LIPID DISTRIBUTION AND METABOLISM IN THE FUNGI

A Dissertation
Presented to the
Faculty of the Department of Biology
College of Arts and Sciences
University of Houston

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

by
John Donald Weete

May, 1970

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ABSTRACT

Hydrocarbon and fatty acids extracted from the spores of the phytopathogenic fungi Ustilago maydis, Sphacelotheca reiliana, Urocystis agropyri, Tilletia caries, Tilletia controversa, Tilletia foetida, Puccinia graminis tritici, Puccinia stripiformis, Rhizopus arrhizus, Aspergillus niger, and Curvularia sp. were analyzed by gas chromatography. Many of the lipid constituents were characterized by combination gas chromatography-mass spectrometry.

Heptane eluates from silica gel column chromatography revealed the presence of the predominance of n-alkane hydrocarbons ranging in chain length from C_{21} to C_{33}. The hydrocarbon chains contain a predominance of odd-number of carbon chains. The even-numbered carbon chains were found in low concentrations. Several species contained branched-chain structural isomers of the C_{27}, C_{29}, and C_{31} compounds found in low relative concentrations. This distribution of hydrocarbons was found only for spore samples obtained from diseased plant material and not from spore material obtained from synthetic media. For example, no detectable hydrocarbons were found in sporangiospores of R. arrhizus.

The total fatty acid components of fungal spores follow the same distribution as that of higher plants. The carbon chain length ranges from C_{12} to C_{24} in most of the fungi analyzed. Chain lengths of fatty acids greater than C_{20} are seldom reported. The length of the fatty acid chains typically contains an even number of carbon atoms but odd-

numbered carbon chain acids are reported in lesser concentrations. With few exceptions the saturated fatty acids are in predominant concentrations in both spore and mycelial material. Generally, oleic ($C_{18:1}$) was the predominant unsaturated acid and palmitic (C_{16}) was the predominant saturated acid.

The surface ultrastructure of the chlamydospores of Urocystis agropyri, Ustilago maydis, Sphacelotheca reiliana, and Tilletia caries are illustrated using the freeze-etch technique. Each spore possesses a unique surface architecture characteristic for each species. The value of spore internal and external ultrastructure in taxonomy is illustrated in differentiating the three Tilletia species: T. caries, T. foetida, and T. controversa.

The presence of natural methyl esters of long chain fatty acids is reported for the first time in the fungi. Chlamydospores of U. maydis and R. arrhizus mycelia contain these natural methyl esters. Only in Rhizopus mycelia were the methyl esters demonstrated as a fungal metabolic product. The natural methyl esters represent approximately 4 per cent of the total lipid extract of Rhizopus mycelia.

Total lipid analysis of Rhizopus mycelia reveal large concentrations of polar lipids (44%) followed by the triglycerides (22%), sterols (16.7%), free fatty acids (16.7%) and hydrocarbons (1%). The only extractable hydrocarbon from the mycelia of Rhizopus is identified as squalene. Total fatty acid analysis revealed saturated and unsaturated chain lengths ranging from C_{10} to C_{24}. In addition, a detailed fatty

acid analysis of the complex lipids reveal a predominance of unsaturated over the unsaturated acids with oleic ($C_{18:1}$) being the predominant unsaturated acid. The predominant saturated acid was palmitic (C_{16}) for the total methyl ester acids and polar lipids, whereas stearic (C_{18}) was the major acid of triglycerides. Spore and mycelia total fatty acids had very similar distributions except the spore contained 2 fold concentrations of oleic ($C_{18:1}$) acid and the mycelia contained over 2 fold concentrations of arachidic acid (C_{20}).

Hydrocarbon and fatty acid analyses of sclerotia of S. sclerotiorum revealed the influence of natural host substrate and synthetic media on the distribution of the lipid components. The sclerotia from pea field samples contained a single unknown hydrocarbon component whereas no hydrocarbons were detected in the sclerotia from synthetic cultures. The fatty acid distribution of the pea field sclerotia samples contained a 1:1 ratio of linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids which is in contrast to the 1:1 ratios of oleic ($C_{18:1}$) and linoleic acids in samples from PDA and synthetic media.

Incorporation of ^{14}C-labeled acetate and stearate into the lipids of R. arrhizus follows patterns consistant with the relative concentrations of the respective lipid classes reported for this organism. Acetate incorporation into the single hydrocarbon is consistant with its identification as squalene. Preliminary incorporation studies reveal that stearate is preferred to myristate in the synthesis of natural methyl esters of fatty acids. Methanol stimulates this incorporation suggesting a precursor-product relationship.

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Plant diseases caused by fungal organisms result in the loss of hundreds of thousands of dollars annually. All the information we can gain concerning the life cycle, host-parasite relationship, infection process, and chemical nature of these plant pathogens is necessary for their control.

It is speculated that lipid compounds play an important role in the survival of fungal organisms. So that our knowledge of the lipid constituents of fungi may be broadened, the primary purpose of this study is to analyze the fatty acid and hydrocarbon components of sclerotia, spores, and mycelia of several fungal species causing plant disease. Preliminary studies involving the incorporation of ^{14}C-labeled lipid precursors into the lipid classes of Rhizopus arrhizus to determine synthetic relationships are undertaken.

In addition to the chemical differences, morphological differences may also be used to delimit fungal taxa. One of the objectives is to correlate the ultrastructure of the fungal spore surface with the lipid components in distinguishing differences at the generic and specific level.

VIII.

INTRODUCTION

INTRODUCTION

The fungi are a diverse group of saprophytic and parasitic organisms. The life cycle of most fungal species consists of distinct phases: the vegetative mycelial phase and the reproductive spore phase. The spore phase is of primary importance because it represents the agent for dissemination, mechanism of survival, and the primary agent of infection. Airborne fungal spores are the primary mechanism by which the fungi are dispersed. The distribution and survival of fungal spores are dynamic factors in the occurrence of plant and animal diseases (4).

The lipid components of spores are important in survival, particularly in relation to maintaining water balance and providing a barrier to penetrating substances. From a medical point of view the external lipid components of spore walls come in direct contact with inner surfaces of respiratory tissues whenever spores are inhaled (4). The effects of lipid components on these tissues is not known. A knowledge of the composition of the lipid fraction should be useful in predicting what interactions occur. In relation to plant diseases, an understanding of the nature of spore lipids and their formation could be valuable in developing a practical means of controlling diseases caused by the fungi.

At the present time little is known of the nature of the lipids of fungal spores. The only lipid component investigated to any extent

has been the fatty acids in spores of rusts and smuts (68, 69, 70). In the case of hydrocarbons there are two reports of their presence in fungal spores (5, 51).

The waxes of higher plants have been extensively investigated and reviewed (15,16). The waxes of algae, fungi, and bacteria have received less attention.

In higher plants, a surface which is exposed to the atmosphere usually has a layer containing wax and is called the cuticle. The cuticle is a layer of cutin composed of cross-linked hydroxy fatty acids bound by a layer of wax on the outside and pectin on the inside, between the wax and the cellulose wall. Pectin is composed of polygalacturonic acid chains with methylated or free carboxyl groups capable of reacting with divalent calcium ions (19). The surface waxes are complex mixtures of long chain alkanes, alcohols, ketones, aldehydes, acetals, esters, and acids. The characteristic hydrocarbons in higher plants include aliphatic molecules with an odd number of carbon atoms ranging in chain length from C_{19} to C_{33}. Hydrocarbon molecules containing an even number of carbons and branched-chains are present in lesser concentrations. Predominate fatty acids contain an even number of carbon atoms ranging in chain length from C_{14} to C_{22}. In addition, odd and branched-chain fatty acids are found in lesser concentrations (15).

The wax components make up to 15 per cent of the dry weight of the leaf. Because of the positioning and number of functional groups,

degree of chain branching and unsaturation, and with the increasing number of other constituents identified, the picture is complicated. It has been established by light and electron microscopy that the waxy covering of higher plants is made up of minute closely packed plates or rods. The wax, when present, serves to preserve the water balance of the plant. Its other protective functions may include minimizing mechanical damage and inhibiting fungal and insect attack. It may also serve as light scattering and absorbing functions shielding the inner cells from excessive ultraviolet radiation (15).

Bacterial fatty acids have been somewhat less extensively investigated. The predominate fatty acids contain an even number of carbon atoms ranging in chain lengths from C_{12} to C_{18}. Of four marine bacteria analyzed, three were rich in branched-chain acids while one had the common straight-chain acids (55). An organism containing odd-chain fatty acids is Corynebacterium acnes, where the predominate acid is an iso-C_{15} (50). Unusual fatty acids have been reported in bacteria. For example, rugose Vibrio cholerae is found to contain cyclopropane C_{17} and C_{19} acids representing a major proportion of the fatty acid constituent (8, 9). The degree of unsaturation is not commonly found beyond the monoenoic fatty acids.

Predominant bacterial hydrocarbons range from C_{22} to C_{29} all showing methyl branching. For example, Sarcina lutea contained branched-chain and unsaturated compounds with an anteiso- -C_{25} present in the greatest concentrations. As in the fatty acids neither even- nor odd- numbered carbon chains predominated (54).

Prior to 1965, information on the fatty acid distribution of blue-green algae was unreported. The fatty acid composition of eleven species (8 marine) was determined. Their distribution was simple but showed a pronounced qualitive variation among different species. For example, Anacystis nidulans contained no polyunsaturated acids, whereas A. marina, Oscillatoria williamsii, and Lyngbya lagerhaimii contained linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids. Trichodesmium erythaeum was the only species found to contain $C_{10:0}$ in concentrations up to 50 per cent of the total fatty acids. Odd-chain acids were found in low concentrations and branched-chain acids were completely absent in blue-green algae (55, 56).

Fatty acid distributions of the higher algae follow similar patterns common to the higher plants.

Fatty acids of the fungi also show the typical distribution found in higher plants. Interest has been concentrated on the yeast-like fungi showing chain-lengths ranging from C_{14} to C_{22}. In addition, fatty acids with branched and odd numbered carbon chains, and various degrees of unsaturation are found in the fungi (45, 46, 62, 76). Unusual fatty acids are also present in some fungi. For example, cis-9,10-epoxyoctadecanoic acid is found to be one of the predominant fatty acids of Puccinia graminis uredospores (68, 69, 70).

Hydrocarbons have received much less attention with interest being concentrated primarily on fungal spores. Aliphatic hydrocarbons were reported for the first time in Puccinia striiformis uredospores (5).

The predominant hydrocarbons show an odd-carbon number preference ranging in chain length from C_{21} to C_{33} (5, 51). Molecules containing an even number of carbon atoms and branched-chains are also reported in lower concentrations.

It has been suggested by some investigators that the wax constituents of plants may be of taxonomic value (15,16). The hydrocarbons appear to have universal occurrence in the plant kingdom and represent a stable end-product isolated from the active metabolism of the plant. In addition, with modern microanalytical techniques and simplicity of sampling, the plant waxes could serve as a taxonomic tool to aid in the differentiation of taxa at the specific level. When Eglinton and his colleagues examined the leaf waxes of related genera of the subfamily Sempervivoideae (Crassulaceae) they concluded that from alkane carbon-number patterns, in Aeonium species, such comparisons could serve to confirm relationships between closely related species but the differences between closely related genera were often insufficiently discriminating (15).

The use of other chemical classes have been successively employed chemotaxonomically. For example, Alston, was able to distinguish species and their hybrids in the Baptista genera using paper chromatograms of polyphenolic compounds (3).

The wax composition of fungal spores could also be applied to differentiation of fungal genera and species as well as in the higher plants (39, 73). The sensitivity of new instrumentation such as gas

chromatography and mass spectrometry require only minute concentrations obtained from the small quantities of spores available.

External spore morphology has long been the primary method for differentiating fungal species. Now with the development of the electron microscope and application of the freeze-etch technique both external and internal ultrastructure of fungal spores and mycelia can be readily observed (22,47,48,61). Electron photomicrographs of frozen-etched fungal material illustrate the presence or absence of surface wax layers and surface architecture. In addition, the presence, number, location, and nature of wall layers may be observed. Protoplasmic particles such as vacuoles, nuclei, membrane elements, and lipid bodies may be identified. Frozen-etched spores illustrating the surface architecture have been used to taxonomically differentiate Penicillium conidiospores (22,61). It was found that conidiospores of 10 representative Penicillium species contained a unique interwoven fibrous texture on their wall surface.

The position, number, and nature of spore wall layers are thought to be taxonomically important and of evolutionary significance. For example, phycomycete sporangiospores generally contain one wall layer producing a second during germination which is continuous with the germ tube. Conidiospores of higher fungi are found to contain two or more wall layers. These spores generally do not produce a new wall during germination, the inner wall being continuous with the germ tube. The conidiospore of higher fungi is considered to be a

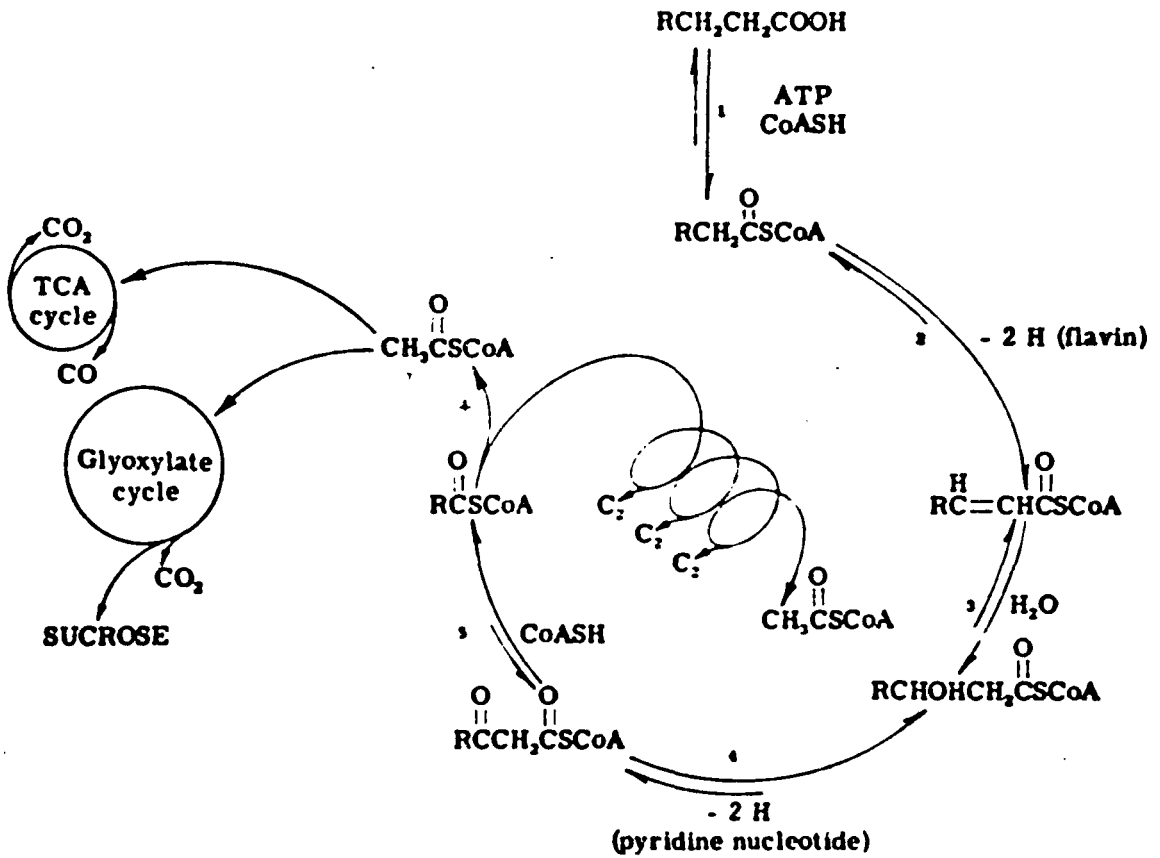
single spored sporangium, the outer wall representing the spore wall. However, this viewpoint is not shared by all mycologists (43).

The metabolism of fatty acids and hydrocarbons have been extensively investigated. In 1904, Knoop proposed the beta-oxidation theory for degradation of fatty acids by the successive removal of C_2 units. Further investigations by Munoz and Leloir, Weinhouse, Lehninger, Kennedy, Lipmann, and Lynen improved this theory which is diagramed in Figure 1 (12). Another degradation pathway for the fatty acids has been described for plant systems, this is the alpha-oxidation pathway diagramed in Figure 2 (7).

Fatty acid synthesis has been investigated in some detail using three systems: E. coli by Vagelos, yeast by Lynen, and pigeon liver by Wakil (44). The synthesis of fatty acids is not the reverse of beta-oxidation as first suspected. For example, Lynen has described a multi-enzyme complex of fatty acid synthetase (Figure 3). This synthetase complex contains the seven required enzymes spatially arranged to carry out the reactions subsequent to the condensation of the activated acetate and malonate molecules resulting in an activated butyrate molecule (42). Recycling of these enzymatic steps results in the synthesis of long chain fatty acids given in Figure 4.

The metabolism of waxes is not understood as well as that of the fatty acids. Although they have been the subject of several reviews the exact pathways remain uncertain. At the present only two biosynthetic routes are seriously being considered: the elongation-decarboxylation and condensation mechanisms (15).

FIGURE 1 . Schematic diagram of the beta-oxidation pathway for degradation of fatty acids by the sequential removal of C_2 units (7).



1 Fatty acid thiokinases
2 Fatty acyl-CoA dehydrogenases
3 Enoyl hydrase
4 β Hydroxyacyl dehydrogenase
5 β-Ketoacyl thiolase

FIGURE 2 . Schematic diagram for the degradation of fatty acids by the alpha-oxidation pathway. This illustrates the sequential removal of a C_1 unit (7).

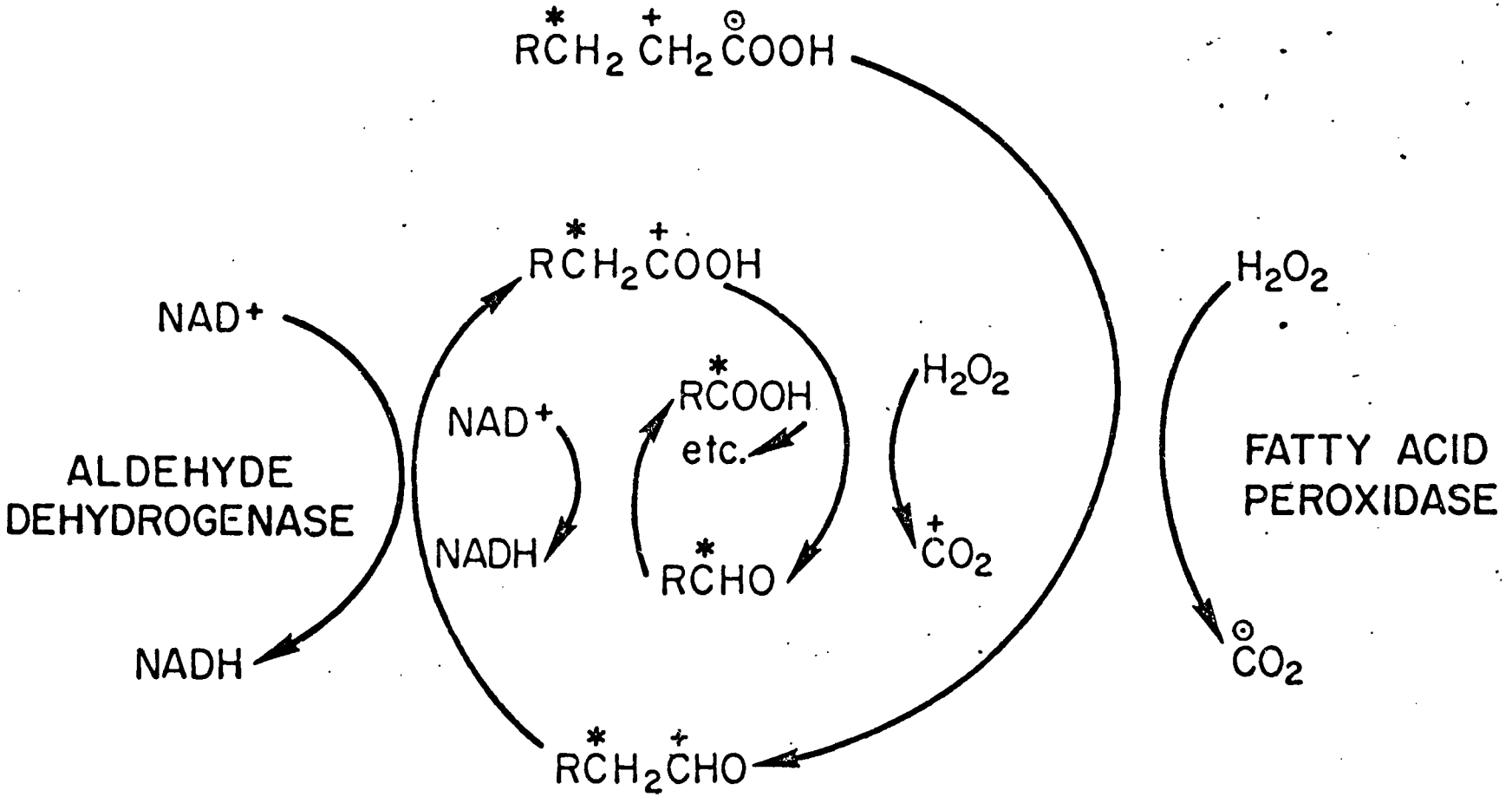

RCH2 CH2 COOH
RCH2 COOH
NAD+
NAD+
NADH
RCOOH
etc.
RCHO
H2O2
CO2
H2O2
FATTY ACID
PEROXIDASE
CO2
ALDEHYDE
DEHYDROGENASE
NADH
RCH2 CHO

FIGURE 3. Schematic diagram of Lynen's fatty acid synthetase complex (43).

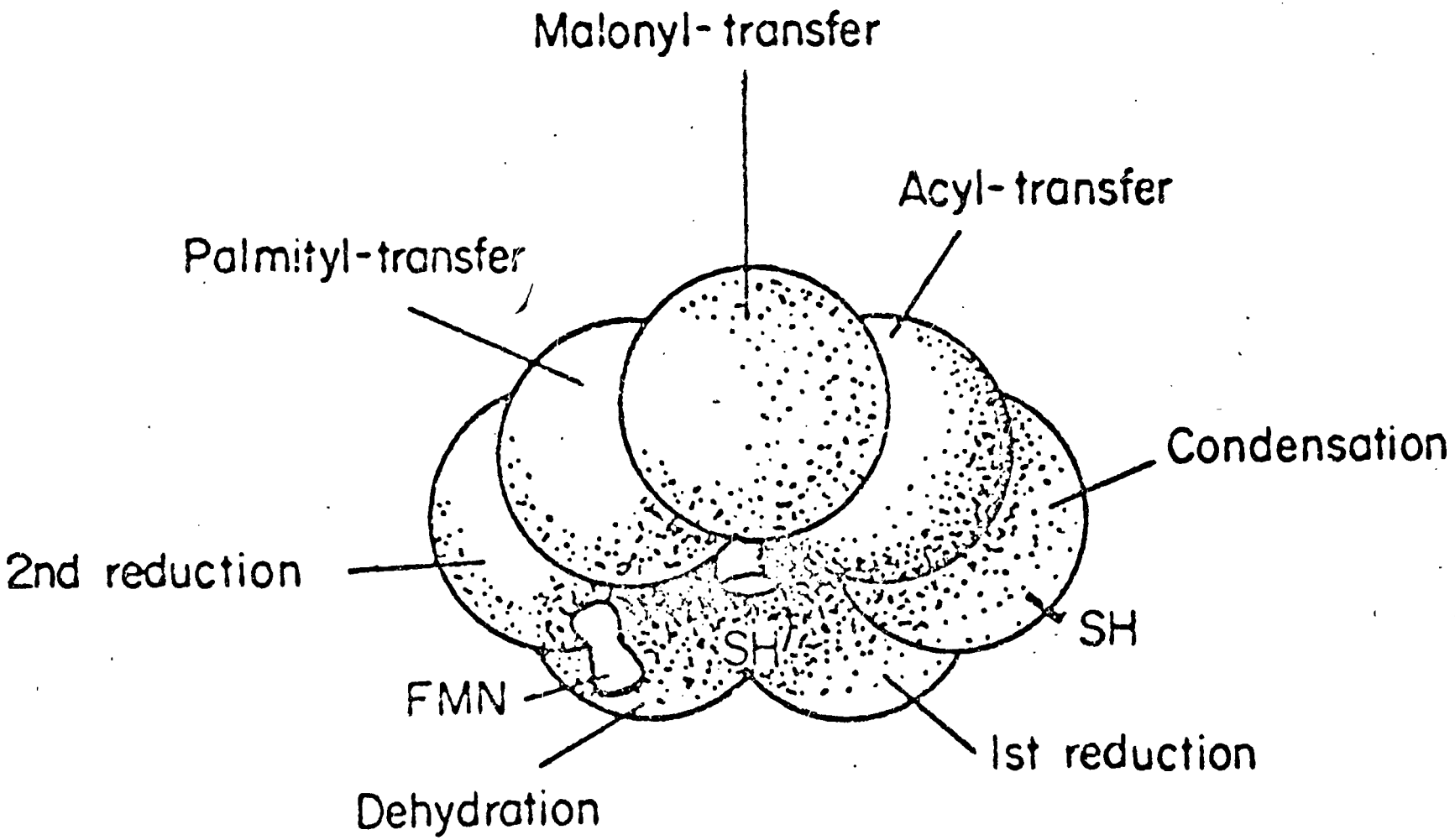

Malonyl-transfer
Acyl-transfer
Palmityl-transfer
Condensation
2nd reduction
SH
SH
FMN
Ist reduction
Dehydration

FIGURE 4. Sequential arrangement of the reactions involved in the synthesis of long chain fatty acids (43).

$$\text{acetyl CoA} + \text{ACP—SH} \xrightarrow{\text{transacetylase}} \text{acetyl—S—ACP}$$

$$\text{acetyl—S—ACP} + \text{malonyl—S—ACP} \xrightarrow[\text{enzyme}]{\text{condensing}} \text{acetoacetyl—S—ACP} + CO_2$$

$$\text{acetoacetyl—S—ACP} + \text{NADPH} + H^+ \xrightarrow[\text{dehydrogenase}]{\beta\text{-ketoacy}} \text{D}(-)\text{-}\beta\text{-hydroxylbutyryl—S—ACP} + \text{NADP}^+$$

$$\text{D}(-)\text{-}\beta\text{-hydroxylbutyryl—S—ACP} \xrightarrow[\text{dehydrase}]{-H_2O} \text{crotonyl—S—ACP}$$

$$\text{crotonyl—S—ACP} + \text{NADPH} + H^+ \xrightarrow[\text{reductase}]{} \text{butyryl—S—ACP} + \text{NADP}^+$$

$$\text{butyryl—S—ACP} + \text{CoA} \xrightarrow[\text{transacylase}]{} \text{butyryl CoA} + \text{ACP—SH, etc.}$$

The former mechanism has received the greatest support by P. E. Kolattukudy. Preliminary investigations found that 60 per cent of the wax fraction of Brassica oleracea (cabbage) contains three compounds: nonacosan-15-one, nonacosan-15-ol, and nonacosane (28, 29, 30, 37, 38). Specificity studies indicate that there is no precursor-product relationship between the three compounds. The biosynthesis of n-nonacosane (C_{29}) and n-hentriacontane (C_{31}) have been the subject of intensive studies to illustrate the elongation-decarboxylation pathway.

Light and inhibitor (trichloroacetate) studies indicate two sites of synthesis for fatty acids of chain length C_4-C_{16} and paraffins. The site of synthesis for fatty acids of chain length to C_{16} are known to occur in the chloroplast, but inhibitor studies indicate a different site for longer chain acids. Trichloroacetate inhibits the synthesis of both very long chain fatty acids and paraffins. Tissue studies indicate that synthesis of these compounds is in the epidermal region of the leaf. This suggests that the very long chain fatty acids and paraffins are biosynthetically related, but do not show a precursor-product relationship (30).

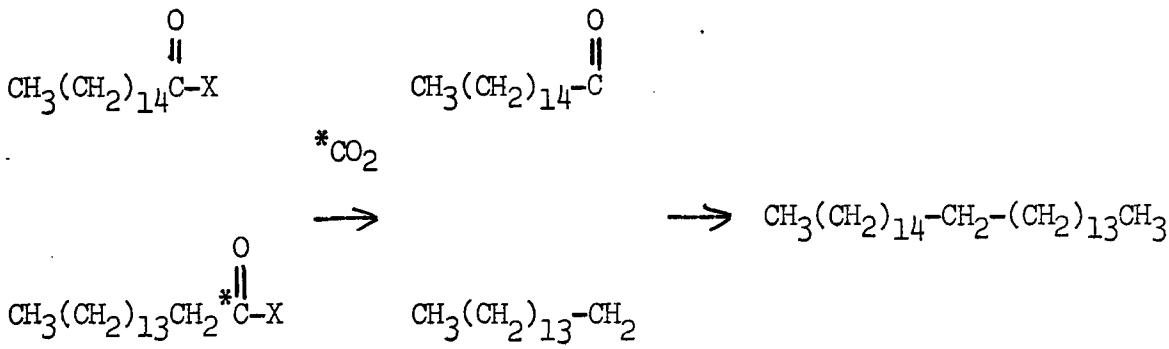
Other evidence supporting the elongation-decarboxylation mechanism includes the fact that the doubly labelled dodecanoic acid (C_{12}) moiety maintains its original $^{14}C/^{3}H$ ratio when incorporated into long chain paraffins. Thus condensation of the C_{12} moiety with an octadecanoic acid molecule (which does not lose its carboxyl carbon atom when incorporated into paraffins) and loss of the C_{12} carboxyl carbon did not occur in the synthesis of the C_{29} paraffin (32).

The condensation mechanism which is also considered by some investigators (Kaneda) to be operative in plant systems was tested by Kolattukudy (34). He used young pea leaves (Pisum sativum) and spinach leaves (Spinacia oleracea) which contained 90 and 98 per cent n-hentriacontane (C_{31}) respectively. For head to head condensation to occur producing C_{31} either two C_{16} molecules or a C_{18} and C_{14} molecule would be required. Figure 5 diagrams the presumed pathway for head to head condensation. But incorporation studies revealed that both palmitic-1-^{14}C and palmitic-UL-^{14}C acids were equally well incorporated. Also, it was found that the carbonyl carbon of the C_{31} ketone was produced from a preformed saturated carbon atom. Similar results are consistant with the condensation-decarboxylation hypothesis for synthesis of long chain paraffins.

Kolattukudy describes an alternative pathway which is consistant with evidence presented thus far. As diagramed in Figure 6 this pathway involves the condensation of two activated acyl moieties with a malonyl-enzyme complex. Subsequent reduction of the acyl carbonyl carbons and decarboxylation of the malonyl carboxyl group would give rise to the long chain alkane. If reduction does not occur a diketone would result. Diketone compounds are prevalent in the waxes of higher plants.

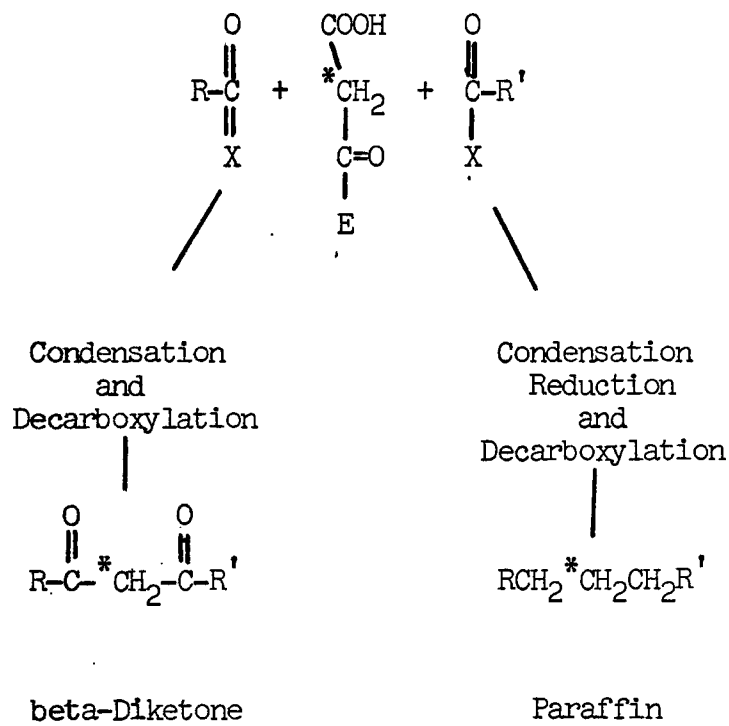
When labelled branched amino acids (L-leucine, L-valine, and L-isoleucine) are used as starter compounds the branched fatty acids and paraffins are also labelled. Valine is incorporated into the odd-

FIGURE 5. Condensation and subsequent reactions leading to the synthesis of n-hentriacontane (34).



palmitic acid patmitone n-hentriacontane

FIGURE 6 . A diagram illustrating the synthesis of long chain paraffins and diketones. By the condensation of two activated long chain acid molecules with an enzyme complexed malonyl moiety, either paraffin or diketone molecules can be synthesized (34).


Condensation
and
Decarboxylation
Condensation
Reduction
and
Decarboxylation
beta-Diketone
Paraffin

carbon numbered iso-C_{29}, C_{31}, and C_{33} paraffins and isoleucine into the even-carbon numbered anteiso-C_{30} and C_{32} paraffins (32).

The literature concerning hydrocarbon biosynthesis has been dominated by the investigations of P. E. Kolüttukudy. Figure 7 illustrates his proposed relationship between the synthesis of fatty acids, very long chain fatty acids and paraffin compounds (34).

As described before paraffins appear to be universally distributed throughout the plant kingdom. It seems logical that nature would provide a mechanism for degradation of these compounds. It has been demonstrated that natural paraffins are catabolized by soil microbes and by animal organisms (20, 27). Kolattukudy found that young leaves of broccoli, pea, and tobacco possess the enzymes necessary for the oxidation of long chain n-alkanes, specifically hexadecane. The proposed pathway is as follows: alkane—alcohol—acid—phospholipid (Figure 8). The alkane is oxidized to the primary alcohol followed by further oxidation to the fatty acid. The fatty acid is readily incorporated into the phospholipid fraction of the lipids (27).

Waxy esters such as pentadecanyl and hexadecanyl esters of long chain fatty acids are detected in waxes of higher plants, insects, and the oils of certain fish (33). The exact mechanism of their synthesis remains uncertain but Kolattukudy has shown that broccoli (Brassica oleracea) leaves contain enzymes capable of synthesizing waxy esters from fatty alcohols by direct esterification with fatty acids and by acyl transfer from phospholipids and acyl coenzyme A (33). The mech-

anism of synthesis for the methyl esters of long chain fatty acids reported here for the first time as a fungal product does not appear in the literature.

FIGURE 7. A schematic diagram illustrating the metabolism routes of fatty acids, very long chain fatty acids, and paraffins (34).

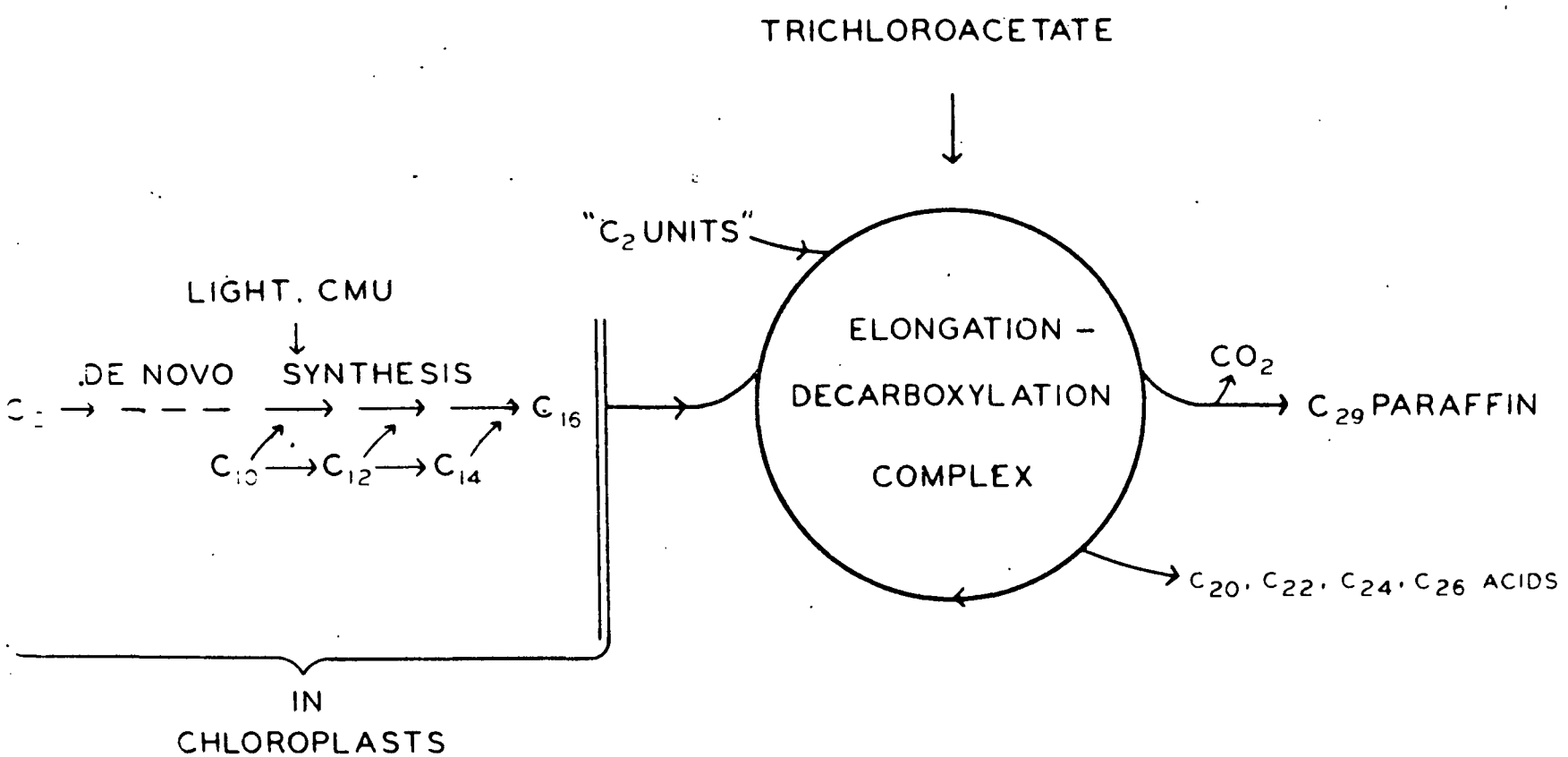

TRICHLOROACETATE
"C2 UNITS"
LIGHT, CMU
DE NOVO SYNTHESIS
C2
C10
C12
C14
C16
ELONGATION -
DECARBOXYLATION
COMPLEX
CO2
C29 PARAFFIN
C20, C22, C24, C26 ACIDS
IN
CHLOROPLASTS

FIGURE 8. Proposed pathway for the biological degradation of long chain paraffins (34).

$$R\text{-}CH_3 \longrightarrow R\text{-}CH_2\text{-}OH \longrightarrow R\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}OH \longrightarrow \text{phospholipid}$$

IX.

METHODS AND MATERIALS

METHODS AND MATERIALS

SPORE COLLECTION

Rust and Smut Spores: Rust and smut spores were carefully removed from the infected host plant and passed through a number 100 sieve (U. S. standard sieve with 149u openings). The spores were examined with a light microscope to establish the purity and type of spore. Great care was taken to ensure the complete separation of host materials from the spore materials. All samples were stored at 4° C. In some cases uninfected host tissue was obtained for analysis.

Other Spores: The spores cultured in our laboratory for analysis were harvested in the following manner: After removing the agar, cultures were suspended in distilled water in 1 liter flasks, vigorously shaken, filtered through cheese cloth, and the filtrate centrifuged to obtain a spore pellet. After removal of the supernatant all spores were lypholyzed prior to analysis.

All fungal organisms used for analysis in this study and their source are listed in Table 1.

EXTRACTION PROCEDURE

During this investigation various modified extraction procedures have been employed, each suited to its particular purpose (18).

When using small quantities, the material to be extracted was first placed in 50 ml of a benzene:chloroform (3:1) solution for 30

TABLE 1. FUNGAL ORGANISMS USED FOR LIPID AND BIOSYNTHETIC ANALYSES IN THIS STUDY

FUNGI	SOURCE	DISEASE-CROP
Sphacelotheca reiliana (Kuhn) Clint.	R. A. Fredericksen, Texas A&M Univ.	Sorghum Smut
Urocystis agropyri Fisch v. Waldh.	L. H. Purdy, USDA, Washington State Univ.	Flag Smut
Ustilago maydis (D.C.) Cda.	Don Mathre, Montana State Univ. Jack Paxton, University of Illinois	Corn Smut
Tilletia caries (D.C.) Tul. (Race T-6)	C. S. Holton and Jim Hoffman, U.S.D.A., Washington State Univ.	Bunt of Wheat Host Variety 50077 (CI 13561)
Tilletia foetida (Wallr.) Lira (Race L-10)	" " " "	Bunt of Wheat Host Variety 50077 (CI 13561)
Tilletia controversa (Kuhn) [Race D-5 (36)]	" " " "	Dwarf Bunt of Wheat Host Variety 50077 (CI 13561)
Rhizopus arrhizus Fisher		Soft Rot, Sweet Potato
Puccinia graminis tritici		Black Stem Rust of Wheat
Puccinia stripiformis		
Culvularia sp.	Isolated from atmosphere	Saprophyte
Helminthosporium sp.	" " "	"
Aspergillus niger	" " "	"
Sclerotinia sclerotioium	Dr. D. LeTourneau University of Idaho	Diseases of Vegetable Crops

minutes at 40° C. The mixture was subjected to low centrifugation for 3 minutes. The residual material was re-extracted with a second 50 ml volume of n-heptane under the same conditions and followed by centrifugation. The combined extracts were taken to dryness under a stream of prepurified nitrogen. The total lipid extract was then ready for fractionation.

For total lipid extractions of larger amounts of material such as mycelia, the same method was followed except the procedure was carried out at room temperature, overnight, and with constant agitation by a magnetic stirrer.

Lipid extraction from cell free synthesis systems again followed the same procedure as the above except the solution was first lypholyzed.

FRACTIONATION PROCEDURES

Column Chromatography: The total lipid residue was dissolved in n-heptane and applied to the top of a silica gel column (1 x 20 cm). The silica gel had been prepared by treatment in an electric furnace for 10 hours at 425° C and washed with four volumes of n-heptane prior to use. The column sample was eluted with 20 ml of n-heptane, then washed with 20 ml of benzene, followed by 20 ml of methanol. Each fraction was collected separately and dried at 40° C under a stream of prepurified nitrogen. Each fraction was dissolved in 20 ul of benzene before injection into the gas chromatograph. In the total lipid analysis of *R. arrhizus* the column was also eluted with 20 ml of chloroform between the benzene and methanol eluates.

Thin-layer Chromatography: Thin-layer plates were prepared using Silica gel G:H_2O (2:1) suspension placed on a 20 x 20 cm glass plate. The stationary phase was approximately 250 u in thickness. The silica gel G was activated by heating at 100° C for 1 hour immediately prior to use. Sample and standard solutions were placed at the origin with 10 ul micropipettes. The plate was allowed to develop until the solvent front reached to within 1 inch of the top. After drying the compounds were detected with ultraviolet light and Rhodamine 6G stain. The solvent system used unless otherwise indicated was n-heptane:ethyl ether:acetic acid (85:20:2).

Gas Chromatography: All column eluates were analyzed in a Perkin Elmer 900 (Figure 10) or Barber Colman 5000 gas chromatograph equipped with a hydrogen flame ionization detector. The columns, source, and compounds for which they were employed are given in Table 2. The specific conditions for each gas chromatographic separation are given in the description of each chromatogram.

The major alkane and fatty acid components were identified by chromatographic techniques by comparison with retention times of authentic standards chromatographed under identical conditions. All standard compounds were purchased from Applied Science Laboratories. The major alkane and fatty acid components were verified by analysis with a combination LKB 9000 gas chromatograph-mass spectrometer where indicated (59, 60). Throughout this study all procedures were checked by the use of suitable solvent blanks. All quantitative data was calculated from peak area values obtained with an Infotronics Digital Integrator.

TABLE 2. STATIONARY PHASE, SIZE, SOURCE, AND APPLICATION OF EACH COLUMN* USED IN THIS STUDY FOR GAS CHROMATOGRAPHIC ANALYSES ARE GIVEN IN THIS TABLE.

	STATIONARY PHASE	SIZE	COMPOUNDS SEPARATED	SOURCE
I.	Apiezon L "Q"	30 m x 0.025 cm	Hydrocarbons, Fatty Acids	Perkin-Elmer, Norwalk, Va.
II.	Igepal CO-880	200 m x 0.076 cm	Fatty Acids	General Aniline and Film Corp. New York, N.Y.
III.	SE-30	2.7 m x 3 mm	Hydrocarbons	Applied Science Laboratory Incorporated, State College, Penn.
IV.	Apiezon L	40 m x 0.025 cm	Hydrocarbons	" " "
V.	Ethylene Glycol Succinate	10 ft. x 1/8 in.	Fatty Acids	" " "
VI.	OV-1	60 ft. x 0.01 in.	Hydrocarbons	" " "

* All columns used in this study were stainless steel.

All glassware was washed and rinsed in distilled water followed by thorough washing in dichromate:sulfuric acid cleaning solution. They were again rinsed in distilled water. In all cases, spectrograde chemicals were used for extractions and cleaning.

Sample Preparation for Gas Chromatography: Heptane (alkane) and benzene (natural methyl esters) eluates from silica gel columns and thin-layer chromatograph spots were taken up in 10-30 ul of benzene and injected directly into the gas chromatograph.

Two methods were employed for preparation of fatty acids for gas chromatographic separation. First, the methanol fraction from column chromatography was placed directly into a 3 ml mixture of boron trifluoride-methanol per 100 mg of fatty acids present, as described by Morrison and Smith (49). This solution was boiled in a water bath for 3 minutes. The methylated acids were extracted in a separatory funnel containing 30 ml of n-heptane and 20 ml of water. The n-heptane phase was removed and taken to dryness under prepurified nitrogen at 40° C.

Total fatty acids were prepared by first refluxing a portion of the total lipid extract for 1 hour in 6 per cent KOH (w/v) in methanol: water (5:2 v/v). The non-saponifiable material was first extracted with 3 equal volumes of n-heptane followed by extraction of the saponifiable material with 3 equal volumes of ethyl ether after acidifying to pH 2 with n-HCl. Each fraction was evaporated to dryness under prepurified nitrogen at 40° C (76). The saponifiable material was esterified by the boron trifluoride:methanol method described above. The fatty acid methyl esters were dissolved in 10-30 ul of benzene for injection into the gas chromatograph.

Freeze-etch Procedure: Spores were wet by a solution of Teepol detergent (one drop of detergent in 10 ml of water) then washed with distilled water. The spores were centrifuged into a pellet and a drop of water containing spores was placed on a small (3mm) copper disc, previously scratched to ensure greater adherence. The water drop containing spores was then immediately frozen in liquid Freon 22 and transferred to liquid nitrogen. The disk with the frozen drop was then placed on a precooled (-100° C.) table in the Balzers 360 M apparatus (Balzer A. G. Lichtenstein), and frozen-etched as described by Moor and Muhlethaler and Moor. After etching, the discs containing the specimens were removed, warmed to room temperature, and dipped into distilled water to remove replicas from the specimens. The replicas were treated with 70% sulfuric acid (1 hour), a distilled water rinse, Chlorox (1 hour), and a final distilled water rinse to remove any spores that remained attached to the replicas. After the replicas were picked up on formvar-coated grids and allowed to dry, they were examined in the electron microscope.

CULTURE CONDITIONS

Rhizopus arrhizus Culture for Lipid Analysis and Cell Free Synthesis: Sporangiospores harvested from potato dextrose agar plates were inoculated into a proline germination buffer prepared by adding Na_2HPO_4 (14 g/liter) to KH_2PO_4 (13 g/liter) until a pH of 6.5 was reached. To this was added proline to make a 0.01 M solution. Spore suspensions of 100 ml per 250 ml flask were incubated at 30° C on a rotatory

shaker for 8 hours. Ten ml of the germinated spore suspension were pipetted into 90 ml of the following modified Fothergill's and Yeoman's media: glucose-40 g/l, K_2HPO_4 (0.048 M)-8.36 g/l, $MgSO_4$:7 H_2O (0.012 M)-3.0) g/l, $(NH_4)_2SO_4$ (0.025 M)-3.3 g/l, Zn, Fe, Mn (2 ppm) 2 ml/l. When solid media was required, 15 g/l of Bacto-agar was added. The resulting spore inoculum was incubated for 24 hours on a rotatory shaker at 30° C.

Cultures were harvested in a Buchner funnel followed by washing with distilled water. The cultures were pressed dry, frozen, and disiccated by lyopholyzation.

Preparation of Sclerotia: Sclerotia of *S. sclerotiorum* free from plant tissue were obtained from dry peas from commercial cleaning operations. The peas were grown in commercial fields near Filer, Idaho. Most of the extraneous dust and debris clinging to surface of the sclerotia was removed by a stream of air. The sclerotia were placed in a cheesecloth and rapidly immersed in three changes of distilled water to remove remaining dust and fine materials. They were then immediately dried in a vacuum oven (55° C , 28-inch of vacuum).

Sclerotia were also grown from an isolate of *S. sclerotiorum* that was originally obtained from a hyphal tip of a surface sterilized germinating sclerotia. Cultures were grown at 25° C on potato dextrose agar in plastic petri dishes (100 x 15 mm). Sclerotia appeared about 1 week after seeding and two months after seeding the sclerotia were removed from the dried agar surface using tweezers.

Sclerotia were obtained in a similar manner except the cultures were grown on a glucose or sucrose salts medium. The medium contained glucose or sucrose-15 g, agar-10 g, KH_2PO_4-1.5 g per liter of solution. The iron-minor element stock solution provided the final concentration per liter of medium as follows: Fe-0.2 g, Cu-0.1 mg, Zn-0.1 mg, Mo-0.02 mg, and Mn-0.02 mg. The sclerotia were ground in a mortar and pestle prior to lipid analysis.

INCORPORATION OF ^{14}C-LABELED ACETATE, STEARATE, AND MYRISTATE INTO THE LIPIDS OF RHIZOPUS ARRHIZUS MYCELIA

Rhizopus arrhizus cultures were grown on Czapek-Dox liquid and solid media as described earlier in this section. The isotope solution was placed directly on top of the solid media prior to inoculation or added directly to the liquid media. All isotopes were purchased from New England Nuclear Corporation, Boston Massachusetts. Each isotope was checked for purity by thin-layer chromatography. Cultures were harvested and washed as previously described in this section. Lipid extracts were obtained and separated by thin-layer chromatography. Methods for detection of the radioactive lipid components are described later in this section.

The fresh mycelia used for cell-free incorporation studies were ground in neutral alumina by mortar and pestle in a phosphate buffer at pH 6.2. Five ml of the crude extract was pipetted into 50 ml flasks containing 0.5 ml of the isotope solution. To the respective liquid culture and cell-free flask was added 2.5 uc acetate, 0.25 uc stearate,

and 0.31 uc myristate. Each isotope was placed in 3 liquid culture flasks to obtain enough mycelia for detection of low lipid levels. Only one flask for each isotope was used for the cell-free incorporation. The control was placed in a boiling water bath for 15 minutes and allowed to cool before adding the isotope solution. All manipulations for the cell-free incorporation studies were carried out in ice cool equipment.

Due to the water insolubility of the long chain fatty acids a solubilizer was used for placing them into solution. The acid was first placed in 3 ml of ethyl ether followed by the addition of 2 drops of Tween 80. After evaporation of the ether, warm water was added resulting in a clear to slightly turbid fatty acid solution.

Preliminary centrifugal fractionations of the mycelial preparation was carried out in a refrigerated Servall Ultracentrifuge and Beckman Model L Ultracentrifuge. The crude extract was centrifuged for 10 minutes at 4000 rpm. The supernatant was then centrifuged at 14,000 rpm for 30 minutes followed by centrifugation of the resulting supernatant at 38,000 rpm for 90 minutes. This remaining supernatant is considered the soluble enzyme preparation.

DETECTION OF RADIOACTIVITY

Liquid Scintillation: Determination of radioactivity of liquid samples was done on a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003. Eluates from column or silica gel G from thin-layer plates were placed into glass scintillation vials each containing 10 ml of Pre-Mix "P" in 5 g/liter of toluene (PPO-98%, POPOP-2%) (72).

Thin-layer Chromatography-Autoradiography: Thin-layer chromatographic methods were carried out as described above. After drying each plate containing radioisotopes, the plate was covered by a 20 x 20 cm piece of Kodak No-Screen X-ray film. The plate and film were placed in the dark for 2-4 weeks before development (72). Sample and standard spots were located by dichromate-sulfuric acid spray followed by heating at 100° C for 30 minutes.

X.

SECTION I

HYDROCARBONS, FATTY ACIDS, AND ULTRASTRUCTURE OF SMUT SPORES

INTRODUCTION

The nature and distribution of paraffinic hydrocarbons and fatty acids of several fungal species were studied. The following organisms were investigated: *Ustilago maydis*, *Urocystis agropyri*, *Sphacelotheca reiliana*, *Tilletia caries*, *Tilletia controversa*, *Tilletia foetida*, *Puccinia graminis*, *Puccinia stripiformis*, *Aspergillus niger*, *Rhizopus arrhizus*, and *Curvularia sp*. The contributator of each fungal species and the plant diseases caused by them are given in Table 1. The objective of this study was to illustrate the distinct wax patterns obtained from each fungal species. In addition, the morphological surface differences of four representative smut species using electron microscopy and the freeze-etch technique are illustrated.

RESULTS

The n-heptane fraction of spore extracts contained primarily n-alkane compounds. The major components consist of straight-chained molecules containing an odd number of carbon atoms. Smaller amounts of branched hydrocarbons and chains containing an even number of carbon atoms were detected.

The hydrocarbon pattern of U. agropyri is shown in Figure 9. The gas chromatogram illustrates the type of resolution obtained and the complexity of the n-heptane fraction. While the major peaks are easily detected, it should be noted that minor peaks are also numerous. Each species analyzed had a distinct pattern of hydrocarbons with the exception of the three Tilletia species. While each Tilletia species contained identical alkane patterns, the Tilletia group contained a distinct pattern from the other species. While the chromatograms are valuable in illustrating the resolution of the sample into individual components, it is difficult to compare the concentrations of different peaks of the chromatograms. Therefore, for comparison purposes the concentration of each peak was determined for four of the species analyzed and illustrated in histograms based on the per cent total of the hydrocarbon components (Figure 14 A to D).

The predominant hydrocarbons of U. agropyri were C_{29} and C_{31} (Figure 14 A). The n-heptane fraction of U. maydis was analyzed and its histogram and chromatogram are illustrated in Figures 14 C and 12 re-

spectively. The predominant hydrocarbon present was C_{27}. The per cent of branched-chain alkanes is indicated by the solid bar lines in each histogram (Figure 14D). The spores of S. reiliana had a high content of hydrocarbons in comparison to other spores, and its per cent distribution is shown in Figure 14D. In S. reiliana the major hydrocarbon was C_{29}, with a rather simple pattern as illustrated in Figure 11. The hydrocarbon fraction of all Tilletia species contained a high concentration of C_{27}, C_{29}, and C_{31}. The per cent concentration of the hydrocarbons of T. caries is found in the histogram of Figure 14B and that of T. foetida and T. controversa is found in Table 3 . The chromatograms of the three Tilletia species are given in Figure 35.

The alkane distribution of P. graminis and P. stripiformis uredospores are almost identical. The predominant hydrocarbon chain lengths are C_{27}, C_{29}, and C_{31}, with C_{29} present in the greatest concentrations. The relative per cent distribution of each component is given in Table 3 and their chromatograms in Figures 10 and 13 respectively.

Sporangiospores of R. arrhizus did not contain a significant concentration of hydrocarbons. In all extractions similar quantities of spores were extracted (2-5 g) unless otherwise indicated.

The methanol fraction that contained the free fatty acids showed differences between each species, yet the differences were not as distinct as those from the n-heptane fraction (Table 4). In all species detectable concentrations of palmitic acid (C_{16}) were present. The only species that did not contain palmitoleic acid ($C_{16:1}$) was U. agropyri. All species contained sizable quantities of myristic acid (C_{14}), with

FIGURE 9. An example of gas chromatographic separations of the heptane fraction of *Urocystis agropyri*. Separation was carried out on stainless steel capillary tubing (30 m x 0.025 cm) coated with OV-1. Helium pressure 145 g/cm^3; no split. Perkin-Elmer 900 instrument equipped with a flame ionization detector. Range 1; attenuation 128. Temperature programed from 150° C to 300° C at 10° C/min. In each case approximately 5 g of spores were extracted, and 1/30 of the sample injected.

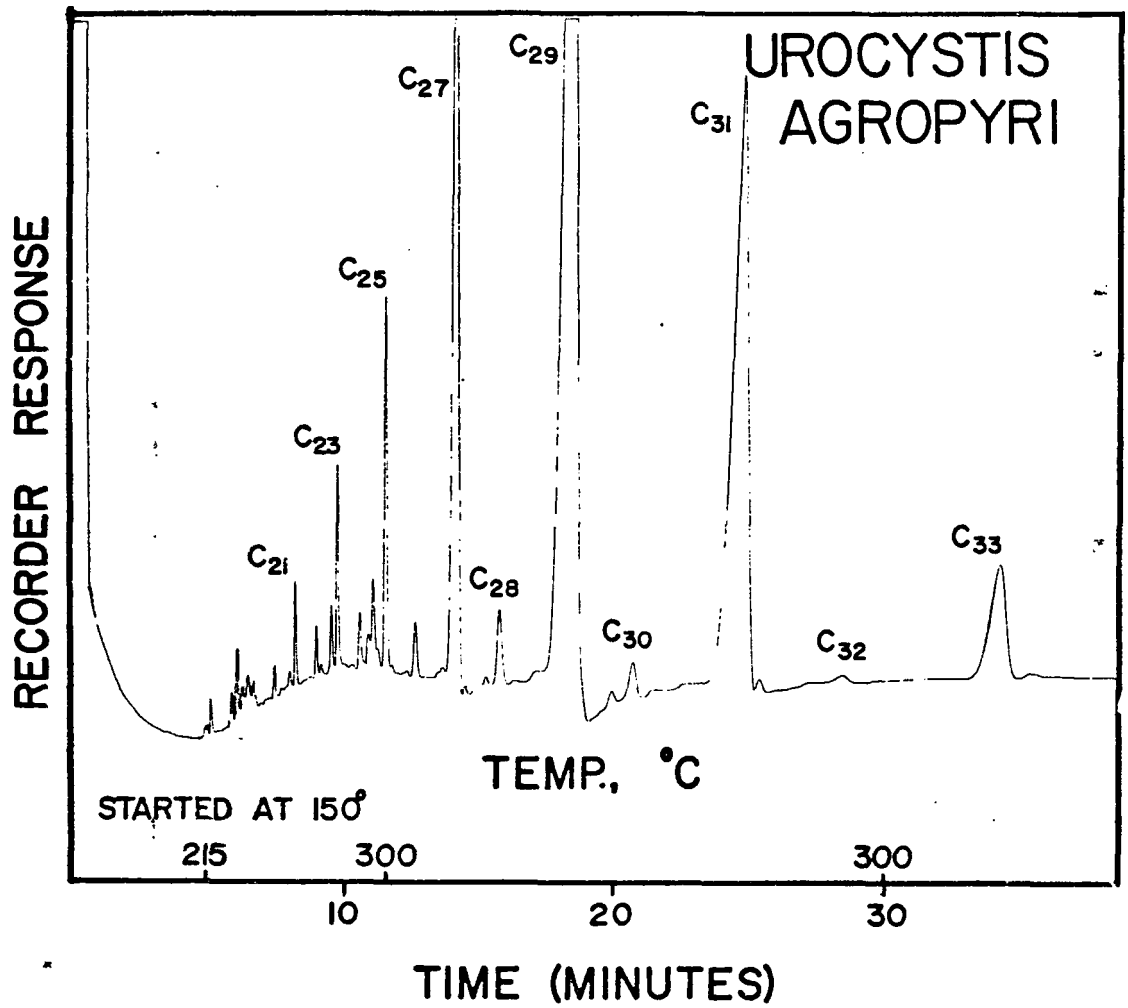

UROCYSTIS
AGROPYRI
RECORDER RESPONSE
C21
C23
C25
C27
C28
C29
C30
C31
C32
C33
TEMP., °C
STARTED AT 150°
215
300
300
10
20
30
TIME (MINUTES)

FIGURE 10. A gas chromatogram illustrating the separation of the alkane fraction from uredospore extracts of *Puccinia graminis tritici*. Separation was carried out in 100 ft. x 0.01 in. capillary stainless steel tubing coated with Apiezon L using a Barber Coleman gas chromatograph with N_2 pressure 2430 g/cm^2, no split. Temperature was programed from 150° C - 300° C at 10° C per minute.

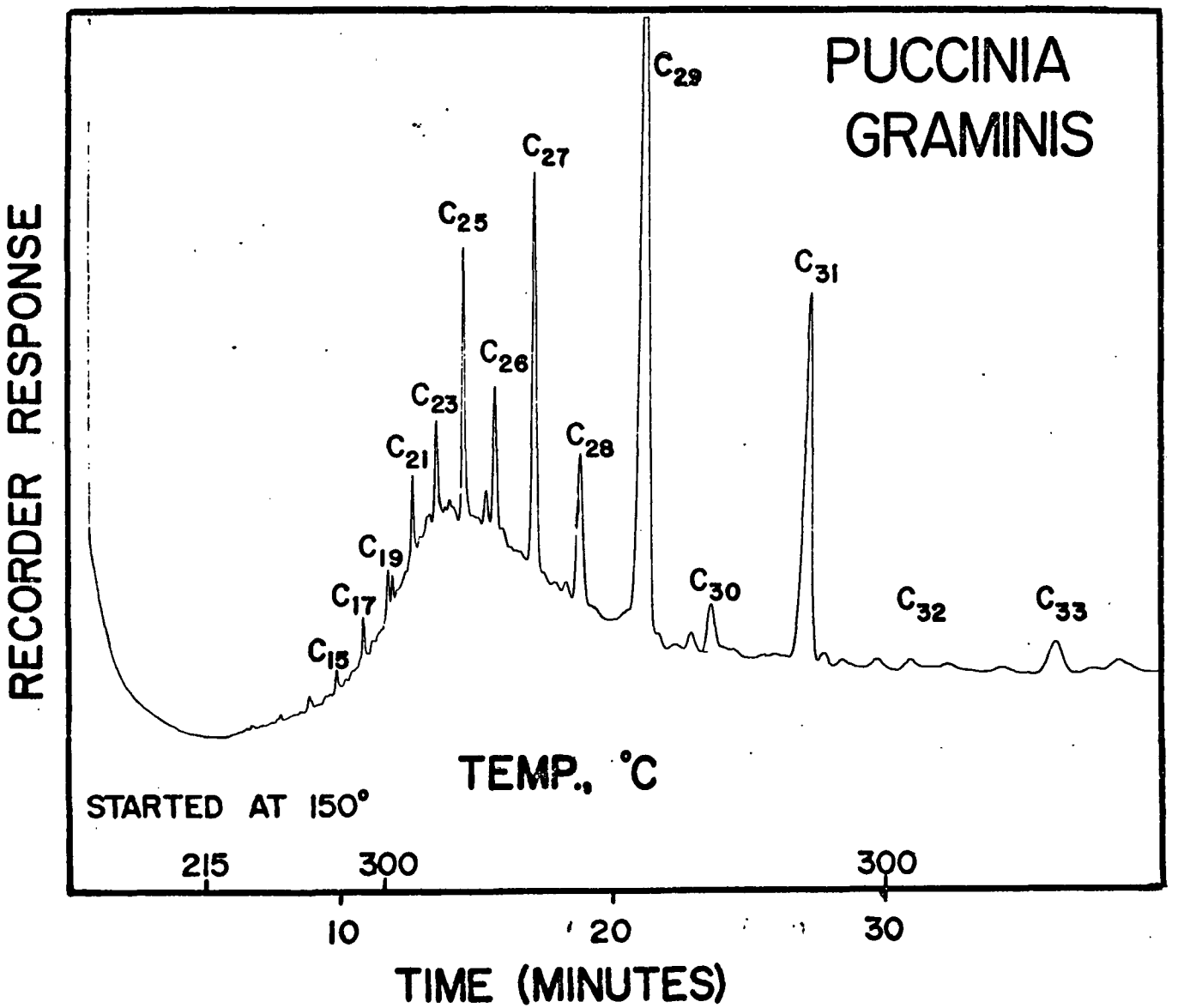

PUCCINIA
GRAMINIS
RECORDER RESPONSE
C15
C17
C19
C21
C23
C25
C26
C27
C28
C29
C30
C31
C32
C33
TEMP., °C
STARTED AT 150°
215
300
300
10
20
30
TIME (MINUTES)

FIGURE 11. A gas chromatogram illustrating the separation of the heptane fraction of chlamydospores of Sphacelotheca reiliana. Using a Perkin-Elmer the separation of alkanes was accomplished in 60 ft. x 0.03 in. stainless steel capillary tubing coated with OV-1. The temperature was programed from 150° C - 270° C at 4° C per minute under Helium pressure of 8 psi, no split.

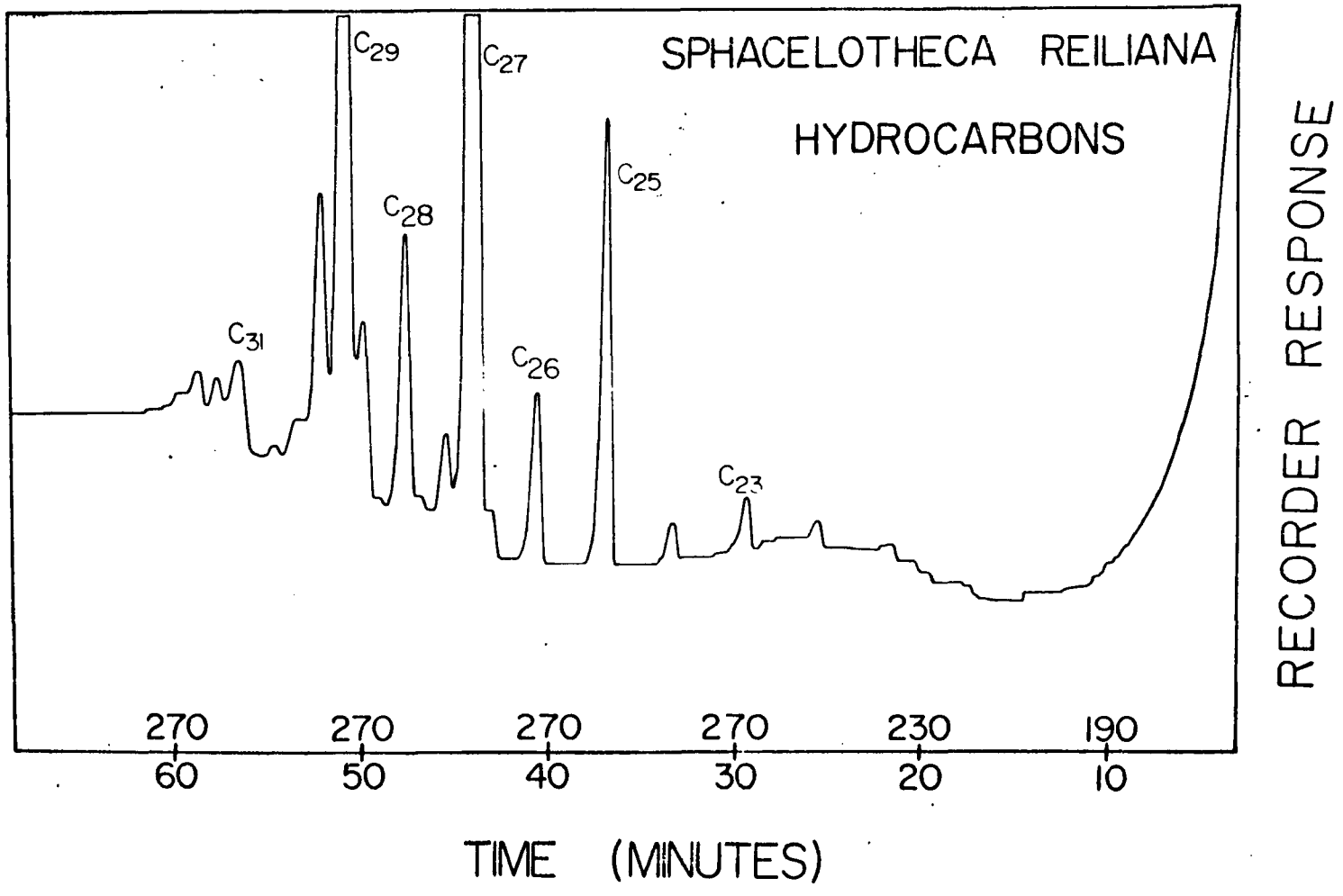

SPHACELOTHECA REILIANA
HYDROCARBONS
C29
C27
C28
C25
C31
C26
C23
270
270
270
270
230
190
60
50
40
30
20
10
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 12. A gas chromatographic separation of heptane extracts of chlamydospores of *Ustilago maydis*. The separation was carried out on a Perkin-Elmer gas chromatograph equipped with 60 ft. x 0.03 in. stainless steel tubing coated with OV-1. Helium pressure 10 psi. Temperature programed from 150° C to 270° C. No split.

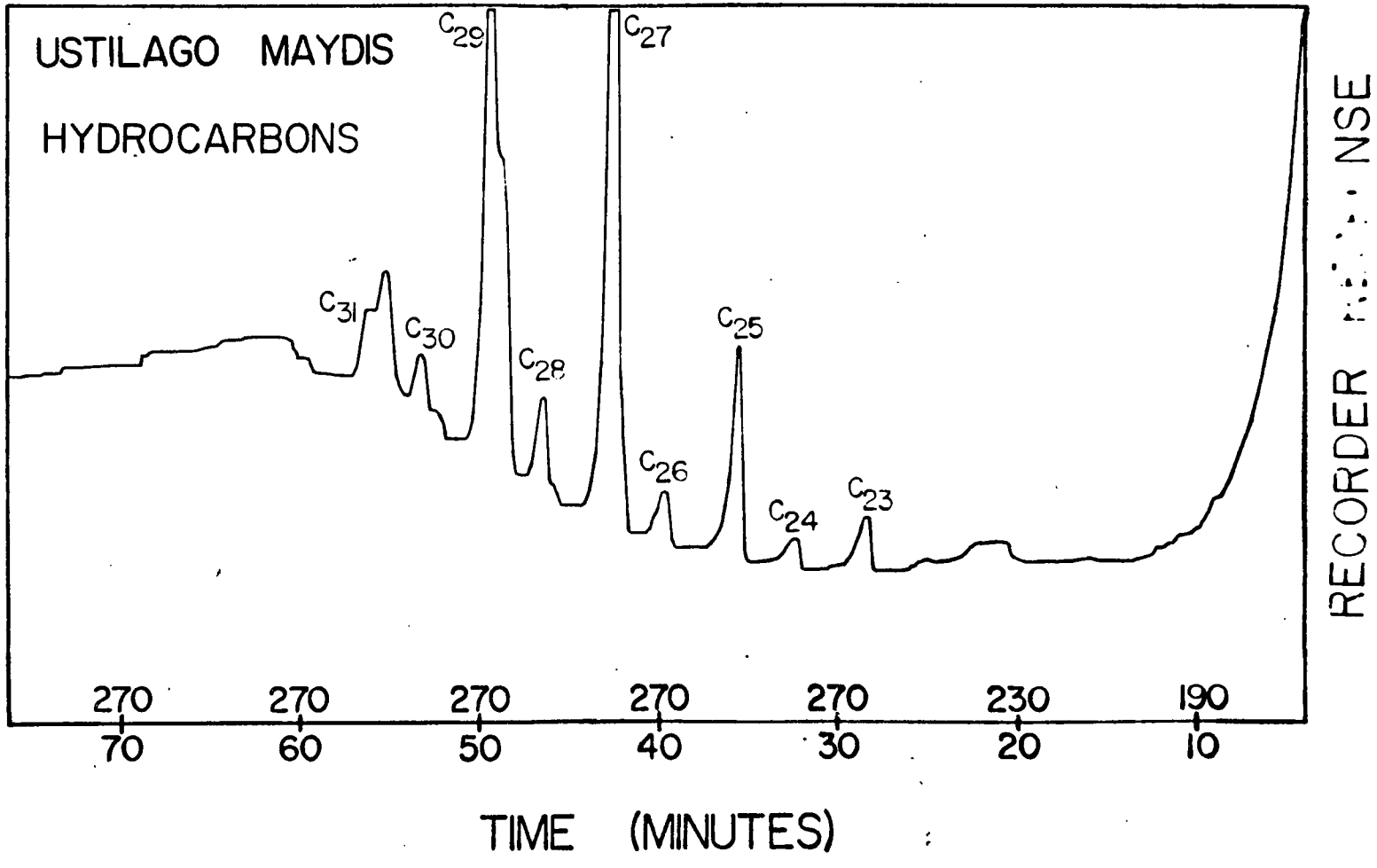

USTILAGO MAYDIS
HYDROCARBONS
C29
C27
C31
C30
C28
C25
C26
C24
C23
RECORDER
270
270
270
270
270
230
190
70
60
50
40
30
20
10
TIME (MINUTES)

FIGURE 13. A gas chromatographic separation of heptane extracts of chlamydospores of *Puccinia stripiformis*. The separation was carried out on a Perkin-Elmer gas chromatograph equipped with 60 ft. x 0.03 in. stainless steel tubing coated with OV-1. Helium pressure 10 psi. Temperature programed from 150° C to 270° C. No split.

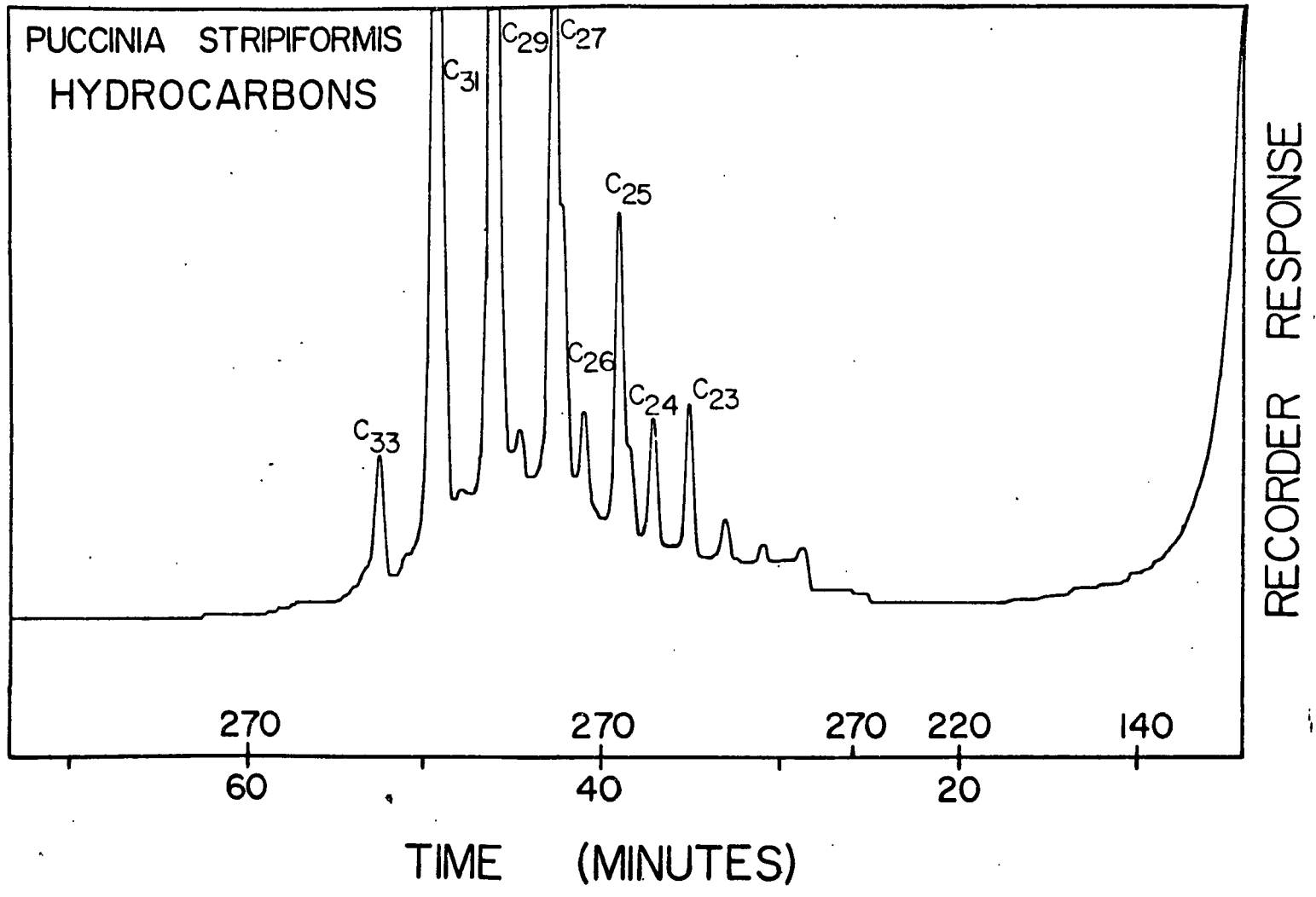

PUCCINIA STRIPIFORMIS
HYDROCARBONS
C33
C31
C29
C27
C26
C25
C24
C23
RECORDER RESPONSE
270
270
270
220
140
60
40
20
TIME (MINUTES)

FIGURE 14. Histograms illustrating the per cent distribution of the heptane fraction (hydrocarbons) of A) *Urocystis agropyri*, B) *Tilletia caries*, C) *Ustilago maydis*, and D) *Sphacelotheca reiliana*. The compounds were separated by gas chromatography, and characterized by mass spectrometry.

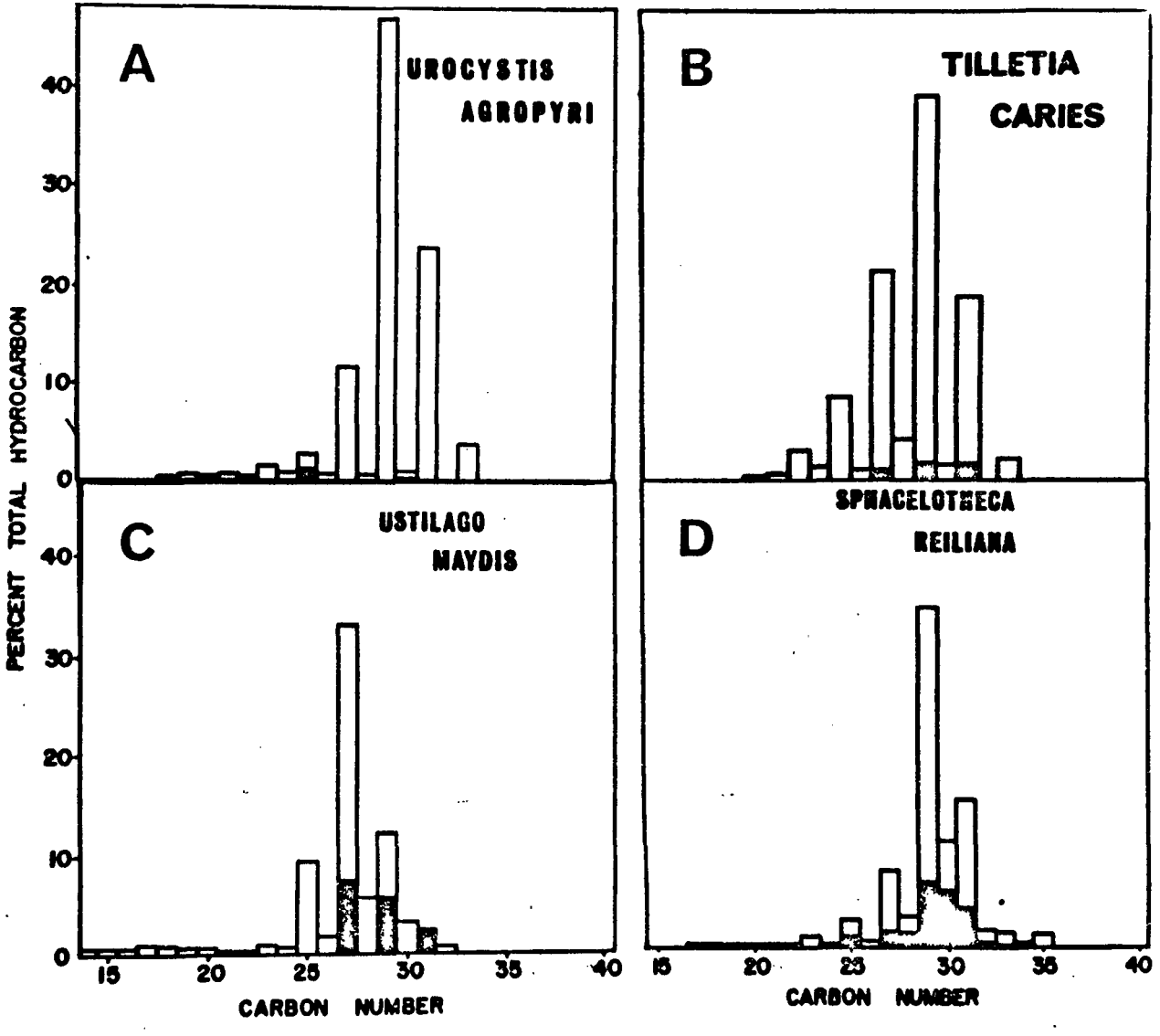

A
UROCYSTIS
AGROPYRI
B
TILLETIA
CARIES
C
USTILAGO
MAYDIS
D
SPHACELOTHECA
REILIANA
PERCENT TOTAL HYDROCARBON
0
10
20
30
40
15
20
25
30
35
40
CARBON NUMBER

TABLE 3. PER CENT DISTRIBUTION OF THE PARAFFINIC HYDROCARBONS EXTRACTED FROM SPORES REPRESENTING FOUR SPECIES AND TWO GENERA OF BASIDIOMYCETES.

FUNGI	HYDROCARBON CHAIN															
	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}	C_{26}	C_{27}	iC_{27}	C_{28}	C_{29}	iC_{29}	C_{30}	C_{31}	iC_{31}	C_{32}	C_{33}
Puccinia graminis	-	-	3.10	7.20	7.30	8.10	17.60	-	7.30	29.40	-	1.30	16.20	-	-	2.00
Puccinia stripiformis	-	-	3.90	3.20	7.90	2.20	20.50	-	2.50	23.90	-	-	23.90	-	-	3.40
Tilletia controversa	0.38	0.15	3.97	0.89	11.41	0.38	24.73	0.76	2.66	25.57	1.90	1.33	21.46	1.71	-	2.66
Tilletia foetida	0.30	0.34	3.21	1.02	11.98	1.28	24.69	0.85	5.66	28.81	1.62	1.97	15.18	1.28	-	1.71

the exception of U. agropyri. In all species, detectable concentrations of stearic acid (C_{18}) were found. The greatest variability between these species was in content and concentrations of unsaturated fatty acids: oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), and linolenic acid ($C_{18:3}$). S. reiliana contained only oleic and linoleic acids in almost equal concentrations (Figure 16). U. maydis contained all three unsaturated acids from oleic acid to linolenic acid (Figure 31). T. caries contained predominantly linoleic acid, with lesser concentrations of both oleic and linolenic acids. U. agropyri contained only oleic and linoleic acids, with oleic acid predominating. Of greater interest in this species was the presence of two additional saponifiable compounds in the C_{20} and C_{22} range (designated Unknown No. 1 and Unknown No. 2 in Table 4). These compounds were not identified because of the lack of suitable standards at the time of analysis. The Unknown No. 1 compound was the predominant component of the methanol fraction of this species (Table 4).

Puccinia stripiformis contained the most unusual free fatty acid distribution of the five species analyzed (Figure 18). The predominant fatty acid is behenic (C_{22}) acid with a 24.8 per cent relative concentration (Table 4). Other major saturated acids in high concentrations are palmitic (C_{16}) and arachidic (C_{20}) acids in relative concentrations of 16.5 and 12.4 per cent respectively. Linolenic acid ($C_{18:3}$) represented the unsaturated acid in greatest concentration but was not a significant constituent (Figure 18). The C_{18} acids, both saturated and unsaturated were in unusually low relative concentrations.

TABLE 4. PER CENT DISTRIBUTION OF THE FREE FATTY ACIDS IN THE METHANOL FRACTION OF LIPID EXTRACTS OF SIX FUNGAL SPECIES.

FUNGI	HYDROCARBON CHAIN LENGTH																
	C_{14}	$C_{14:1}$	C_{15}	C_{16}	$C_{16:1}$	C_{17}	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	C_{20}	C_{21}	C_{22}	$C_{22:1}$	C_{24}	Unk. # 1	Unk. # 2
Sphacelotheca reiliana	2.3	0.6	1.1	25.4	2.8	3.9	0.1	33.9	29.1	0.4	-	-	-	-	-	-	-
Tilletia caries	3.2	1.6	-	26.2	17.9	-	3.9	10.4	34.2	2.7	-	-	-	-	-	-	-
Urocystis agropyri	-	-	-	8.6	-	-	6.3	18.0	7.7	-	-	-	-	-	-	41.4	18.0
Puccinia stripiformis*	2.2	1.9	1.7	16.5	3.1	-	6.2	3.3	2.6	5.7	12.4	2.0	24.8	2.6	7.6	-	-
Ustilago maydis	3.7	13.8	13.9	28.9	3.9	3.9	1.3	12.4	5.7	-	5.8	-	-	-	-	-	-
Puccinia graminis	15.1	-	-	35.8	0.7	-	3.9	35.7	15.9	5.8	-	-	-	-	-	-	-

*Contained low concentrations of C_{10}.

FIGURE 15. A gas chromatographic separation of the free fatty acids extracted from chlamydospores of *Urocystis agropyri*. Separation was accomplished on a Barber Coleman gas chromatograph equipped with 200 ft. x 0.02 in. stainless steel tubing coated with CO-880. The temperature was 200° C isothermal. No split.

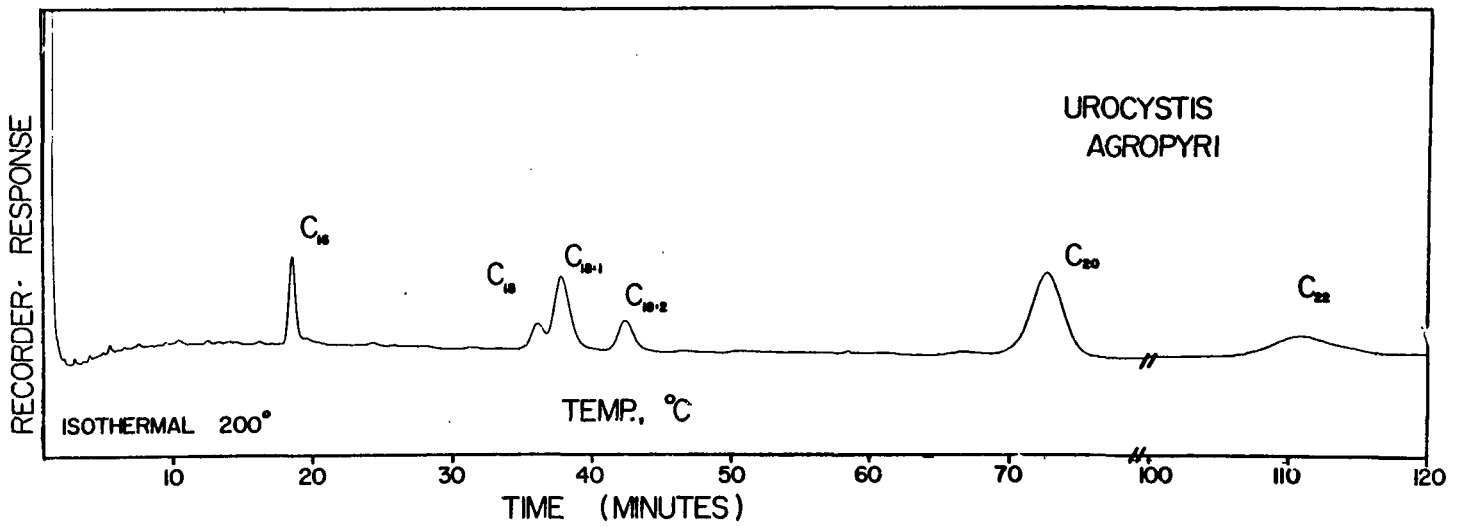

UROCYSTIS
AGROPYRI
C_{16}
C_{18}
$C_{18:1}$
$C_{18:2}$
C_{20}
C_{22}
RECORDER- RESPONSE
ISOTHERMAL 200°
TEMP., °C
10
20
30
40
50
60
70
100
110
120
TIME (MINUTES)

FIGURE 16. A gas chromatogram of the free fatty acids extracted from the chlamydospores of Sphacelotheca reiliana. The separation was carried out on a Barber Coleman gas chromatograph equipped with 200 ft. x 0.02 in. stainless steel tubing coated with CO-880. The temperature was run at 200° C isothermal. No split.

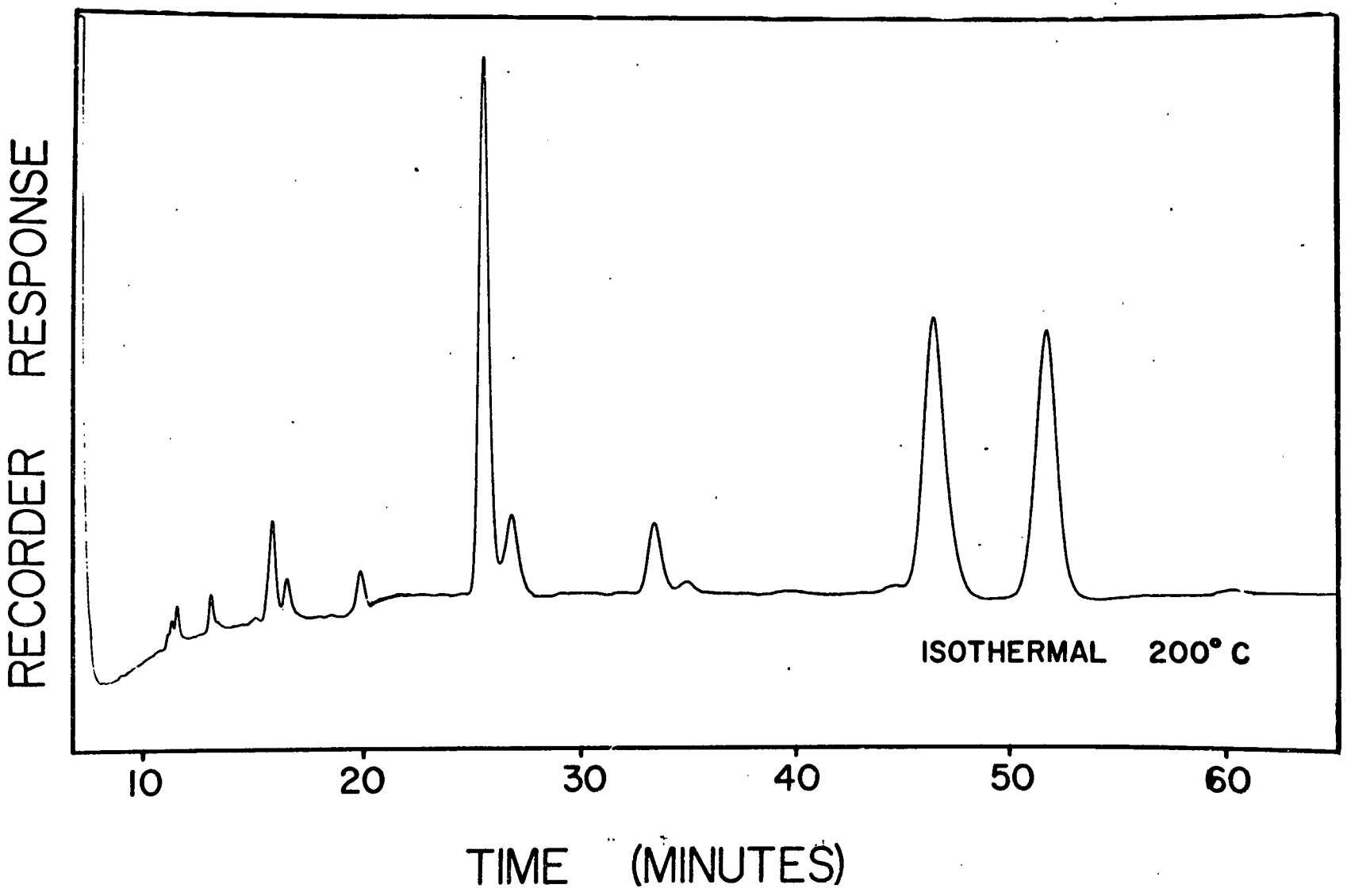

RECORDER RESPONSE
ISOTHERMAL 200° C
10
20
30
40
50
60
TIME (MINUTES)

FIGURE 17. A gas chromatogram of the free fatty acids extracted from uredospores of *Puccinia graminis*. The separation was carried out on a Barber Coleman gas chromatograph equipped with 200 ft. x 0.02 in. stainless steel tubing coated with CO-880. The temperature was run isothermal at 200° C. No split.

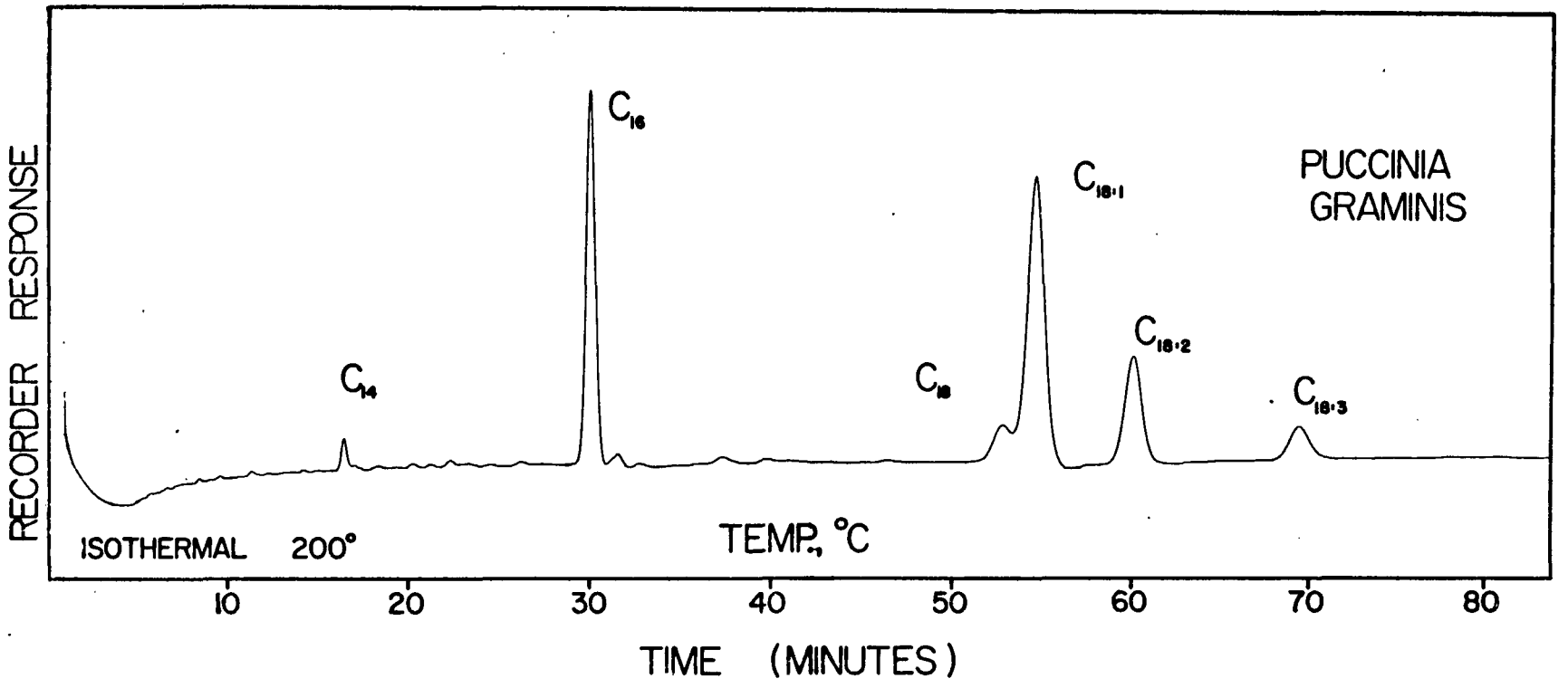
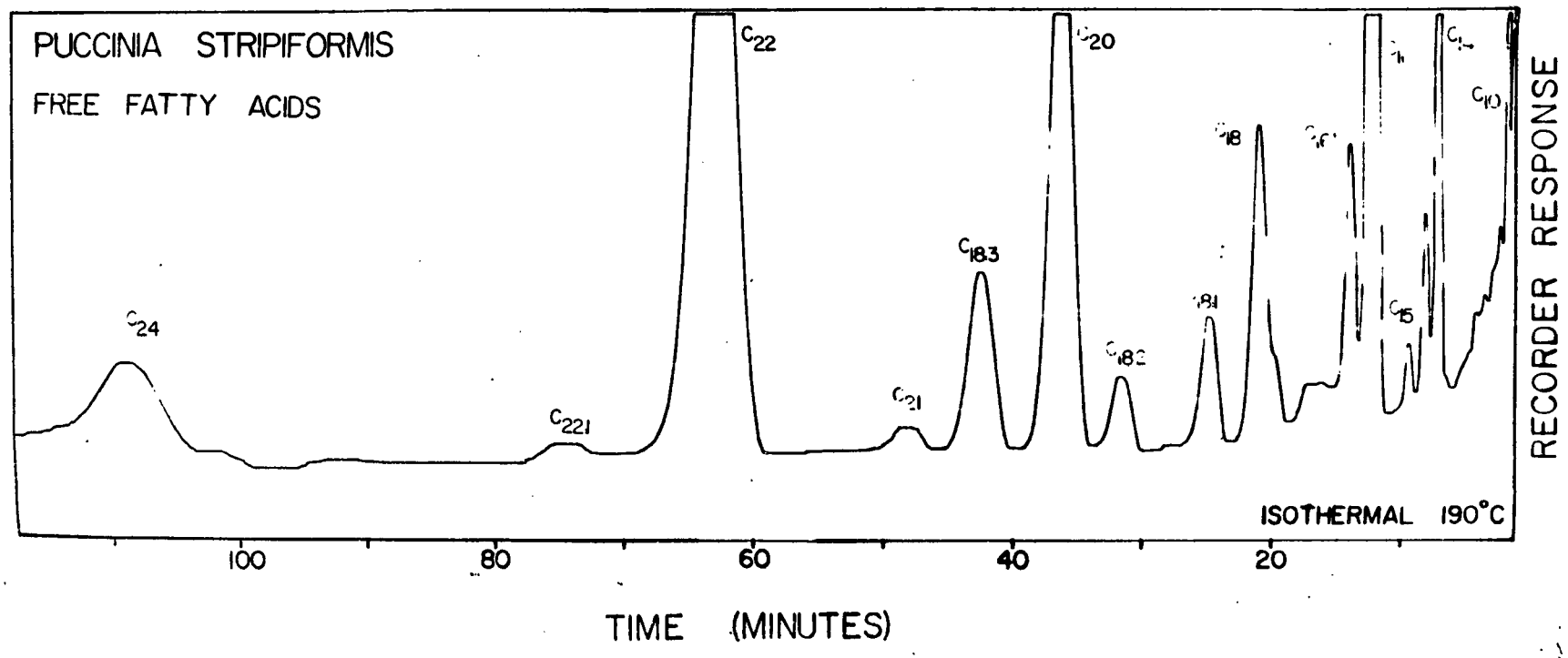

RECORDER RESPONSE
C_{14}
C_{16}
C_{18}
$C_{18:1}$
$C_{18:2}$
$C_{18:3}$
PUCCINIA GRAMINIS
ISOTHERMAL 200°
TEMP., °C
10
20
30
40
50
60
70
80
TIME (MINUTES)

FIGURE 18. A gas chromatographic separation of the free fatty acids from the lipid extracts of *Puccinia stripiformis* uredospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.


PUCCINIA STRIPIFORMIS
FREE FATTY ACIDS
C24
C22:1
C22
C21
C18:3
C20
C18:2
C18:1
C18
C15
C10
ISOTHERMAL 190°C
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

Puccinia graminis contained a very narrow range of free fatty acids. The predominant saturated acid is palmitic (C_{16}) while the major unsaturated acid is oleic ($C_{18:1}$) in concentrations of 35.8 and 35.7 per cent respectively (Table 4). The gas chromatogram is given in Figure 17.

In contrast, alkaline hydrolysates of the total lipid extracts of fungal spores contain a considerably larger range of fatty acids. The longer chain (C_{20}-C_{24}) fatty acids are present where they were not generally detected in the methanol fraction. For example, all species contained lignoceric acid (C_{24}) with the exception of Curvularia sp. The per cent distribution of the total fatty acids for the nine fungal species analyzed is given in Table 5 and 6.

Sporangiospores of R. arrhizus contained oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) for the predominant unsaturated and palmitic (C_{16}), stearic (C_{18}), and arachidic acids (C_{20}) as the major saturated fatty acids. The unsaturated acids are in higher concentration than the saturated acids (Table 7) having a saturated to unsaturated ratio of 0.80 (Table 8). The gas chromatogram is given in Figure 19.

The three Tilletia species had remarkably similar total fatty acid distributions. The hydrolysates contained a single predominant fatty acid, linoleic acid ($C_{18:2}$), in relative concentrations of 44.9, 46.7, and 41.2 for T. caries, T. foetida, and T. controversa respectively. Other major fatty acids include oleic ($C_{18:1}$), nervonic ($C_{24:1}$), and palmitic (C_{16}) acids in similar concentrations. These are the only species that contained detectable concentrations of $C_{24:1}$ (Table 5). The

unsaturated acids were found in higher concentrations than the saturated acids (Table 7). The saturated to unsaturated ratios are 0.29, 0.31, and 0.25 for T. caries, T. foetida, and T. controversa respectively. The gas chromatographic separations of the three species are given in Figures 20, 21, and 22 respectively.

Chlamydospores of S. reiliana contained an unusual distribution of total fatty acids. As with the hydrocarbon components, the total fatty acid pattern (Figure 23) illustrates a simple distribution. The 1:1 ratio of the predominant unsaturated acids, oleic (29.0%) and linoleic (27.5%) acids, was maintained (as compared to the free fatty acids). The saturated acids, palmitic (C_{16}), heptadecanoic (C_{17}), and stearic (C_{18}), were in similar relative concentrations. The total fatty acid preparation revealed the presence of longer chained fatty acids in low concentrations (Table 5). The unsaturated acids are in greater concentrations than the saturated (Table 7) with the saturated to unsaturated ratio being 0.49 (Table 8).

Chlamydospores of U. maydis contain a range of total fatty acids similar to the free acids. The predominant unsaturated components are oleic (23.1%) and linoleic (22.6%) acids whereas the major saturated acids are palmitic (C_{16}) followed by pentadecanoic (C_{15}), heptadecanoic (C_{17}), and stearic (C_{18}) in similar concentrations (Table 6). As before, the longer chain acids (C_{22}-C_{24}) appear in the total fatty acid preparation which is in contrast to the methanol fraction. The unsaturated acids were in greater concentrations than the saturated acids (Table 7). The

saturated to unsaturated ratio is 0.72 (Table 8). The gas chromatogram is given in Figure 25.

Puccinia graminis uredospores contain a similar total fatty acid distribution to the smut and sporangiospores described above. The major difference is the linolenic acid ($C_{18:3}$) content reaching relative concentrations to 21.8 per cent (Table 5). This is followed by oleic acid ($C_{18:1}$) with 15.1 per cent as the other major unsaturated acid (Table 5). The saturated fatty acids in the greatest relative concentrations are stearic (C_{18}) and palmitic (C_{16}). The unsaturated acids are in higher concentrations than the saturated acids (Table 7). The saturated to unsaturated ratio is 0.66 (Table 8).

The total fatty acid content of conidia from two members of the Fungi Imperfecti were compared. The fatty acids of the two species, Aspergillus niger and Curvularia sp., contained considerable variation in their distribution. A. niger contained a relatively simple pattern (Figure 26) containing oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) in concentrations of 35.9 and 29.9 respectively (Table 6). The major saturated acids were palmitic (C_{16}) and stearic (C_{18}). The concentration of linoleic acid ($C_{18:3}$) was considerably low. The long chain fatty acids (C_{20}-C_{24}) were also in low or undetectable concentrations. The unsaturated acids were in greater concentrations than the saturated acids (Table 7) with the saturated to unsaturated ratio being 0.46 (Table 8).

Conidiospores of Curvularia sp. contained a more complex pattern of fatty acids (Figure 27). The conidia of this species contained a large

concentration of total fatty acids per weight extracted (0.5 g) compared to the other fungal species in this study. In Curvularia sp., oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids are in predominant concentrations. The major saturated acid is palmitic (C_{16}) with a concentration of 27.9 per cent (Table 6). The unsaturated acids are in larger concentrations than the saturated acids (Table 7) with the saturated to unsaturated ratio being 0.70 (Table 8). The conidia of this species contained three unknown compounds, two of which are found in several of the other species studied, but the compound designated Unknown No. 1 is unique to Curvularia sp. (Figure 27). Its relative retention time suggests that this compound contains a C_{15} or C_{16} carbon skeleton. Plausible speculations as to the nature of this compound would be a branched or unsaturated C_{15} molecule.

The ultrastructure of the spore surface is shown in Figure 28 A to D. Figure 28 A shows a surface view of U. maydis. The surface is covered with small echinulations, and the surface between the echinulations has a rough appearance. T. caries spore surfaces (Figure 28 B) are covered with small ridges that form a reticulated pattern. The spore surface appears to be smooth, with occasional hair-like projections. Spore surfaces of S. reiliana (Figure 28 C) are similar to U. maydis spore surfaces, although the echinulations of S. reiliana are smaller and more numerous. Figure 28 D shows a cross fracture of U. agropyri spore. The central cell contains the organelles of the spore, with sterile cells surrounding the central cell. A cross-fractured view is shown to illustrate the relationship of the inclusion bodies to the central cell.

The surfaces of these spores are very rough, with prominent projections. These results demonstrate that these four species of smut spores differ markedly in their surface morphology.

The ultrastructure of the external surface of spores after extraction with n-heptane and benzene was examined. The results indicated little change in the external morphology of the spore surface in comparison to the unextracted spores. No photographs of the extracted spores are included in this report because the appearance of the spores is the same as that of the unextracted spores.

TABLE 5. PER CENT DISTRIBUTION OF THE TOTAL FATTY ACIDS OF HYDROLYZED LIPID EXTRACTS FROM THE SPORES OF FIVE FUNGAL SPECIES.

HYDROCARBON CHAIN LENGTH	FUNGI				
	P. graminis	*T. caries*+	*T. foetida*+	*T. controversa*	*S. reiliana*
C_{13}	-	-	-	-	-
C_{14}	2.3	0.3	0.9	0.4	-
$C_{14:1}$	-	-	-	-	-
C_{15}	0.8	0.3	-	0.2	-
C_{16}	9.4	13.3	15.0	11.1	5.9
$C_{16:1}$	0.5	2.5	2.6	1.9	1.8
C_{17}	-	0.4	0.3	0.3	8.0
C_{18}	12.2	1.3	1.7	0.5	8.0
$C_{18:1}$	15.1	12.9	12.3	15.8	29.0
$C_{18:2}$	5.8	44.9	46.7	41.2	27.5
$C_{18:3}$	21.8	1.5	1.8	1.2	2.4
C_{20}	-	0.8	1.1	0.7	0.9
$C_{20:1}$	-	1.5	1.8	1.5	-
C_{21}	-	1.4	0.8	1.9	-
C_{22}	6.7	1.6	1.7	1.7	2.4
$C_{22:1}$	6.3	1.0	0.8	3.1	-
C_{24}	1.5	2.3	2.4	2.2	4.6
$C_{24:1}$	-	8.7	9.0	8.5	-
Unk.	*	*	*	*	*

+Contained slow concentrations of C_{10} and $C_{10:1}$.

*Each species contains unidentified components. These are normally in low insignificant concentrations but in significant instances are described in the text.

TABLE 6. PER CENT DISTRIBUTION OF THE TOTAL FATTY ACIDS OF HYDROLYZED LIPID EXTRACTS FROM THE SPORES OF FOUR FUNGAL SPECIES.

HYDROCARBON CHAIN LENGTH	FUNGI			
	U. maydis	*Curvularia sp.*	*A. niger*	*R. arrhizus*
C_{13}	-	0.6	-	-
C_{14}	1.5	0.5	1.9	1.1
$C_{14:1}$	1.2	1.4	-	-
C_{15}	4.1	0.6	1.1	0.8
C_{16}	15.0	27.9	13.3	16.8
$C_{16:1}$	1.9	3.3	1.3	1.7
C_{17}	6.7	-	0.9	-
C_{18}	6.4	9.4	13.4	19.9
$C_{18:1}$	23.1	21.7	35.9	42.4
$C_{18:2}$	22.6	27.8	29.9	7.7
$C_{18:3}$	3.5	1.8	0.6	0.1
C_{20}	2.8	0.7	0.8	6.9
$C_{20:1}$	-	-	-	-
C_{21}	-	-	-	-
C_{22}	1.5	tr	-	0.3
$C_{22:1}$	tr	-	-	-
C_{24}	tr	-	tr	tr
$C_{24:1}$	-	-	-	-
Unk.	*	*	*	*

+Contained slow concentrations of C_{10} and $C_{10:1}$.

*Each species contains unidentified components. These are normally in low insignificant concentrations but in significant instances are described in the text.

TABLE 7. RELATIVE DISTRIBUTION OF TOTAL SATURATED AND UNSATURATED FATTY ACIDS OF NINE FUNGAL SPECIES.

CARBON CHAIN	FUNGAL ORGANISM*							
	R. arrhizus	*P. graminis*	*T. caries*	*T. foetida*	*T. controversa*	*U. maydis*	*Curvularia sp.*	*A. niger*
Saturated	43.9	32.9	21.7	23.9	19.0	38.0	39.7	31.4
Unsaturated	51.9	49.5	73.0	75.0	73.2	52.3	56.0	67.7

*These values do not include the unknown compounds of each species.

TABLE 8. RATIO OF SATURATED TO UNSATURATED TOTAL FATTY ACIDS OF THE SPORES OF THE NINE FUNGAL SPECIES.

FUNGI	RATIO (Saturated/Unsaturated)
Rhizopus arrhizus	0.80
Puccinia graminis	0.66
Tilletia caries	0.29
Tilletia foetida	0.31
Tilletia controversa	0.25
Sphacelotheca reiliana	0.49
Ustilago maydis	0.72
Curvularia sp.	0.70
Aspergillus niger	0.46

FIGURE 19. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of Rhizopus arrhizus sporangiospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with helium pressure at 44 lbs. No split.

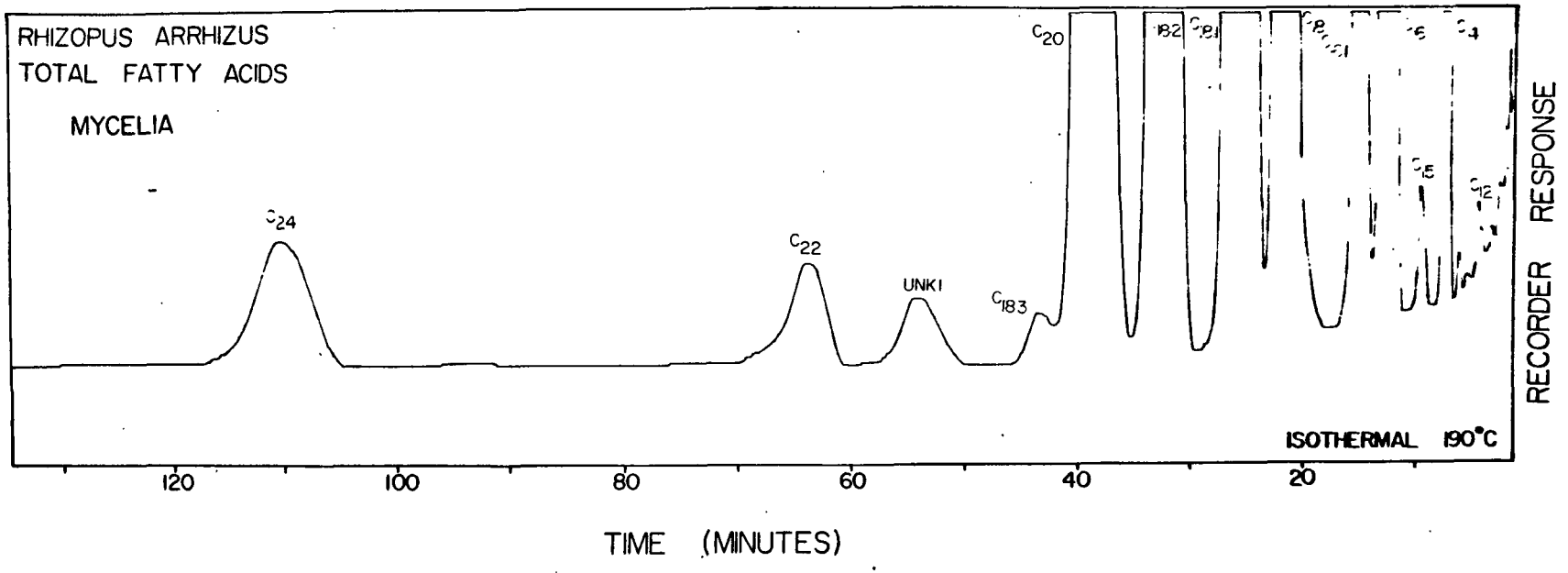

RHIZOPUS ARRHIZUS
TOTAL FATTY ACIDS
MYCELIA
C24
C22
UNK1
C183
C20
C182
C181
ISOTHERMAL 190°C
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 20. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of Tilletia caries chlamydospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.

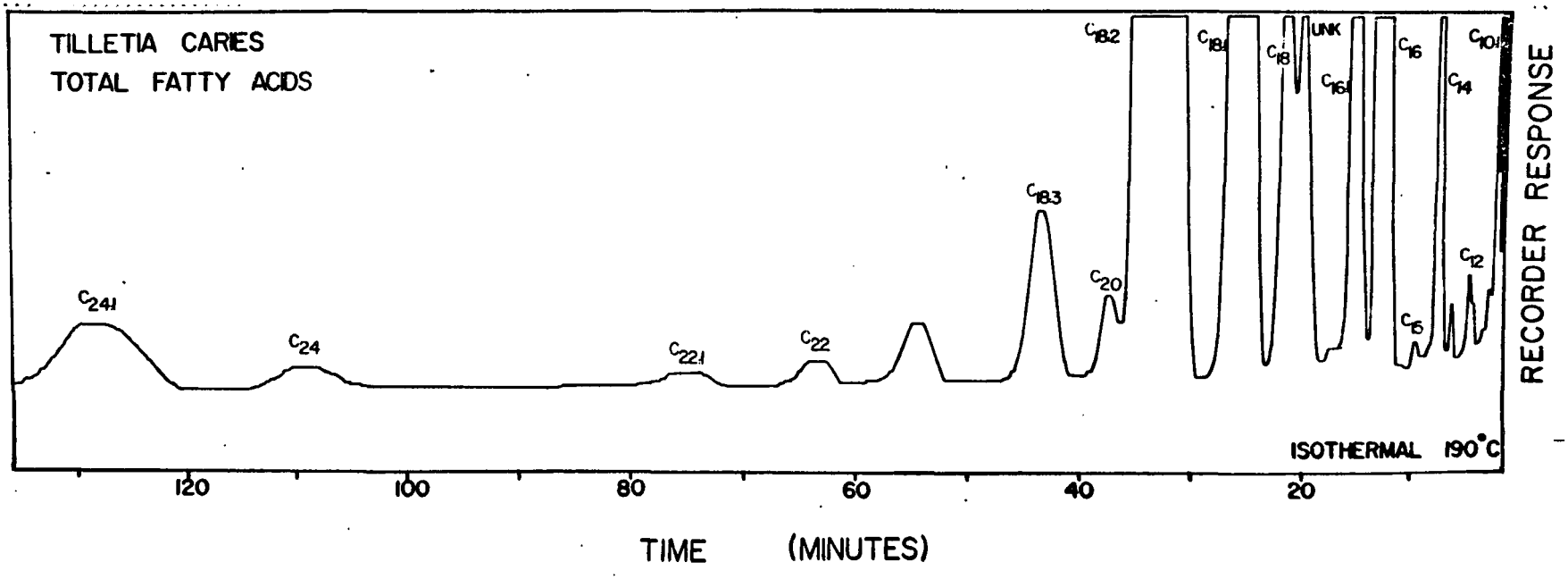

TILLETIA CARIES
TOTAL FATTY ACIDS
C24:1
C24
C22:1
C22
C18:3
C20
C18:2
C18:1
C18
UNK
C16:1
C16
C15
C14
C12
C10:1
ISOTHERMAL 190°C
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 21. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of Tilletia foetida chlamydospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.

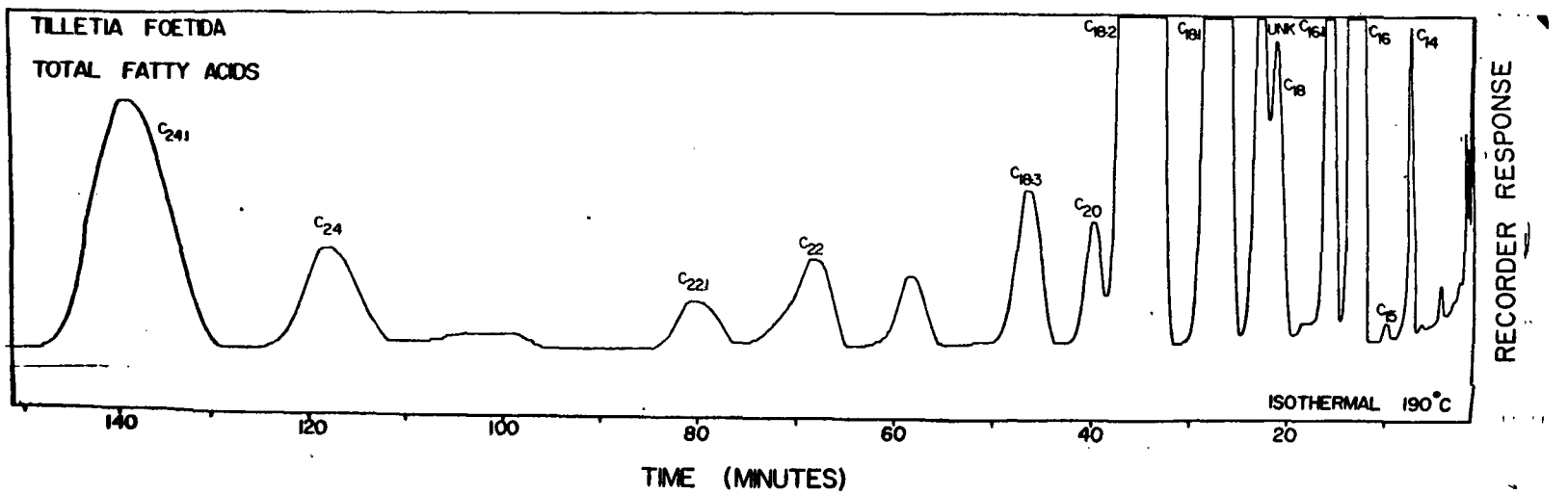

TILLETIA FOETIDA
TOTAL FATTY ACIDS
C24:1
C24
C22:1
C22
C18:3
C20
C18:2
C18:1
UNK
C16:1
C18
C16
C15
C14
ISOTHERMAL 190°C
140
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 22. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of Tilletia controversa chlamydospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium at 44 lbs. No split.

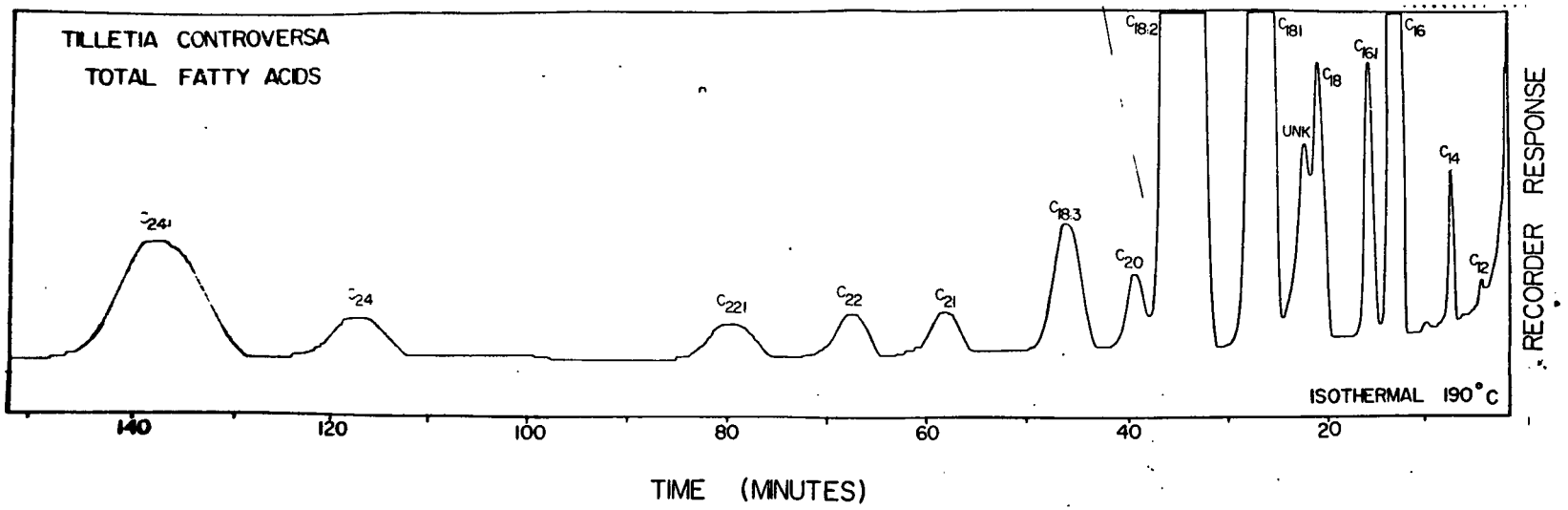

TILLETIA CONTROVERSA
TOTAL FATTY ACIDS
C24:
C24
C221
C22
C21
C18:3
C20
C18:2
C181
UNK
C18
C161
C16
C14
C12
ISOTHERMAL 190°C
140
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 23. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of Sphacelotheca reiliana chlamydospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.

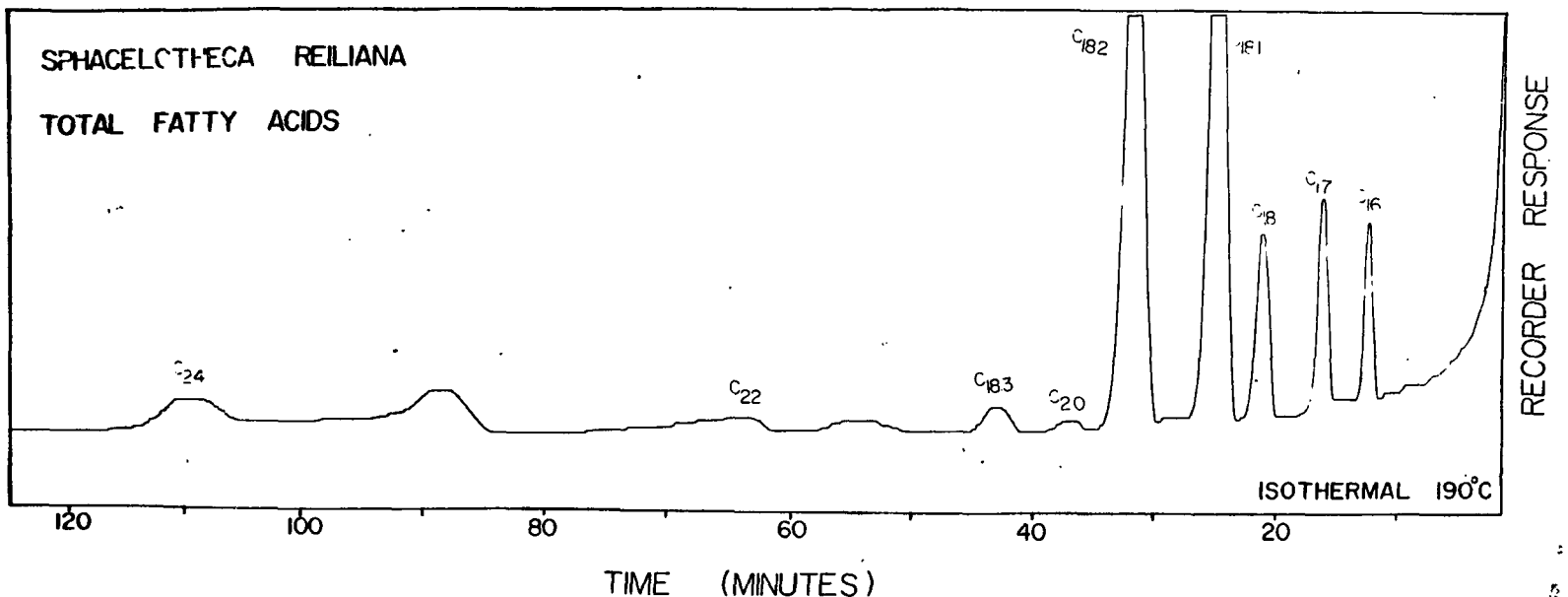

SPHACELOTHECA REILIANA
TOTAL FATTY ACIDS
C24
C22
C18:3
C20
C18:2
C18:1
C18
C17
C16
ISOTHERMAL 190°C
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 24. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of *Puccinia graminis* uredospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.

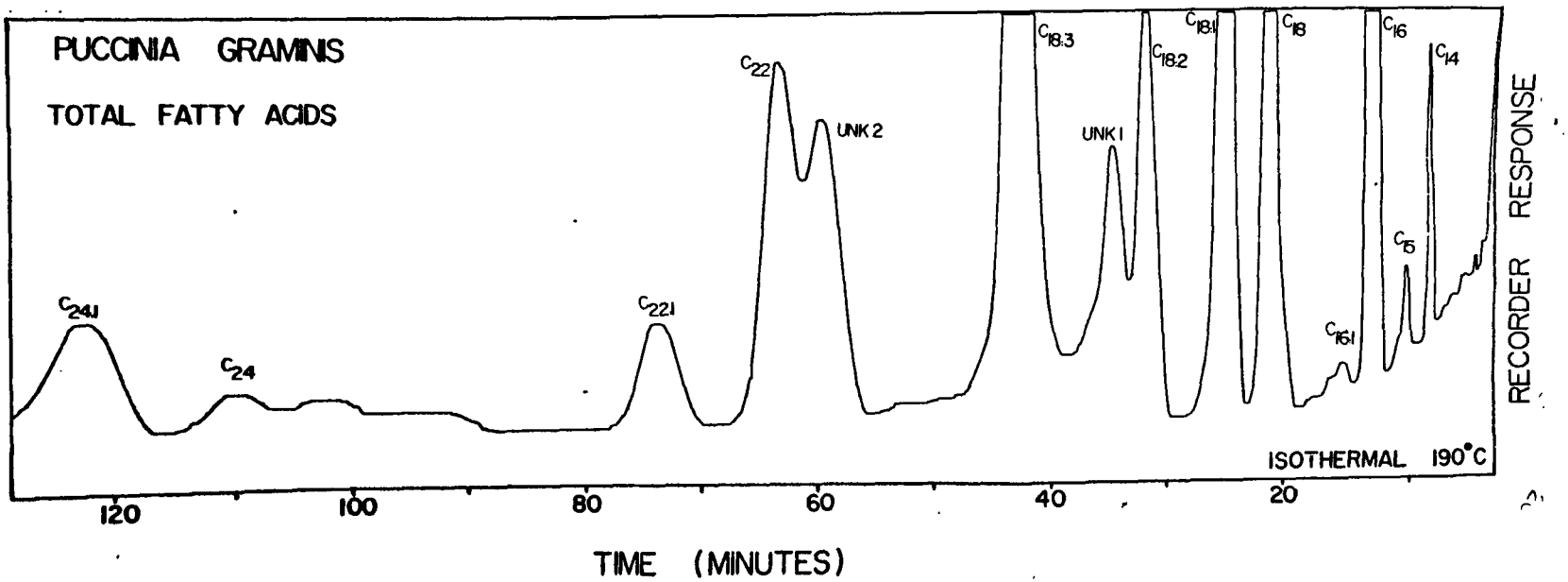

PUCCINIA GRAMINIS
TOTAL FATTY ACIDS
C24:1
C24
C22:1
C22
UNK 2
C18:3
UNK 1
C18:2
C18:1
C18
C16:1
C16
C15
C14
ISOTHERMAL 190°C
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 25. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of Ustilago maydis chlamydospores. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.

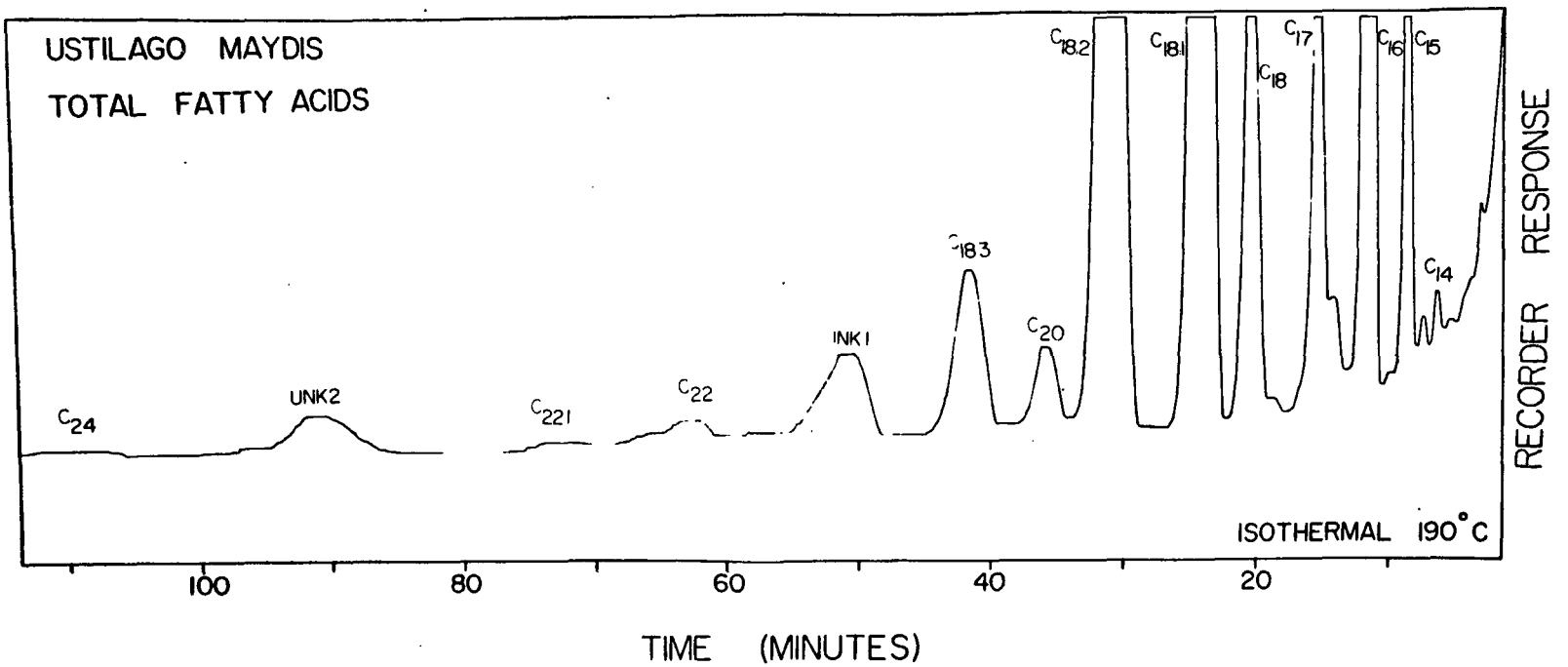

USTILAGO MAYDIS
TOTAL FATTY ACIDS
C24
UNK2
C221
C22
INK1
C183
C20
C182
C181
C18
C17
C16
C15
C14
ISOTHERMAL 190°C
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 26. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of *Aspergillus niger* conidium. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.

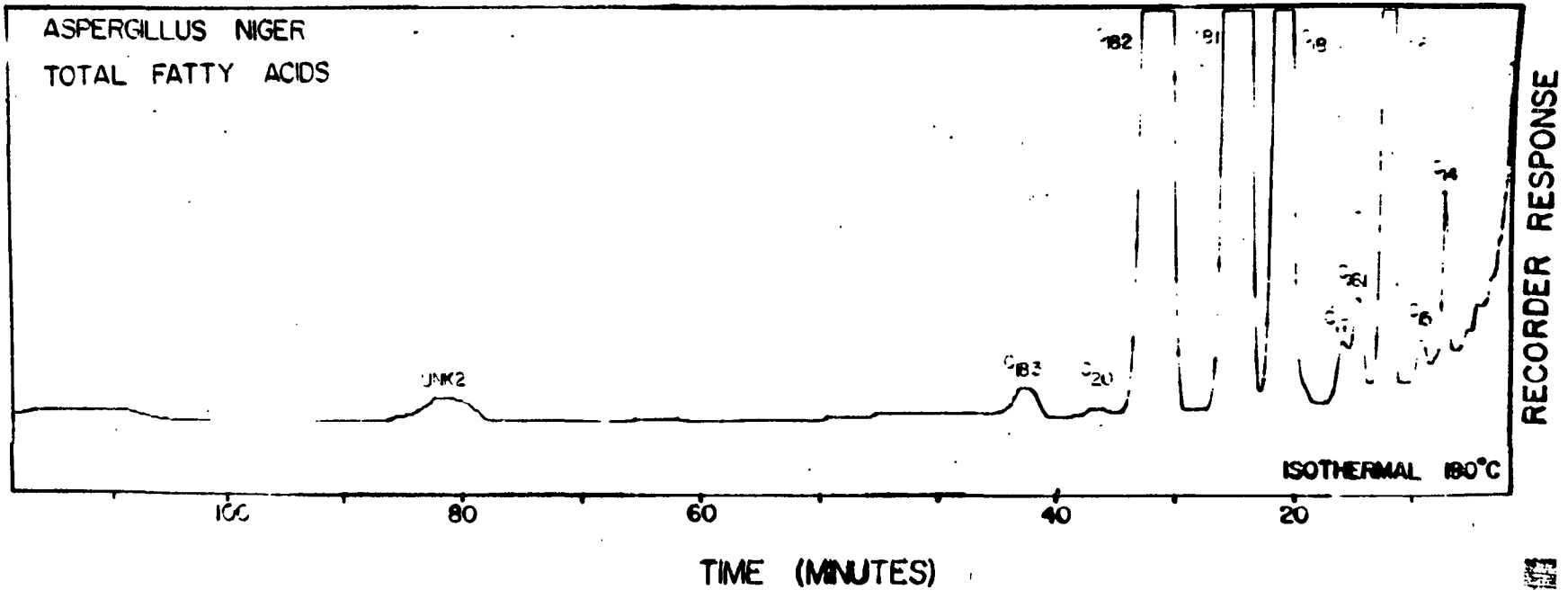

ASPERGILLUS NIGER
TOTAL FATTY ACIDS
UNK2
C18:3
C20
C18:2
C18:1
C18
C16:1
C16
C14
ISOTHERMAL 180°C
RECORDER RESPONSE
100
80
60
40
20
TIME (MINUTES)

FIGURE 27. A gas chromatogram illustrating the separation of total fatty acids obtained from alkaline hydrolysis of the total lipid extracts of *Curvularia sp.* conidium. Using a Perkin-Elmer gas chromatograph, separation was accomplished with a 10 ft. x 1/8 in. stainless steel column packed with diethylene glycol succinate coated on Chromosorb W. The temperature was isothermal at 190° C with Helium pressure at 44 lbs. No split.

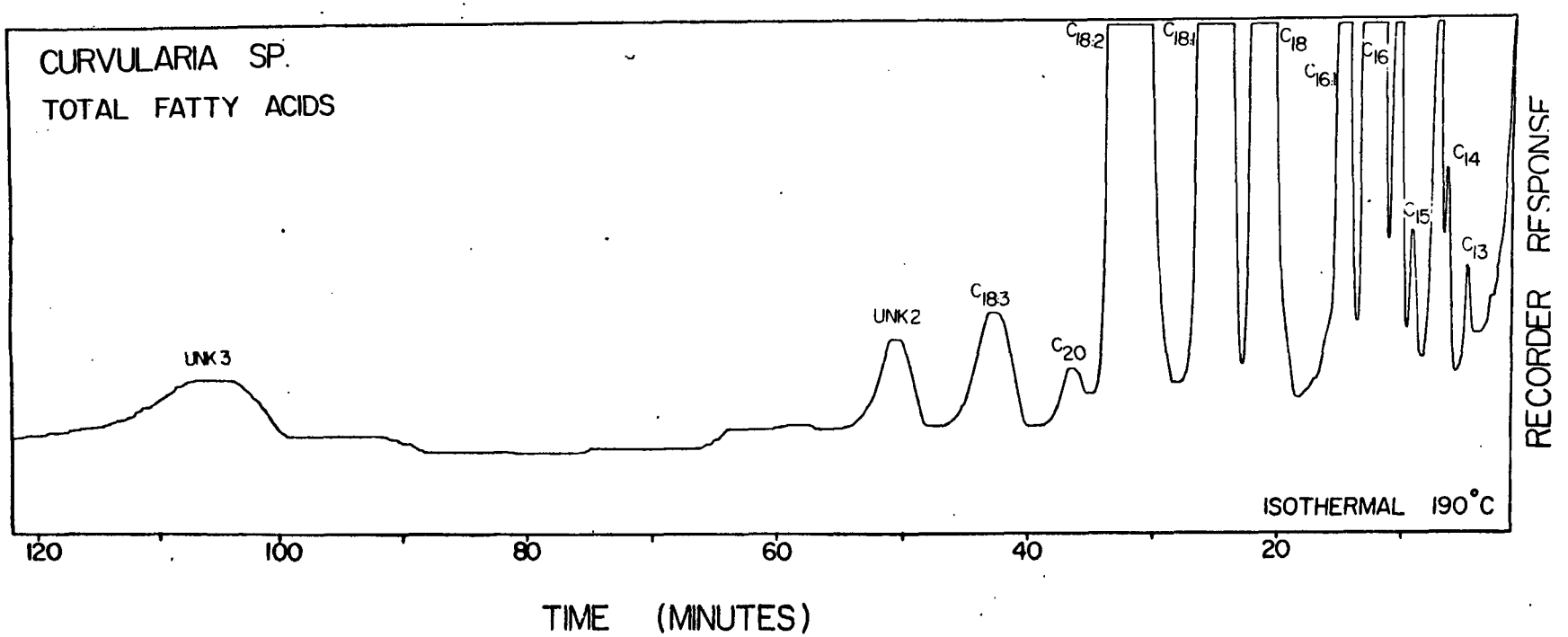
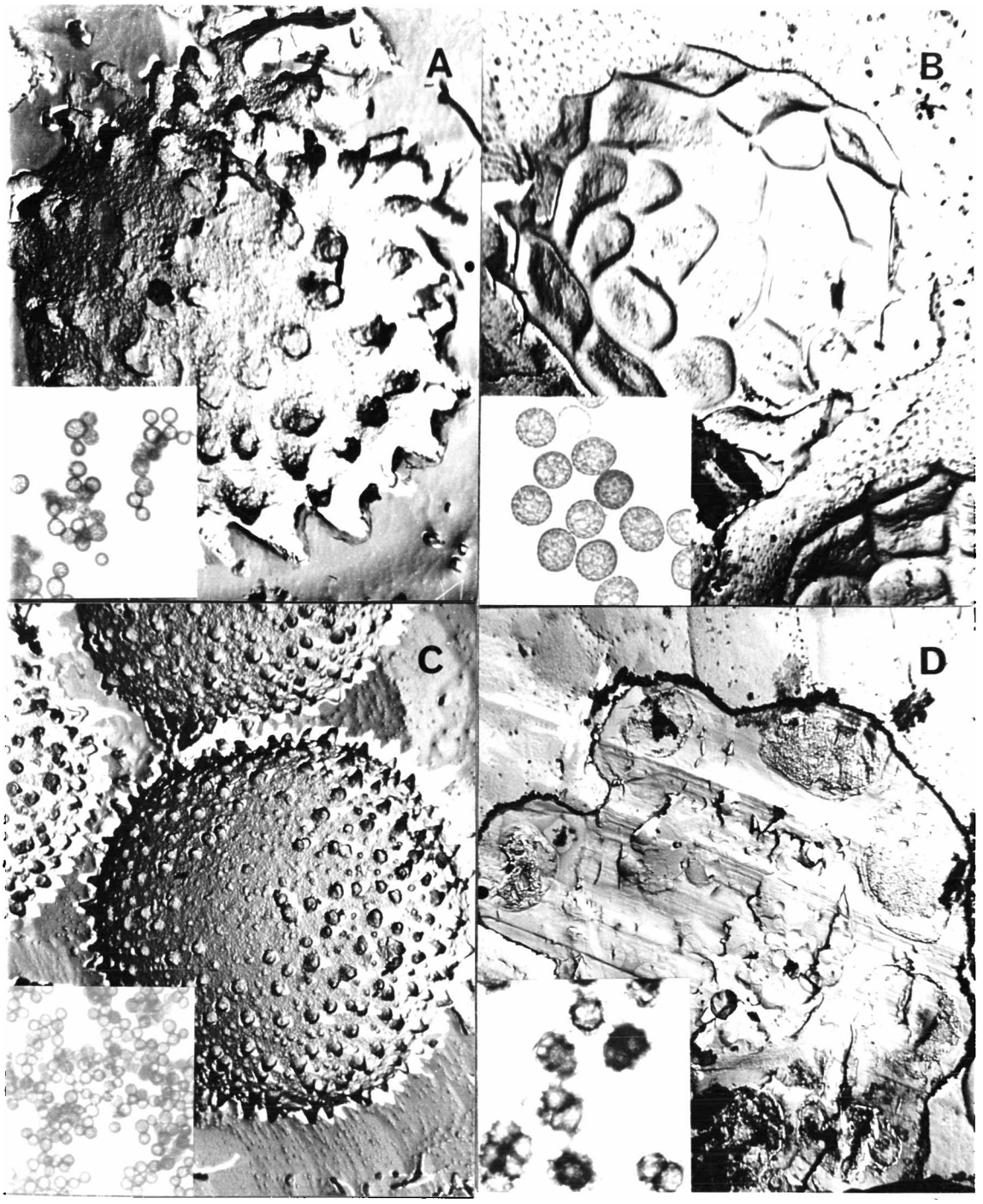

CURVULARIA SP.
TOTAL FATTY ACIDS
UNK 3
UNK 2
C18:3
C20
C18:2
C18:1
C18
C16:1
C16
C15
C14
C13
ISOTHERMAL 190°C
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 28. Electron photomicrographs illustrating the surface structure chlamydospores prepared by the freeze-etch method. A) *Ustilago maydis* chlamydospore x 17,900; B) *Tilletia caries* spore surface x 7,800; C) *Sphacelotheca reiliana* spore surface x 17,900; and D) cross-section of *Urocystis agropyri* spore x 6,700. All small inserts represent spores photographed at the light microscope level (x 2,800). (Electron microphotographs compliments of Dr. Bill Hess.)


A
B
C
D

DISCUSSION

The hydrocarbon analyses of Sphacelotheca reiliana, Ustilago maydis, Urocystis agropyri, Tilletia caries, Tilletia foetida, Tilletia controversa, Puccinia graminis, Puccinia stripiformis, and Rhizopus arrhizus indicate the following: rather complex patterns of n-alkanes and branch-chained alkanes can be detected in the wax fraction of most fungal spores. These compounds have been characterized by mass spectrometry, and their structures are known. Just how useful hydrocarbon patterns will be as a taxonomic tool will depend upon the consistency of the patterns from a large number of samples. In the present study, the chromatograms were distinct and seemed to fingerprint each species. The hydrocarbon patterns of the lipid fraction of fungal spores may be more consistent than those obtained by Eglinton (15,16) in higher plants.

The hydrocarbons in higher plants (15,16) are n-alkanes with predominantly odd chain lengths ranging from C_{21} to C_{35}, whereas bacteria (52) contain paraffinic hydrocarbons with generally shorter chain lengths (C_9 to C_{19}). From a comparative point of view, the spores of the higher fungi having n-alkanes ranging from C_{17} to C_{33} were more like higher plants than bacteria. The significance of the branched-chain alkanes in certain fungal spores is not clear at this time.

It is interesting to note that with only one exception, R. arrhizus, all species contained similar hydrocarbon distributions and all belong to the class Basidiomycetes. Rhizopus arrhizus, a Phycomycete, contained

essentially no hydrocarbons when a comparable quantity of spores was extracted. It would be interesting to know if these results are universal throughout the Phycomycete class. Evolution of the capacity for alkane synthesis throughout the fungal division could have phylogenetic significance. However, it should be kept in mind that in all cases the basidiomycetous fungi analyzed in this study were obtained from their natural host whereas Rhizopus arrhizus was obtained from synthetic culture medium. The exact interrelationships in lipid metabolism of host-parasite associations are unknown.

The fatty acids common to fungal spores do not differ greatly from those of other organisms. It has been suggested (64) that there is a turnover of these compounds, yet a consistant pattern appears to be maintained. The saturated fatty acids appear to be the same throughout the species studied with palmitic being the major acid in most species. The differences in unsaturated acids are both qualitative and quantitative. The greatest variation in free fatty acids of fungal species lies in the unsaturated acids with oleic generally the predominant component. It is interesting to note the differences in the free and total fatty acid distributions.

The total lipid hydrolysates contain the total fatty acids whereas the methanol fraction contains the unbound acids that are actively involved in metabolic processes. In general, the species analyzed in this study contained many similarities yet each contained small relative quantitative differences. The closely related Tilletia species contained remarkable similarity in the relative distributions of their total fatty

acids. The comparative aspects of the free and total fatty acids of the Tilletia species is discussed in more detail in Section III of this dissertation.

Generally, the longer chain fatty acids are found in the total hydrolysates indicating a preference of incorporation into the complex neutral and polar lipids. It is found that in each species the saturated to unsaturated ratio is less than one, showing a predominance of the unsaturated. This is true in spite of the fact that linolenic acid is less than 4 per cent of the total fatty acid fraction in all the species with the exception of P. graminis. The significance of this one sided distribution of mono- and dienoic:trienoic acids is uncertain.

The total fatty acid analyses reported here are consistant with rust and smut spore analyses previously reported (68, 69, 70). For example, the total fatty acid distribution of P. graminis was qualitatively and quantitatively similar to that previously reported (68). Relative concentrations of the fatty acids reported were very similar with the exception of that for palmitic acid. Their value was 41.3 per cent in contrast to 9.4 for C_{16} reported here. They also report the presence of cis-9,10-epoxyoctadecanoic acid in relative concentrations to 19 per cent of the total fatty acids. The results reported here show two unidentified compounds, one (Unknown No. 1) of which has a retention time similar to that of stearic acid. This suggests a close affinity to cis-9,10-epoxyoctadecanoic acid or a breakdown product, but it cannot be identified without suitable standards or mass spectrometry. The other (Unknown No. 2) compound has a similar retention time to behenic acid. The rel-

ative concentrations of either unknown does not reach that for the previously reported epoxy acid. In addition, lignoceric acid (C_{24}), previously unreported in P. graminis is shown to be present in low relative concentrations.

The relative per cent values reported here for the Tilletia species and Ustilago maydis conform closely to those previously published for species of the same genera. For example, T. caries, T. foetida, and T. controversa containing exceptionally high concentrations of linoleic acid is consistant with that reported for T. foetens (68). Also, lignoceric (C_{24}) and nervonic ($C_{24:1}$) acids are reported for the first time in Tilletia species (the identification of $C_{24:1}$ is tentative). The values reported for U. zeae, U. tritici, U. nigra, and U. levis are very close to those reported here for U. maydis. The former species generally contain a 2:1 ratio for oleic:linoleic acids whereas a 1:1 ratio is illustrated here for the same acids in U. maydis (68).

The hydrocarbon fingerprints of fungal spores offer greater possibilities as a taxonomic characteristic than the fatty acid patterns, although unusual fatty acids may be characteristic of the taxa. From these results it is evident that the spore surfaces of the four species are very distinctive. From a taxonomic point of view, it would appear that the use of the freeze-etch method would provide a means to delimit species.

The electron microscopy of the external surfaces of spores revealed the complexity of the spore walls. Of particular interest are the two wall layers of T. caries which are discussed in more detail in Section III. The chlamydospores of U. agropyri have been described as having an

incomplete investment of the spore ball by sterile cells (13). Electron microscopy reveals that the sterile cells are granular regions embedded in a spore wall of uneven thickness. The granular texture, which is in contrast to the smooth spore wall, is similar to the wall layer referred to as W_1 in Tilletia controversa (Section III). Exactly what role, if any, these granular regions play in spore survival is uncertain.

The comparison of the normal and extracted spores indicate that there is no surface wax layer analogous to that of higher plants and lipids are not structural components of the spore wall. It appears that more refined methods such as radioautography in combination with electron microscopy will be necessary to determine the location of extractable lipids of fungal spores.

XI.

SECTION II

ALKANES, FATTY ACID METHYL ESTERS, AND FREE FATTY ACIDS IN SURFACE WAX OF *USTILAGO MAYDIS*

INTRODUCTION

The data presented here describes the alkane and fatty acid distribution in *Ustilago maydis* chlamydospores. Of primary importance is the characterization of natural methyl esters of long chain fatty acids for the first time as a fungal product. Natural methyl esters of this type have been reported on one previous occasion in the pollen of corn.

RESULTS

The heptane fraction was found to contain, as determined by gas chromatographic retention times, both normal and branched-chain alkanes as shown in Figure 29. The major alkane components were C_{25}, C_{27}, and C_{29}. Heptacosane was found in the largest concentrations of chlamydospores from two different sources. The histogram in Figure 30 illustrates the consistancy of the hydrocarbon distribution of spores harvested from two different geographical locations.

The benzene fraction contained predominantly natural methyl esters of long chain fatty acids shown in Figure 31 with chain lengths ranging from C_{12} to C_{20}.

The methanol fraction, following treatment producing methyl esters of fatty acids, were characterized, as well as the paraffinic hydrocarbons and natural methyl esters, by gas chromatography-mass spectrometer combination. The predominant saturated fatty acids were pentadecanoic and palmitic acids, whereas the predominant unsaturated fatty acids were myristoleic and oleic acids (Figure 31). The results are similar to those previously reported by Tulloch and associates (68, 69, 70). Table 9 represents a comparison between the free fatty acids and the corresponding free ester by carbon chain number.

FIGURE 29. Gas chromatographic separation of the n-alkanes extracted from chlamydospores of *Ustilago maydis*. Perkin-Elmer gas chromatograph equipped with a 50 ft. x 0.03 in. capillary column coated with 2% SE-30 (high temperature silicone grease from Applied Science Laboratories, State College, Penn.). Helium pressure 8 psi, temperature program 150° C. to 270° C., no split.

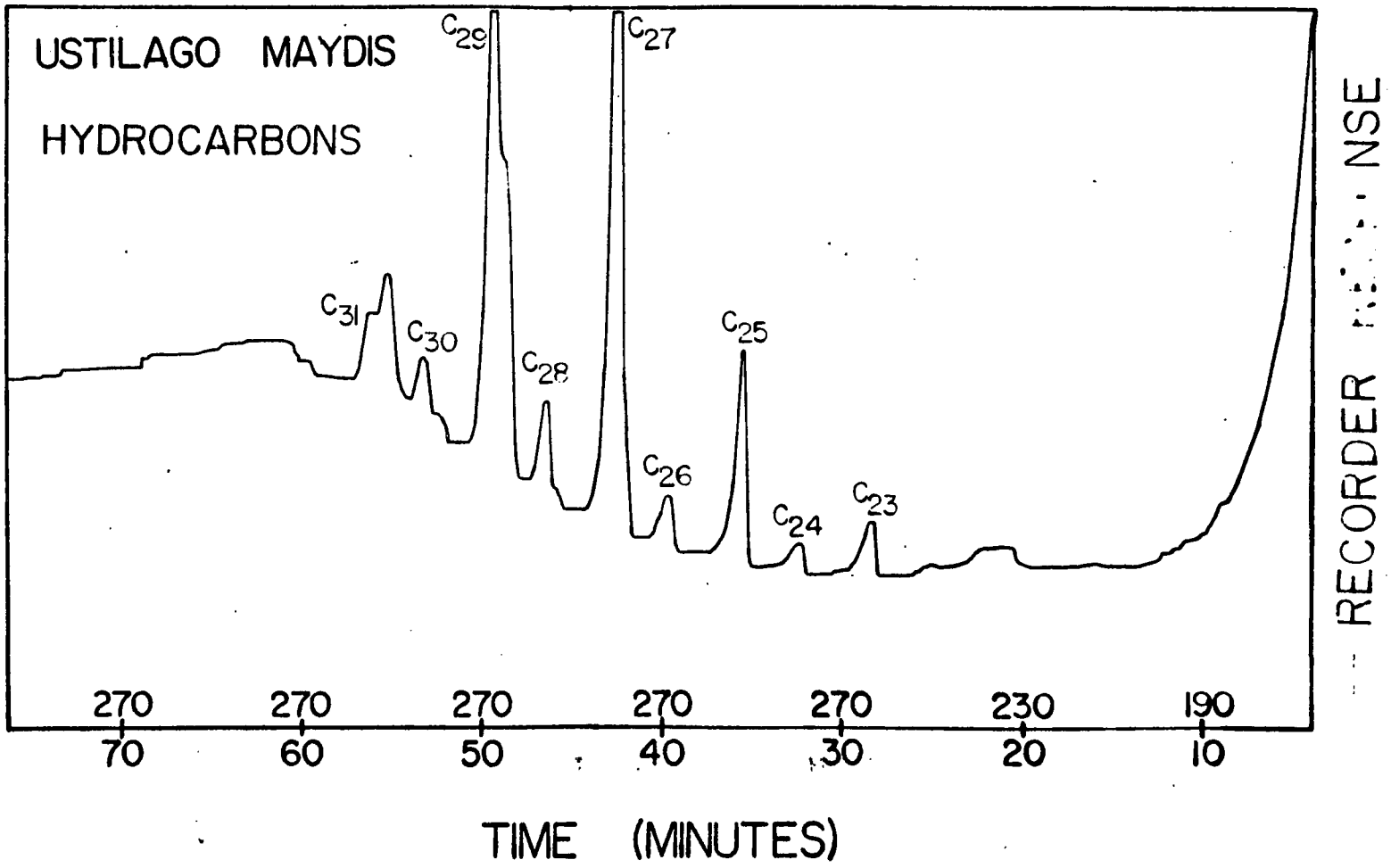

USTILAGO MAYDIS
HYDROCARBONS
C29
C27
C31
C30
C28
C25
C26
C24
C23
270
270
270
270
270
230
190
70
60
50
40
30
20
10
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 30. The carbon number distribution of normal (open bars) and branched-chain (solid bars) alkanes in *U. maydis* spores from two sources as per cent total Hydrocarbons extracted. (A from California, collected in 1965 season; B from Illinois, collected in 1966 season.)

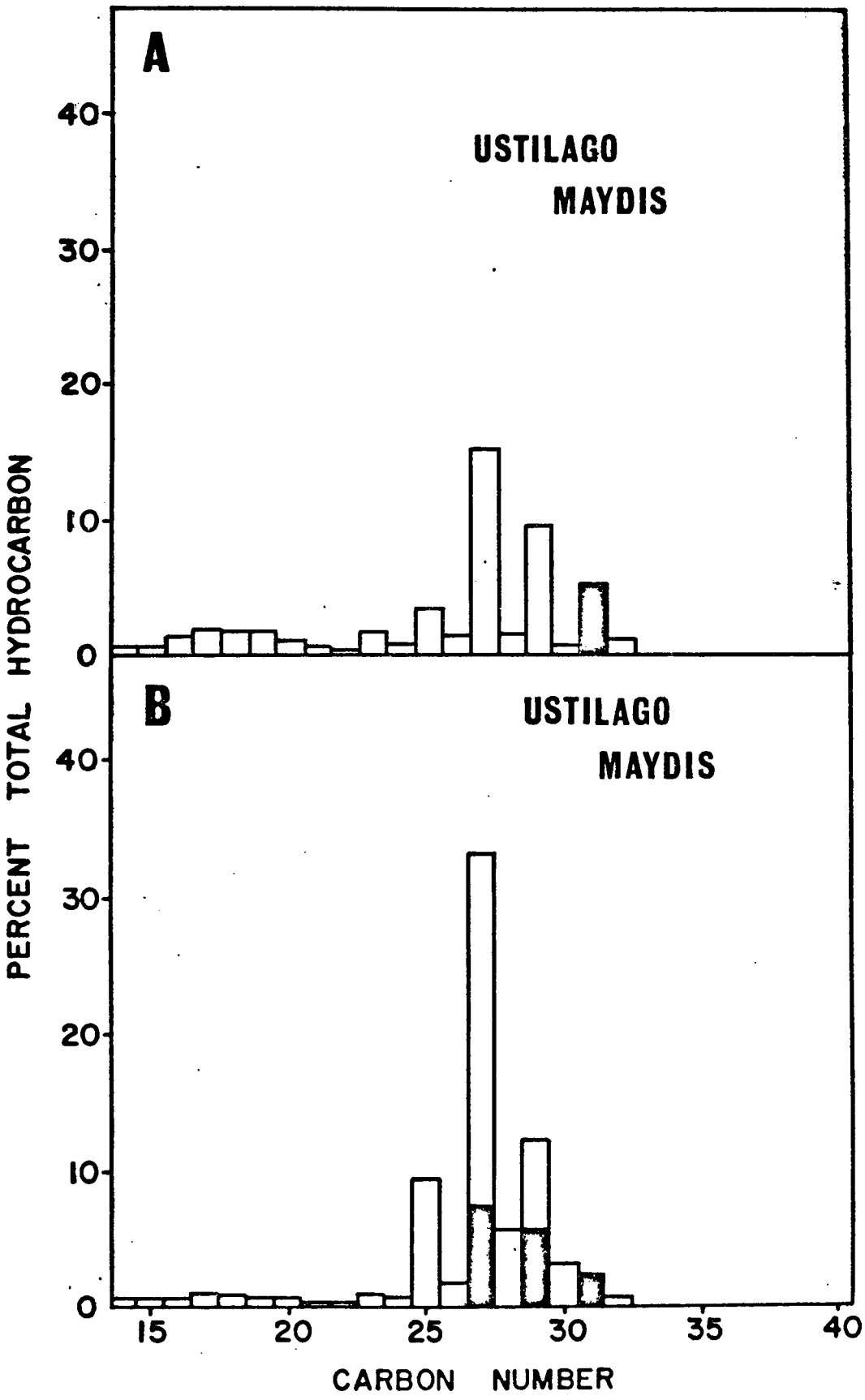

A
USTILAGO
MAYDIS
B
USTILAGO
MAYDIS
PERCENT TOTAL HYDROCARBON
0
10
20
30
40
15
20
25
30
35
40
CARBON NUMBER

FIGURE 31. (A) Gas chromatographic separation of methylated free fatty acids of U. maydis spores on stainless steel tubing (200 m x 0.076 cm) coated with Igepal CO-880. Nitrogen pressure, 2196 g/cm^2; no split. Barber Colman 5000 instrument equipped with a flame ionization detector. Range, 1; attenuation, 10. Temperature held isothermal at 200°. From 2.77 g of spores extracted, 1/40 of the sample injected. (B) Gas chromatographic separation of natural methyl esters of fatty acids of U. maydis spores separated under the same conditions with approximately 1/50 of the sample injected.

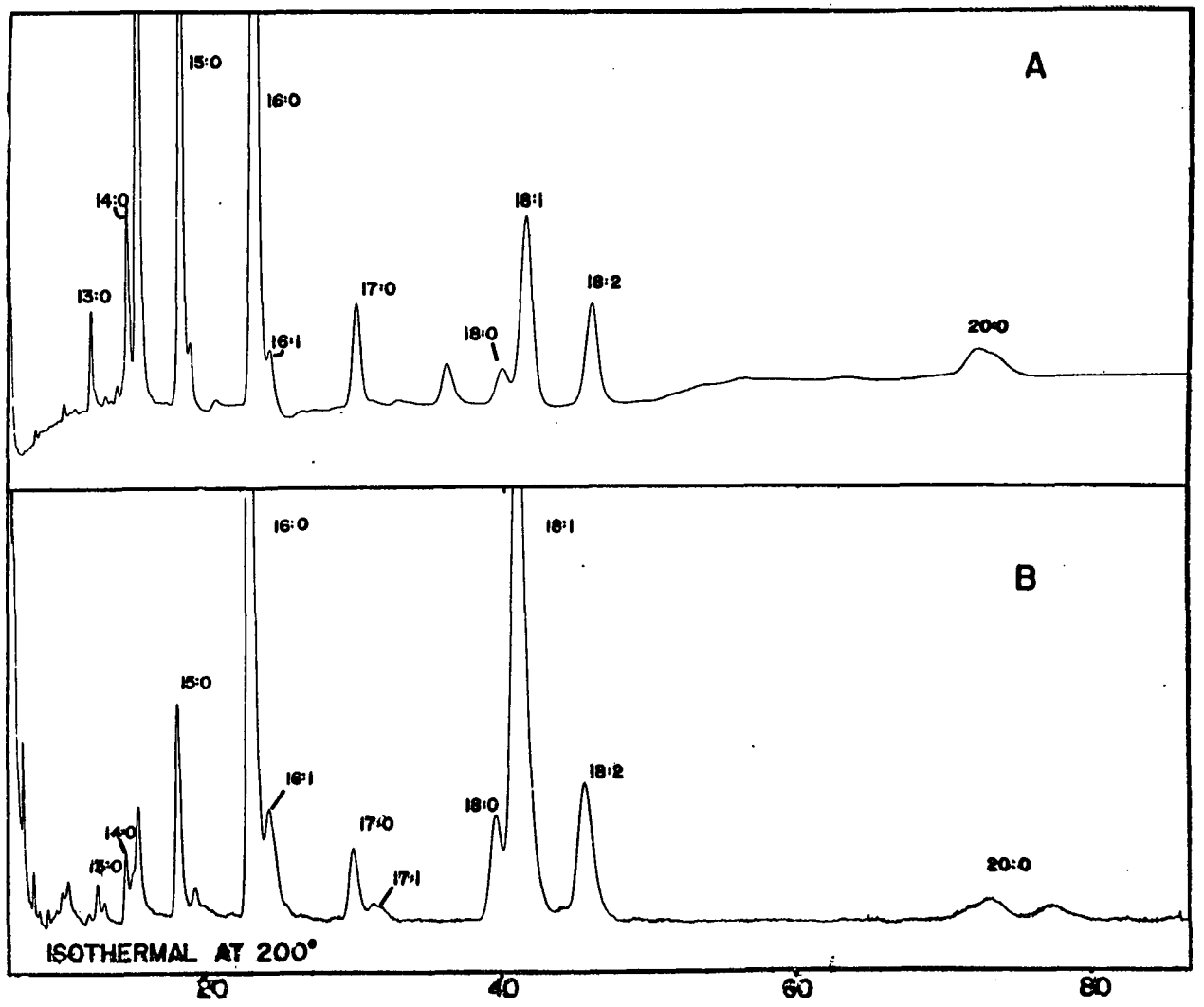

A
13:0
14:0
15:0
16:0
16:1
17:0
18:0
18:1
18:2
20:0
B
13:0
14:0
15:0
16:0
16:1
17:0
17:1
18:0
18:1
18:2
20:0
ISOTHERMAL AT 200°
20
40
60
80

TABLE 9. PERCENT DISTRIBUTION OF CARBON CHAINS BETWEEN THE FREE FATTY ACIDS AND THE NATURALLY OCCURRING FATTY ACID METHYL ESTERS IN WAX OF USTILAGO MAYDIS SPORES

Carbon chain number	Percent free fatty acids (A)	Percent fatty acid methyl esters (B)
13:0	1.75	0.70
14:0	3.74	1.86
14:1	13.80	3.26
15:0	13.90	6.28
15:1	2.92	1.20
16:0	28.90	20.69
16:1	3.92	7.89
17:0	3.86	3.26
17:1	1.21	1.01
18:0	1.29	6.20
18:1	12.40	32.70
18:2	5.65	9.36
18:3		
19:0		
20:0	5.84	4.85

DISCUSSION

While the naturally occurring methyl ester and free fatty acid patterns are similar in Ustilago maydis, some distinct differences can be noted.

In comparing the percentage distribution between the free acids and methyl esters in Table 9 it becomes apparent that methylation of the C_{18} carbon chains is favored. The greatest increase was noted for oleic acid ($C_{18:1}$), which was over 2.5 times the concentration of the corresponding free acid. Methyl palmitoleate ($C_{16:1}$) also appeared to be somewhat favored in methylation. The remainder of the fatty acids appeared to undergo methylation to a lesser degree except heptadecanoic (C_{17}) and heptadecenoic ($C_{17:1}$) acids which yielded the same percentage distribution in both fractions. Just what relationship exists between the methyl esters of the long chain fatty acids is not clear, but one would anticipate that a metabolic interchange does occur. It is likely that the methyl esters of fatty acids and the free fatty acids are associated in the wax complex similar to the methyl esters of galacturonic acid and free galacturonic acids in pectin. However, it has been demonstrated that a laminated cutinous layer does not exist in some fungal spores (39).

A recent report (58) suggests that methyl esters appear to serve as a reserve energy supply in Euglena. Methyl esters of short chain carboxylic acids are present as volatile flavoring constituents of the

pineapple (17). It has been demonstrated that methyl esters of long chain fatty acids, both saturated and unsaturated, promote auxin and gibberellin-induced elongation. Thus, these methyl esters could be considered another natural growth substance (65, 66).

In considering the presence of such esters in fungal spores coupled with the fact that an energy supply is required for germination, it is possible that the natural esters may function as a reserve energy source utilized during that process.

XII.

SECTION III

CHEMOTAXONOMIC AND ULTRASTRUCTURAL STUDIES ON THREE SPECIES OF *TILLETIA* OCCURRING ON WHEAT

INTRODUCTION

The difficult problem of species delimitation between various members within the genus *Tilletia* has received much attention (23, 24, 25, 57). Three species of particular significance are *T. foetida* (Wallr.) Lira (smooth spore surface), *T. caries* (DC) Tul. (reticulate spore surface), both of which cause the commonly observed bunt of wheat, and *T. controversa* (Kühn), (possessing a very reticulate spore surface) which causes dwarf bunt of wheat. Spore morphology, host reaction, spore germination, and characteristics of the life cycle have all been used to differentiate between various types. There is experimental evidence to indicate that hybrids between *T. caries* and *T. foetida* produce intermediate types in size, spore morphology, and other distinguishing characteristics (24). This problem was discussed at length by Holton and Kendrick (23). They concluded that wide variation exists in spore wall markings within species of *Tilletia*, but that natural hybridization between these species has compounded the problem of species differentiation. It was suggested that through the use of classical taxonomic criteria additional species of *Tilletia* on wheat might be recognized, and that a revision of the species concept was paramount because of the inherent potential variability within the *Tilletia* group. Other work from our laboratory suggests that differ-

ences exist in the waxes found in fungal spores. We analyzed the hydrocarbons in fungal spores of several genera in addition to those listed above: Ustilago nuda (Jens.) Kellerm. and S. W., Ustilago avenae (Pers.) Roste., and Puccinia graminis Pers. The distribution pattern of hydrocarbons was distinct for each genus, and indicated that these variations may be of value in distinguishing between spore types from various species.

Spore morphology has long been used to distinguish species. With the development of the freeze-etch preparative technique, it is possible to observe the fine details of the outer surface of spores with the electron microscope (22,61). In the case of Tilletia it was of interest to determine whether or not a detailed observation of the ultrastructure of spore walls might reveal additional distinguishing characteristics that could be related to taxonomy, as well as to provide evidence for the location of waxes produced by the spores.

This investigation explores the possibility of using lipids found in the spore walls and the ultrastructural appearance of the spore walls by use of the freeze-etch technique to differentiate between closely related species of Tilletia.

RESULTS

ULTRASTRUCTURE OF SPORE WALLS

TILLETIA FOETIDA

At the light microscope level T. foetida spores have smooth surfaces (Fig. 32). With the electron microscope the spore walls also appeared smooth by comparison to the other spores, as shown in Figs. 33 and 34. The oblique section of the spore shown in Fig. 32 (3) indicates the spore contains two wall layers with a partition layer between them. It is also possible to see the plasma membrane in the electron micrograph.

TILLETIA CARIES

The spores of T. caries have a reticular surface as seen with light microscopy (Fig. 33(4). In the electron microscope the spore wall appears to have an outer surface that contains ridges and depressions to give a convoluted surface as seen in Fig.33(5). The outer surface contains a very limited amount of hair-like projections and two wall layers with a partition layer between them. The cross sections of the spores shown in Figs.33-6 and 33-7 reveal thicker walls than T. foetida spores. The reticulation appears to be limited to the W_2 wall layer. It is also possible to see the plasma membrane and many structures in the protoplasm of the spore as shown in Fig. 33 (6).

TILLETIA CONTROVERSA

The spores of T. controversa shown in Fig. 34 contain reticular surfaces with many hair-like projections. The frozen-etch replicas of

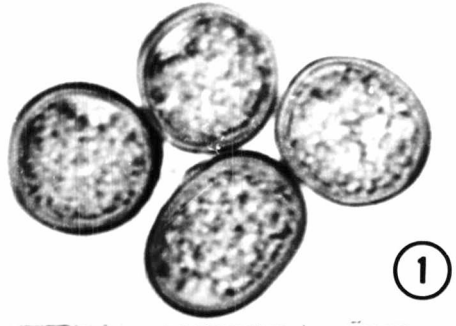
these spores when observed in the electron microscope show that the spore walls are very thick and contain many ridges and depressions similar to those of T. caries (Fig.34-9). The fracture surface of the spore wall (Fig.34-9) contains fibrous-appearing areas in the spore wall labelled W_1. In an oblique view of this spore it is possible to see three wall layers (W_1, W_2, and W_3) as well as a partition layer, plasma membrane, and other internal organelles (Fig.34-10). The W_2 layer in T. controversa contributes to the reticular appearance of the spore. It is more reticulate than T. caries and contains many more hair-like projections on the surface. At increased magnifications as shown in Fig. 34 (11) the fibrous-appearing areas labelled W_1 become rather large and granular whereas W_2 wall layer has a smooth texture. The third wall layer (W_3) is uniform and not reticulate. T. controversa has the thickest wall, the most reticulate surface, and the most hair-like projections on the outer wall when the three species are compared.

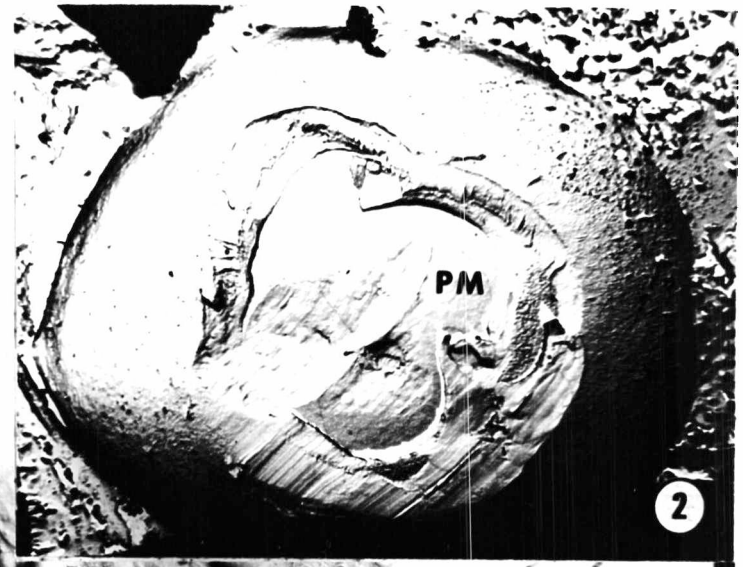
CHEMICAL ANALYSES OF SPORE LIPIDS

HEPTANE FRACTION

The heptane fraction contained n-alkanes, ranging in length from C_{21} to C_{33}. The hydrocarbon pattern of each of the three species was qualitatively similar as shown in Fig. 35. The extraction and analysis of a number of spore samples were repeated several times with identical results. All of the identified peaks were characterized with the LKB 9000 gas chromatograph-mass spectrometer combination. Uninfected wheat kernels [variety 50077 (CI 13561)] were also analyzed for hydrocarbon content, and the pattern obtained was qualitatively similar to those

FIGURE 32. Tilletia foetida spores. (1) Photomicrograph. x 3500. (2) Replica of the outer surface of a spore showing the plasma membrane (PM) where the wall layers have been fractured away. x 5300. (3) Replica of a fractured spore showing the spore surface (SS), two wall layers (W_2 and W_3) with a partition layer (PL) between them, the plasma membrane (PM), lipids (L), and a vacuole (V). x 14,400. (Electron microphotographs compliments of Dr. Bill Hess.)


1


PM
2

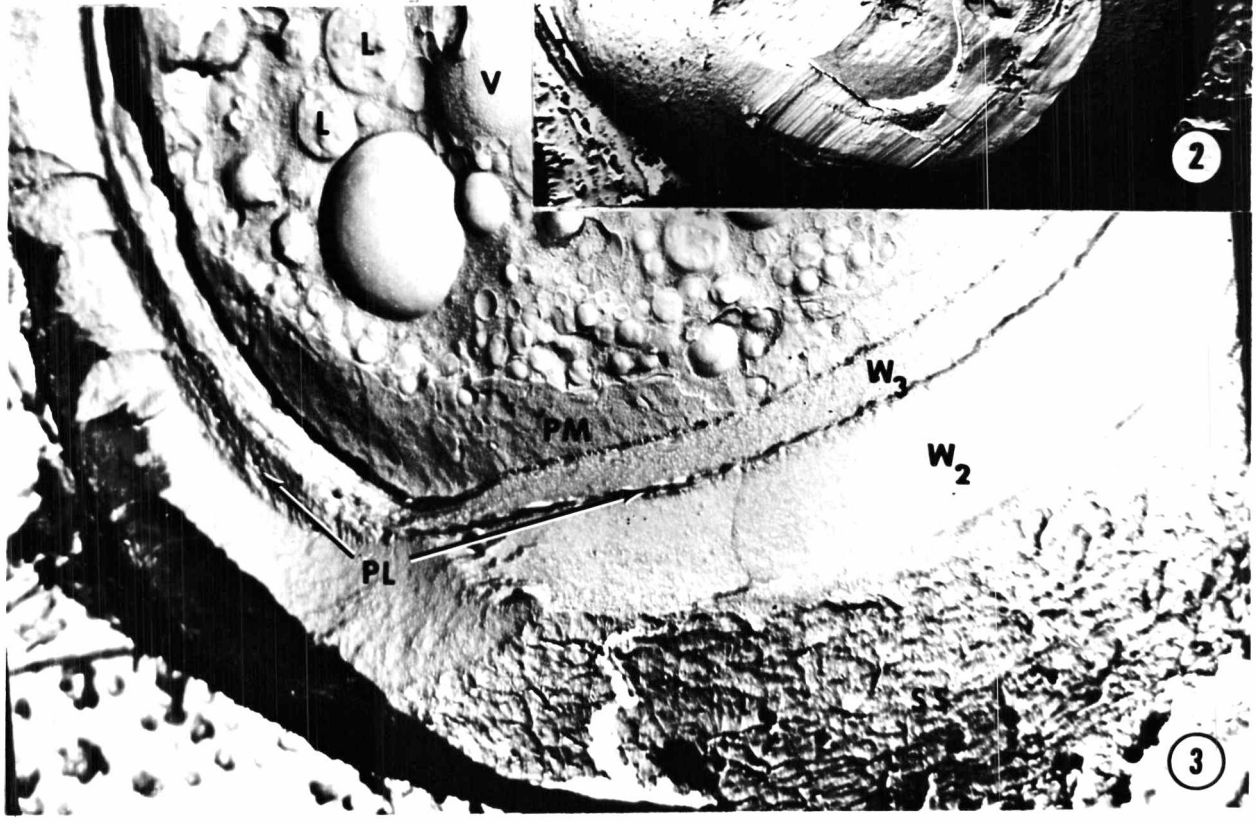

L
L
V
PM
PL
W_3
W_2
3

FIGURE 33. Tilletia caries spores. (4) Photomicrograph. x 3500. (5) Replica of the outer surface of a spore. x 5300. (6) Replica of a fractured spore showing two wall layers (W_2 and W_3) with a partition layer (PL) between them. The plasma membrane (PM) has been partially fractured away exposing underlying organelles. x 17,100. (7) Replica of a cross-fractured spore showing the spore surface (SS), two wall layers (W_2 and W_3) with a partition layer (PL) between them. The cross-fractured plasma membrane (PM), lipids (L), and vacuoles (V) are also seen. x 27,900. (Electron microphotographs compliments of Dr. Bill Hess.)

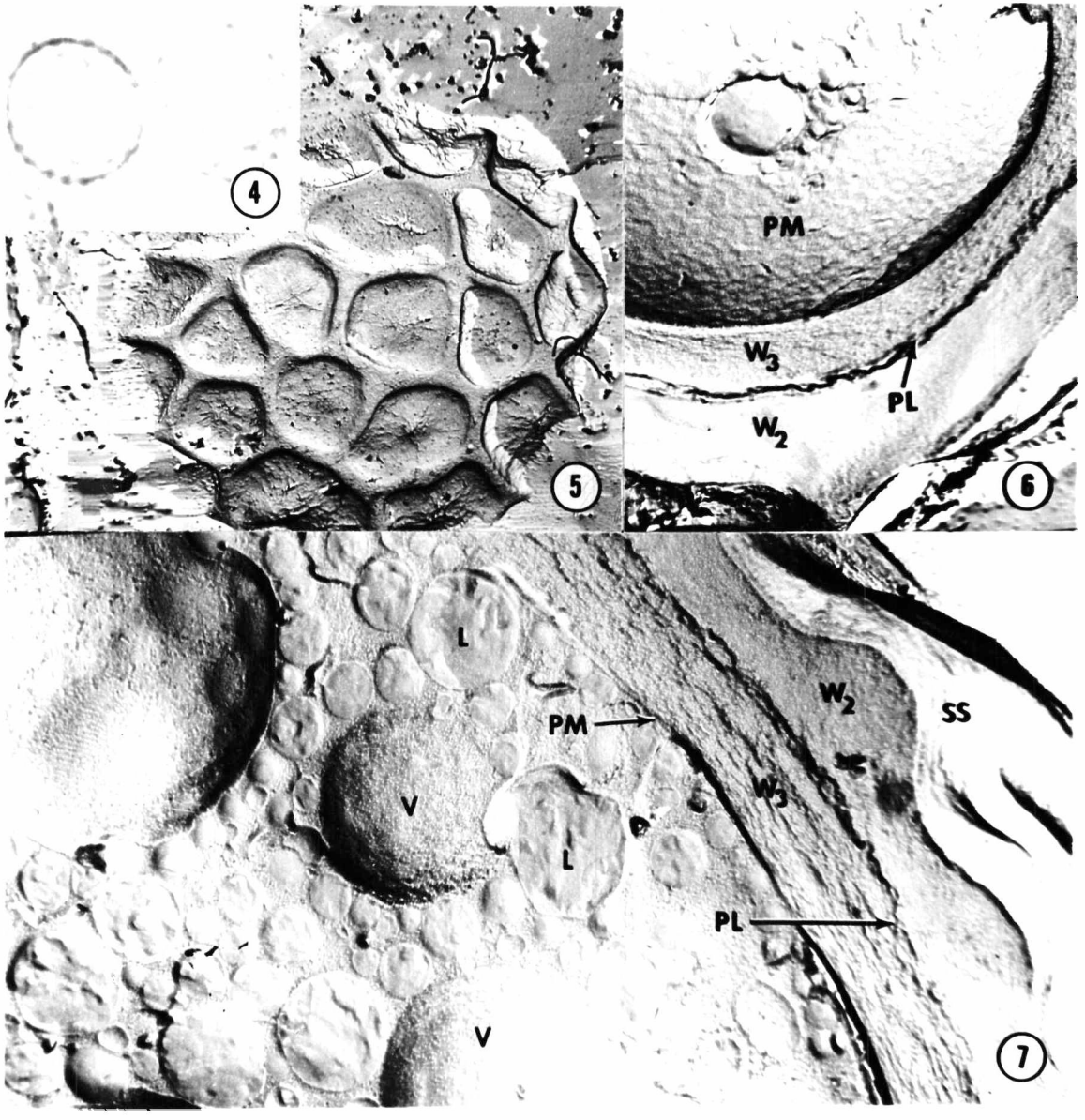
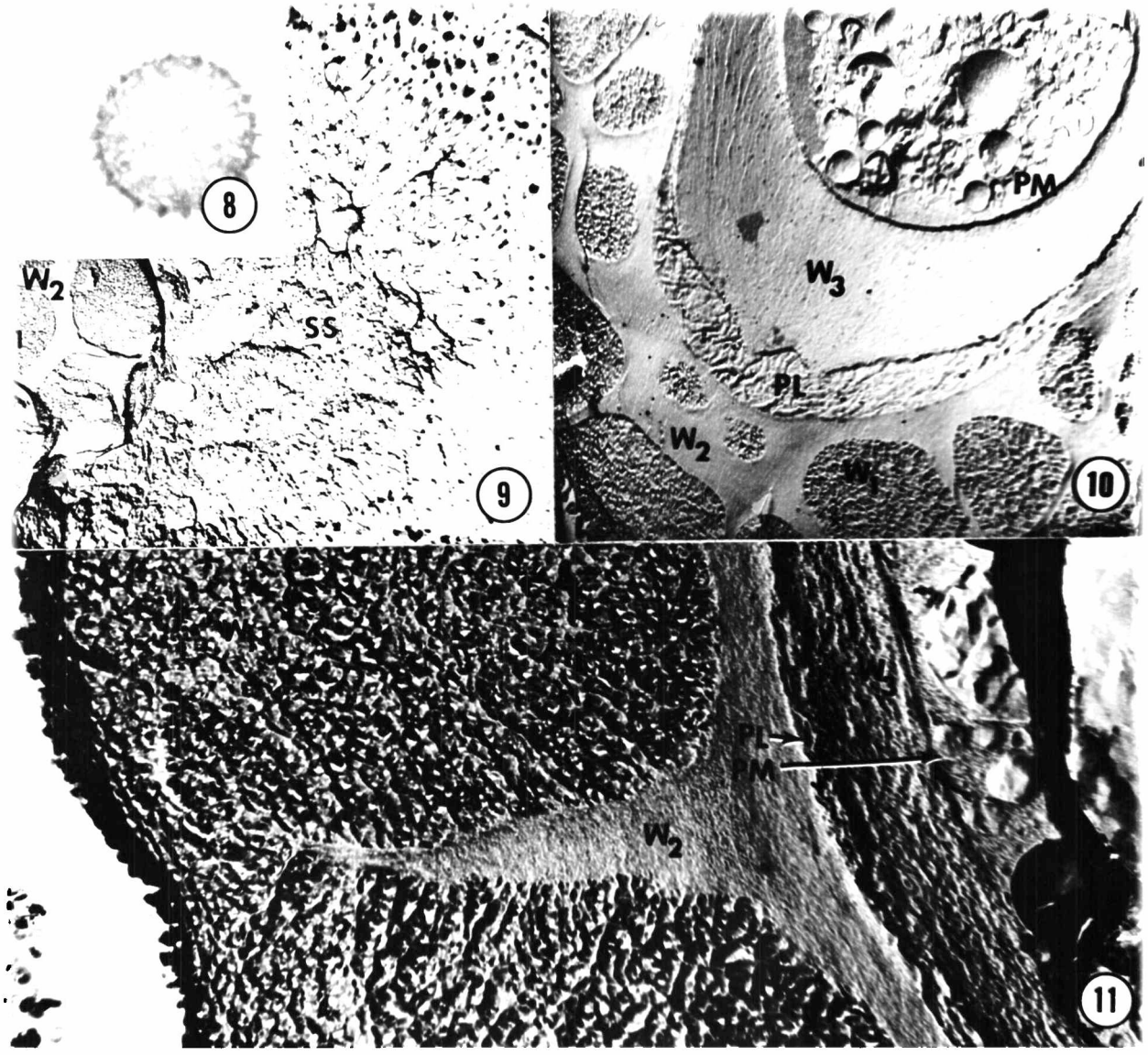

4
5
PM
W3
PL
W2
6
L
PM
V
L
W2
SS
W3
PL
V
7

FIGURE 34. Tilletia controversa spores. (8) Photomicrograph. x 3500. (9) Replica of the outer surface of a spore (SS) showing portions of the outer wall layers (W_1 and W_2) which have been exposed by fracturing. x 5300. (10) Replica of a cross-fractured spore showing three wall layers (W_1, W_2, and W_3) and the irregular nature of the partition layer (PL) between the two inner layers of the wall. The plasma membrane (PM) and organelles are also seen. x 9000. (11) Replica of a cross-fractured spore wall showing the various wall layers (W_1, W_2, and W_3), the partition layer (PL) between the two inner wall layers, and the plasma membrane (PM). x 27,900. (Electron microphotographs compliments of Dr. Bill Hess.)


8
W2
SS
9
PM
W3
PL
W2
W1
10
PL
PM
W2
11

obtained from the Tilletia spores (Fig. 36). In each example studied the odd-carbon-numbered alkanes predominated, as has been found characteristic of all biological materials which contain alkanes. Both the spores and wheat kernels had a marked preponderance of nonacosane (n-C_{29}). It should be noted that branched-chain isomers of each carbon chain length were also commonly present. The relative per cent distribution of the alkanes of each of the three Tilletia species are given in Table 10.

METHANOL FRACTION

The methanol fraction contained saturated and unsaturated free fatty acids. The fatty acids were also characterized by mass spectrometer and the results are shown in Table 11. The predominant saturated free fatty acid is palmitic in each of the species. The predominant free unsaturated fatty acid for T. foetida was oleic (18:1); for T. controversa and T. caries it was linoleic (18:2), although in the case of T. caries considerable linolenic (18:3) was also present. In comparing the total free saturated fatty acids, T. foetida contains the highest quantity followed by T. controversa, with T. caries containing the least. On the other hand, with the free unsaturated fatty acids, the situation was reversed, with T. caries containing the highest quantity, then T. controversa, and finally T. foetida.

It is obvious (Table 11) that the free and total fatty acid distributions differ considerably. There is a shift in the predominant saturated palmitic acid (C_{16}) of the free acids to the unsaturated linoleic acid ($C_{18:2}$) of the total fatty acids. In each of the three Tilletia

species $C_{18:2}$ is in relative concentrations greater than 50 per cent. The relative concentrations of all the total fatty acids are remarkably similar. Predominant fatty acids in lesser concentrations are the saturated palmitic and unsaturated oleic. All are in similar concentrations in each of the three species. In addition, many fatty acids appear in lesser concentrations that do not appear in the free fatty acid fraction.

The total fatty acid fraction also contained a compound that was very difficult to resolve from stearic acid (C_{18}). The identity of this compound could be ricinoleic acid. This is a hydroxylated $C_{18:1}$ fatty acid found in some fungal organisms. Also, several high molecular weight compounds with long retention times were eluted from the column. Their identity is unknown.

The saturated acids predominate in the free fatty acid fraction of T. foetida and T. controversa (Table 12) giving saturated to unsaturated ratios greater than one in each case (Table 13). Tilletia caries contained greater quantities of unsaturated acids reversing the predominance from the saturated condition. The saturated to unsaturated ratio was 0.70 (Table 13). The total fatty acid extracts show the opposite condition having a more consistant distribution. The unsaturated ratios are 0.26, 0.24 and 0.21 for T. foetida, T. controversa, and T. caries respectively (Table 13).

FIGURE 35. Comparison of gas chromatographic separations of the heptane eluates from a silica gel column of three species of Tilletia. Separation of alkanes was carried out on stainless steel capillary tubing (30 m x 0.050 cm) coated with OV-1. Helium pressure 145 g/cm^2; no split. Perkin-Elmer 900 instrument equipped with a flame ionization detector. Range 1; attenuation 128. Temperature programmed from 120° C to 260° C at 4° C per minute. In each case approximately 5 g of spores was extracted and 1/30 of the sample injected.

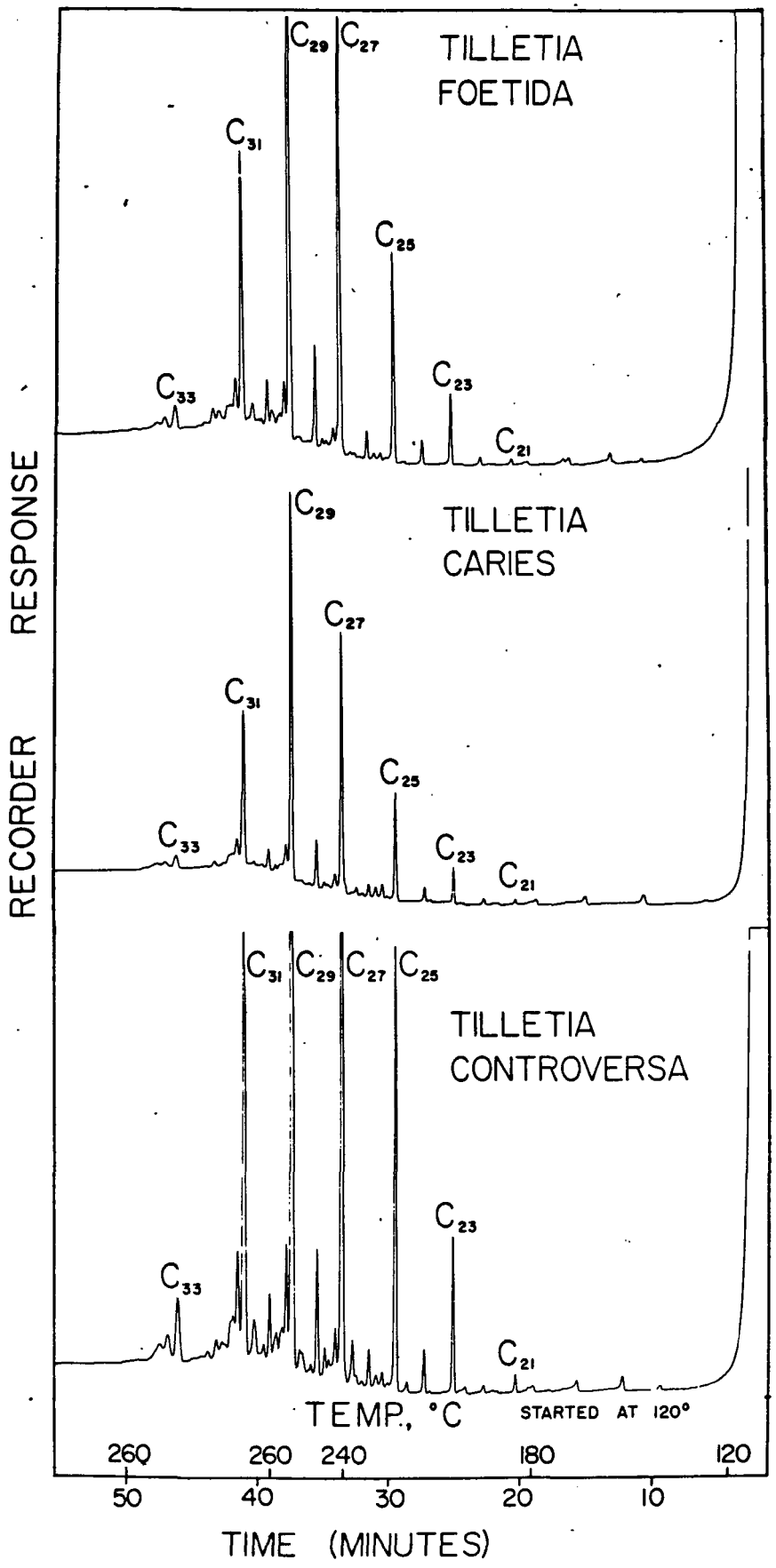

TILLETIA FOETIDA
TILLETIA CARIES
TILLETIA CONTROVERSA
C_{33}
C_{31}
C_{29}
C_{27}
C_{25}
C_{23}
C_{21}
RECORDER RESPONSE
TEMP., °C
STARTED AT 120°
260
260
240
180
120
50
40
30
20
10
TIME (MINUTES)

FIGURE 36. Gas chromatographic separation of the hydrocarbon components extracted from non-infected wheat. Separation was carried out on a stainless steel capillary column (30 m x 0.050 cm) coated with OV-1. Helium pressure 145 g/cm^2; no split. Perkin-Elmer 900 instrument equipped with a hydrogen flame detector. Temperature programmed from 120° C to 260° C at 4° C per minute.

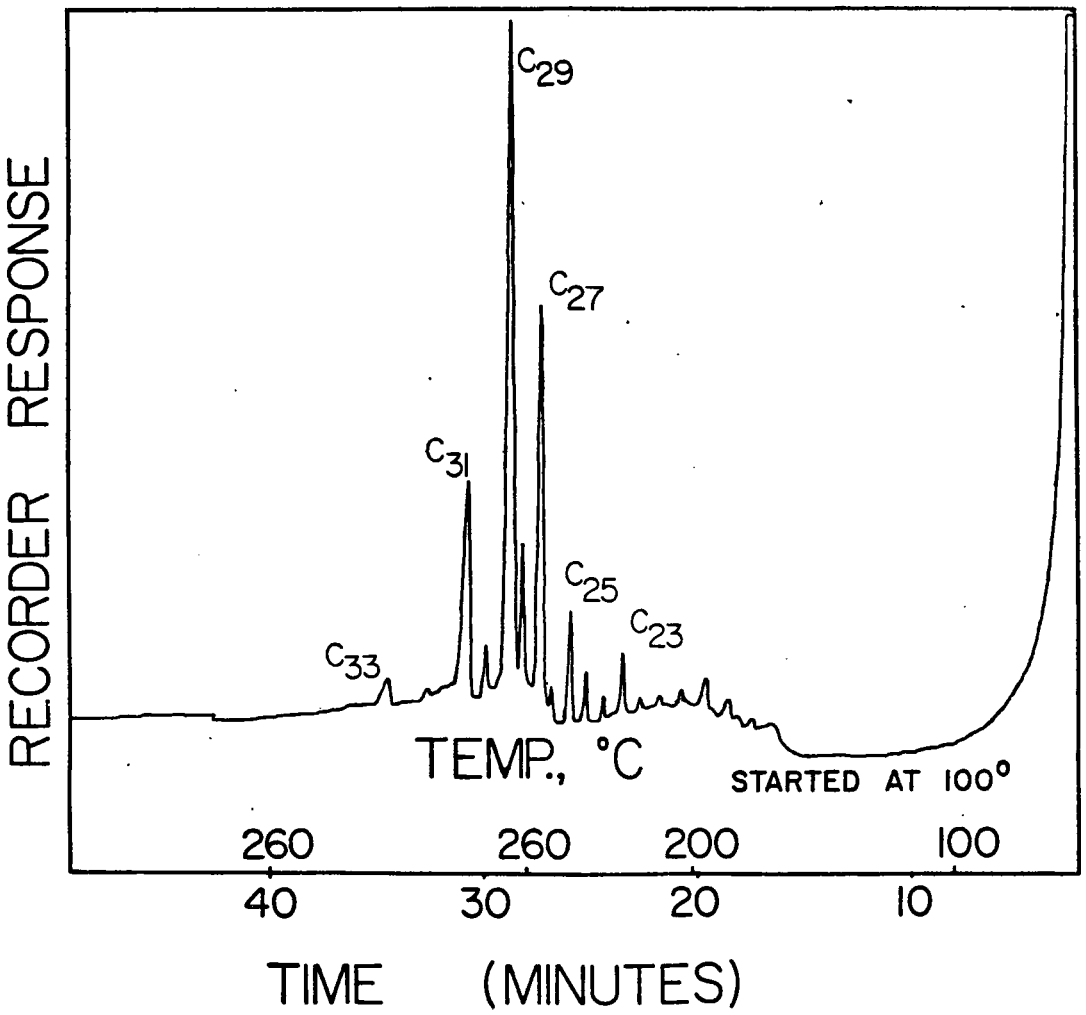

RECORDER RESPONSE
C29
C27
C31
C25
C23
C33
TEMP., °C
STARTED AT 100°
260
260
200
100
40
30
20
10
TIME (MINUTES)

TABLE 10. RELATIVE ALKANE DISTRIBUTION OF THE LIPID EXTRACTS FROM CHLAMYDOSPORES OF THE *TILLETIA* SPECIES.

TILLETIA SPECIES	HYDROCARBON CHAIN-LENGTH																
	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}	iC_{25}	C_{26}	C_{27}	iC_{27}	C_{28}	C_{29}	iC_{29}	C_{30}	C_{31}	iC_{31}	C_{32}	C_{33}
foetida	0.30	0.34	3.21	1.02	11.98	-	1.28	24.69	0.85	5.66	28.81	1.62	1.97	15.18	1.28	-	1.71
controversa	0.38	0.15	3.97	0.89	11.41	-	0.38	24.73	0.76	2.66	25.57	1.90	1.33	21.46	1.71	-	2.66
caries	0.13	0.18	5.39	0.87	9.70	-	0.97	23.45	1.01	4.28	34.77	1.34	1.61	13.74	1.07	-	1.48

TABLE 11. MAJOR FREE AND TOTAL FATTY ACIDS COMPUTED FROM GAS CHROMATOGRAPHIC ANALYSIS (AS METHYL ESTERS) EXTRACTED FROM CHLAMYDOSPORES OF THREE *TILLETIA* SPECIES.

FATTY ACID	*TILLETIA* SPECIES *foetida*		*controversa*		*caries*	
	free	total	free	total	free	total
dodecanoic acid C_{12}	—	0.1	—	0.2	—	0.1
myristic acid C_{14}	5.6	0.7	3.3	0.4	2.0	0.2
myristoleic acid $C_{14:1}$	—	0.9	—	0.9	—	0.7
pentadecanoic acid C_{15}	—	—	—	0.2	—	0.2
palmitic acid C_{16}	67.3	15.3	63.1	10.9	36.9	13.2
palmitoleic acid $C_{16:1}$	7.6	2.5	9.7	1.8	26.6	2.4
heptadecanoic acid C_{17}	—	0.3	—	0.7	—	0.3
stearic acid C_{18}	5.0	2.1	4.2	0.5	2.4	1.5
oleic acid $C_{18:1}$	7.6	14.0	4.9	16.7	4.6	12.9
linoleic acid $C_{18:2}$	5.8	56.9	11.2	51.2	14.3	54.9
linolenic acid $C_{18:3}$	0.3		3.6		13.2	
arachidic acid C_{20}	—		—		—	
eicosenoic acid $C_{20:1}$	—	2.3	—	2.6	—	
heneicosanoic acid C_{21}	—	0.9	—	2.1	—	1.5
behenic acid C_{22}	—	1.1	—	1.8	—	1.7
erucic acid $C_{22:1}$	—	0.9	—	3.1	—	1.0
Unknown		1.7		2.3		1.9

TABLE 12. RELATIVE DISTRIBUTION OF MAJOR SATURATED AND UNSATURATED FREE AND TOTAL FATTY ACIDS EXTRACTED FROM CHLAMYDOSPORES OF THREE *TILLETIA* SPECIES.

CARBON CHAIN	*TILLETIA* SPECIES *foetida*		*controversa*		*caries*	
	free	total	free	total	free	total
Saturated	72.9	20.7	70.6	16.8	41.3	18.5
Unsaturated	21.8	77.7	29.4	78.6	58.7	78.6

TABLE 13. RATIO OF SATURATED TO UNSATURATED FREE AND TOTAL FATTY ACIDS OF LIPID EXTRACTS OF CHLAMYDOSPORES OF THREE *TILLETIA* SPECIES.

FUNGAL SPECIES	RATIO (SATURATED/UNSATURATED)	
	free	total
Tilletia foetida	3.34	0.26
Tilletia controversa	2.40	0.21
Tilletia caries	0.70	0.23

DISCUSSION

CHEMICAL ANALYSES OF SPORE LIPIDS

Because of previous experiences in comparing spores of different smut genera in which each contained a distinct hydrocarbon distribution pattern, it was somewhat surprising to find that the three species contained essentially the same pattern. Even on a quantitative basis there appear to be only small differences in the total amount of hydrocarbons present in each of the three species. It was even more surprising to find that a similar hydrocarbon pattern could be obtained from the uninfected wheat kernels. This suggests that the species of Tilletia remove hydrocarbons directly from the host and incorporate them into the spores. This is in contrast to our earlier work which reported that the hydrocarbon distribution in U. maydis spores was distinctly different from the hydrocarbon pattern of normal corn. In addition we found that the infected corn contained a distinctly different pattern from the normal corn and the pattern obtained from the fungal spores of U. maydis. In reference to the possibility that the hydrocarbon pattern represents contamination from an external source we feel that the distinct predominance of odd-numbered hydrocarbons is highly characteristic of biological material, whereas commercial oils and petroleum contain a random distribution of even and odd hydrocarbons. To avoid the possibility of contamination, all these spores were obtained from a nursery specifically designed for the culturing of fungal spores on wheat, and all glassware was cleaned carefully with hot chromic acid cleaning solution before use.

The benzene eluates contain a number of unknown low molecular weight lipophilic compounds in addition to the triglycerides normally found there.

In reference to the free fatty acids that are present in Tilletia spores, it is of interest to note that a definite pattern exists between the concentration of saturated versus unsaturated free fatty acids. T. foetida contains the highest concentration of saturated fatty acids; in T. controversa the concentration is intermediate, and in T. caries it is lower, whereas in the unsaturated fatty acids the condition was just reversed. It is interesting to note, however, that in each case the concentration in T. controversa was intermediate between that of T. foetida and T. caries. This would suggest that T. foetida and T. caries are farther apart on a species level with respect to the chemical analysis, with T. controversa being an intermediate or hybrid form.

The total fatty acid analyses reveals more information concerning their true distribution. The results indicate a preference of incorporation of the longer unsaturated fatty acids into the complex neutral lipids (tri-, di-, monoglycerides) and the polar phospholipids. Although the longer chain acids are in low relative concentrations in the total acid fraction they were absent from the free acid fraction. This conforms to results obtained from investigations of other biological systems (64). Free fatty acids represent a comparatively small fraction of the total lipid fraction which is also consistant with reports of plant and animal systems (64). The consistancy of percentages of the total fatty acids reveal little with respect to species differentiation.

ELECTRON MICROSCOPY ANALYSIS OF SPORES

Results of the electron microscope indicate strongly the potential value of the freeze-etch technique for studying the external morphology of spores. Such information should help elucidate many of the problems which result from difficulties in resolving external morphology under the light microscope. Several points were brought out by the electron microscope study. It is evident that fungal spore walls are thick and rather complex, containing several layers. Perhaps of most interest is the fibrous-appearing material which is present in the cell walls of T. controversa. It will be of special interest to determine the chemical nature of this material as well as the other cell wall layers. Preliminary studies at the light microscope level indicate that no observable change in morphology occurs after extraction of the spores with lipo-solvents. This suggests that the lipids of Tilletia spores do not represent a major factor in determining spore wall architecture. However, it is recognized that the freeze-etch fracturing method may remove a thin layer from the surface of the spores, but this is unlikely because long periods of etching do not reveal additional layers on areas of spores which have been exposed to etching.

In comparing the three species on the basis of external morphology it appears that T. foetida is the simplest having a thinner wall, two wall layers, and a smooth surface; whereas T. controversa has the most complex cell walls, containing three wall layers and a reticulate surface with numerous hair-like projections.

XIII.

SECTION IV

FATTY ACID AND HYDROCARBON COMPONENTS OF SCLEROTIA OF *SCLEROTINIA SCLEROTIORUM* (LIB)DBY

I N T R O D U C T I O N

Sclerotia are dormant masses of compact mycelium that are resistant to unfavorable conditions, but can germinate in a favorable environment. Little is known about their chemical composition especially the lipid components. Chet and Henis (10) have analyzed sclerotial walls of *Sclerotium rolfsii* and found the components to be chitin and beta-1,3-glucan. They observed (11) that the lipid content (15%) was higher in the sclerotia walls as compared to the mycelial walls (8%), and the amino acid composition was different in sclerotia walls. The fatty acids of sclerotia of *Sclerotium rolfsii* were found to contain linoleic (56%), palmitic (17%) and oleic (7%) by Howell Fergus (26). The fatty acid fraction of sclerotia of *Claviceps purpurea* contained ricinoleic (34%), palmitic (24%), oleic (21%), and linoleic (12%) (6).

LeTourneau (41) previously determined that trehalose and mannitol were the principal carbohydrates in sclerotia of *Sclerotinia sclerotiorum*.

In order to increase our understanding of the chemical nature of sclerotia, the lipid fraction of sclerotia of *Sclerotinia sclerotiorum* was investigated. The results are reported in this dissertation.

RESULTS

SCLEROTIA OF PEA

The fatty acid and hydrocarbon components from 1.2 g of sclerotia of S. sclerotiorum obtained from infected pea plants were analyzed. The heptane fraction contained only one detectable hydrocarbon component (figure 37). Its identity is uncertain, but assuming a typical straight-chain alkane moiety the carbon length is in the C_{25}-C_{29} range as compared by known paraffin standards.

The benzene fraction contained no compounds detectable with the column typical for fatty acid methyl ester analysis (ethylene glycol succinate). The methanol fraction contained free fatty acids, as illustrated in Table 14 and Figure 39 D.

SCLEROTIA FROM POTATO DEXTROSE AGAR

The lipid analysis of 1 g sclerotia grown on PDA was different from that obtained from the pea field. Both the n-heptane and benzene fractions contained no compounds detectable by gas chromatography. The fatty acid patterns obtained from the methanol fraction is shown in Figure 39 B The predominant components were oleic (31.1%) and linoleic (31.8%) acids. The per cent composition of each fatty acid is given in Table 14.

SCLEROTIA FROM SUCROSE SALTS MEDIUM

The n-heptane and benzene fractions contained no compounds detectable by gas chromatography. The methanol fraction contained a number

of fatty acids as shown in Figure 39C. The predominant acids were oleic (37.5%) and linoleic (37.9%) (Table 14). The lipids were extracted from 0.76 g of sclerotia.

SCLEROTIA FROM GLUCOSE SALTS MEDIUM

Again the n-heptane and benzene fractions contained no compounds detectable by gas chromatography. The methanol fraction contained fatty acids with oleic (41.6%) and linoleic (44.3%) acids being the major components (Figure 38A). The lipids were extracted from 0.54 g of sclerotia.

FIGURE 37. Gas chromatogram of the single hydrocarbon found in the lipid extract of *Sclerotinia sclerotiorum*. Separation was carried out on a stainless steel capillary column (50 ft. x 0.03 in.) coated with OV-1. Helium pressure 10 lbs; no split. Perkin-Elmer 900 instrument equipped with flame ionization detector. Range 1; attenuation 64. Temperature programed from 100° C. to 270° C. at 8° C/min. Four grams of sclerotia was extracted, and 1/10 of the sample injected.

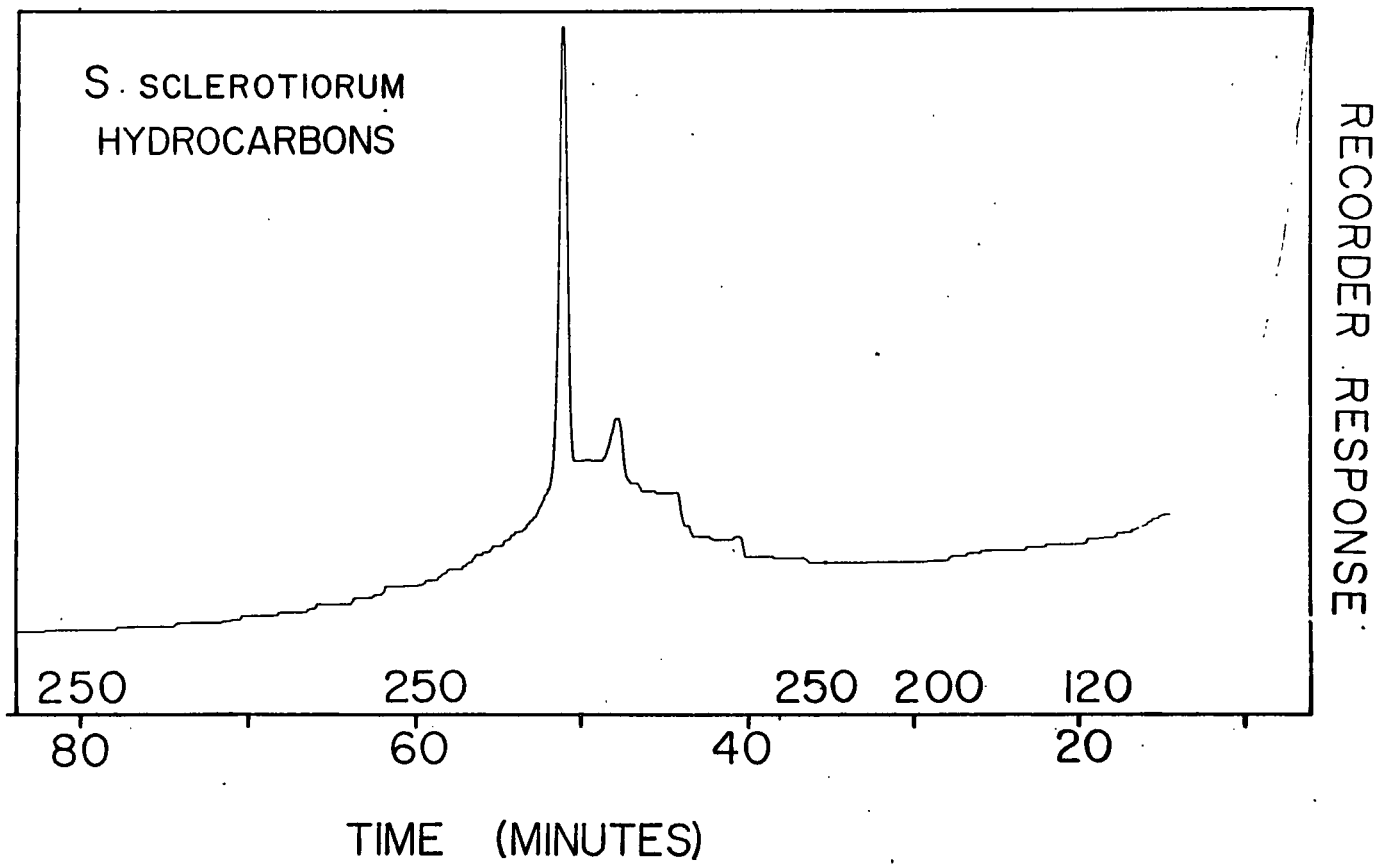

S. SCLEROTIORUM
HYDROCARBONS
250
250
250
200
120
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 38. (A) Gas chromatogram illustrating the free fatty acid distribution from 0.5 g of sclerotia of *S. sclerotiorum* grown on glucose salts media. (B) Gas chromatogram of free fatty acids from sclerotia of the same organism grown on PDA media. Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel column packed with chromosorb W coated with ethylene glycol succinate. Temperature 180° C. isothermal, no split, Helium 21 psi.

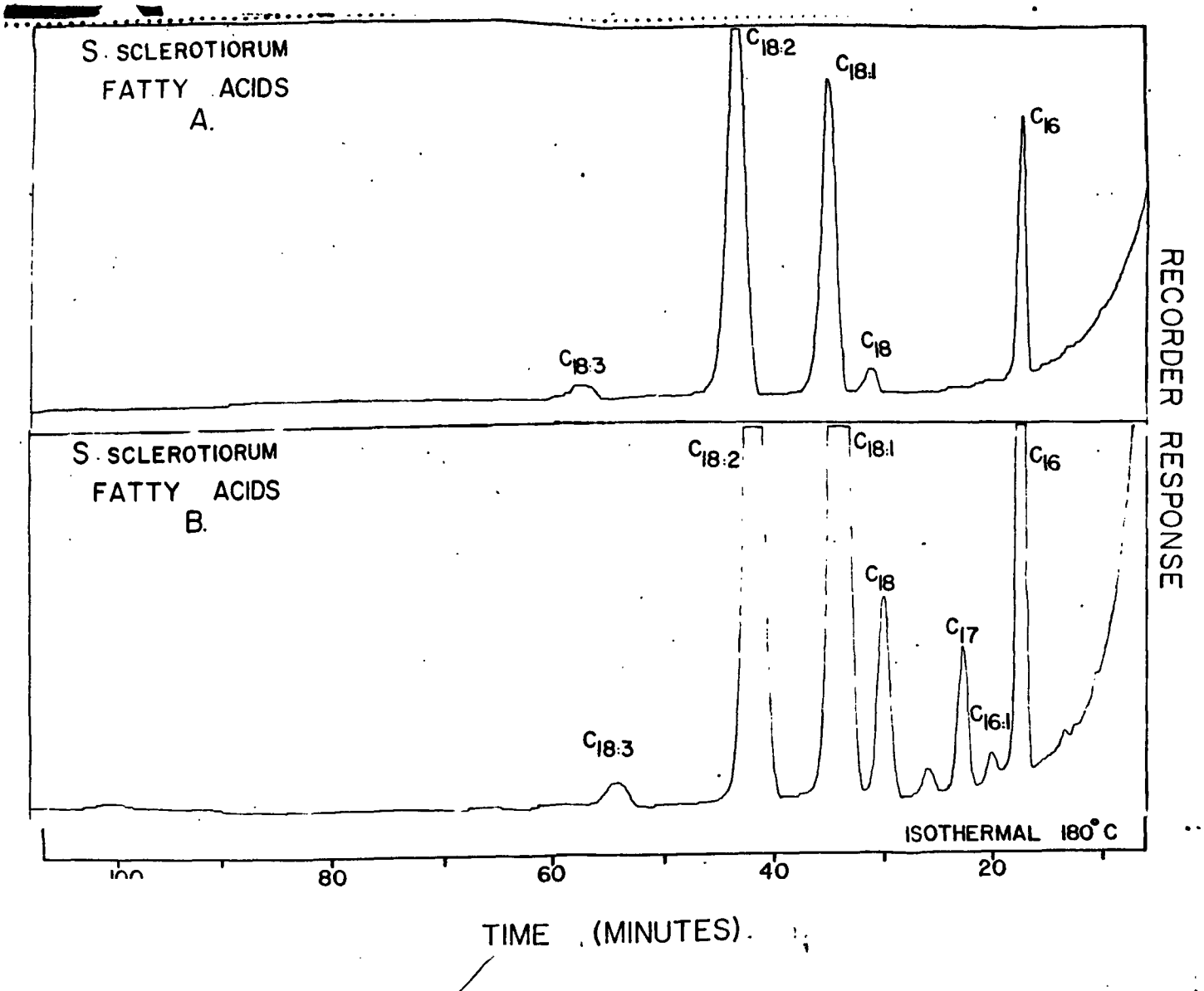

S. SCLEROTIORUM
FATTY ACIDS
A.
C18:2
C18:1
C16
C18:3
C18
S. SCLEROTIORUM
FATTY ACIDS
B.
C18:2
C18:1
C16
C18
C17
C16:1
C18:3
ISOTHERMAL 180° C
RECORDER RESPONSE
100
80
60
40
20
TIME (MINUTES)

FIGURE 39. (C) Gas chromatographic separation of the free fatty acids from sclerotia of S. sclerotiorum grown on sucrose salts media. (D) Gas chromatographic separation of the free fatty acids from sclerotia of the same organism collected from pea field samples. Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel column packed with chromosorb W coated with ethylene glycol succinate. Temperature 180° C. isothermal, no split, Helium 21 psi.

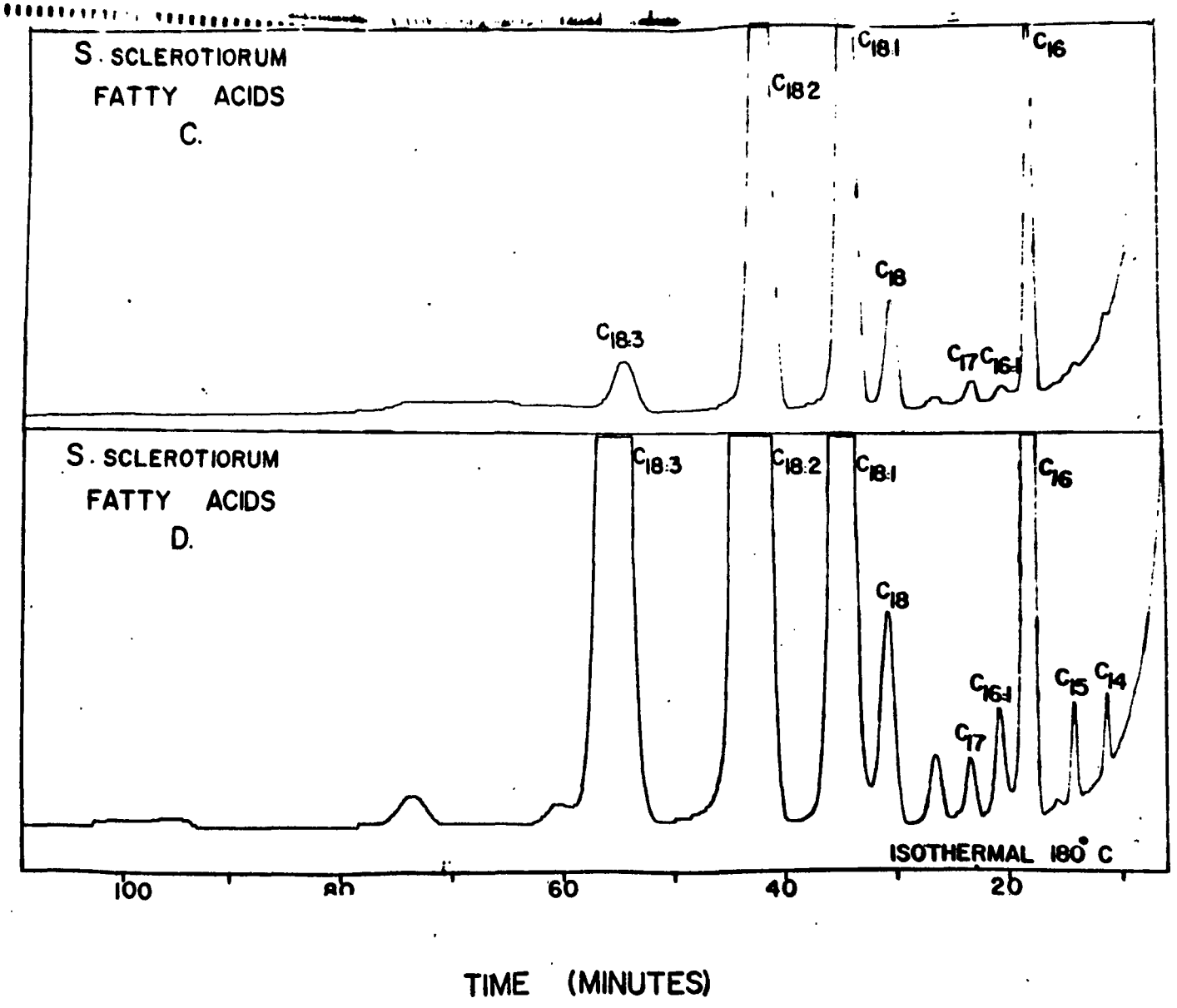

S. SCLEROTIORUM
FATTY ACIDS
C.
C18:3
C18:2
C18:1
C18
C17
C16:1
C16
S. SCLEROTIORUM
FATTY ACIDS
D.
C18:3
C18:2
C18:1
C18
C17
C16:1
C16
C15
C14
ISOTHERMAL 180° C
100
80
60
40
20
TIME (MINUTES)

TABLE 14. PERCENT FATTY ACID COMPOSITION OF SCLEROTIA FROM *S. SCLEROTIORUM* GROWN ON DIFFERENT MEDIA.

MEDIA	C_{14}	C_{15}	C_{16}	$C_{16:1}$	C_{17}	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	C_{20}
Potato Dextrose Agar	tr.*	tr.	14.6	0.7	7.2	12.4	31.1	31.8	1.9	-
Sucrose Salts	tr.	tr.	12.9	0.5	1.3	5.0	37.5	37.9	4.7	-
Glucose Salts	-	-	11.3	-	-	1.5	41.6	44.3	0.1	-
Pea Field	0.6	1.4	16.3	1.9	1.2	5.9	16.6	28.8	26.7	0.7

*tr. - trace

TABLE 15. RELATIVE PERCENT* COMPOSITION OF THE MAJOR FREE FATTY ACIDS OF SCLEROTIA FROM *SCLEROTINIA SCLEROTIORUM* GROWN ON FOUR SUBSTRATES.

CULTURE MEDIA	FATTY ACID CHAIN LENGTH			
	C_{16}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Potato Dextrose Agar	18.4	39.1	39.8	2.3
Sucrose Salts	13.8	48.5	40.6	5.0
Glucose Salts	11.5	42.5	45.2	0.1
Pea Field	18.4	18.7	32.5	30.3

*All other fatty acid components were disregarded for these calculations.

DISCUSSION

The data presented here indicates that substrates do influence the relative distribution of free fatty acids. The data at first glance may be misleading because it appears that natural or undefined media (pea and PDA) give greater concentrations of fatty acids. However, larger quantities of sclerotia were extracted in these cases. Less material from the salts synthetic media was available for analysis.

For accurate interpretation, only the major fatty acid constituents should be considered because of these differences. Only C_{16}, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ will be discussed. All other constituents were disregarded for calculations. Relative percentages of only these acids from each sample are given in Table 15. It is obvious from these calculations that sclerotia of S. sclerotiorum obtained from its natural substrate has a widely different fatty acid distribution from that grown on PDA and synthetic media. There is essentially no significant difference in the relative concentrations of palmitic acid in each of the sclerotia samples. Also, the percentages of oleic and linoleic acids varied no greater than eight percentage units in sclerotia from PDA, sucrose salts, and glucose salts. Essentially a 1:1 ratio is found for oleic and linoleic acids in each of the same samples. The dramatic change in the fatty acid distribution of sclerotia from pea field samples is noticed by an increase in the oleic:linoleic acid ratio (1.7) and approximate 7-fold increase in linolenic acid. Uninfected pea tissue was unavailable for

analysis thus the relationship between it and the sclerotia fatty acid distribution is not known. Exactly what interaction of the fatty acid metabolism in this host-parasite relationship is uncertain.

The field sclerotia contain a single hydrocarbon component whereas the other sclerotia did not contain alkane compounds. It is possible that some of the compounds in the field sclerotia were contaminates from the pea plant. However hydrocarbons in higher plants normally range from C_{25} to C_{35} (15). Bacteria are usually very low in hydrocarbon content and it would be difficult to believe surface bacteria on the sclerotia could account for the hydrocarbons. A more reasonable explanation is that the fungus growing on the pea plant breaks down some of the waxy materials and some of the hydrocarbons remain in the newly formed sclerotia. In the defined media the fungus apparently does not synthesize to any extent hydrocarbons or benzene fraction compounds.

XIV.

SECTION V

LIPIDS OF *RHIZOPUS* *ARRHIZUS* SPORANGIOSPORES AND MYCELIA

INTRODUCTION

Rhizopus arrhizus is a phycomycetous fungus belonging to the Order Mucorales (2). Species of this genera are known to cause the disease soft rot of fruits of several plant species. Sporangiospores are common in the atmosphere causing the familiar black bread mold. As indicated before, preliminary lipid analysis revealed the presence of natural esters of long chain fatty acids. This stimulated further interest in determining the distribution of these esters in mycelial and spore material. Previous studies involving *R. arrhizus* reports palmitic, palmitoleic, stearic, oleic, linoleic, and gamma-linolenic acids. It was speculated by Shaw that gamma-linolenic acid is a vestigial character in phycomycetes, relating these fungi phylogenetically to certain protozoa (62).

In addition to the comparative aspects spore and mycelial methyl esters and fatty acids, a more complete fatty acid and general lipid analysis is reported here.

RESULTS

The lipid components from 8 g of Rhizopus arrhizus mycelia have been isolated and separated by thin-layer chromatography. The relative per cent distribution of each major lipid class is reported in Table 16. The lipid classes in the greatest concentrations are the polar lipids (44.4%) followed by the triglycerides (22.1%), sterols (16.7%), and the free fatty acids (16.7%). Thin-layer chromatographic separation is diagramed in Figure 40. This diagram illustrates the simple pattern of lipid components of R. arrhizus. Approximately 5.3 mg of extractable lipids were obtained from 1 g wet weight of mycelia (Table 16).

The lipid extract was subjected to alkaline hydrolysis to obtain the total fatty acids present. A very broad range of acids was found beginning with traces of decanoic acid (C_{10}) and going up through lignoceric acid (C_{24}) (Figure 42). The predominant unsaturated fatty acids are oleic (29.4%) and linoleic (16.3%) and the major saturated acids are palmitic (18.4%), stearic (11.0%), and arachidic (16.2%). The characteristic low relative proportions of linolenic acid (0.2%) was present (Table 17). There appears to be an even distribution of saturated to unsaturated total fatty acids in the mycelia (Table 18).

Heptane eluates from silica gel column fractionation contained a single hydrocarbon component (Figure 41). This compound is tentatively identified as squalene, a C_{30} branched-chain molecule serving as a precursor for sterol synthesis. This identification is made on the basis of

identical gas chromatographic retention data and preliminary mass spectra interpretation confirming the poly branched nature of this compound. This hydrocarbon constituent represents a small portion of the total lipid components (Table 10).

Following are the results of a detailed fatty acid analysis of the free acids and those incorporated into the complex lipids such as triglycerides, natural methyl esters and polar lipids. All comparisons are with the total fatty acids obtained by hydrolysis as described before.

The benzene eluates contain both the natural esters of long chain fatty acids and triglycerides. The predominant unsaturated esters are oleic (34.4%) and linoleic (21.2%) with the major saturated ester being palmitic (20.1%). Figure 43 illustrates the natural methyl ester distirbution. The relative concentrations of the natural esters are reported in Table 17. The fatty acids incorporated into natural methyl esters follow the same general distribution of the total acids with the exception of arachidic acid (Table 17). Methyl arachidate was not present in detectable concentrations. A decrease in stearic acids was also observed. There appears to be a slight tendency favoring incorporation of oleic and linoleic acids into natural methyl esters. There is a 44 per cent shift of the saturated to unsaturated acid ratio from 1.0 for the total acids to 0.56 for the methyl esters (Table 18). Methyl esters are the only complex lipids in which detectable concentrations of lignoceric acid was found.

TABLE 16. RELATIVE PER CENT DISTRIBUTION OF *RHIZOPUS ARRHIZUS* MYCELIAL LIPID COMPONENTS.

LIPID CLASS	PER CENT COMPOSITION
Hydrocarbons	< 1.0
Free Fatty Acids	16.7
Fatty Acid Methyl Esters	4.1
Triglycerides	22.1
Polar Lipids	44.4
Sterols	16.7
Total lipid weight mg/g wet weight mycelia	5.3

FIGURE 40. Thin-layer chromatographic separation of the extractable lipid components of *Rhizopus arrhizus* mycelia. Solvent system: Heptane:ethyl ether:acetic acid (85:20:2). (A) polar lipid; (B) cholesterol; (C) fatty acid; (D) triglyceride; (E) methyl ester of fatty acid; (F) hydrocarbon; sample.

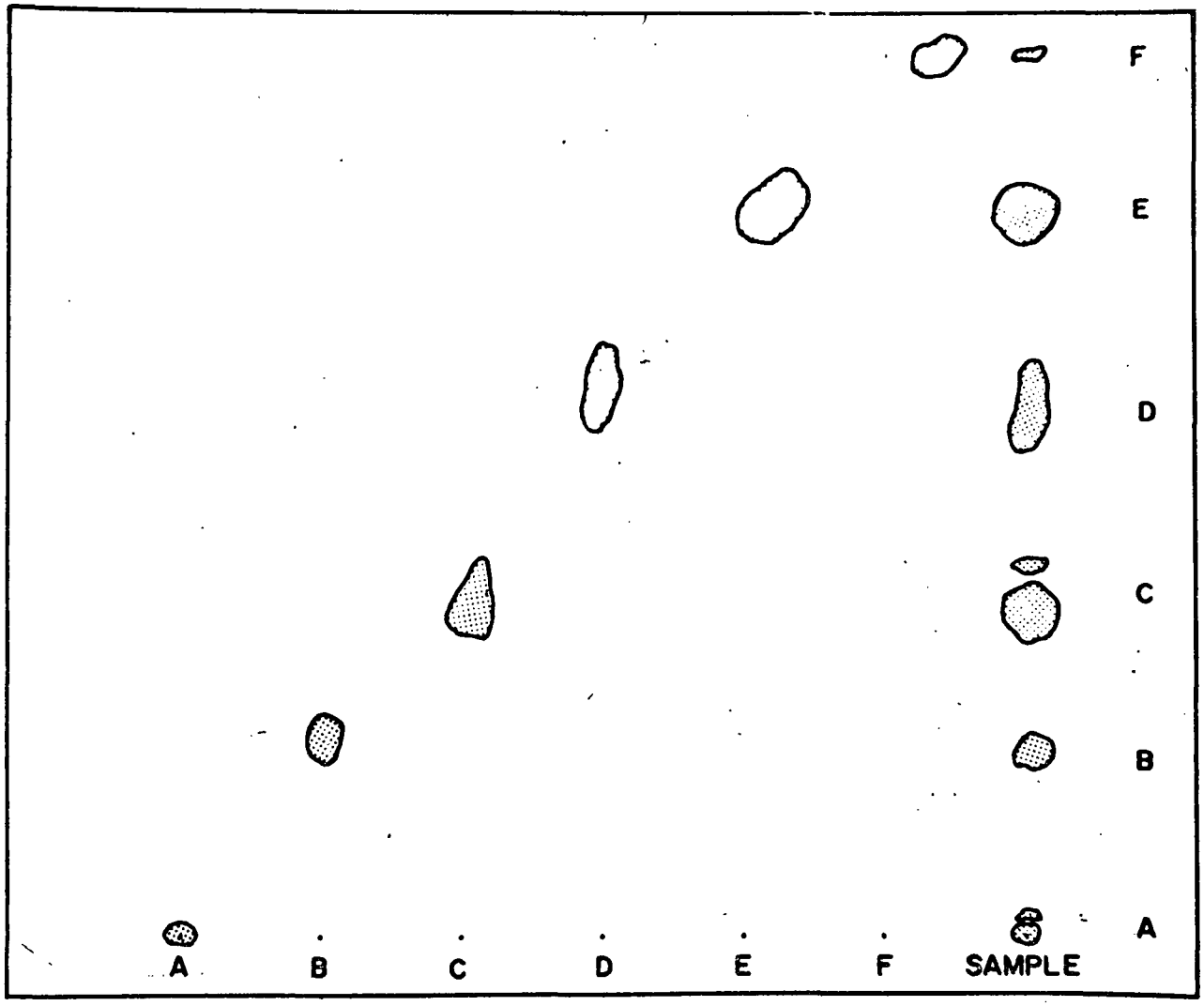

F
E
D
C
B
A
A
B
C
D
E
F
SAMPLE

FIGURE 41. A gas chromatogram illustrating the single hydrocarbon component in the heptane fraction of *Rhizopus arrhizus* mycelia. The analysis was carried out on a Perkin-Elmer gas chromatograph equipped with a 60 ft. x 0.03 in. stainless steel tubing coated with OV-1. Temperature was programed from 100° C to 250° C. No split. Helium pressure 10 psi.

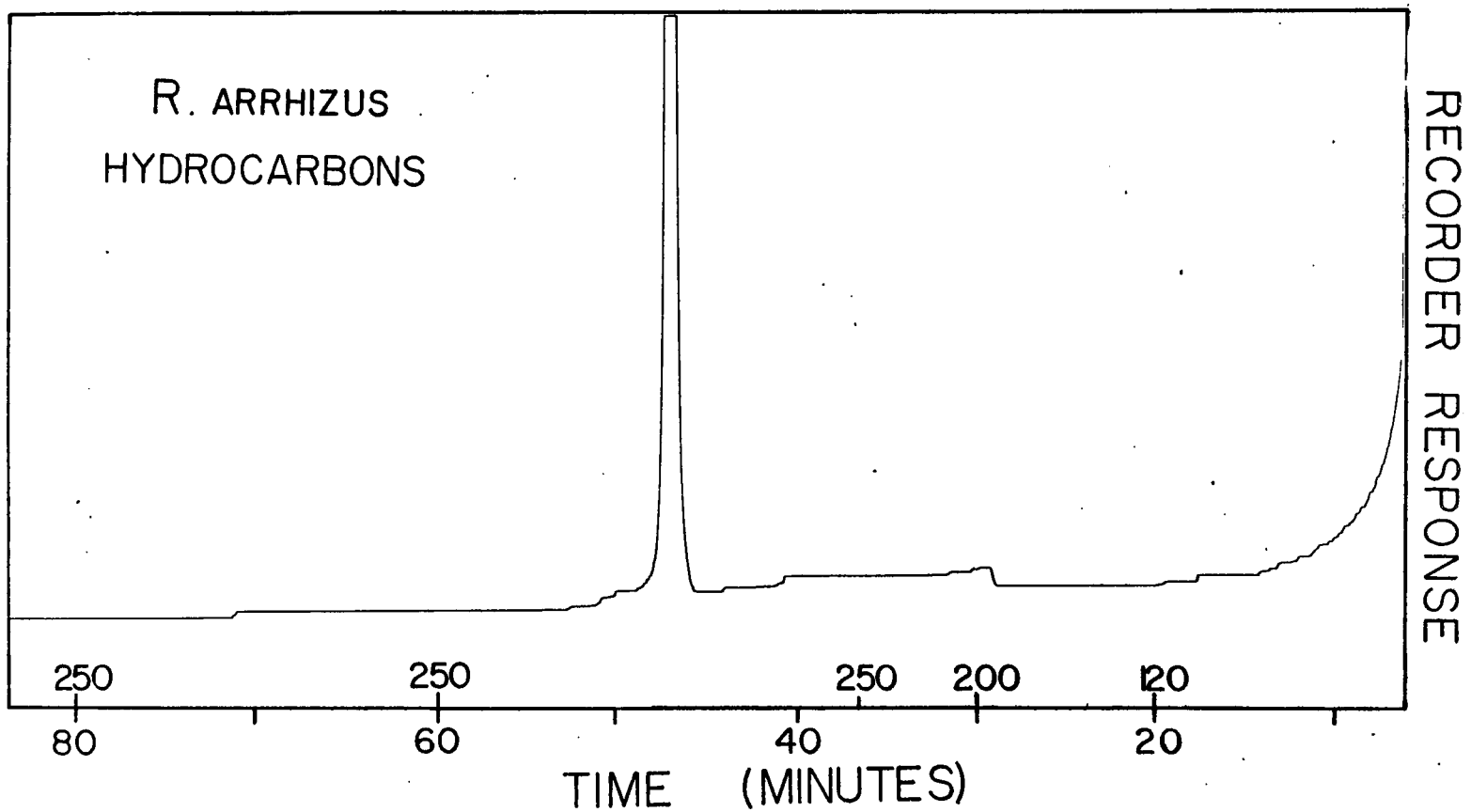
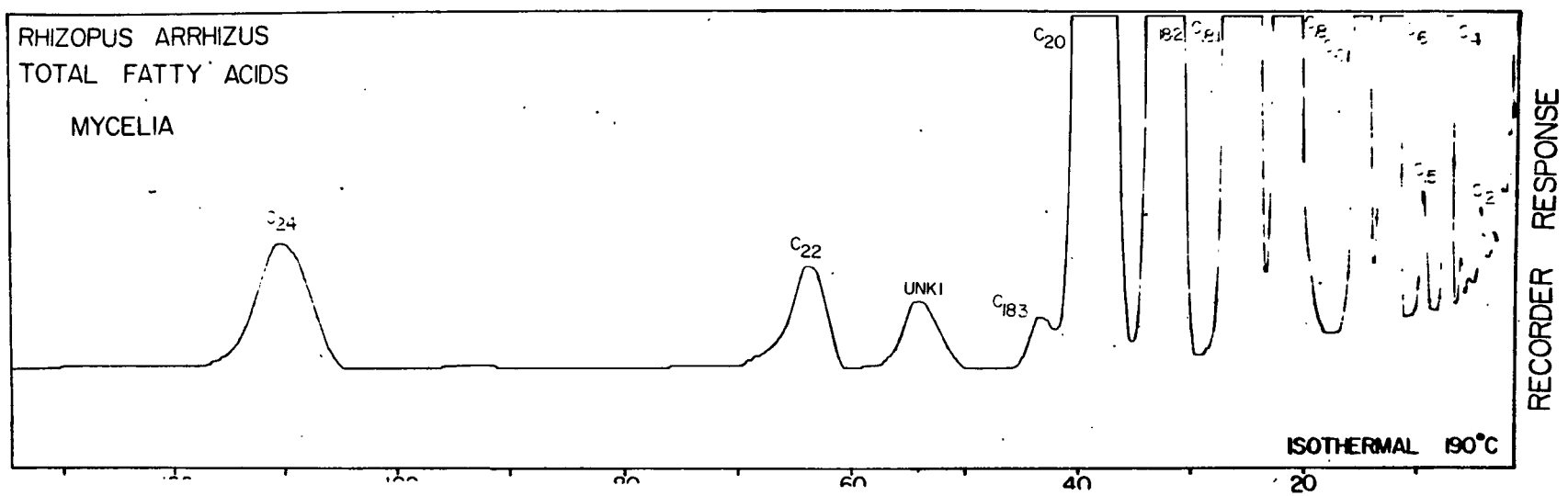

R. ARRHIZUS
HYDROCARBONS
RECORDER RESPONSE
250
250
250
200
120
80
60
40
20
TIME (MINUTES)

FIGURE 42. A gas chromatographic separation of the total fatty acids from lipid hydrolysates of Rhizopus arrhizus mycelia. Analysis was carried out on a Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel tubing packed with Chromosorb W coated with Diethylene glycol succinate. Temperature remained 190° C isothermal. Helium pressure 42 psi. No split.


RHIZOPUS ARRHIZUS
TOTAL FATTY ACIDS
MYCELIA
C24
C22
UNK1
C183
C20
ISOTHERMAL 190°C
RECORDER RESPONSE
40
20

Triglycerides of the benzene eluates were separated by thin-layer chromatography and hydrolyzed to obtain the fatty acid constituents (Figure 44). Oleic (43.1%) appears to be the major unsaturated acid in significant concentrations whereas palmitic (20.5%), stearic (24.5%), and arachidic (3.9%) are the significant saturated acids. There seems to be a slight tendency for the saturated acids to be incorporated into triglycerides (Table 18).

The methanol fraction contains primarily the free fatty acids, polar lipids and sterols. The polar lipids consist mainly of phospholipids. These three lipid classes were separated by thin-layer chromatography as before. No further analysis was carried out with the sterol isolate, but the free fatty acids (Figure 45) and fatty acids of the polar lipid hydrolysates (Figure 46) were separated and identified. The free fatty acids represent a considerable proportion of the lipid constituents (Table 16). Almost two fold increases in stearic (21.6%) and oleic (41.5%) acids are observed along with a considerable decrease in linoleic (6.8%) acid as compared with the total fatty acid distribution (Table 17). Although large shifts in the distribution of free fatty acids occur in comparison with the total acids, only a 9 per cent shift in the saturated to unsaturated ratio is observed. The free acids had a 0.91 ratio compared to that of 1 for the total acids (Table 18).

The fatty acid distribution obtained from polar lipid hydrolysates showed the most significant change from the total acid distribution. Oleic is the predominant unsaturated acid at a relative concentration of 44.0 per cent, showing an approximate 1.5 fold increase over that of the

FIGURE 43. A gas chromatographic separation of the natural methyl esters of long chain fatty acids extracted from mycelia of Rhizopus arrhizus. Separation was carried out on a Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel tubing packed with Chromosorb W coated with Diethylene glycol succinate. The temperature was isothermal at 190° C. No split. Helium pressure was 42 psi.

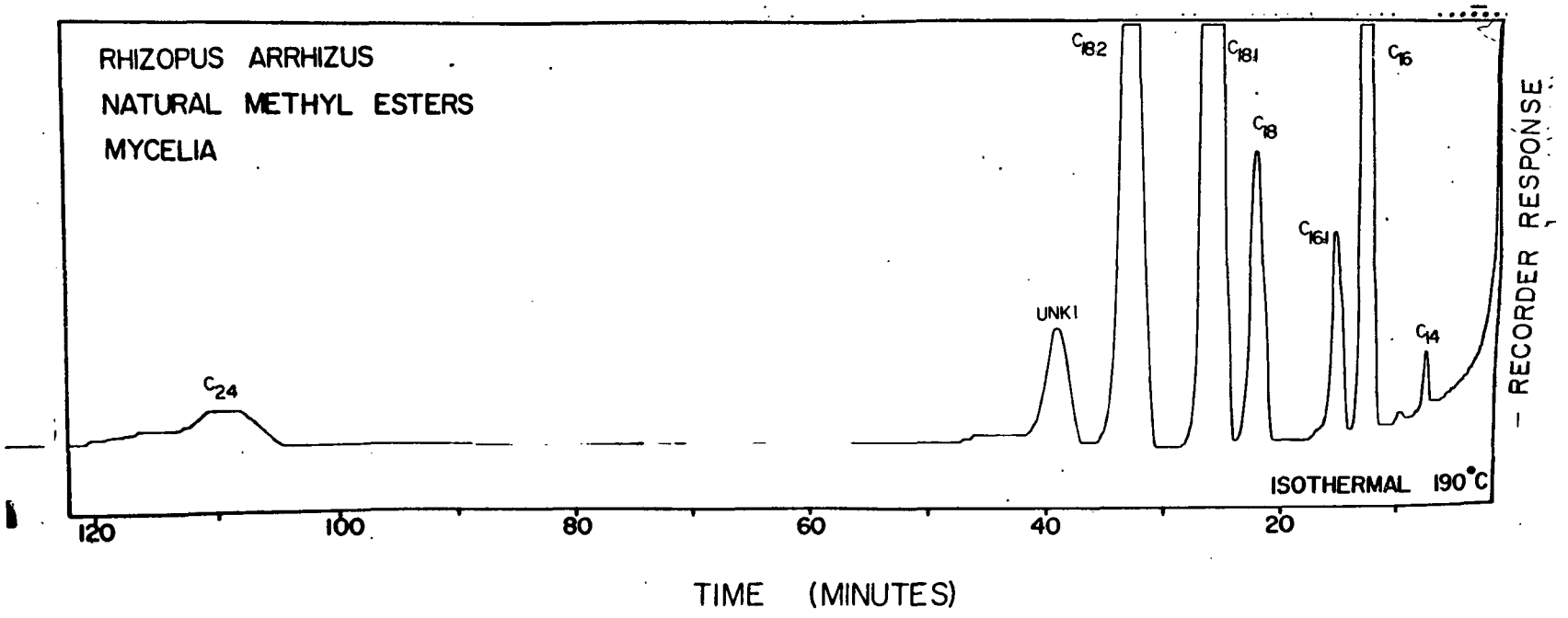
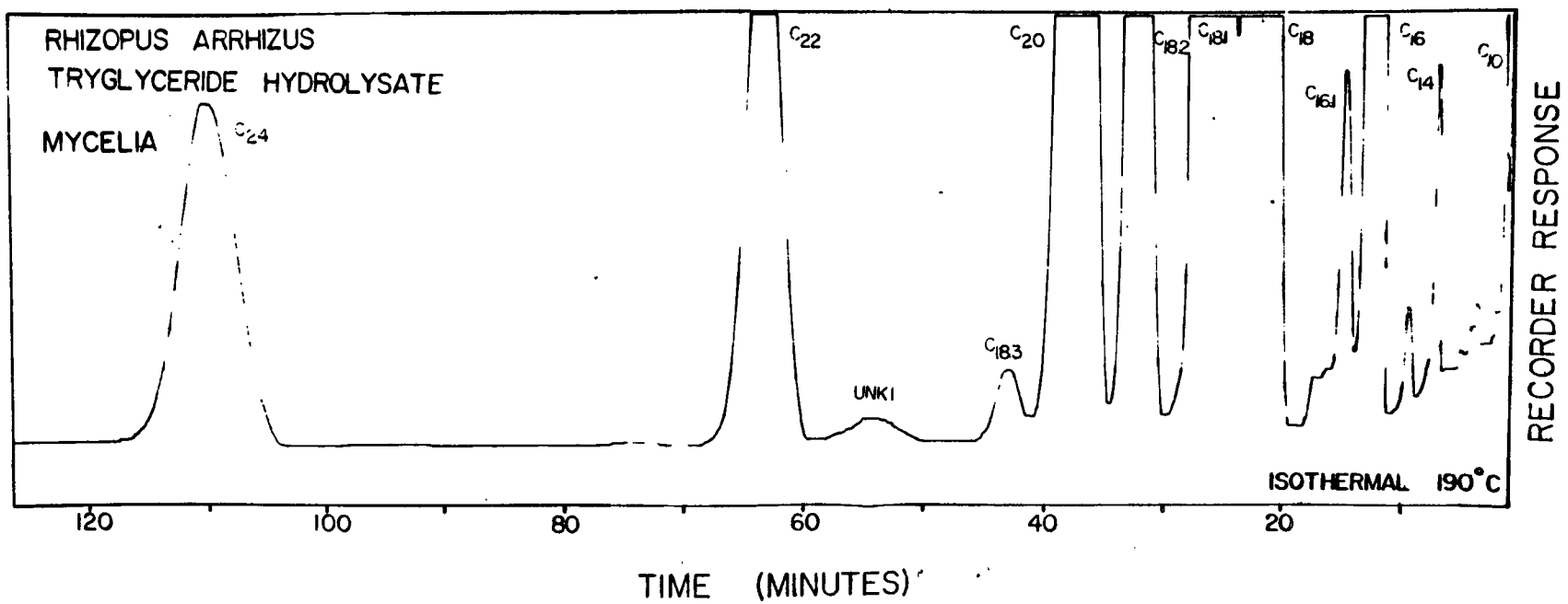

RHIZOPUS ARRHIZUS
NATURAL METHYL ESTERS
MYCELIA
C24
UNK1
C182
C181
C18
C161
C16
C14
ISOTHERMAL 190°C
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 44. A gas chromatographic separation of the fatty acids from hydrolysates of the triglycerides of lipid extracts of Rhizopus arrhizus mycelia. Separation was carried out on a Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel tubing packed with Chromosorb W coated with Diethylene glycol succinate. The temperature was isothermal at 190° C. No split. Helium pressure was 42 psi.


RHIZOPUS ARRHIZUS
TRYGLYCERIDE HYDROLYSATE
MYCELIA
C24
C22
UNK1
C183
C20
C182
C181
C18
C161
C16
C14
C10
ISOTHERMAL 190°C
120
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

total acid (Table 17). A decrease in stearic acid was observed. The acids incorporated into the polar lipid fraction shows the greatest shift in the saturated to unsaturated ratio from 1.0 for the total acids to 0.39 (Table 18).

In all cases oleic acid was the major unsaturated constituent in variable concentrations from one lipid class to the other. Palmitic acid was the predominant saturated acid showing little variation in relative concentrations from one lipid class to the other (Table 17).

The values given in Table 17 for the unknown compound, designated Unknown No. 1, represents the same constituent in each lipid class. It appears that in only the methyl ester (5.5%) and polar lipid (8.5%) fractions does it make a significant contribution to the acid fraction.

To contrast the distribution of total fatty acids in mycelial and sporangiospore material, the total acids from hydrolysates of spore lipids were prepared. The proportion of minor constituents is very similar for the mycelia and spore total acids (Table 19). The relative concentrations of palmitic acid were also similar for spore (16.8%) and mycelial (18.4%) material. There is considerable variability among C_{18} acids. Stearic and oleic acid concentrations of the spore samples increased considerably over that of the mycelia with relative values of 19.9 and 42.4 per cent respectively. Values for the same components of mycelia are 11.0 and 29.4 per cent respectively. Oleic acid is the predominant component for each sample. Linoleic and arachidic acids are in almost equal concentrations in both samples while the concentrations of both acids are decreased by approximately one-half in the spore samples (Table 19).

FIGURE 45. A gas chromatographic separation of the free fatty acids obtained from lipid extracts of *Rhizopus arrhizus* mycelia. Separation was carried out on a Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel tubing packed with Chromosorb W coated with Diethylene glycol succinate. The temperature was isothermal at 190° C. No split. Helium pressure was 42 psi.

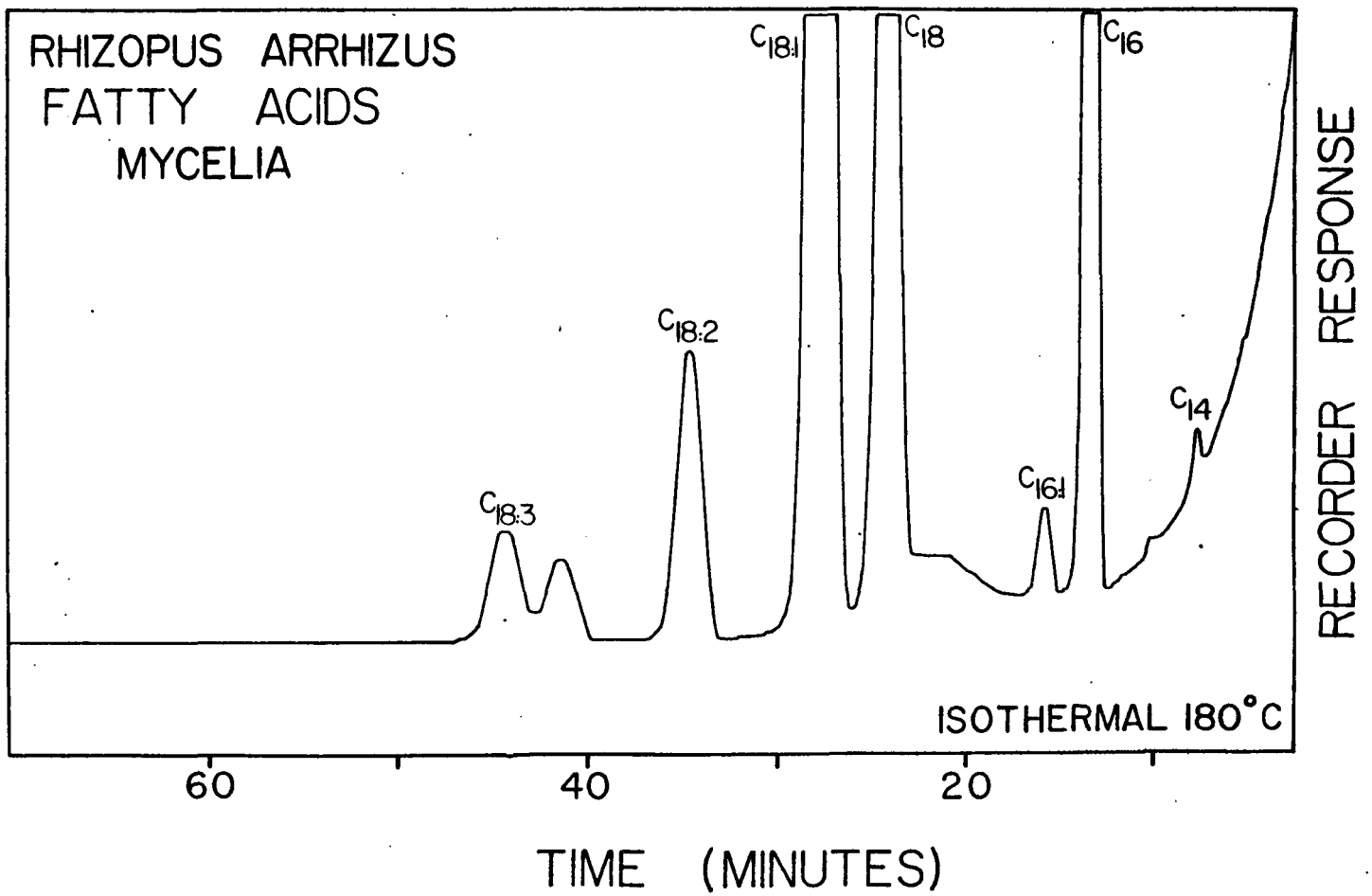
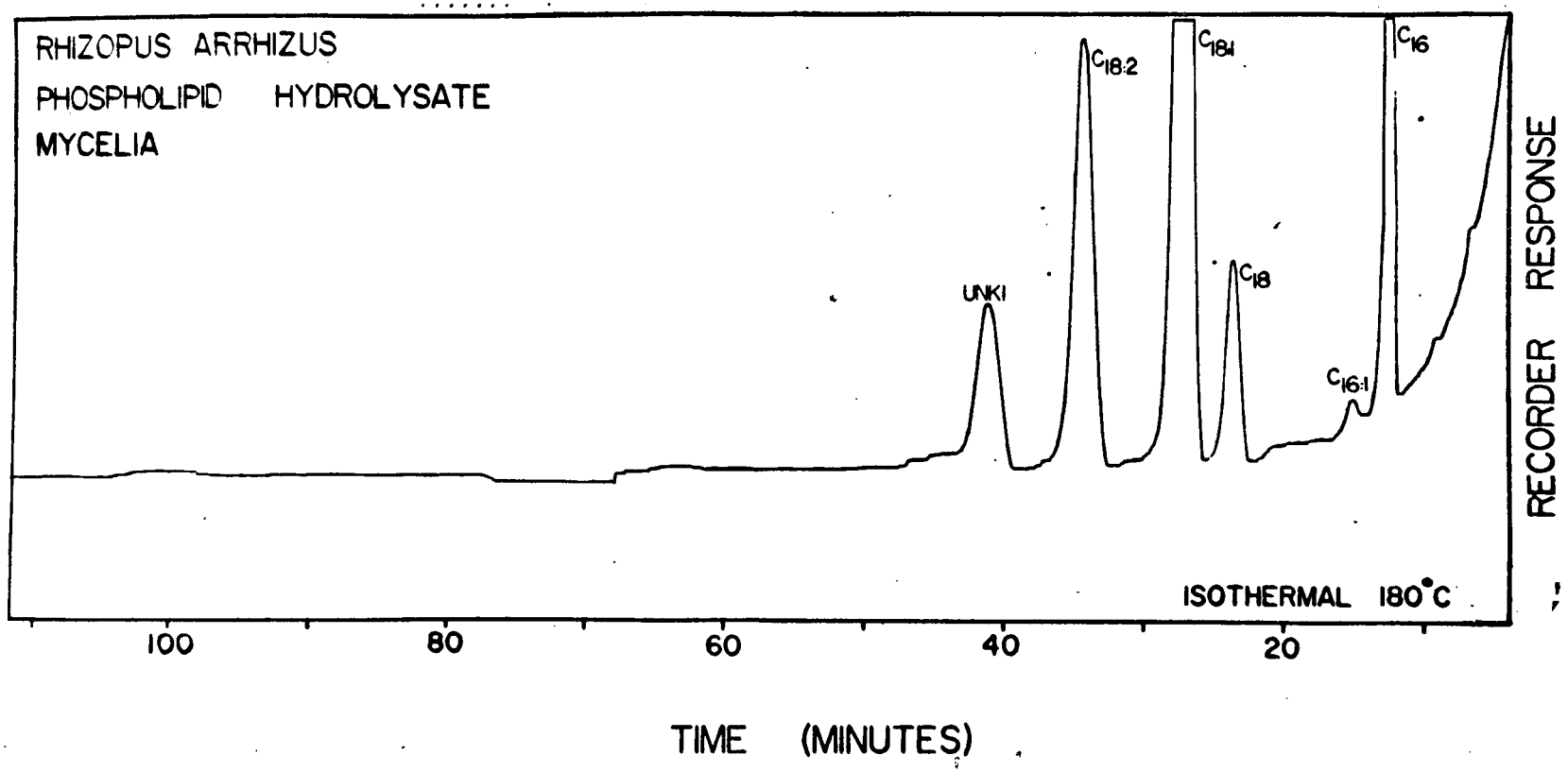

RHIZOPUS ARRHIZUS
FATTY ACIDS
MYCELIA
C18:1
C18
C16
C18:2
C14
C16:1
C18:3
ISOTHERMAL 180°C
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

FIGURE 46. A gas chromatographic separation of the fatty acids obtained from hydrolysis of polar lipid extracts of Rhizopus arrhizus mycelia. Separation was carried out on a Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel tubing packed with Chromosorb W coated with Diethylene glycol succinate. The temperature was isothermal at 190° C. No split. Helium pressure was 42 psi.


RHIZOPUS ARRHIZUS
PHOSPHOLIPID HYDROLYSATE
MYCELIA
UNKI
C18:2
C18:1
C18
C16:1
C16
ISOTHERMAL 180°C
100
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

The same unknown saponifiable compound reported for lipid classes of mycelia is also found in spore total fatty acids (Table 19). The gas chromatographic separation of the spore total fatty acids is illustrated in Figure 47.

Analysis of the culture media both before and after incubation revealed that no methyl esters were present. Thus, methyl esters are not transported into the fungus during growth and they are not secreted into the medium.

TABLE 17. RELATIVE PER CENT DISTRIBUTION OF THE FREE AND TOTAL FATTY ACIDS AND THOSE BOUND AS METHYL ESTERS, TRIGLYCERIDES, AND POLAR LIPIDS EXTRACTED FROM MYCELIA OF RHIZOPUS ARRHIZUS.

FATTY ACID* CHAIN LENGTH	LIPID CLASSES				
	Total Fatty Acids	Free Fatty Acids	Methyl Esters (Natural)	Triglycerides	Polar Lipids
C_{10}	tr	-	-	-	-
C_{12}	tr	-	-	-	-
C_{14}	1.2	tr	3.0	0.3	-
$C_{14:1}$	-	-	-	-	-
C_{15}	0.2	-	0.2	tr	-
C_{16}	18.4	22.8	20.1	20.5	17.9
$C_{16:1}$	3.7	2.6	4.4	0.8	4.1
C_{17}	-	-	-	tr	-
C_{18}	11.0	21.6	6.3	24.5	7.7
$C_{18:1}$	29.4	41.5	34.4	43.1	44.0
$C_{18:2}$	16.3	6.8	21.2	5.5	17.5
$C_{18:3}$	0.2	-	-	-	-
C_{20}	16.2	3.0	-	3.9	-
$C_{20:1}$	-	-	-	-	-
C_{21}	-	-	-	-	-
C_{22}	0.9	-	-	1.0	-
$C_{22:1}$	-	-	-	-	-
C_{24}	1.7	-	3.8	tr	tr
Unknown	0.7	2.2	5.5	1.8	8.5

*Bound fatty acids were obtained by hydrolysis and analyzed as methyl esters.

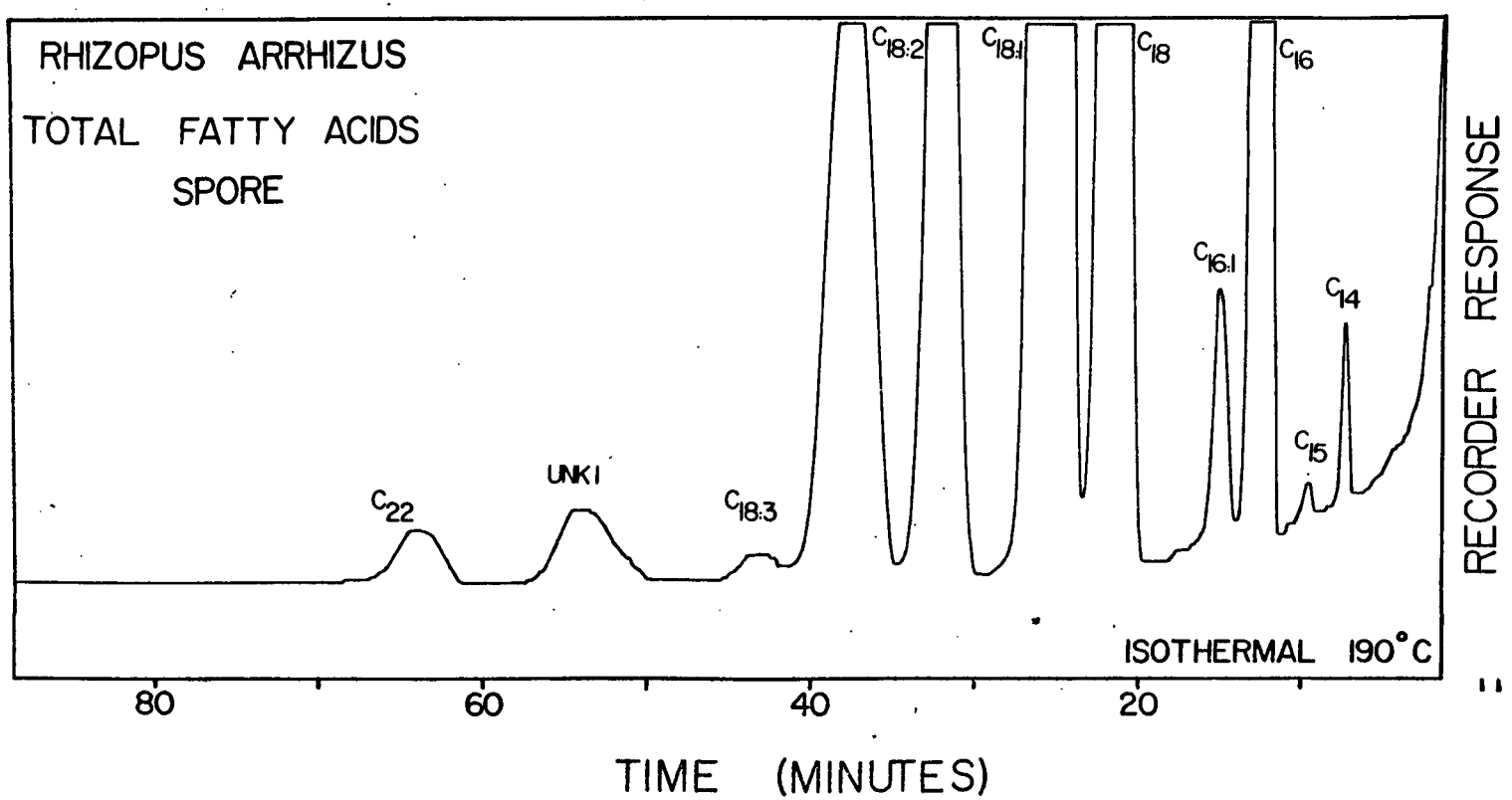
TABLE 18. SATURATED TO UNSATURATED COMPARISON OF THE TOTAL, FREE, AND FRACTIONATED LIPID FATTY ACIDS OF *RHIZOPUS ARRHIZUS*.

LIPID CLASSES	SATURATED	UNSATURATED	RATIO
Total Fatty Acids	49.6	49.6	1
Free Fatty Acids	47.4	50.9	0.91
Methyl Esters (Natural)	33.6	60.0	0.56
Triglycerides	50.2	49.4	1.21
Polar Lipids	25.6	65.6	0.39

TABLE 19. A COMPARISON OF THE TOTAL FATTY ACIDS OF EXTRACTABLE LIPID HYDROLYSATES FROM MYCELIA AND SPORANGIOSPORES OF *RHIZOPUS ARRHIZUS*.

FATTY ACID CARBON CHAIN LENGTH	MYCELIA %	SPORE %
C_{14}	1.2	1.1
$C_{14:1}$	-	-
C_{15}	0.2	0.8
C_{16}	18.4	16.8
$C_{16:1}$	3.7	1.7
C_{17}	-	-
C_{18}	11.0	19.9
$C_{18:1}$	29.4	42.4
$C_{18:2}$	16.3	7.7
$C_{18:3}$	0.2	0.1
C_{20}	16.2	6.9
C_{22}	0.9	-
C_{24}	1.7	-
Unknown No. 1	0.7	0.3

FIGURE 47. A gas chromatographic separation of total fatty acids obtained by alkaline hydrolysis of lipid extracts of Rhizopus arrhizus sporangiospores. Separation was carried out on a Perkin-Elmer gas chromatograph equipped with a 10 ft. x 1/8 in. stainless steel capillary tubing packed with Chromosorb W coated with Diethylene glycol succinate. Temperature was 190° C isothermal. Helium pressure was 42 psi. No split.


RHIZOPUS ARRHIZUS
TOTAL FATTY ACIDS
SPORE
C22
UNK I
C18:3
C18:2
C18:1
C18
C16:1
C16
C15
C14
ISOTHERMAL 190°C
80
60
40
20
TIME (MINUTES)
RECORDER RESPONSE

DISCUSSION

The lipid classes and fatty acids obtained in the extractable lipids from mycelia of Rhizopus arrhizus are reported here. The relative distribution of the lipid classes given is of little significance because their relative proportion is a function of culture conditions such as age, temperature, etc. (62). Of greatest importance is the recognition of the major lipid classes present in mycelia of R. arrhizus. Included are some classes not previously reported, such as the natural methyl esters, sterols, and hydrocarbons.

The fatty acid results given here are consistant with those previously reported for this organism; however, early reports did not show the presence of acids above the polyunsaturated C_{18} level (62, 63). Mycelial lipid analyses reported here illustrate the presence of arachidic (C_{20}), behenic (C_{22}), and lignoceric (C_{24}) acids. Previous articles also report the presence of gamma-linolenic acid as characteristic of the class Phycomycete (63). The presence of this positional isomer in contrast to the typical alpha-linolenic acids is not confirmed in this study. However, it is possible that the unknown compound found in all fatty acid fractions of R. arrhizus in this study could be gamma-linolenic acid. This accounts for the lower concentrations of $C_{18:3}$ reported here. The elution sequence for linoleic acid and Unknown No. 1 reported here are consistant with previous gas chromatographic separations. The Unknown No. 1 component

immediately follows $C_{18:3}$ having a retention time 8 minutes longer than the latter.

This investigation presents for the first time in R. arrhizus the presence of natural methyl esters of long chain fatty acids. This represents the second report of a fungal organism containing esters of this type. The first report is presented in Section I of this dissertation (36). There does not appear to be a widespread distribution of the natural methyl esters of long chain fatty acids in fungal organisms. Esters of this type are found here in concentrations less than 5 per cent; however, age of the culture affects this proportion (36). This may account for their not being previously reported. Only two of the organisms of this investigation, Ustilago maydis and Rhizopus arrhizus, are found to produce the natural methyl esters. Methyl esters of long chain fatty acids have also been reported in the pollen of Zea mays (17). Because U. maydis is a parasite of corn, some question is raised as to whether the esters are produced by the parasite or absorbed from the host. Unreported results in this study fail to detect the presence of natural methyl esters in the immature male inflorescence of corn. To further determine the distribution of natural methyl esters of long chain acids, a systematic study of the mycelia of fungal organisms should be made.

The role of methyl esters of long-chain fatty acids in fungal metabolism is uncertain. Stowe (65, 66) found growth promotion by methyl esters (saturated and unsaturated) in the split pea bioassay above that obtained by plant hormones. He also found the same re-

sponse to a lesser degree to triglycerides such as triolein and monoolein. He suggests their normal plant growth regulation may be exerted through a synergistic effect on hormones rather than as hormones themselves.

In comparing the incorporation of fatty acids into their respective methyl esters, very similar relative concentrations are found in both U. maydis and R. arrhizus. The major difference is linoleic acid with larger concentrations being found in R. arrhizus. Also, greater concentrations of odd chain acids and esters are present in U. maydis (Section II).

Generally, oleic appears to be the predominant component of the free fatty acids and complex lipids. Palmitic remains in relatively stable concentrations in all the lipid classes and is remarkably consistant with other reports (62, 63). The other major acids tend to be somewhat variable from one lipid class to another.

Preference appears to be given the unsaturated acids by the fungus, favoring their incorporation into the natural esters and polar lipids. Considering the effect of methyl esters on pea stem growth, the unsaturated esters gave the most significant results (65, 66). Favoring incorporation of the unsaturated acids into the polar lipids is consistant with the polar nature of the primary constituents of this fraction (phospholipids).

The distribution of total fatty acids in the spores and mycelia of R. arrhizus shows many similarities. The large concentrations of oleic and small concentrations of linoleic acid are consistant with

results of spore analysis of several fungal organisms in this study. The low concentrations of linolenic acid are also consistant with the spores of almost all of the fungal organisms analyzed here.

These consistencies remain true even though environmental conditions are completely different for the mycelia and spore cultures. Because of the nature of shake culture conditions, the mycelia remains completely submerged in the medium prior to harvesting. On the other hand, for sporulation to occur the mycelia must remain at the liquid: air surface several days prior to harvesting.

XV.

SECTION VI

PRELIMINARY STUDIES ON THE INCORPORATION OF ^{14}C-LABELED FATTY ACIDS INTO THE LIPIDS OF *RHIZOPUS ARRHIZUS*

INTRODUCTION

Studies on the content and distribution of lipids from the spores and mycelia of *Rhizopus arrhizus* are reported in previous sections. This section reports the results obtained in investigations on the incorporation of ^{14}C-labeled acetate, stearate, and myristate into the lipid components of *R. arrhizus*. Of primary interest is ^{14}C-label found in the natural methyl esters of long chain fatty acids from the mycelia of this organism. These lipid components represent a potential problem for biosynthesis. The natural methyl esters identity has been confirmed by mass spectrometry and the incorporation of precursors for their synthesis will be examined in more detail.

RESULTS

Rhizopus arrhizus was grown on Czapek-Dox solid media in the presence of sodium acetate-1-^{14}C. The cultures were allowed to incubate 3 weeks to ensure complete sporulation and maturation. It was assumed that an equilibrium in the relative proportions of lipid components was reached at this age.

The total lipid extract was partially fractionated into the major lipid classes by silica gel column chromatography resulting in three fractions: the n-heptane fraction containing the hydrocarbons, benzene fraction containing the triglycerides and natural methyl esters, and the methanol fraction. The methanol fraction containing the polar lipids (phospholipids) was further fractionated by thin-layer chromatography. Approximately 0.23 per cent of the total radioactivity off the Rhizopus cultures was found in the mycelial lipid extracts. The thin-layer chromatogram in Figure 48 illustrates the major lipid classes of mycelia of R. arrhizus and the distribution of radioactive components in each of the fractions. There was not a significant incorporation of acetate-1-^{14}C into the hydrocarbon component of the heptane fraction (Table 20) which contained approximately 0.03 per cent label of the lipid extract. No radioactivity was detected in this fraction as assayed by thin-layer chromatography-autoradiography (Figure 48).

The benzene fraction contained approximately 22.0 per cent of the ^{14}C found in the lipid components (Table 20). The primary constituents of this fraction are the natural methyl esters of long chain fatty acids and the triglycerides (Figure 48 B and A respectively). Both of these components contained ^{14}C-label (Table 21). Only 18 per cent of the total radioactivity of the benzene fraction could be detected. Calculations indicated that 25.4 per cent of the benzene fraction was found to be the natural methyl esters and the remaining 74.6 per cent were triglyceride compounds (Table 21). Using the radioactivity data in Tables 20 and 21, the relative per cent distribution of the major lipid classes were computed (Table 22). The values obtained were consistant with a few exceptions. The major difference was in the free fatty acid content (9.6%) which was approximately 35 per cent less than values obtained in the previous section (Section V). The radioactivity of the polar lipids was not determined but the value obtained for the remainder of the activity corresponds to that calculated for the polar lipids of Section V (Table 22).

The methanol fraction contained the polar lipids (origin), sterols (C), and free fatty acids (D) all of which contained ^{14}C-label (Figure 48). The free fatty acids represented approximately 17.3 per cent of the methanol fraction (Table 21) and 9.6 per cent of the total lipid fraction (Table 21). The sterols were approximately 12.2 per cent of the methanol fraction (Table 22) and 13.5 per cent of the total lipid

FIGURE 48. Diagram illustrating the separation of three lipophilic fractions of Rhizopus arrhizus mycelia and the location of standard compounds. The mesh spots indicate the location of the radioactive components and the circles indicate the location of the non-radioactive components and standards. [(1) stearyl alcohol, (2) heptane fraction, (3) benzene fraction, (4) methanol fraction, (5) palmitic acid, (6) tripalmitin, (7) cholesterol, (8) methanol fraction, (9) acetate-1-^{14}C].

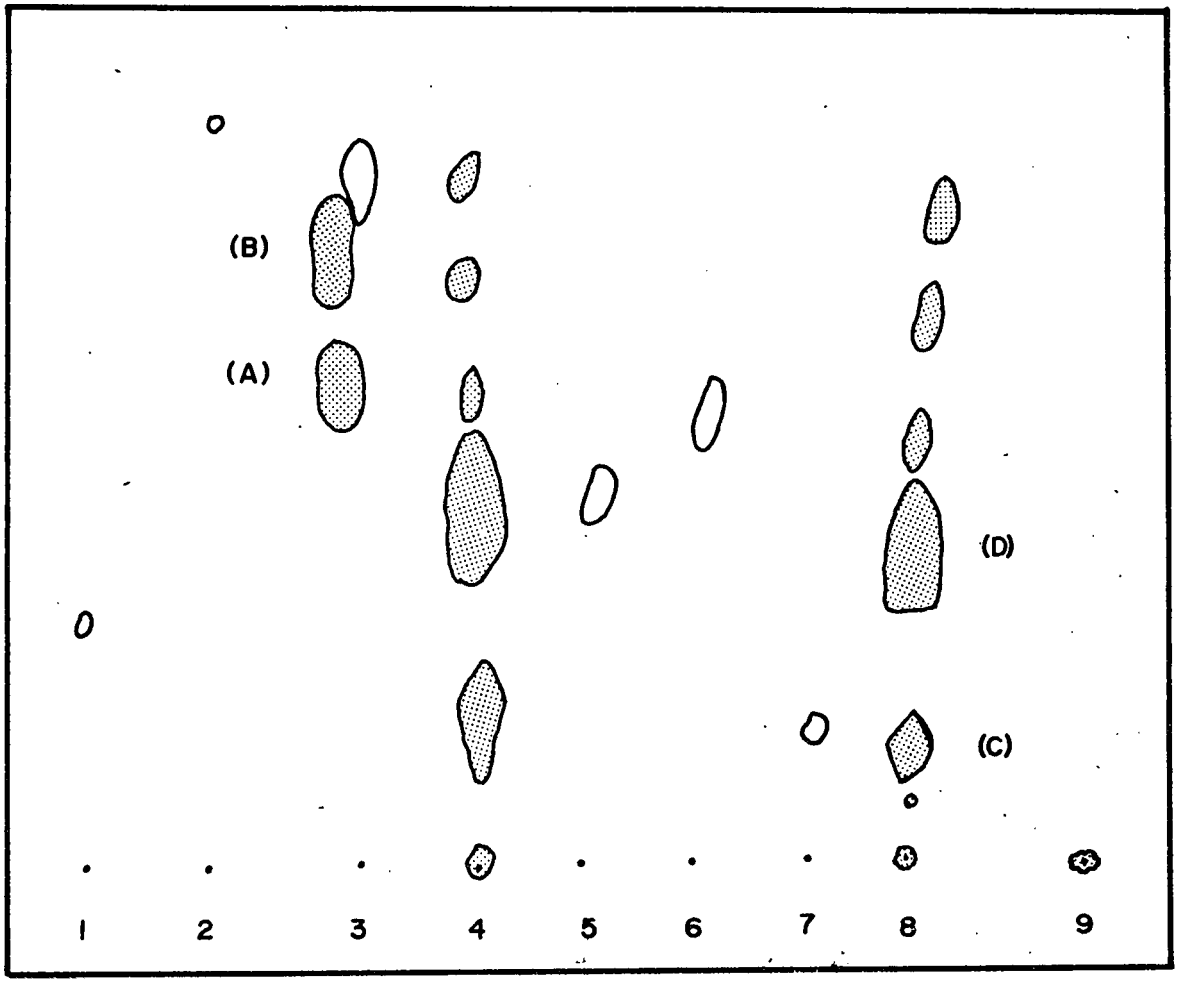

(B)
(A)
(D)
(C)
1
2
3
4
5
6
7
8
9

TABLE 20. TOTAL RADIOACTIVITY FROM THE HEPTANE, BENZENE, AND METHANOL ELUATES FROM MYCELIAL LIPID EXTRACTS OF *RHIZOPUS ARRHIZUS*.

FRACTION	CPM+	BKG*	CORRECTED COUNT (CPM)	DILUTION FACTOR	TOTAL COUNT (CPM)	PER CENT RADIOACTIVITY PER FRACTION (%)
Heptane	57	26	31	2.5	77	0.03
Benzene	4704	26	4677	10	46774	22.0
Methanol	16572	26	16545	10	165448	78.0

*Bkg - Background

+Cpm - Counts per minute

TABLE 21. RADIOACTIVITY OF THE INDIVIDUAL LIPID CLASSES IN THE BENZENE AND METHANOL FRACTIONS ISOLATED FROM A THIN-LAYER CHROMATOGRAM.

SPOT	FRACTION	CPM	BKG	CORRECTED COUNT (CPM)	DILUTION FACTOR	TOTAL COUNT (CPM)	PER CENT OF FRACTION (%)
A	Benzene	238	25	214	10	2134	25.4
B	"	651	25	626	10	6262	74.6
C	Methanol	597	25	572	10	5720	17.3
D	"	439	25	414	10	4140	12.2

TABLE 22. RELATIVE PER CENT DISTRIBUTION OF THE MAJOR LIPID CLASSES CALCULATED FROM RADIOACTIVITY DATA.

LIPID CLASS	RADIOACTIVE DATA (%)	NON-RADIOACTIVE+ DATA (%)
Hydrocarbon	trace	1
Methyl esters (Natural)	5.0	4.1
Triglycerides	16.7	22.1
Free fatty acids	9.6	16.7
Sterols	13.5	16.7
Polar lipids	*	44.5
Other≠	45.2	-

+These were taken from Table .

*The polar lipids were not counted

≠Primarily the polar lipids

fraction (Table 22). The polar lipid radioactivity was not determined. Other radioactive components were present in the methanol eluate two of which correspond to the triglyceride and natural methyl ester spots. These three spots above the fatty acids represent residual material not eluated with the benzene. Sodium acetate-1-^{14}C was also subjected to the same solvent system to illustrate isotope purity (Figure 48).

The incorporation of sodium acetate-1-^{14}C into the lipid fraction was also followed using liquid shake cultures of R. arrhizus. Cultures were harvested at the end of a 36 hour incubation period. Two per cent of the total radioactivity was recovered in the extractable lipid components when ^{14}C-labeled acetate was added to the culture medium. The greatest amount of the recovered radioactivity was in the polar lipids which contained 39.4 per cent (Table 23). The lipid class containing the next highest amount of ^{14}C-label were the sterols with 25.8 per cent. The hydrocarbons and natural methyl esters contained the lowest activity with 2.6 and 0.2 per cent respectively (Table 23).

Stearic-18-^{14}C acid was fed to Rhizopus cultures under the same conditions. Approximately 26.5 per cent of the total radioactivity was recovered in the extractable lipids from mycelia grown in the stearate. The triglyceride fraction contained the greatest amount of ^{14}C-label with 40.2 per cent followed by the free fatty acids with 22.8 per cent (Table 24). The polar lipid and sterol fractions contained similar amounts of activity with 18.5 and 17.5 per cent respectively. The natural methyl ester and hydrocarbon fractions contained very low relative incorporation with 0.3 and 0.35 per cent respectively.

TABLE 23. DISTRIBUTION OF ^{14}C-LABEL IN THE MAJOR LIPID CLASSES OF *RHIZOPUS ARRHIZUS* MYCELIA GROWN ON LIQUID SHAKE CULTURES WITH SODIUM ACETATE-1-^{14}C.

LIPID FRACTION	CPM	BKG	CORRECTED COUNT (CPM)	DILUTION FACTOR	TOTAL RADIOACTIVITY (CPM)	PER CENT TOTAL
Natural Methyl Ester	47	25	22	10	220	0.2
Triglyceride	2341	25	2316	10	23160	17.9
Free Fatty Acid	1814	25	1789	10	17890	13.8
Sterol	3393	25	3368	10	33680	25.8
Hydrocarbon	366	25	341	10	3410	2.6
Polar Lipid	5148	25	5123	10	51230	39.4

TABLE 24. DISTRIBUTION OF ^{14}C-LABEL IN THE MAJOR LIPID CLASSES OF RHIZOPUS ARRHIZUS MYCELIA GROWN ON LIQUID SHAKE CULTURE IN THE PRESENCE OF STEARIC-18-^{14}C ACID.

LIPID FRACTION	CPM	BKG	CORRECTED COUNT (CPM)	DILUTION FACTOR	TOTAL RADIOACTIVITY (CPM)	PER CENT TOTAL
Natural Methyl Ester	163	25	138	10	1380	0.3
Triglyceride	18312	25	18287	10	182870	40.2
Free Fatty Acid	10478	25	10453	10	104530	22.8
Sterol	8012	25	7987	10	79870	17.5
Hydrocarbon	204	25	179	10	1790	0.35
Polar Lipids	8487	25	8462	10	84620	18.5

An experiment was conducted to compare the incorporation of Stearic-18-^{14}C and myristic-1-^{14}C acids into lipids of R. arrhizus mycelia in the presence and absence of methanol. The mycelia were grown in liquid shake cultures for 36 hours. Approximately 1.3 per cent of the total radioactivity was recovered in the lipid fraction. Radioactivity was found predominantly in the free C_{18} acid in relative amounts of 59.1 per cent. The greatest amount of activity was incorporated into the polar lipids and triglycerides with percentages of 25.5 and 9.6 respectively (Table 25). Again low relative amounts of activity were found in the hydrocarbon and natural methyl ester fractions with percentages of 1.5 and 3.3 respectively.

When 0.1 ml of 100 per cent methanol was added to the medium there appeared to be a general decrease in the relative incorporation of ^{14}C-labeled stearate into the complex lipids with one exception. There was a 1.5 per cent increase in the natural methyl ester components (Table 25). Approximately 72.4 per cent of the radioactivity was located in the free fatty acid fraction. Only 0.75 per cent of the total activity was in the lipid extract.

The mycelial lipids of R. arrhizus contained a similar distribution of ^{14}C-label when grown in the presence of myristic-1-^{14}C acid. Again the free acid was present in the greatest relative quantities in the cultures with and without the methanol. Remarkable similarities were also noted for the polar lipids and triglycerides (Table 25). Compared to the cultures grown in the presence of stearate, there were

TABLE 25. DISTRIBUTION OF ^{14}C-LABEL IN THE LIPIDS OF *RHIZOPUS ARRHIZUS* MYCELIA GROWN IN THE PRESENCE OF STEARIC-18-^{14}C ACID ALONE AND WITH METHANOL.

LIPID CLASSES	CPM	BKG	CORRECTED COUNT (CPM)	DILUTION FACTOR	TOTAL RADIOACTIVITY (CPM)	PER CENT TOTAL
C_{18}						
Hydrocarbon	94	25	69	5	345	1.5
Natural Ester	174	25	149	5	745	3.3
Triglyceride	458	25	433	5	2165	9.6
Free Fatty Acid	2712	25	2687	5	13435	59.1
Polar Lipid	1171	25	1146	5	5730	25.5
C_{18} + Methanol						
Hydrocarbon	36	25	11	5	55	0.5
Natural Methyl Ester	130	25	105	5	525	4.8
Triglyceride	187	25	162	5	810	7.5
Free Fatty Acid	1603	25	1578	5	7790	72.4
Polar Lipid	339	25	314	5	1570	14.6

smaller relative concentrations of myristate incorporated into the hydrocarbons and natural methyl esters (Table 26).

When fresh mycelia were ground in alumina and incubated in the presence of stearic-18-^{14}C acid alone and with methanol the following results were obtained: the control flask containing the boiled preparation demonstrated essentially no significant amounts of enzymatic activity as illustrated by the low levels of radioactivity detected (Table 27). The incubation flask containing the ^{14}C-labeled stearate without methanol showed similar levels of enzymatic activity illustrated by the similar levels of radioactivity in each of the lipid classes. There was an 8-10 fold increase in radioactivity over that of the control (Table 27). The flask containing the ^{14}C-labeled stearate and methanol maintained the same 8-10 fold increase over the control in each of the lipid classes with the exception of the natural methyl ester fraction. This fraction contained a 32 fold increase in radioactivity over natural ester fraction of the control (Table 27).

TABLE 26. DISTRIBUTION OF ^{14}C-LABELED IN THE LIPIDS OF *RHIZOPUS ARRHIZUS* MYCELIA GROWN IN THE PRESENCE OF MYRISTIC-1-^{14}C ACID ALONE AND WITH METHANOL.

LIPID FRACTION	CPM	BKG	CORRECTED COUNT (CPM)	DILUTION FACTOR	TOTAL COUNT (CPM)	PER CENT TOTAL
C_{14}						
Hydrocarbon	31	25	6	5	30	0.23
Natural Methyl Ester	47	25	22	5	110	0.84
Triglyceride	378	25	352	5	1765	13.5
Free Fatty Acid	1578	25	1553	5	7765	59.5
Polar Lipid	702	25	677	5	3385	25.9
C_{14} + Methanol						
Hydrocarbon	32	25	7	5	35	0.24
Natural Methyl Ester	32	25	7	5	35	0.24
Triglyceride	444	25	419	5	2095	14.4
Free Fatty Acid	1855	25	1830	5	9150	63.2
Polar Lipid	657	25	632	5	3160	21.8

TABLE 27. INCORPORATION OF STEARIC-18-^{14}C INTO THE VARIOUS LIPID CLASSES BY A CRUDE FRESHLY GROUND MYCELIAL PREPARATION FROM *RHIZOPUS ARRHIZUS*.

PREPARATION	TOTAL CPM IN EACH LIPID FRACTION			
	Free Fatty Acid	Methyl Ester	Triglyceride	Polar Lipid
Control (Boiled enzyme)	13801	33	40	35
C_{18} alone	17721	385	298	205
C_{18} + methanol	22327	1087	307	277

DISCUSSION

The results indicate that the incorporation of ^{14}C-labeled acetate by R. arrhizus is typical with respect to lipid metabolism. Radioactivity from the C_1 carbon of acetate is found in the free fatty acids, sterols, triglycerides, polar lipids (primarily phospholipids), hydrocarbons, and natural methyl esters of long chain fatty acids. The acetate is preferentially incorporated into the fatty acids and sterols, with subsequent incorporation of the fatty acids into the phospholipid and triglyceride fractions. They are found to a lesser extent in the natural methyl ester components. Similar incorporation patterns are observed when the fungus is grown in the presence of ^{14}C-labeled acetate under different culture conditions. It is also found that a similar distribution of ^{14}C-label is found in the lipid classes when grown in the presence of stearic-18-^{14}C acid. This similarity would be expected because acetate is incorporated into the triglycerides, phospholipids, and natural methyl esters via the fatty acid route. The notable exception found here is the lower activity level of the sterol fraction. Acetate is not incorporated into the sterol via the fatty acid route. Radioactivity in the sterol fraction can be accounted for through oxidative degradation of the acid moiety to acetate. Acetate may then be incorporated into the sterol moiety through the mevalonic acid pathway (44). A preference of incorporation of the stearate moiety into the triglycerides over that of the phospholipids is noted by

the two fold difference in activities. This is consistant with the fatty acid distribution in the complex lipids of R. arrhizus given in Section V. The mycelial triglycerides show a three fold greater concentration of the saturated stearic acid (C_{18}) over that of the phospholipids.

The low levels of radioactivity in the hydrocarbon fraction are consistant with the low concentration of the single component found there (Section V). The exact structure of this hydrocarbon component has not been confirmed by mass spectrometry. However, gas chromatographic data reveals that it has a retention time identical with that of squalene. The structure of squalene, a highly branched unsaturated C_{30} molecule, is consistant with preliminary mass spectra interpretation. Thus, the single hydrocarbon component of R. arrhizus incorporates acetate through the mevalonic acid pathway leading to the synthesis of sterols. Low levels of radioactivity were found in the hydrocarbon fraction subsequent to incubation with ^{14}C-labeled fatty acids. This can be explained by the oxidative degradation of the fatty acid molecule to the acetate level followed by incorporation via the mevalonic acid pathway.

Of primary interest are the natural methyl esters of long chain fatty acids reported here for the first time in R. arrhizus. Studies involving the synthesis of esters of this type are unreported but the synthesis of pentadecanyl and hexadecanyl esters of long chain fatty acids are the subject of one report (33). The exact mechanism for

their synthesis remains uncertain but the immediate precursors are the respective alcohol and acid moieties. The preliminary studies reported here show that both acetate and stearate are incorporated into the natural methyl ester components in both the intact and freshly ground mycelial preparation. It also appears that the presence of methanol stimulates an increase in the relative concentrations of natural methyl esters in both systems suggesting a precursor-product relationship. Incorporation data suggests that the C_{14} moiety of myristic-1-^{14}C acid is not favored for incorporation into the natural methyl ester fraction. This is supported by the results given in Section V revealing the low concentrations of methyl myristate (C_{14}) in the mycelia of R. arrhizus.

Centrifugal fractionation of the crude ground mycelia preparation has yielded only partial success. Inconsistant results indicate the enzyme or enzymes responsible for the esterification of long chain fatty acids are located in a soluble extract obtained by removing particles at 39,000 rpm. This supernatant does not contain the natural methyl esters in solution. After incubation with unlabeled palmitic acid (C_{16}), methyl palmitate could be extracted from the solution. These results could not be repeated with labeled substrates.

XVI.

GENERAL DISCUSSION

DISCUSSION

Lipid extracts from the fungal spores of the following phytopathogenic fungi were analyzed: Sphacelotheca reiliana, Tilletia caries, Tilletia foetida, Tilletia controversa, Puccinia graminis tritici, Puccinia stripiformis, Ustilago maydis, and Rhizopus arrhizus. The heptane eluates from silica gel column fractionation revealed predominant concentrations of straight chain n-alkane containing an odd number of carbon atoms. This is characteristic for all biological materials containing paraffinic hydrocarbons. Alkane chains containing an even number of carbon atoms and branched-chain structural isomers are present in low concentrations. The detectable chain lengths generally ranged from C_{21} to C_{33} (5, 35, 36, 39, 40, 73, 74). The only exception to this typical hydrocarbon distribution is the sporangiospores of Rhizopus arrhizus. No detectable alkanes were detected. This is the only spore analyzed that was not obtained from its natural host

The origin of these extractable hydrocarbon components of fungal spores is uncertain. The general carbon length range is the same as that found in the waxes of higher plants (15,16). Results presented here show that only the spore material obtained from its natural host tissue contained the hydrocarbon components described above. The exact host-parasite relationship with respect to lipid metabolism is unknown at this time. There are only two reports comparing the host and fungal hydrocarbon distribution (35, 39). Laseter, et. al. report that chan-

ges do occur in the hydrocarbon distribution of normal and diseased corn infected with Ustilago maydis (35). The paraffin distribution of the noninfected wheat kernel and the chlamydospores of Tilletia species causing bunt of wheat are identical (39). This indicates that the alkane components are taken directly from the host plant by the disease causing fungus. From this information it appears that the influence fungal infection has on hydrocarbon metabolism is specific for the host-parasite relationship. Radioactive tracer studies on the infected plant would be required to determine the exact interrelationships existing between the fungal parasite and the host.

The fatty acids reported here are similar to those reported for other fungal species, higher plants, and animals (1, 64). It is interesting to note the dramatic qualitative and quantitative differences in the free and total fatty acid components of fungal spores. Free fatty acid extracts generally contain components with hydrocarbon chain lengths of C_{14} to C_{18} whereas acids with longer chain lengths appear in the total fatty acid extracts. Generally, the literature reports fatty acid chain lengths up to C_{18} omitting the longer chain C_{20} to C_{24} acids which are commonly referred to as animal fatty acids (21, 46, 62, 63, 64, 68). Reported here for the first time in many fungal organisms are arachidic (C_{20}), behenic (C_{22}), and lignoceric (C_{24}) acids. The presence of fatty acids with longer chain lengths is uncertain due to the lack of suitable standards. Most fungal species contain oleic ($C_{18:1}$) as the major unsaturated and palmitic (C_{16}) as the major saturated acid. Thus, the saturated and unsaturated fatty acids in fungal

spores obtained from natural host tissue and synthetic culture, contain chain lengths ranging from C_{12} to C_{24}. The even-numbered carbon chains are in predominant concentrations with lesser quantities of odd-numbered acids. In almost every species the unsaturated acids are in higher concentrations than the saturated.

Methyl esters of long chain fatty acids are reported here for the first time as a fungal product (36). The distribution of natural methyl esters of this type do not appear to have a wide spread distribution in fungal organisms. The chlamydospores of Ustilago maydis and mycelia of Rhizopus arrhizus are the only species of this study containing the natural methyl esters. From spore analysis it cannot be determined if the esters were produced by U. maydis or absorbed from the host tissue (corn) which is reported to contain esters of the same type (17). Analysis of the immature corn androecium by this author revealed no long chain natural methyl esters (71). Thus, it cannot be stated that the natural methyl esters are metabolic products of U. maydis. On the other hand, it is demonstrated without a doubt that natural methyl esters of long chain fatty acids are metabolic products of Rhizopus arrhizus representing approximately 4 per cent of the total mycelial lipids. In both organisms, U. maydis and R. arrhizus, the natural methyl ester distribution was similar to the fatty acid distribution of the respective species. However, some preference of synthesis was indicated for the C_{18} and longer chain esters (36). The metabolic or structural function of these newly reported fungal products is unknown. In spite of

the several speculations reported in the literation, they probably represent an available energy source for growth and spore germination (17, 58, 65, 66).

The chlamydospore ultrastructure of six species of smut fungi was reported here (37, 73). It was determined that by using the freeze-etch preparative technique, electron microscopic studies of the spore surface of Ustilago maydis, Urocystis agropyri, Tilletia caries, and Sphacelotheca reiliana reveal a unique architecture for each species. Another study comparing the three closely related Tilletia species, T. caries, T. foetida, and T. controversa, reveals surface as well as internal structural differences. Each species differs with respect to surface architecture with the smooth wall of T. foetida differing from the reticulated surfaces of T. caries and T. controversa. Numerous hair-like fibers present on the spore surface of T. controversa differentiate it from T. caries. Spore cross-section studies reveal close similarities in the smooth texture and number of spore wall layers in T. caries and T. foetida. However, a third wall layer of a granular consistancy was noted in T. controversa chlamydospores. Thus, it is demonstrated here how the ultrastructure of fungal spores may be of taxonomic value in differentiating closely related species as well as species belonging to different genera. When electron microscopic examination using the freeze-etch technique becomes routine, a reference library of electron photomicrographs of identified fungal spores should be of value for convenient and positive identification.

The value of hydrocarbon and fatty acid constituents as chemotaxonomic tools are implicated in this study. The hydrocarbon components of fungi represent the greatest potential for taxonomic purposes because they represent metabolic end products. However, the extent to which these compounds are produced by plant pathogenic fungi needs to be determined before definite conclusions can be drawn. On the other hand, mycelia of R. arrhizus obtained from synthetic culture contain a single hydrocarbon tentatively identified as squalene. Whereas, sclerotia of Sclerotinia sclerotiorum obtained from synthetic culture produced no detectable hydrocarbon constituents (75). The value of n-alkane hydrocarbons as chemotaxonomic characters is uncertain. But hydrocarbon patterns vary according to the host tissue in non-specific parasites, the characteristic alkane pattern may be used to identify the source of the spore sample.

The use of fatty acids as a potential taxonomic character is not considered important. Fatty acids do not represent a metabolic end product, but is subject to change with environmental fluctuations (15,16). In a few cases the presence of unusual fatty acids such as 9,10-epoxyoctadecanoic acid (68, 69, 70) and cyclopropane fatty acids (8, 9) may be of some taxonomic value.

Also presented here are results illustrating the effect of substrate media on the hydrocarbon content and distribution of free fatty acids of Sclerotinia sclerotiorum sclerotia. Heptane extracts revealed the presence of a single unknown hydrocarbon component of sclerotia

collected from its natural pea substrate, whereas, no hydrocarbon components were detected from sclerotia grown on synthetic media. Fatty acid analysis of sclerotia from PDA, glucose, and sucrose substrates revealed identical fatty acid distributions, whereas, sclerotia obtained from the natural substrate showed a dramatic shift in the ratio of monoenoic and dienoic C_{18} acids. Large increases in linolenic acid appeared in sclerotia from pea field collections. It cannot be ascertained from lipid analysis whether the shift in the relative concentrations of the unsaturated fatty acids is due to an uptake from the host or an upset of the metabolic balance within the fungal organism.

The total lipid analysis of Rhizopus arrhizus mycelia illustrates the presence of lipid constituents not reported until this time (62, 63). Mycelia from liquid shake cultures contain polar lipids in predominant concentrations followed by the triglycerides. Of special interest is the presence of natural methyl esters of long chain fatty acids representing a minor proportion of the total lipid fraction. Also of special interest, is the presence of squalene as the lone hydrocarbon constituent of the heptane extract.

The total mycelial fatty acids are consistant with those of other organisms of this study, having chain lengths ranging from C_{14} to C_{24}. The even-numbered carbon chains predominated with lesser concentrations of the odd-numbered carbon chains. The unsaturated acids predominated with high concentrations of oleic and linoleic. The saturated acids in highest concentration are palmitic and arachidic. Also, presented

here for the first time in Rhizopus are significant relative concentrations of the longer chain acids: arachidic, behenic, and lignoceric.

As in the total fatty acid fraction, oleic acid is the major component of the free fatty acids and complex lipids. Palmitic acid remains in relatively stable concentrations in all the lipid classes. The unsaturated acids remain in predominant relative concentrations in the free fatty acids, natural methyl esters, and phospholipids. Total spore fatty acids have a similar distribution to the mycelial lipids with few exceptions. For example, oleic acid is found in much larger relative concentrations, accompanied by a significant drop in linoleic acid. The significance of these differences is uncertain.

Sodium acetate-1-^{14}C and stearic-18-^{14}C acid are both incorporated into the mycelial lipids of R. arrhizus. The lipid classes containing the greatest amounts of radioactivity when grown in the presence of acetate are the polar lipids (phospholipids) and sterols followed by the triglyceride and free fatty acid fractions. The lowest amounts of radioactivity are the hydrocarbons and natural methyl esters. The relative amounts of radioactivity found in these lipid classes closely correspond to the relative concentrations of these lipids in mycelial material. When grown in the presence of ^{14}C-labeled stearate the incorporation pattern closely agreed with the distribution of the saturated stearate moiety reported for the mycelial lipids. Greater concentrations were found in the triglyceride fraction with lesser quantities located in the polar fractions.

It is interesting to note that only when ^{14}C-labeled acetate was added to the culture medium was the radioactivity of the hydrocarbon fraction highest. When ^{14}C-labeled stearate was added to the medium the hydrocarbon fraction radioactivity level was lowered significantly. This is consistant with the identification of the single hydrocarbon constituent, squalene, found in Rhizopus mycelial lipids. Squalene is normally synthesized through the acetate pathway via mevalonic acid.

Incorporation of ^{14}C-labeled stearate into the natural methyl ester fraction occurred in R. arrhizus grown on liquid shake culture and with crushed mycelial mixtures. Cultures incubated under the same conditions showed an increase of incorporation of stearate-^{14}C into the methyl esters when methanol was included in the system. The same results were not obtained when ^{14}C-labeled myristic acid was added to similar systems. These results suggest that both stearic acid and methanol could be precursors for the synthesis with the C_{18} moiety showing preference over the C_{14} moiety.

Preliminary fractionation of the crude mycelial enzymes suggest potential value for the soluble extracts for a cell-free synthesis system.

XVII.

CONCLUSIONS

CONCLUSIONS

1. The spores of plant disease causing fungi obtained from their natural host contain straight chain n-alkane hydrocarbon compounds with major chain lengths ranging from C_{21} to C_{33}. These paraffin molecules show an odd-numbered carbon chain preference.

2. The hydrocarbon extracts of mycelial material and spores from synthetic media did not contain this typical range of hydrocarbon components.

3. The spores and mycelia of fungi contain a distribution of total fatty acids different from that previously reported. The fatty acid chain length ranges from C_{12} to C_{24} with the even-numbered carbon chains in predominant concentrations.

4. Fungal tissue contains unsaturated fatty acids in larger concentrations than the saturated acids.

5. In most cases oleic is the predominant unsaturated fatty acid and palmitic is the predominant saturated acid.

6. Methyl esters of long chain fatty acids were detected for the first time as a fungal metabolic product.

7. The ultrastructure of the internal and external architecture of fungal spores using the freeze-etch technique represents a potential taxonomic character for differentiating closely related taxa.

8. Hydrocarbon and fatty acid distributions of sclerotia depended upon the type of substrates from which the sclerotin were obtained.

9. Incorporation of ^{14}C-labeled acetate and stearate into mycelial lipids of Rhizopus arrhizus was similar to incorporation patterns obtained for other plant and animal organisms.

10. There is a preference for the incorporation of the C_{18} moiety into the triglyceride components over that of other lipid fractions.

11. Acetate-1-^{14}C was incorporated into squalene, the single hydrocarbon component of Rhizopus mycelia.

12. The C_{18} moiety of stearic acid is preferentially incorporated into the natural methyl esters of fatty acids over that of the C_{14} moiety of myristic acid.

13. Methanol stimulates the incorporation of stearate into the natural methyl esters of fatty acids.

XVIII.

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