in addition.

- (c) If atropine is given, both these effects are abolished.
 - (d) If now a large dose of acetyl choline is given, it will cause a rise of blood pressure due to the release of adrenaline from the suprarenal gland and to stimulation of the cells of the postganglionic fibres of the sympathetic ganglia. The cells send impulses to the blood vessels and constrict them. This is the nicotine action, for nicotine has the same effect.
2. It increases intestinal movements and secretions.
 3. It constricts the pupil and the bronchioles.
 4. It causes contraction of the skeletal muscle easily obtainable in the frog.
 5. It stimulates the receptors in carotid body, and the impulses set up cause increased respiration."||

Details of biological tests

All tests were performed on male albino rabbits weighing about 2 Kg. The ambient temperatures were between 75° and 80°F. Light intensities varies between 51 and 20 foot candles in the experimental area.

Depending on the solubility characteristics of the materials screened, they were prepared for intravenous administration either by dissolving in sterile, pyrogen-free 0.9% NaCl solution or by suspension in sterile, pyrogen-free polyvinylpyrrolidone solution.

Relevant data concerning the weight of dry solids in each sample and the fresh mushroom weight corresponding to each fraction and also solubilities and other characteristics were supplied to the pharmacologists.

With the above data each material was screened first. Control observations were made. Biological assays were then carried out. Three to six male albino rabbits were studied at each dose level, with negative and positive controls. A group of animals which received no treatment formed the negative control and a group which received only the menstruum in which the test material was administered served as positive control.

At frequent intervals before and after administration of each sample, pupil diameter was measured with a millimeter scale and rectal temperature determined with a clinical thermometer. Observations were made of the general behaviour of the animals. Frequency of occurrence of excessive salivation, lacrimation, the dose at which death occurred etc. were recorded. These observations were continued for at least four hours after dosing.

For each material, for all dose levels and for all animals at each dose level, the mean maximum increase or decrease in pupil diameter was plotted against the logarithm of the corresponding dose. For each material, the mean maximum change in body temperature for all animals at a given dose level, less the mean temperature change in the positive control group was plotted against the logarithm of the corresponding dose. Straight lines best fitting the data were determined by the method of least squares, or the method of averages. From the computed (by interpolation) dose-effect-curves, effective doses and units were defined thus:

- Doses: 1) The dose of material in mg (of dry solid) per Kg. of body weight required to increase the pupil diameter 1 mm (mydriatic effect) or decrease the pupil diameter 1 mm (miotic effect).
- 2) The dose of material in mg (of dry solid) per Kg. of body weight required to increase the body temperature 1.5°C.

- Units: 1) Unit mydriatic activity was defined as the activity of a material, 1 mg. of which per Kg. of body weight caused pupillary dilatation of 1 mm.
- 2) Unit miotic activity was possessed, by definition, by a material which in dose of 1 mg/Kg caused pupillary constriction of 1 mm.
- 3) Unit hyperpyrexia activity was defined as the activity of a material which in a dose of 1 mg/Kg caused increase in rectal temperature of 1.5°C.

in a male albino rabbit of about 2 Kg. under the conditions of tests.

Table III

FRACTIONS FROM PART I

Frn.	Ref.	Dry Mushroom Weight	Amt. isolated	Amt. for B.T.*	Dose*	Objective Symptoms
A.M. 1 (I.1)	Pt. 1; basic frn. (Prol- lius fluid)	20g	40.8mg	9 mg	-	-
A.M. 2 (I.2)	Charts 1,2; basic frn.	75g	35 mg (appx)	33.3 mg	-	-
A.M. 3 (I.3)	Chart 1; acidic frn. from Skelly (Soxhlet)	25g	32.3mg	32mg	173mg/Kg	none
A.M. 4 (I.4)	Charts 1,2; acidic frn.	75g	50mg (appx)	38.4mg	-	-
A.M. 5 (I.5)	Chart 2; neutral frn.	50g	707.4mg.	44.53mg	5.2mg/Kg	none
A.M. 6 (I.6)	Chart 2; Neutral frn. insoluble in acetone	50g	1.1813mg.	23.02mg	-	-
A.M. 7 (I.7)	Chart 1; Neutral frn. from Soxhlet extractive	25g	836mg.	39.91mg	9.2mg/Kg	none
A.M. 8 (I.8)	Chart 2; Acetone in- soluble frn. from the methanolic extractive of defatted mushroom	50g	95mg	46.40mg in 3 ml of water	3.5mg/Kg	At 10mg/ Kg level, hypoactivity, flaccidity, slow-labored breathing

Table II (continued)

Frn.	Ref.	Dry Mushroom Weight	Amt. iso- lated	Amt. for B.T.*	Dose *	Objective Symptoms
A.M.9 (I.9)	Chart 2; Acetone soluble fraction from the methanolic extractive of defatted mushroom	50g	1g (appx)	52.58mg	1.53 mg/Kg	Dilated pupils salivation, rapid res- piration at higher dose levels
A.M.10 (I.10)	Chart 2 pp. Final water extract of marc.	50g	12ml (conc.) (0.2g total solids)	4 ml.	0.019ml per Kg	Hypoactivity, flaccidity slow labour- ed breath- ing, partial paralysis, miosis side position & convulsions, at 0.1 mg/Kg Death after 3.8 hrs.

*B. T. indicates Biological Tests

*Dose stands for amount required to raise body temperature to 1.5°C
Fractions A.M.1, A.M. 2, A.M. 4 and A.M. 6 were not tested.
For the dry mushroom itself a dose of 20mg/Kg was reported to be
required to raise body temperature by 1.5°C. Salivation and miosis
were then noticed.

Fractions A.M. 10, A.M. 9 and A.M. 8, in order, were potent in
producing hyperpyrexia.

2) Crystalline substance, A. M. 20, X₁.20 (Potassium salt from water extract of 'marc' from 150 g. of dry mushroom):

Quantity isolated: 606 mg.

Quantity sent to Dr. Pelican: 426 mg.

<u>Symptoms</u>	<u>ED-50</u> <u>mg/Kg</u>
Hyperactivity	22.4
Hyperpyrexia	2.9
Rapid Respiration	9.6
Tremors	22.4
Ataxia of Gait	22.4

Dose required to raise body temperature to 1.5°C: 5.2 mg/Kg

Dose required to raise body temperature to 3.0°C: 123 mg/Kg
(extrapolated)

The following experiments were carried out at the campus, Bozeman, on male mice with the kind cooperation of the Staff of Veterinary Research Institute, on January 16, 1959, 3:30-4:00 P. M.

<u>Mouse</u> <u>Marked</u>	<u>Dose of A. M. 20 in</u> <u>physiological saline</u>	<u>Observations on</u> <u>behaviour until</u> <u>Jan. 22, 1959.</u>
(1)	0.81mg/0.1 ml, intravenous	normal
(2)	0.91 mg/0.1 ml, intravenous	normal
(3)	0.41 mg/0.1 ml, intravenous	normal
(4)	0.41 mg/0.1 ml, intravenous	normal
(5)	0.20 mg/0.1 ml, intravenous	normal
(6)	0.20 mg/0.1 ml, intravenous	normal
(7)	2.1 mg/1 ml, intraperitoneal	normal

The mice were fed regularly (with mice food borrowed from Dr. B. L. Johnson's Laboratory) and given water with the dropping bottle on the cage periodically. No changes were observed until January 22, 1959 evening.

At 5 p.m., on January 22, 1959, 10 mg. (approximately) of the aqueous mother liquors of A. M. 20 were dissolved in the water bottle. The mice were normal until 24th evening. At 11:30, on the night of the 24th, mouse (5) was found sluggish, but showed reflex action. When poked with a splinter, the muscles twitched. It died about 12:15, noon of 25th. About 1:30, mice (6) and (7) showed signs of approaching death. By 3:15 in the afternoon, excepting mice (2) and (3), all the other five were dead.

The survivors were observed regularly. They, both, were active and moved about normally in the cage, until 11:00 a.m. of 26th. By noon, about 12:15, they died, after shivering.

Dr. William H. Sippel, Physician, Bozeman, administered me 3.2 mg. of A. M. 20 in 2 ml. of normal physiological saline intramuscularly at 4:52 p.m., Jan. 17th, 1959. Dr. Sippel took careful notes, for nearly three hours, of my body temperature and pupil size. No appreciable changes were detected.

At about 10:30 p.m. in the night, I felt feverish. The temperature appeared to raise slowly for about 2 hours. It gradually receded to normal by early dawn of 18th. My sleep was disturbed. I experienced no hallucinations. My only feelings were uneasiness and anxiety. On

the 18th I was treated with penicillin as a prophylactic at the Student Health Center.

The above experiments, on looking back, appear to be crude. They reflect my impetuosity owing to the small quantity of dry mushroom, 255g., available for investigations for nearly nine months (June 1958 - March 1959) and the delay in obtaining pharmacological results from Dr. Pelican.

3. Fractions from Part II (Chart 3) Isolated from fresh mushroom, 6.5 Kg, corresponding to 488 g (rounded to 500 g) on dry weight basis: Tables III, IV & V.

The weights recorded are approximate as the mushrooms examined were dropped by the collectors into methanol immediately after picking. Accurate weight of fresh and dry mushrooms, with standard deviations, will have to be ascertained in future work. However, other investigators have recorded that 100 g. of fresh mushroom on drying yielded 5 to 6 g. of the dried material.

Table III

Frn.	Amt. Iso- lated	Fractions from Part - <u>II</u>			
		Sample for B.T.	Total Solids in Sample	Wt. of Fresh Mushroom Equivalent to Sample	Wt. of Dry Mushroom Equivalent to Sample
A.M. 11 (<u>II.1</u>)	34 g	2.4 g in 10 ml water	2.4 g	460 g	35 g
A. M. 12 (<u>II.2</u>)	20.412 g	4.555 g in 20 ml water	4.555 g	112 g	1.45 Kg
A. M. 13 (<u>II.3</u>)	11.03 g	6.9675 g in 25 ml water	6.9675 g	318 g	4.14 Kg
A. M. 14 (<u>II.4</u>)	150 g	37.0795 g	37.0795 g	123 g	1.6 Kg
A. M. 15 (<u>II.5</u>)	18.95 g	6.9948 g	6.9948 g	184 g	2.4 Kg

<u>Fraction</u>	<u>Dose required to raise body temperature to 1.5°C</u>
A. M. 11	0.162 mg/Kg
A. M. 12	18.2 mg/Kg
A. M. 13	4.23 mg/Kg
A. M. 14	21.4 mg/Kg
A. M. 15	6.6 mg/Kg

Table IV

Fractions from Part II: biological data

Symptoms	ED-50 mg/Kg				
	A.M. 11 (II.1)	A.M. 12 (II.2)	A.M. 13 (II.3)	A.M. 14 (II.4)	A.M. 15 (II.5)
Hypoactivity	6.3	-	32.4	59.6	16.2
Hyperpyrexia	0.05	10.5	3.6	16.6	4.1
Rapid Respiration	5.6	45.7	15.8	144.5	12.0
Convulsions	120.2	-	141.3	117.5	43.6
Death (24 hrs.)	31.6	-	46.8	-	43.6
Death (4 hrs.)	89.1	-	128.8	117.5	-
Salivation	5.2	45.7	11.2	44.7	11.2
Lacrimation	17.8	-	-	-	-
Slowing of Respiration	17.0	-	-	-	-
Prone Position	89.1	83.1	44.8	-	16.2
Loss of Righting Reflex	38.0	-	104.7	117.5	-
Miosis	16.6	57.5	66.1	87.1	32.4
Vasodilatation	16.6	-	-	-	-
Pupillary Dilatation	-	-	37.2	-	-
Ataxia of gait	-	-	-	-	53.7

The Comparative potencies of the five fractions in producing hyperpyrexia, mydriasis and miosis are given in Table V.

Table V

Comparative potencies of fractions from Part II

Fraction	Hyperpyrexia potency (units/g)	Mydriatic potency (units/g)	Miotic potency (units/g)
A. M. 11	20820	33330	833
A. M. 12	58	95	17
A. M. 13	301	2105	16
A. M. 14	47	0	16
A. M. 15	152	83	36

Table VI

Fractions from Part III

Fraction	Amt. iso- lated	Sample for B.T.	Total Solids in Sample	Wt. of Fresh Mush- room Equivalent to Sample
A.M. 19/20 'A' (<u>III</u> .121)	260 g	8.5 g	8.5g (oil)	6.2 lbs.
A.M. 19/20 A/1 (<u>III</u> .1211)	58 g	10.1 g	10.1 g (light yellow oil)	33.1 lbs.
A.M. 19/20 A/2 (<u>III</u> .1212)	124 g	13.1 g	13.1 g (brown oil)	20 lbs.
A.M. 19/20 'C' (<u>III</u> .111)	160.5 g	22.3 g	6.516 g	26.4 lbs.
A.M. 19/20 'D' (<u>III</u> .112)	250 g	13.0 g	4.267 g	9.88 lbs.
A.M. 19/20 'F' (<u>III</u> .1222)	155 g	23.5 g	6.017 g	28.8 lbs.

Table VII

Fractions from Part III: biological data

Symptoms	ED-50 mg/Kg					
	(III.121)	(III.1211)	(III.1212)	(III.111)	(III.112)	(III.1222)
Hypoactivity	2.09	224	200	1.84	1.28	0.4
Hyperpyrexia	100	1700	50	≈ 4000	≈ 200	≈ 250
Rapid Respiration	-	-	-	0.24	1.28	1.2
Convulsions	> 400	-	-	0.95	-	1.23
Death	> 400	275	316	1.54	2.17	1.29
Slowing of Respiration	-	-	-	0.24	1.28	1.2
Prone position	-	-	-	1.15	-	-
Loss of Righting Reflex	> 400	275	316	0.97	1.27	1.23
Miosis	-	-	-	0.08	1.28	0.52
Vasodilation	-	-	-	2.8	16.98	2.87
Ataxia of gait	> 400	316	200	0.9	1.64	1.20
Mydriasis	3400	32 to 100	76	-	-	-

The crystalline substance, III.X₁, m.p. 196-97°C. (dec) (fumaric acid?) was reported to produce only hyperpyrexia and not mydriasis or miosis. The comparative potencies of the various fractions, in units per gram of dry solid are shown in Table VIII.

Table VIII
Comparative potencies of fractions from Part III

Material	Hyperpyrexia potency (units/g)	Mydriatic potency (units/g)	Miotic potency (units/g)
A.M. 19/20 'A' (<u>III</u> .121)	8.3	54	0
A.M. 19/20 A/1 (<u>III</u> .1211)	0.6	59	0
A.M. 19/20 A/2 (<u>III</u> .1212)	18	17	0
A.M. 19/20 'C' (<u>III</u> .111)	0*	0	3510
A.M. 19/20 'D' (<u>III</u> .112)	14		378
A.M. 19/20 'F' (<u>III</u> .1222)	0*	0	2445
<u>III.X₁</u> (fumaric acid?)	103	0	0

*Fractions III.111 and III.1222 exhibited only hypothermia

Table IX
Fractions from Part IV

Fraction	Reference	Amount isolated	Sample for B.T.	Total Solids in Sample	Wt. of Fresh Mushroom Equivalent to Sample
Ab. 'A' <u>IV.1</u>	Chart 15, Aq. Methanol Concentrate	3628 g	69.59 g	47.73 g	777 g
Ab. 'B-R' <u>IV.211</u>	Chart 18 Ether insoluble from absolute methanol concentrate	252.3 g	71.18 g	36.64 g	11.42 Kg
Ab. 'C' <u>IV.3</u>	Charts 16, 20 Water concentrate of the 'marc'	3288 g	59.84 g	0.8486 g	≈ 1 Kg
Ab. 'A' <u>III</u> <u>IV.2121</u>	Chart 18 yellow oil on chromatography	25.98 g	2.92 g	2.92 g	4.55 Kg
Ab. A <u>IV</u> <u>IV.2122</u>	Chart 18 reddish brown oil on chromatography	54.62 g	3.272 g	3.272 g	2.43 Kg
Ab. A <u>V</u> <u>IV.2123</u>	Chart 18 brown oil on chromatography	12.67 g	2.048 g	2.048 g	6.55 Kg

Table X

Biological activities of fractions,

Ab. A (IV.1), Ab. B-R (IV.211) and Ab. C (IV.3)

Symptoms	ED-50 mg/Kg		
	Ab. A IV.1	Ab. B-R IV.211	Ab. C IV.3
Hypomotility	1.48	1.50	0.39
Ataxia of Gait	2.15	1.65	-
Loss of Righting Reflex	2.32	2.19	-
Prone Position	2.15	-	-
Miosis	1.03	1.38	-
Salivation	1.04	1.02	-
Lacrimation	1.04	< 2.40	-
Hyperpyrexia	1.01	2.40	1.53
Slowing of Respiration	1.01	1.63	0.40
Convulsions	2.32	2.40	-
Death	2.32	2.25	more than 0.65 less than 0.90
Opisthotonos	2.32	2.37	-
Proptosis	2.15	-	-
Hypotonia	-	1.71	-
Mydriasis	-	-	1.5

Opisthotonos: Tetanic spasm in which the body or trunk is arched, dorsally concave, with the head and posterior bent both toward one another.

Proptosis: Protrusion or prolapse especially of the eyeball.

Table XI

Biological data on oil fractions from Part IV

Symptoms	Corresponding doses in mg/Kg		
	Ab. A. <u>III</u> <u>IV.2121</u>	Ab. A. <u>IV</u> <u>IV.2122</u>	Ab. A. <u>V</u> <u>IV.2123</u>
Hypomotility	10, 100, 316 562	56.2, 100, 316, 562	562
Mydriasis	(2 m.m. max.) 10, 100, 316	(2 m.m. max.) 56.2, 100, 316, 562	(1.5 mm. min.) at 3.16, 10.0, 100.00, 316, 562*
Ataxia of Gait	100, 316, 562	316, 562	562
Prone Position	316, 562	316, 562	
Laboured Respiration	316, 562	316, 562	316, 562
Loss of Righting Reflex	316, 562	316, 562	
Death	316, 562	562	none
Hypothermia	(1.3°C) at 562	(1.6°C) at 316 (0.4°C) at 56.2 (0.9°C) at 562	(0.1°C) at 10 (0.5°C) at 316 (0.85°C) at 562
Hyperpyrexia	-	(0.5-0.8°C) at 10 & 100	(0.2°C) at 3.16 & 100

*Mydriasis increased with dose in the case of oil, IV.2123

Maximum dilation, 1.0 mm at 1.0 mg/Kg

Maximum dilation, 3.0 mm at 3.16 mg/Kg

Maximum dilation, 4.0 mm at 10, 100 and 316 mg/Kg

Crystalline product, 'Z', m. p. 135-38°C (dec), ($C_{15}H_{30}O_{15}K_4$, provisional), isolated from the water concentrate, Ab. 'C', IV.3:-

Total quantity isolated: 307.4 mg.

Samples sent for biological tests: a) 34.7 mg were lost.

b) 12 mg in 1 ml. of distilled water.

Reports of test at two dose levels

Low level dose: 1.0 mg/Kg.

a) Mydriasis resulted 30 minutes after administration and persisted thence for 30 minutes. Pupil dilation was 2 mm above normal.

b) Three hours after administration, there was a temperature raise of 0.9°C. Hyperpyrexia lasted less than an hour.

High level dose: 6.25 mg/Kg

a) Mydriasis was immediate. It persisted for 30 minutes. Pupil dilation was initially 1.5 mm above normal, increasing to 2 mm. later.

b) Thirty minutes after administration, temperature rose to 0.7°C. Hyperpyrexia persisted for 30 minutes.

c) Fifteen minutes after administration, there was rapid respiration persisting for 2 hours.

d) There were no other symptoms.

Other samples on which pharmacological reports are expected soon:

1) Potassium gluconate; m. p. 186°C (dec) (Part IV, Chart 17)

Quantity isolated: 606.6 mg.

Sample sent for biological tests: 111 mg.

2) B-glucoside, m. p. 106°C (dec) (Part III, Chart 13)

Quantity isolated: 292 mg

Sample sent for biological tests: 30.7 mg

Dr. Pelican's Comments

Hyperpyrexia, per se, is not necessarily accompanied by or necessarily produces mydriasis or miosis. For example, III.X₁ (fumaric acid?) produced hyperpyrexia but neither mydriasis nor miosis. If, then, a material produces mydriasis accompanied by hyperpyrexia it is suggestive of the materials containing a single substance capable of producing a unique drug syndrome (cf. "sympathotonia" of LSD).

From bio-assays, the most efficient extraction of the material with LSD-like effect appears to have been made by the method which yielded fraction A. M. 11., (II.1). The most efficient separation of the muscarine-like material from the material with LSD-like activity was made by the procedures which yielded fractions, A. M. 19/20 'C', III.111 and A. M. 19/20 'F', III.1222. These procedures also gave the highest yields of muscarine-like material.

The lethal dose of Schmiedeberg's muscarine, after intravenous administration to rabbits was apparently of the order of 6-10 mg/Kg. The median lethal dose of A. M. 19/20 'C', III.111, the most potent

muscarine-like material in this series was 5.9 mg/Kg of dry solid. Fraser found that pure muscarine produced transient effects on the blood pressure and the uterus of anesthetized rabbit after intravenous administration of doses ranging from 5-10 micrograms/Kg. Material A. M. 19/20 'O', III.III produced prolonged effects on the pupil after intravenous administration of doses of 285 micrograms/Kg of dry solid. It is obvious that 'O', III.III compares favourably with samples of muscarine isolated from natural sources.

That a single material exists in the fractions, with muscarine-like action is suggested by the fact that all of the materials tested which produced miosis also produced excessive salivation. In contrast, it can be said that neither hyperpyrexia nor the prostration which may accompany it will produce salivation. Excessive salivation was not, for instance, observed after the administration of III.X₁ (fumaric acid) in doses sufficient to produce fever.

Specific pharmacodynamic studies were not performed to identify the mechanism of action of the material in the mushroom responsible for the production of hyperpyrexia-mydrasis drug syndrome. Data obtained during the course of the bio-assays suggest that the material is LSD-like, rather than atropine-like. Fever produced by LSD is produced at doses far lower than the lethal dose; fever produced by atropine-like agents occurs after near-lethal doses. The dose of A. M. 11, II.I required to elevate body temperature 1.5°C was 0.16 mg/Kg. The median lethal dose of A. M. 11 was 26.7 mg/Kg. Ratio of the two doses is of the order to

be expected of LSD-like materials rather than atropine-like materials.

Although precise data are not available, review of the literature shows that, under the conditions of our experiments, LSD itself might be expected to produce hyperthermia after doses of the order of 25-50 micrograms/Kg. According to our definition, pure LSD has a potency of the order of 20,000-40,000 units/g of dry material. The LSD-like material present in the plant extracts studied is of the same order of potency as LSD itself.

Computation of the ratio: Mydriatic potency/miotic potency, from biological data yields a value which measures the degree to which LSD-like material was separated from the muscarine-like material in each sample.

In regard to the oil fractions, the most efficient separation of LSD-like material from muscarine-like material was made in fraction, A. M. 19/20 A/2, III.1212, which unfortunately gave a low yield. Although neither A. M. 19/20 A, III.121 and A. M. 19/20 A/1, III.1211 produced miosis, III.121 produced hypothermia at the highest dose level studied, 400 mg/Kg. III.1211 at the highest dose level studied, 300 mg/Kg. produced an initial hypothermia followed by hyperthermia. Hence, we believe these two oils, III.121 and III.1211 to be less well separated from muscarine-like substance than III.1212.

Our conclusions are based on such assumptions that the separation procedures did not produce chemical alteration of materials present in the fresh plant and that the test materials contained no bacterial pyrogens. The "cleanness" of the results obtained indicates that few,

if any, biologically active contaminants were present in the samples.

SUMMARY: Bio-assays indicate that the mushroom probably contained two, separable, biologically active substances. One substance had LSD-like effects in rabbits; the other had muscarine-like effects. In the materials in which they were present in the highest concentrations, the substances had potencies comparable to those of LSD and muscarine, respectively.

SUMMARY

1. The mushroom, Amanita muscaria, both fresh and dry, has been examined chemically and physiologically.
2. The ultimate water-concentrate of the 'marc' has been found on repeated experimentation, to have LSD-like properties, dilating the pupil and producing hyperpyrexia.
3. From the water concentrate, a potent crystalline material with LSD-like activity has been isolated in small quantity and designated 'Z', Z, m.p. 135-38°C (dec), $C_{15}H_{30}O_{15}K_4$ is being studied with the help of NMR spectroscopy.
4. A fixed oil has been isolated and fractionated. It consists mainly of esters of oleic acid. The oils are being screened for insect repellent and insecticidal properties.
5. Mannitol, fumaric acid, and some 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. Potassium gluconate has been isolated for the first time from the mushroom among the minor constituents.
6. Isolation and fractionation procedures have been worked out in detail, perfecting them for easy repetition in future work.

Indolic components have been located in the water concentrate and are being subjected to thin sheet chromatographic separation.

7. The experimental has been presented under four parts with illustrations. The subject of 'pilzotropine', the elusive hallucinogen, has been discussed and for future research some valuable suggestions have been made.

8. Pharmacological data has been included.

9. A preliminary communication on the above work was presented at the I. U. P. A. C. Symposium on the chemistry of Natural Products, Australia, 1960.

APPENDIX I

List of Publications of A. V. Subbaratnam

1. Carotenoid pigments of pyrethrum flowers.
Subbaratnam and Pillay, J. Sci. Ind. Res. 6B (1947), 100.
2. Alkaloidal constituents of Gloriosa superba Linn.
Subbaratnam, J. Sci. Industri. Res., 11B (1952), 446.
3. Untersuchungen uber die Inhaltsstoffe der Gloriosa superba Linn.
Subbaratnam, Die Pharmazie, 8 (1953), 1041
4. Studies in the alkaloidal constituents of Gloriosa supbera Linn.
Subbaratnam, J. Sci. industr. Res., 13B (1954), 670.
5. Chemical Examination of the Bark of Tabernaemontana dichotoma, Roxb.
Subbaratnam, Current Science, India, 23, 66 (1954)
6. Chemical Examination of Gloriosa superba Linn.
Subbaratnam, Bull. of the Nat. Inst. Sci. (India) No. 4 (1955), 27.
7. Chemical Examination of the Bark of Tabernaemontana heyneana Wall.
Subbaratnam, J. Sci. Ind. Res. 14B (1955), 424.
8. Studies on the Irritant Factor of Pyrethrum Flowers.
Subbaratnam and Siddiqui, J. Sci. Ind. Res. 15B (1956), 243.
9. Chemical Examination of the Seeds of Alangium lamarckii, Thwaites:
Part I - Alkaloidal Constituents of the Seed Kernels.
Subbaratnam and Siddiqui, J. Sci. Ind. Res. 15B (1956), 432.
10. Chemical Examination of the Seeds of Erythrina indica Lam.
Subbaratnam, J. Sci. Ind. Res. 15B (1956), 210.
11. Studies in the Hallucinogenic Principles of Amanita muscaria.
Cook and Subbaratnam, Abstract 50 of papers presented at
I.U.P.A.C. Symposium on the Chemistry of Natural Products, 1960,
Australia.
12. Chemical Examination of the Roots of Cocculus laeaba D. C.
Cook and Subbaratnam, Abstract 136, I.U.P.A.C. Symposium, 1960,
Australia.
13. Structure of Gloriosine (to be published).

Patents and Non-technical Notes

1. Manufacture of mosquito-repellant compositions.
Mitra, Subbaratnam and Siddiqui, Indian Patent No. 34410 (1946).
Non-technical note, J. Sci. Industr. Res. 5 (1946), 28.
2. Improvements in or relating to manufacture of concentrated extracts of pyrethrum.
Siddiqui, Subbaratnam and Bagchi, Indian Pat. No. 35338 (1946).
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APPENDIX 2

From "Drugs and the Mind" by Robert S. de Ropp,⁷⁴ pp. 278-80,
St. Martin's Press, New York, 1957.

"Moving now to a very different part of the world, the icy plains bounded on the north by the Siberian Sea and on the east by the Bering Sea, we find a curious "passport to happiness" in use among the tribes who inhabit these inclement regions. The crimson fly agaric, Amanita muscaria, is known to most people as a decorative fungus which figures in countless illustrations of fairy stories along with dwarfs in red caps, mottled fawns, elves, and other assorted flora and fauna. The primitive peoples of northeastern Asia, the Tungus, Yakuts, Chukches, Koryaks, and Kamachadales, put this fungus to a very different use. It is called muchamor and is eaten as an intoxicant during the long winters when the sun scarcely rises above the horizon and total boredom is an ever present threat. Under these circumstances any form of inebriation may be considered better than none and the Koryak, whose tastes are not refined, prefers the fly agaric to any other agent and will even give a whole reindeer for a single dried mushroom, as the fungus itself does not grow so far north. The fly agaric presents other advantages which appeal to the thrifty, for the active principle is excreted unchanged in the urine and can thus be used again. Thus an agaric orgy among the Koryaks is started by the women, who chew the dried fungus and roll the chewed substance into sausages which are then swallowed by the men. As the party warms up the participants grow lively. Some shout and sing, some hold conversations with imaginary beings, some relate with delight that they have made vast fortunes, some leap to and fro across the room, imagining every tiny obstacle in their path to be so high that they must jump to get over it. Then, when the initial jollity has somewhat worn off, there are shouts of "pass the pot", and one of the women enters with a tin can into which all present urinate with enthusiasm. The can is then passed around and each partakes of the still warm urine, gaiety is restored to the party and the leaping and singing recommence.

This is a form of social gathering which might not appeal to an asethete but, as was mentioned above, the Koryak is not refined. Besides, the precious agarics are very expensive and the winters are long, so there is every inducement to prolong the orgy. Certainly as a euphoriant the fly agaric

leaves much to be desired and excessive indulgence in the drug leads to an attack of raving madness. The hallucinations become destructive and dangerous, ending in acts of violence or, in some cases, self-mutilation. The active principle in fly agaric which produces these hallucinations appears not to have been isolated. The chief toxic substance in this fungus is an alkaloid called muscarine which, in sufficient doses, stops the beating of the heart. But whatever the substance is that affords such joy to Koryaks, Tungus, Chekches, Yakuts, et cetera, it certainly cannot be muscarine."

APPENDIX 3

From the article "On Going Berserk: a Neurochemical Inquiry" by Howard D. Fabing²⁵, The Scientific Monthly (Am. Asscn. Adv. Sci.), 83, 232-37 (1956).

"Drew reports, the patient, a middle-aged tavern keeper, picked some wild mushrooms and ate them at 10:00 o'clock one night in October 1955. These mushrooms were later identified by the botany department as Amanita muscaria. Two hours after ingestion the patient had an explosive onset of diarrhea, profuse sweating, excessive salivation and vertigo. He fell asleep and awakened at 2:00 A.M. completely disoriented, irrational and violent. When he was admitted to University Hospital, Ann Arbor, Mich., his nail beds were dusky and he appeared cyanotic; blood pressure was 150/90, pulse 120, respiration 24 per minute, and his temperature 98.4°. He did not react to deep pain stimulation, but responded to pinprick. He was disoriented in all three spheres. Somnolence alternated with periods of excitement. He thought that he was in hell and identified the interne, nurses, and attending physicians as Christ, Satan, God, or angels. Nursing notes on admission indicate that he was threshing about in bed, talking constantly and irrationally.

As the day wore on, the content of his hallucinatory and illusional output remained almost entirely religious. He constantly misidentified a tall resident physician as Christ. He kept referring to nurses and other attendants as God or angels. He felt that he was in the Garden of Eden, and then in hell. As evening came he cleared up mentally, lost his motor excitement, and felt relaxed. All laboratory tests were within normal limits. He appeared to be recovered on the following morning and was discharged."

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