Antagonistic Effects of *Myxococcus xanthus* on Fungi: II. Isolation and Characterization of Inhibitory Lipid Factors

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ABSTRACT

The chemical composition of the lipophilic excretion of *Myxococcus xanthus* inhibitory to the germination of fungal spores and growth has been investigated. The inhibitory effect was attributed to a mixture of fungistatically acting fatty acids and a component of antibiotic character. The fatty acid mixture has been fully characterized and found to constitute a mixture of saturated (68%) and unsaturated (32%) structures in the C_{13}-C_{17} range. The major part is methyl-branched of the *iso*-type, with 13-methyltetradecanoic acid being the main component (33% of the total). The fungistatic activity of the fatty acid mixture on spore germination is attributed to the structures with *iso*-configuration. The presence of unsaturation is of minor importance. Observed morphological changes of the spores and hyphae in the presence of *iso*-fatty acids suggest that they act on the plasma membrane.

INTRODUCTION

Myxobacteria are terrestrial organisms which are ubiquitous inhabitants of normal soil, bark, and decaying plant material (1). As described in a previous paper (2), *Myxococcus xanthus* secretes into the culture medium organic substances inhibitory to the germination of spores and the growth of fungi. The isolation and characterization of these excreted factors are important, as they possibly may be involved in the phenomenon known as soil mycostatis.

In an early stage of the investigation of the chemical background of these findings, it was found that the responsible components readily dissolved in organic solvents such as ethyl acetate, chloroform or benzene. Recently, Schröder and Reichenbach (3) reported an analysis of the fatty acids of vegetative cells and myxospores of *Stigmatella aurantiaca*. A saturated branched C_{15} acid (~35%) and a monounsaturated C_{16} acid (~20%) were identified as the main fatty acids. No effort was made to locate the position of the branch (assumed to be a methyl group) or the double bond.

This paper deals with extracellular material in the culture medium and not with extracts of cells.

EXPERIMENTAL PROCEDURES

The cultivation of *M. xanthus* has been described previously (2).

Germination experiments: Two wetted and sterilized strips (10 x 10 mm) of cellophane (PT 300) were placed on a sterile glass filter (Jena G1) in a small petri dish half filled with 3 ml test solution. The nutrient medium consisted of glucose (2.0 g) KNO_3 (2.0 g), KH_2PO_4 (2.5 g), $MgSO_4 \cdot 7H_2O$ (1.26 g) and Tween 80 (0.1 ml) in distilled water (1000 ml). The preparations to be examined were added to the medium as methanol solutions. All media had a methanol concentration of 1.0% and pH 6.5. The strips were inoculated with conidia of *Fusarium roseum* (40,000 conidia per strip) from cultures grown for 10 days at 25 C. The strips were removed after 3 and 4 hr incubation at 25 C, mounted on slides, and treated with lactophenol to stain and kill the conidia. The proportions of germinated and nongerminated conidia were then determined in the microscope. On each strip at least 400 randomly chosen conidia were examined.

Analytical gas chromatography (GC): A 50 m long stainless steel capillary tube, ID 0.01 in. (Golay capillary column type R) coated with polypropylene glycol was used. A Perkin-Elmer model 900 gas chromatograph equipped with a standard capillary injector block, split no. 3, and flame ionization detectors was employed. Helium was used as carrier gas, and the gas flow was ca. 3 ml/min at room temperature.

Preparative GC: The column used had a length of 8 m and an internal diameter of 8 mm. It was filled with Versamid 900 (6%) on Gaschrom Z (80-100 mesh) and was initially kept at 275 C during 24 hr in a stream of nitrogen. The chromatography was performed at 190 C on a modified Aerograph (model A-700) gas chromatograph operated manually under isothermal conditions. The flow of helium carrier gas was 100 ml/min, and the vapors were condensed in standard Autoprep flasks filled with stainless steel turnings. The turnings were moistened with 0.5 ml chloroform and the flasks immersed in a cold bath at -80 C.

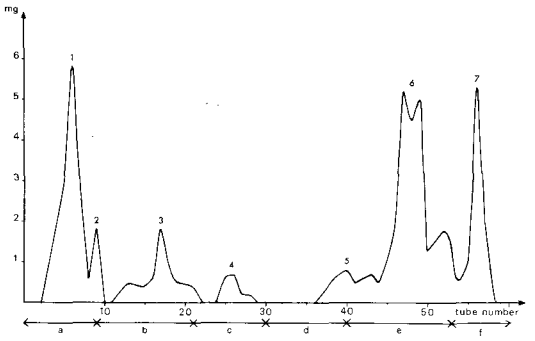


FIG. 1. Liquid chromatogram of the lipophilic excretion of *M. xanthus* on silicic acid. Tube contents are shown as a function of tube number. *a* benzene-ethyl acetate (3:1 v/v), *b* benzene-ethyl acetate (1:1 v/v), *c* ethyl acetate, *d* ethyl acetate-acetone (1:1 v/v), *e* acetone, *f* methanol. Three liters of culture medium.

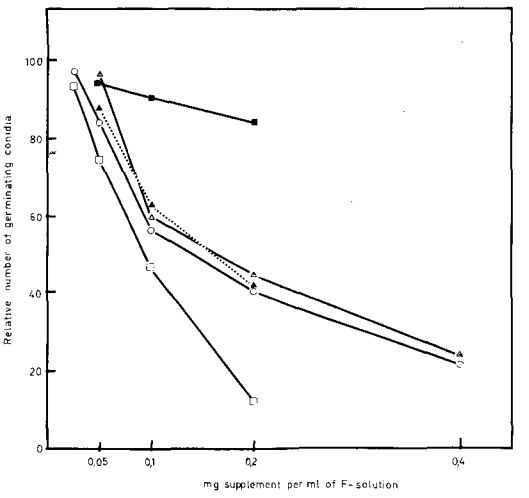


FIG. 2. Relative number of germinating conidia of *F. roseum* after 3 hr incubation at 25 C. Control = 100. Supplement to F-solution: □ lipophilic extract of cell free culture solution of *M. xanthus*, ○ natural fatty acid mixture, △ saturated moiety of natural fatty acid mixture, ▲ unsaturated moiety of natural fatty acid mixture, ■ neutral fraction of the lipophilic extract.

Mass spectrometry (MS): The GC-MS combination instrument described by Stallberg-Stenhagen et al. (4) was used. The temperature of the ion source was 200 C, and the electron energy 70 eV.

Catalytic hydrogenation: In a typical experiment, 4.5 mg of the unsaturated methyl ester mixture was dissolved in 2 ml *n*-heptane in a 25 ml flask. One milligram of Adam's catalyst ($PtO_2 \cdot H_2O$) was added and the flask filled with hydrogen, stoppered, and shaken for 15 min at 25 C. The suspension was centrifuged prior to analysis.

Oxidative degradation: The procedure was illustrated by the oxidative degradation of component XIV; 0.5 mg XIV was dissolved in 50 µl glacial acetic acid in a small test tube. One and eight-tenths milligrams finely pulverized potassium permanganate was added and the mixture heated for 15 min at 40 C. One-tenth milliliter of a solution of sulphur dioxide in water (10%) was added, followed by one drop of diluted sulphuric acid (5%). The mixture was heated on the waterbath until colorless. One-tenth milliliter dichloromethane was added; after shaking and subsequent centrifugation the aqueous layer was discarded and the organic phase washed with water. After evaporation the residue was esterified with diazomethane in ether. The reaction product was investigated on the combined GC-MS.

Preparation of branched chain reference material. Synthesis of 12-methyltridecanoic acid: This previously known acid, see e.g. (5), was prepared via a mixed anodic coupling (Kolbe electrosynthesis) of 4-methylpentanoic acid (Fluka AG, Bucks, Switzerland) and methyl nydrogen decan-1,10-dioate (6). There was obtained 13.5 g of the desired methyl ester of bp 136-140 C, 1 mm, from 64.0 g half ester and 34.4 g 4-methylpentanoic acid. The methyl ester was hydrolyzed and the free acid recrystallized from light petroleum (bp 60-85 C). Yield 10.7 g, mp 52.2-52.8 C of free acid.

13-Methyltetradecanoic acid: This previously known acid (7) was prepared from 12-methyltridecanoic acid by chain lengthening (Arndt-Eistert reaction). The diazoketone obtained from 5.0 g of 12-methyltridecanoic acid was rearranged in methanol in the presence of commercial silver oxide. The crude methyl 13-methyltetradecanoate (5.1 g) was chromatographed on 50 g silicic acid (Mallinckrodt, 100 mesh) with ether-light petroleum (bp 40-60 C) (1:50 v/v). Free acid (2.1 g) mp 49.7-50.3 C was obtained after hydrolysis and crystallization from light petroleum (bp 40-60 C).

RESULTS

Fractionation of the crude inhibitory material: After 4 days of incubation at 30 C, the cells (5.2 g dry weight) were removed from 3000 ml of culture solution by centrifugation. The solution was shaken immediately with five 200 ml portions of ethyl acetate (Analar grade), and the combined organic extracts evaporated to dryness at reduced pressure (25 C). One hundred thirty-five milligrams of brownish viscous residue with a strong odor characteristic of Myxobacteria was obtained. The extract was triturated with 25 ml benzene for 1 hr and the suspension centrifuged. The benzene soluble material weighed 94 mg (69.7%). This was

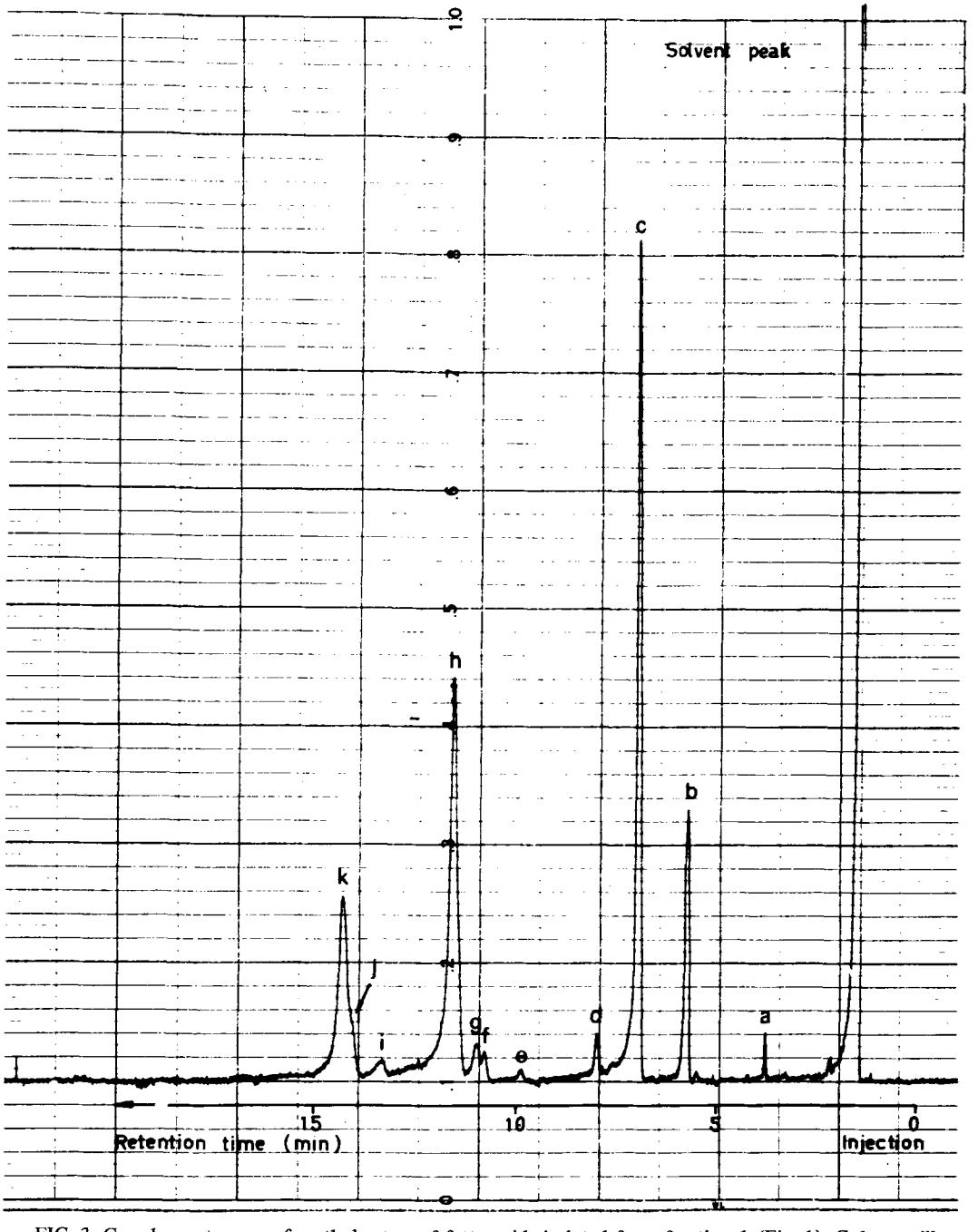
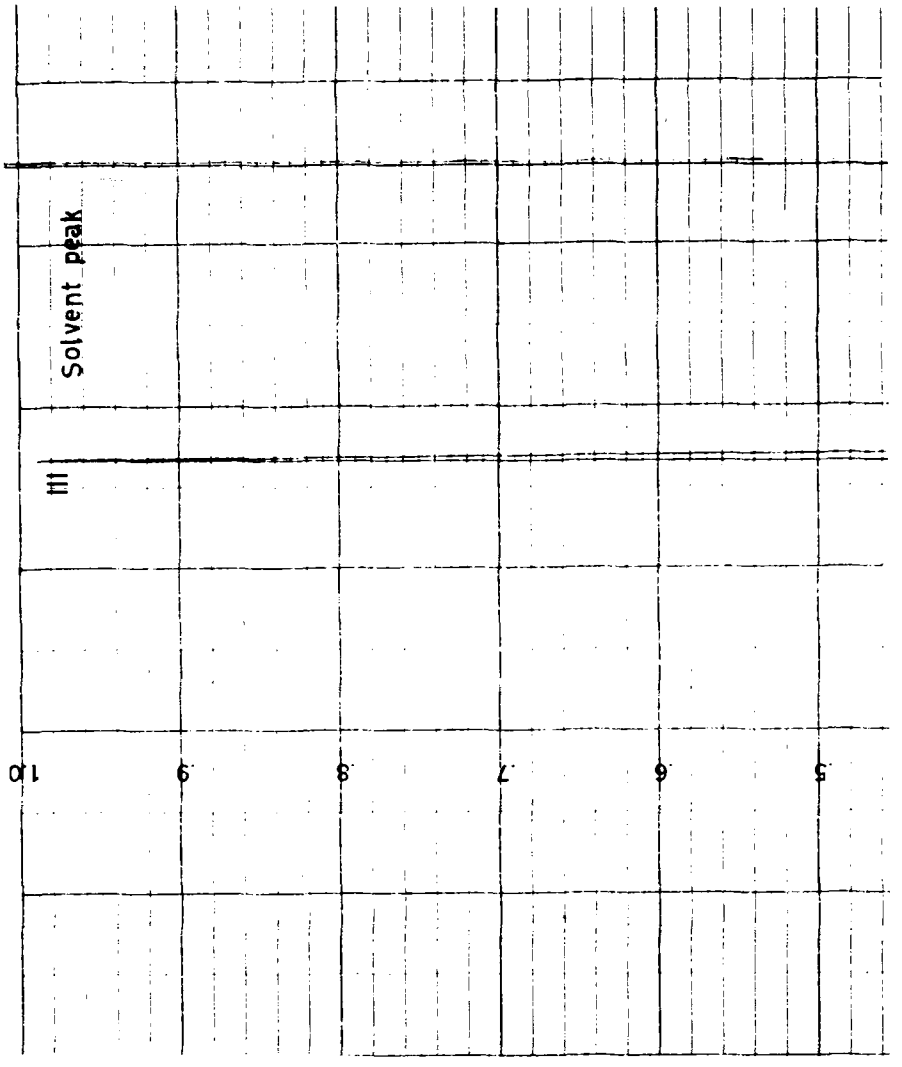


FIG. 3. Gas chromatogram of methyl esters of fatty acids isolated from fraction 1 (Fig. 1). Golay capillary column type R with polypropylene glycol as stationary phase at 185 C with *n*-heptane as solvent.

chromatographed on 5 g silicic acid activated at 110 C overnight (Mallinckrodt 200-325 mesh).

The polarity of the eluant was increased stepwise as follows—20 ml benzene-ethyl acetate (3:1 v/v), 60 ml benzene-ethyl acetate (1:1 v/v), 45 ml ethyl acetate, 50 ml light petroleum (bp 40-60 C)-acetone (1:1 v/v), 65 ml acetone, and 50 ml methanol. Five milliliter fractions were collected in preweighed flasks. The solvents were removed in a stream of nitrogen; 91% of the material was eluted (Fig. 1). The fractions were combined as indicated (1-3) and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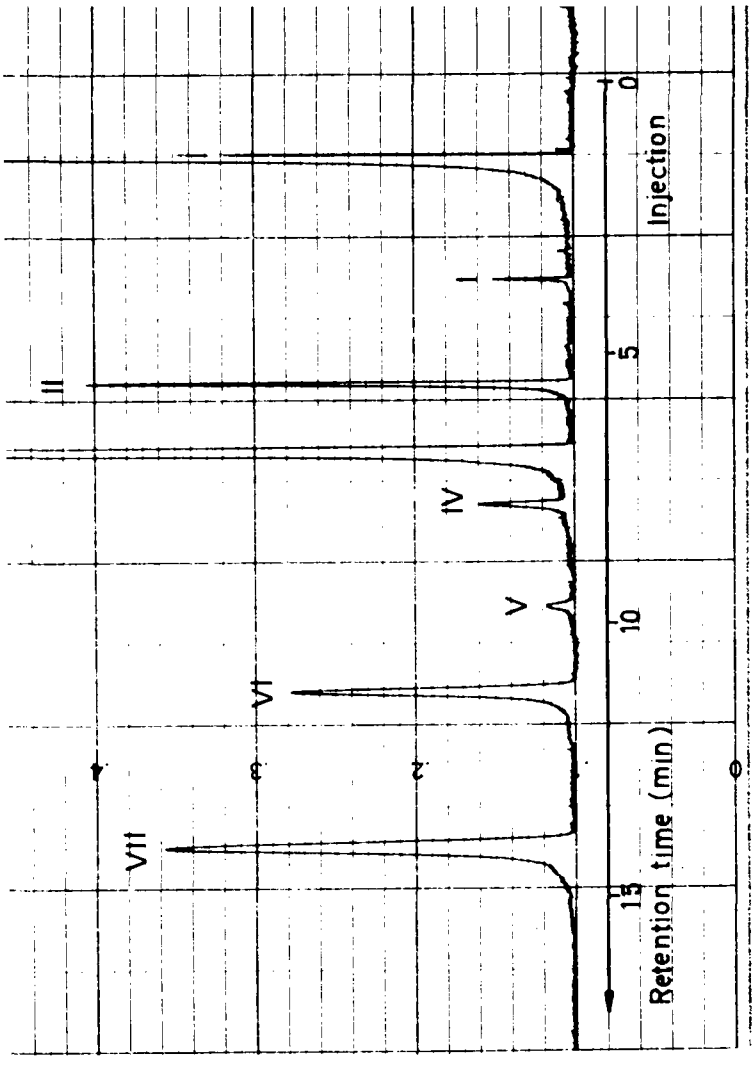

FIG. 4. Gas chromatogram of methyl esters of saturated fatty acids isolated from fraction 1. Conditions identical with those in Figure 3.

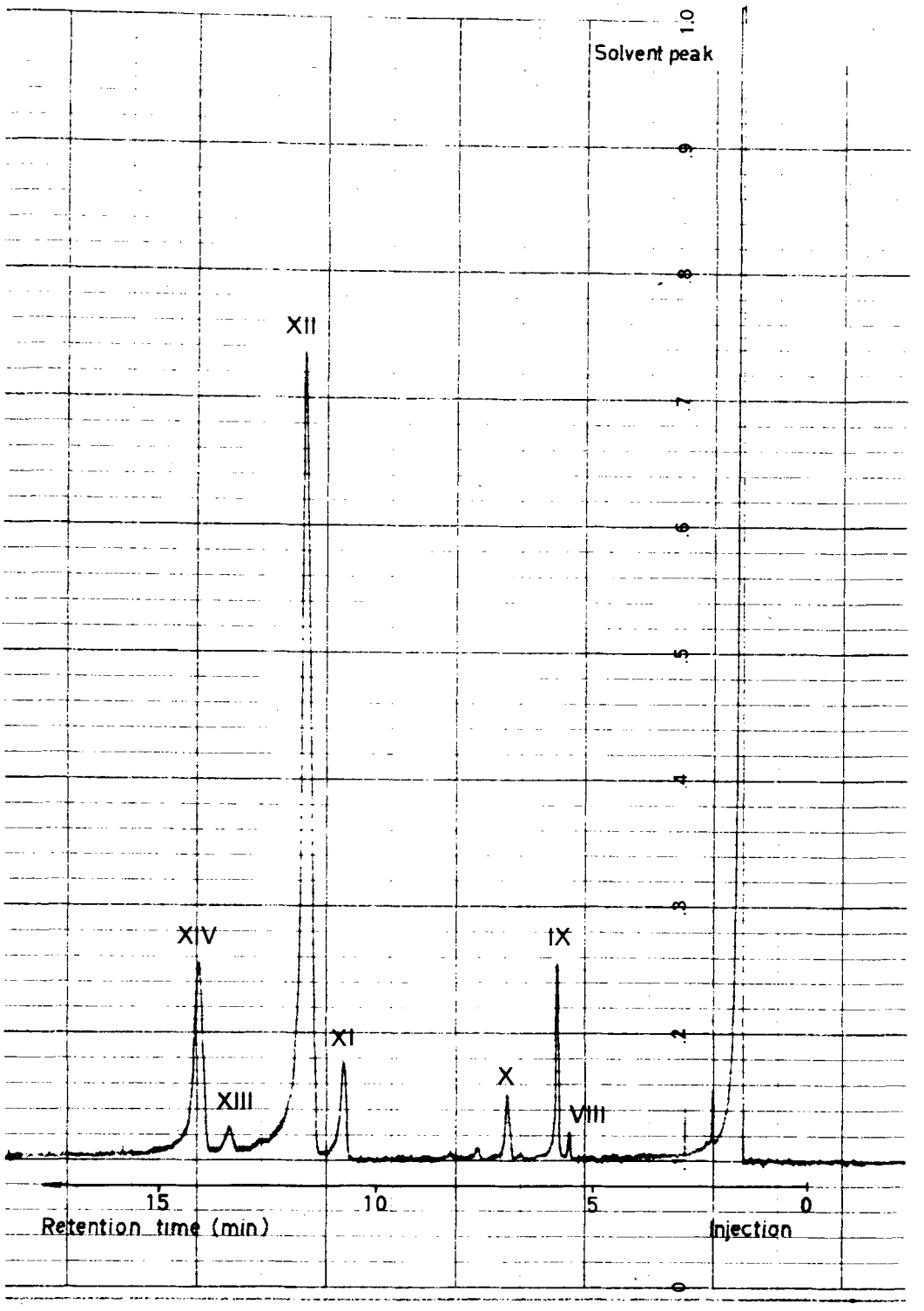

FIG. 5. Gas chromatogram of methyl esters of unsaturated fatty acids isolated from fraction 1. Conditions identical with those in Figure 3.

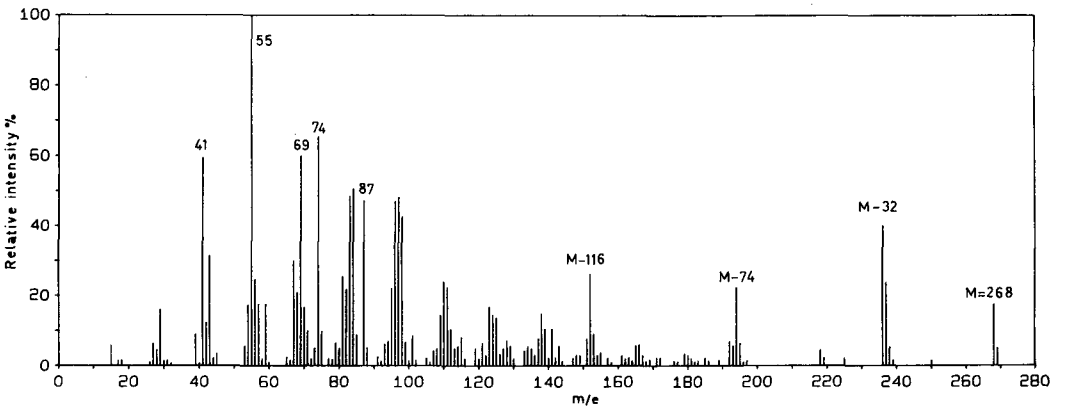

FIG. 6. Mass spectrum of component XII.

tested for inhibitory activity on the germination of *Fusarium* conidia. Fractions 1 and 3 exhibited such activity; whereas, the rest of the material had practically no effect on germination. This paper deals with lipid fraction 1; whereas, fraction 3 will be dealt with in a later paper.

An IR spectrum of fraction 1 indicated the presence of free carboxylic acid groups by characteristic absorptions at 2700-2500 cm^{-1} and 1700 cm^{-1}. The material (86 mg) was dissolved in a small volume of methanol and titrated with sodium methoxide in methanol to pH 9 and subsequently distributed between water and ether. Acidification of the aqueous phase, followed by repeated extraction with ether, afforded the acidic material (64 mg, 77%) in a state sufficiently pure for further analysis. The tests for activity on fungal development indicated that the acidic components accounted for a strong inhibition, while the neutral fraction had practically no such effect (Fig. 2). An IR spectrum of the purified acids showed, in addition to the characteristic absorptions previously mentioned, weak absorption at 1650 cm^{-1} (-C=C-stretching vibrations) given by, for example, unsaturated fatty acids of the nonconjugated type (8).

Fractionation and identification of the fatty acids: The acids subsequently were esterified by means of diazomethane and subjected to analysis on a capillary GC column (Fig. 3). At least 11 components, designated *a-k*, are distinguishable.

The fatty acid methyl esters were separated into saturated and unsaturated fractions by adsorption chromatography on silicic acid impregnated with silver nitrate (9,10). In a typical experiment 177 mg methyl esters were chromatographed on 5.0 g argentated silicic acid. About two-thirds of the material (119 mg) was saturated and eluted with light petroleum (bp 60-70 C)-ether (50:1 v/v) (I-VII, Fig. 4) while the rest (55 mg), eluted with light petroleum-ether (5:1 v/v) consisted mainly of monounsaturated methyl esters (VIII-XIV, Fig. 5).

It is evident from Figures 3-5 that peak *b* in Figure 3 is composed of three structures (II, VIII and IX), peak *c* of III and a small amount of X, and peak *h* of the two structures VI and XII. By adding normal chain C_{13}, C_{14}, C_{15}, and C_{16} methyl esters (Fluka AG, Switzerland) to a sample of the natural saturated methyl esters and by rechromatographing this mixture, peaks II, IV and VI (Fig. 4) were recognized as due to methyl tetradecanoate, methyl pentadecanoate and methyl hexadecanoate, respectively. Mass spectra of the molecules confirmed the findings.

The mass spectra of I, III, V and VII were found to be very similar to those of the methyl esters of normal C_{13}, C_{15}, C_{16} and C_{17} acids. However, as already shown (Fig. 4), the GC retention times were considerably shorter. This suggested that the 4 components belong to the series of *iso*-fatty acids, i.e., acids possessing a terminal isopropyl group (11). The mass spectra and retention times of synthetic (see Experimental Procedures) methyl esters of *iso*-C_{13}, C_{15}, C_{16} and C_{17} acids proved identical with those of components I, III, V and VII, respectively. The findings constituted proof as to the *iso*-configuration.

A sample of the unsaturated material (Fig. 5) was subjected to total hydrogenation. The GC now showed only 4 peaks which were identified by their retention times as the methyl esters of tetradecanoic-, *iso*-pentadecanoic-, hexadecanoic- and *iso*-heptadecanoic acid. The unsaturated acids thus possessed carbon skeletons of the same type as the saturated fatty acids.

Components IX, X, XI, XII and XIV (Fig. 5) were isolated by means of preparative GC. The

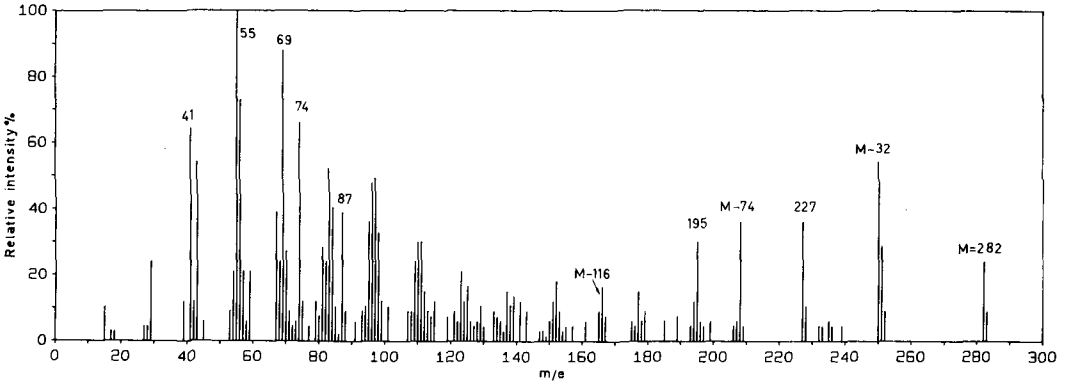

FIG. 7. Mass spectrum of component XIV.

mass spectrum of XII, the main unsaturated component, was reproduced in Figure 6. It indicated a molecular weight of 268 which was that of the methyl ester of a monounsaturated hexadecanoic acid. Characteristic peaks of even mass were observed at m/e 236 (=M-32), m/e 194 (=M-74), and m/e 152 (=M-116). Such peaks also were present in the mass spectrum of, for example, methyl oleate and may be regarded as characteristic for monounsaturated long chain methyl esters (11).

After catalytic hydrogenation as discussed above, sample XII showed a retention time identical with that of methyl hexadecanoate (VI, Fig. 5). The double bond of XII was located by analysis of the products obtained on oxidative degradation with the aid of potassium permanganate. Identification of the fragments obtained with the GC-MS combination revealed the presence of methyl *n*-pentanoate and dimethyl undecan-1,11-dioate. Hence, XII had the double bond in 11:12 position. The absence of absorption at 965 cm^{-1} in the IR (out-of-plane = CH deformation vibrations) generally was taken as proof of *cis*-configuration in esters of long chain unsaturated fatty acids (10). Since XII did not absorb at 965 cm^{-1}, the structure *cis*-hexadec-11-enoic acid is assigned to component XII.

The mass spectrum of XI indicated a mol wt of 266, which was that of the methyl ester of a diunsaturated C_{16}-acid. After catalytic hydrogenation, the GC retention time coincided with that of methyl hexadecanoate. Oxidative degra-

TABLE I

Acids Excreted to the Culture Medium

Designation of gas chromatographic peak, Figures 3 and 4 (methyl ester)	Structure	Relative abundance, %	
Saturated acids			
I	11-Methyldodecanoic acid	0.8	
II	*n*-Tetradecanoic acid	7.7	
III	13-Methyltetradecanoic acid	33.2	
IV	*n*-Pentadecanoic acid	1.9	
V	14-Methylpentadecanoic acid	0.6	
VI	*n*-Hexadecanoic acid	8.3	
VII	15-Methylhexadecanoic acid	15.3	
			67.8
Unsaturated acids			
VIII	*cis,cis*-Tetradec-5,9-dienoic acid	0.1	
IX	*cis*-Tetradec-9-enoic acid	1.5	
X	*cis*-13-Methyltetradec-9-enoic acid	0.8	
XI	*cis,cis*-Hexadec-7,11-dienoic acid	2.1	
XII	*cis*-Hexadec-11-enoic acid	21.4	
XIV	*cis*-15-Methylhexadec-11-enoic acid	5.0	
			30.9
XIII	Unidentified	0.7	
Not designated	Unidentified	0.6	
		100.0	

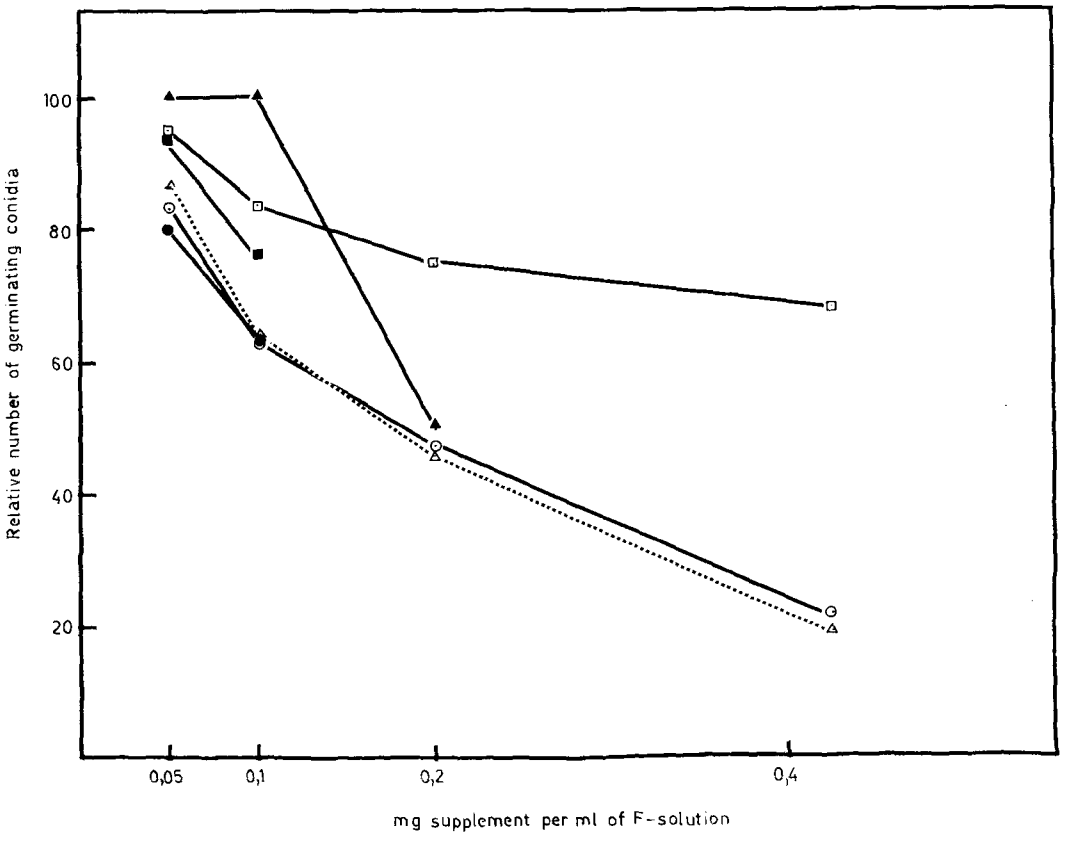

FIG. 8. Relative number of germinating conidia of *F. roseum* after 3 hr incubation at 25 C. Control = 100. Supplement to F-solution: △ synthetic mixture corresponding to the saturated moiety of natural fatty acids, ○ 13-methyltetradecanoic acid, ● *cis*-15-methylhexadec-11-enoic acid, ▲ *cis,cis*-hexadec-7,11-dienoic acid, ■ *cis*-hexadec-11-enoic acid, □ *n*-tetradecanoic acid.

dation, as indicated above, yielded methyl *n*-pentanoate and dimethyl heptan-1,7-dioate accompanied by a small amount of dimethyl succinate. This indicated that the double bonds are situated at positions 7:8 and 11:12. No absorption was observed at 965 cm^{-1} in the IR. Thus, XI must be the methyl ester of *cis,cis*-hexadec-7,11-dienoic acid.

The mass spectrum of component XIV was reproduced in Figure 7. It indicated a mol wt of 282, which was that expected for the methyl ester of a monounsaturated C_{17}-acid. The ions of even mass at m/e 250 (=M-32), m/e 208 (=M-74), and m/e 166 (=M-116), characteristic for long chain monounsaturated methyl esters, were abundant. The mass spectrum differed, however, from the normal chain unsaturated methyl esters, because prominent peaks were observed at m/e M-55 (=227) and m/e M-87 (=195). The former peak was most likely due to loss of a butylene unit from the molecular ion, whereas the latter was due to loss of the $(CH_3O\overset{O}{\overset{\|}{C}}CH_2CH_2-)^+$ ion.

After hydrogenation and subsequent GC examination, it was shown that XIV belonged to the *iso*-series of fatty acids. The products obtained on oxidative degradation showed that the double bond was situated at the 11:12 position. Examination of XIV in the IR indicated *cis* geometry. XIV was, accordingly, the methyl ester of *cis*-15-methylhexadec-11-enoic acid.

The mass spectrum of IX indicated a mol wt of 240. This corresponded to the methyl ester of a monounsaturated C_{14}-acid. In the manner described, the structure was identified as methyl *cis*-tetradec-9-enoate. It was seen from Figure 5 that IX was accompanied by a small amount of a component with a somewhat shorter GC retention time (VIII). MS and GC of the hydrogenated product indicated that VIII was the methyl ester of a normal chain C_{14} diunsaturated acid. It was not possible to

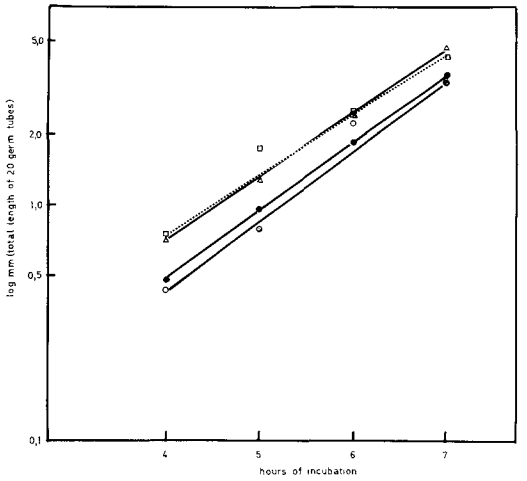

FIG. 9. Growth (total length) of germ tubes, produced by 20 conidia of *F. roseum* at 25 C in F-solution supplemented with: • synthetic mixture corresponding to the saturated moiety of natural fatty acids (0.2 mg/ml), ○ 13-methyltetradecanoic acid (0.2 mg/ml), △ *n*-hexadecanoic acid (0.2 mg/ml), □ non-supplemented F-solution (control).

obtain a sufficient amount of pure VIII for an oxidative degradation experiment. It appears likely that the two double bonds of VIII in analogy with XI were located at the positions 5:6 and 9:10.

The mass spectrum of X indicated a mol wt of 254. This showed that X was the methyl ester of a C_{15}-monounsaturated acid. Degradation experiments and IR examination showed that X possessed the structure of methyl *cis*-13-methyltetradec-9-enoate. The structures, as well as the relative abundance of the components in the inhibitory fatty acid fraction, are summarized in Table I.

Inhibitory effect of the isolated fatty acids on fungi: The biological effect of some lipid fractions and individual fatty acids was examined in germination experiments with conidia of *F. roseum* as the main test material. To facilitate a comparison between different experiments, the germination frequency of the controls was set to 100 and that of the test series was related to this scale. Each point in the diagrams represented the examination of ca. 3000 conidia.

The inhibitory effect of the fatty acid components of the total lipophilic extract of *M. xanthus* and of its saturated and unsaturated moieties was demonstrated in Figure 2. After 3 hr incubation at 25 C, the germination of *Fusarium* conidia was inhibited to the same extent by all three fatty acid fractions. No marked difference in activity between saturated and unsaturated material was observed. Due to

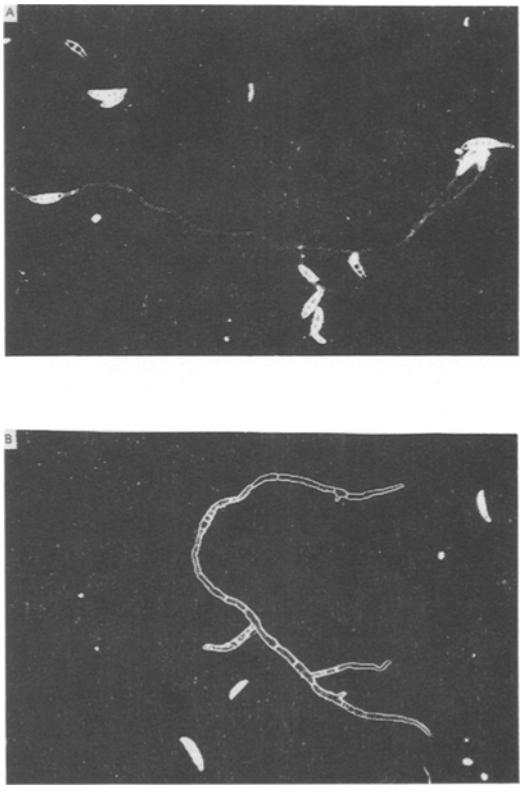

FIG. 10. Hyphae of *F. roseum* grown in F-solution, non-supplemented (a) and supplemented (b) with 0.2 mg/ml F-solution of the natural fatty acid mixture x 400.

the presence of an inhibitory component of nonfatty acid type (fraction 3, Fig. 1), the total lipophilic extract of the culture solution gave a stronger inhibition than the fatty acid mixtures. Clearly, no inhibition was produced by the neutral moiety of fraction 1.

In Figure 8, the inhibitory effect of some of the isolated fatty acids was compared with that of a mixture of synthetic saturated fatty acids. The mixture and the main component of the natural acids, 13-methyltetradecanoic acid, exhibited the same inhibitory effect on conidia germination. Also the branched unsaturated C_{17} acid was markedly inhibitory. In contrast, the straight chain saturated and monounsaturated acids affected the germination to a considerably less degree. In the case of the diunsaturated C_{16} acid, a marked inhibition was observed. Furthermore, tests with 12-methyltridecanoic acid, which was not a natural constituent, showed that this acid acted essentially in the same way as the natural next higher homolog. The results obtained indicated that the fatty acids with *iso*-configuration had an effect on the conidia germination markedly

different from that of the corresponding straight chain structures. The question of saturation or unsaturation appeared to be of minor importance.

Figures 2 and 8 demonstrated the inhibitory effects on the germination of *Fusarium* conidia observed after 3 hr incubation. However, the inhibitions produced by the fatty acids almost were overcome when the incubation period was prolonged to 4 hr. Thus, the natural fatty acid mixture and its active components acted as fungistatic agents.

In another study the fatty acids were investigated with respect to their effect on the growth of the germ tubes. The development of germ tubes produced by 20 randomly selected *Fusarium* conidia in each series was followed by measurements in the microscope during 7 hr (Fig. 9). In the media containing the saturated fatty acid mixture or 13-methyltetradecanoic acid, the germination process was delayed. The development in these series appeared about 1 hr behind that of the control. However, after the germination was over, the germ tubes grew at the same rate in all series.

The fungistatic character of the inhibitory fatty acids also had been established in a number of growth experiments where the development was followed during 15 days. In media supplemented with the relevant fatty acids, used separately or in mixtures and in various amounts, the primary effect was consistently a delayed spore germination, while the subsequent mycelial growth in the test series and control was parallel.

DISCUSSION

Quite a large number of bacteria have been investigated with respect to their lipid content. In particular, the free and bound fatty acids have been analyzed (12). It now has been found that not less than 55% of the free fatty acids excreted by *M. xanthus* possess methyl-branched carbon skeletons. The ability to synthesize such fatty acids sometimes is considered specific to bacteria. It seems, nevertheless, that methyl-branched acids have been observed in only a restricted number of species. In case of the old works, "a reinvestigation of the fatty acid composition is needed with the finer methods available today" (12).

In practically all investigations dealing with lipids of bacteria, the material has been extracted from the bacterial cells. The knowledge of the chemical composition of the excretions to the surroundings by the organisms themselves is consequently very deficient. Since a marked fungistatic action of excreted *iso*-fatty acids has been clearly demonstrated in the case of *M. xanthus,* it appears desirable to investigate the chemical nature of excreted lipids of other soilborne bacteria.

It is not the object of this paper to discuss the mode of action of the *iso*-fatty acids. However, it may be mentioned that the inhibitory fatty acids investigated produced marked morphological changes of both the *Fusarium* conidia and the hyphae developed. The conidia swelled and the hyphae produced were of a short and thick appearance (Fig. 10). It seems very likely that the *iso*-fatty acids act on the plasma membrane. Studies of the mechanism of action will be published elsewhere.

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