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Biochemical Changes During the Growth of Fungi

II. Ergosterol and Fatty Acids in *Penicillium atrovenetum*

JAMES L. VAN ETTEN AND DAVID GOTTLIEB
Department of Plant Pathology, University of Illinois, Urbana, Illinois

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ABSTRACT

VAN ETTEN, JAMES L. (University of Illinois, Urbana), AND DAVID GOTTLIEB. Biochemical changes during the growth of fungi. II. Ergosterol and fatty acids in *Penicillium atrovenetum*. J. Bacteriol. **89**:409–414. 1965.—Changes in the lipid constituents of *Penicillium atrovenetum* were studied during the growth and development of this fungus. The stages in development, as measured by the dry weight, were divided into four phases: lag, log, stationary, and death. The total fatty acids on a dry-weight basis increased from a minimum in the spores to a maximum near the end of the log phase of growth. The major fatty acids were palmitic, stearic, oleic, and linoleic. Myristic, pentadecanoic, palmitoleic, heptadecanoic, linolenic, arachidic, and heptadecenoic acids, together with two unidentified components, were also present in the fatty acid fraction. Compared to ungerminated spores, young mycelium contained a much lower percentage (on the basis of total fatty acids) of linoleic acid. There was a corresponding increase of oleic acid. Except for palmitic acid, which remained constant, the remaining fatty acids increased slightly. During subsequent growth of the fungus, linleic acid decreased, whereas the percentage of palmitic and stearic acids increased steadily from the lag phase to the end of the log phase. Ergosterol was the only sterol detected. The percentage of ergosterol, on a dry-weight basis, increased to a maximum at the start of the log phase and then steadily decreased. The following changes in lipids appear to be associated with the development and aging of fungi: (i) the presence of a relatively high content of nonsaponifiable lipid and ergosterol in the young mycelium, and their later decrease with age, and (ii) a shift from more unsaturated to less unsaturated fatty acids with age.

Though many investigators have identified the fatty acids and sterols in fungi, only a few have studied the changes of lipid constituents during the entire growth cycle. Bernhard, Abisch, and Wagner (1957) reported that very young cultures of *Phycomyces blakesleeanus* contained palmitic, oleic, linoleic, and linolenic acids as the major fatty acids. The only apparent change with age was a decrease in linolenic acid. A similar observation was reported during the growth of *Tricholoma nudum* by Leegwater et al. (1962). Coots (1962) compared the relative rates of synthesis of oleic and linoleic acids in *Penicillium javanicum*. Linoleic acid was preferentially synthesized in the younger cells, whereas oleic acid was preferentially synthesized in older mycelium.

In a previous report on growth and chemical constituents of fungal mycelium, Gottlieb and Van Etten (1964) described the changes in the percentage of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), protein, chitin, total nitrogen, cold trichloroacetic acid-soluble nitrogen, and carbohydrate which occurred during the growth from the spore to the death phase of *P. atrovenetum*. Similar studies have been carried out on the lipids of this fungus, and this paper reports the identification and changes in the fatty acids and sterol composition of *P. atrovenetum* during its growth and development.

MATERIALS AND METHODS

P. atrovenetum IMI 61837 was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. The fungus was grown and collected in the same manner as previously described (Gottlieb and Van Etten, 1964). To protect the lipids, all of the operations following the harvesting of the cultures were carried out in a nitrogen atmosphere and in the absence of light. The dried fungal preparations were subjected to saponification, and the fatty acids and sterols were extracted by a method similar to that of Caltrider, Ramachandran, and Gottlieb (1963). The ether layers from both the saponifiable and nonsaponifiable fractions were dried over anhydrous magnesium sulfate, filtered, and then evaporated. The residues were stored under nitrogen at −20 C until analyzed.

Methyl esters of the fatty acids were prepared

by the diazomethane method of Schlenk and Gellerman (1960). The fatty acid and sterol analyses were made on an F and M gas chromatograph, model 609, by use of a hydrogen flame detector. Two different polyester columns were used for the identification of the fatty acids. One column was made up with 20% diethyleneglycol succinate polyester containing 5% phosphoric acid on Chromosorb W 80–100 mesh packed in a 6-foot stainless-steel tube with an internal diameter of ⅛ in. The injection port, column, and detector block temperatures were 239, 183, and 234 C, respectively. Hydrogen, helium, and oxygen flow rates were 43, 81, and 190 ml per min, respectively. The other column was composed of 15% diethyleneglycol adipate polyester on Chromosorb W 80–100 mesh packed in a 6-ft stainless-steel tube with an internal diameter of ⅛ in. This column temperature was programmed from 170 to 199 C at 9° per min and subsequently held at 199 C. The injection port and detector block temperatures were 239 and 234 C, respectively. Hydrogen, helium, and oxygen flow rates were 43, 78, and 190 ml per min, respectively. Quantitative determinations of the fatty acid methyl esters were made on the diethylene glycol adipate column.

Ergosterol was identified and quantitatively measured on a column composed of 1.5% SE-30 silicone on 100–120 mesh Gas Chrom P (Applied Science Laboratories, Inc., State College, Pa.) packed in a 6-ft stainless-steel tube with an internal diameter of ⅛ in. The column temperature was programmed from 200 to 231 C at 6.4 degrees per min and subsequently held at 231 C. The injection port and detector block temperatures were 285 and 293 C, respectively. Hydrogen, helium, and oxygen flow rates were 41, 80, and 194 ml per min, respectively. The supports of all three columns were acid-washed and pretreated with dimethyldichlorosilane before use.

Thin-layer chromatography was also used to identify the sterol. A 20-g amount of Silica Gel G (Merck & Co., Inc., Rahway, N.J.) was mixed with 45 ml of water and shaken vigorously for 30 sec. The mixture was spread evenly on the glass plates and allowed to air dry for 10 to 20 min. The plates were activated by heating in an oven at 100 C for 1 hr and then stored over a desiccant. The chromatograms were developed in all-glass tanks (diameter, 6 in.; height, 10 in.), lined with filter paper and containing 100 ml of solvent. Two solvent systems of petroleum ether (b.p. 58–69 C):acetone were used for developing the chromatograms; the ratios (v/v) were 4:1 and 6:1. Ergosterol was detected on a silica gel plate by the permanganate spray of Leegwater et al., (1962), and was also identified in the nonsaponifiable fraction by the Liebermann-Burchard test (Stadtman, 1957).

The methyl esters of the fatty acids were identified by comparing the retention times of the extracted acids with known standard fatty acid methyl esters obtained from the National Institutes of Health and the Applied Science Laboratories. The presence of unsaturated fatty acids was verified by the bromination and hydrogenation reactions, according to the methods of Farquhar et al. (1959). The positions of the double bonds in the 18-carbon unsaturated fatty acids were located by the modified permanganate-periodate method of Chang and Sweeley (1962).

After demonstrating that cholesterol could be separated from the other peaks obtained in the nonsaponifiable fraction by gas-liquid chromatography, known amounts of cholesterol were always added together with the mycelium as an internal standard before saponification. The percentage of cholesterol recovered in the nonsaponifiable fraction varied from 60 to 85%. The same percentage of ergosterol was assumed to be recovered from the mycelium, and the data in this paper have been corrected to 100%. Reagent-grade ergosterol from Mann Research Laboratory, New York, N.Y., was used as a standard.

Heptadecanoic acid methyl ester was used as an internal standard for the quantitative determination of all fatty acids in the mycelium. Because *P. atrovenetum* contained a small amount of heptadecanoic acid, the usual method had to be slightly altered. The ratio of the quantity of palmitic acid to heptadecanoic acid was constant when samples from the same mycelial powder were extracted. To determine the amount of added heptadecanoic acid that could be recovered, paired samples were used; a known quantity of heptadecanoic acid was added to only one member of the pair. The ratio of palmitic acid to heptadecanoic acid was determined for the sample to which no heptadecanoic acid was added. The amount of palmitic acid extracted from the mycelial sample to which heptadecanoic acid was added was also determined. Thus, the quantity of endogenous heptadecanoic acid in the latter sample could be calculated. The difference between the calculated endogenous heptadecanoic acid and the amount of heptadecanoic acid actually extracted was due to the added amount. Therefore, the percentage of the added heptadecanoic acid that was recovered in the sample could be calculated; it varied from 50 to 75%. The amount of fatty acids extracted from the fungus was assumed to be the same as the amount of internal standard extracted, and, as a result, all of the values were corrected to 100%. Similar calculations were made by use of the heptadecanoic to linoleic acid ratios, and essentially the same results were obtained.

The term "per cent" was based on mycelial dry weight, unless otherwise stated. The total amount of each lipid constituent per flask was calculated from the percentage of each constituent in the fungus and the total dry weight of mycelium per flask. The peak areas obtained from the gas chromatograph were measured with a planimeter.

RESULTS

The percentage of nonsaponifiable lipids in *P. atrovenetum* increased from 1.6 in the spores to

a maximum of 5.0 in very young hyphae at 24 hr, and then decreased during the remainder of the growth cycle (Fig. 1). The only sterol that could be identified in the nonsaponifiable fraction was ergosterol, which increased from 0.49% in the spores to a maximum of 0.77% at the beginning of the log phase of growth, and then slowly decreased (Fig. 1). The total ergosterol per culture flask reached a maximum at the end of the log phase, and then rapidly decreased.

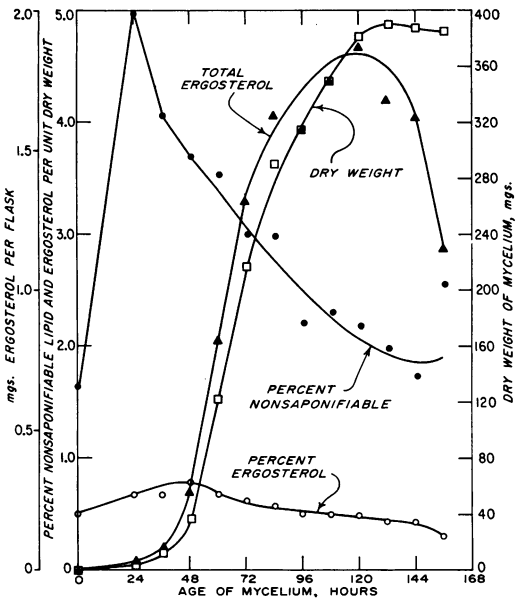

FIG. 1. *Relationship between nonsaponifiable lipids and the age of Penicillium atrovenetum. The nonsaponifiable lipid and ergosterol are expressed as per cent per unit dry weight. Total ergosterol refers to the total amount per flask.*

The nonsaponifiable fraction obtained from *P. atrovenetum* between 24 and 48 hr also contained eight small unidentified peaks. Another single unidentified peak appeared in the nonsaponifiable fraction at 84 hr and was present in this fraction during the remainder of the growth cycle. The unidentified peaks were not caused by squalene or lanosterol, which are probable intermediates in the biosynthesis of ergosterol (Popjak, 1958); nor were they caused by an incomplete extraction of the fatty acids, since none of the fatty acids gave such peaks when they were run on the SE-30 column. All of these transitory peaks in the nonsaponifiable fraction had shorter retention times than ergosterol on the SE-30 column.

At most stages of growth, the major fatty acids in *P. atrovenetum* were palmitic, stearic, oleic, and linoleic (Table 1). Other identifiable fatty acids which appeared at some stage during the growth cycle were myristic, pentadecanoic, palmitoleic, heptadecanoic, linolenic, and arachidic acids (Table 1). Since azelaic acid was the only dicarboxylic acid obtained after the oxidation of the fatty acids by the permanganate-periodate reaction, the common C-18 unsaturated fatty acids (oleic, linoleic, and linolenic) were the only ones present in the fungus.

Three inconclusively identified peaks, U-1, U-2, and U-3, appeared in the fatty acid fraction at some time during the growth of the organism

TABLE 1. *Percentage* of the fatty acids of Penicillium atrovenetum during growth and development*

Fatty acid	Growth cycle (hr)									
	0†	24	36	48	60	72	84	108	132	156
Myristic	0	0.011	0.006	0.007	0.008	0.009	0.025	0.036	0.030	0.028
Pentadecanoic	0.014	0.019	0.015	0.016	0.016	0.033	0.060	0.095	0.088	0.066
Palmitic	0.023	0.78	0.64	0.55	0.62	1.25	1.86	2.87	2.83	2.27
Palmitoleic	0.015	0.084	0.051	0.029	0	0	0	0	0	0
Heptadecanoic	0.029	0.028	0.026	0.021	0.032	0.044	0.039	0.061	0.059	0.048
U-1‡	0	0.006	0.012	0.009	0.006	0	0	0	0	0
U-2	0	0.21	0.24	0.031	0	0	0	0	0	0
Stearic	0.030	0.16	0.11	0.13	0.21	0.61	1.10	1.80	1.68	1.47
Oleic	0.21	1.83	1.65	1.24	1.32	2.18	3.11	4.17	3.95	3.61
Linoleic	1.39	2.11	1.79	1.77	1.87	2.43	2.95	4.39	4.23	3.72
Linolenic	0	0.065	0.054	0.017	0	0	0	0	0	0
Arachidic	0.022	0.060	0.057	0.042	0.070	0.090	0.157	0.161	0.273	0.334
U-3	0	0.107	0.196	0.006	0	0	0	0	0	0
Total	1.94	5.47	4.85	3.87	4.15	6.65	9.30	13.58	13.14	11.55

* The per cent is calculated on a unit dry weight of mycelium.
† The zero time value is for the spores that were used as inoculum.
‡ U-1 is probably a heptadecenoic acid.

(Fig. 2). Compound U-1 was tentatively identified as a heptadecenoic acid. This peak disappeared when the sample was brominated or hydrogenated, indicating that this compound was unsaturated. An increase in the heptadecanoic acid peak following hydrogenation could not be conclusively established because of the small quantities of U-1 present in the extracts. Compounds U-2 and U-3 are completely unknown at the present time. Their peak heights and retention times were not affected by bromination or hydrogenation. A plot of the retention time (log 10) vs. the number of the carbons in the fatty acids (Farquhar et al., 1959) did not help to identify these peaks.

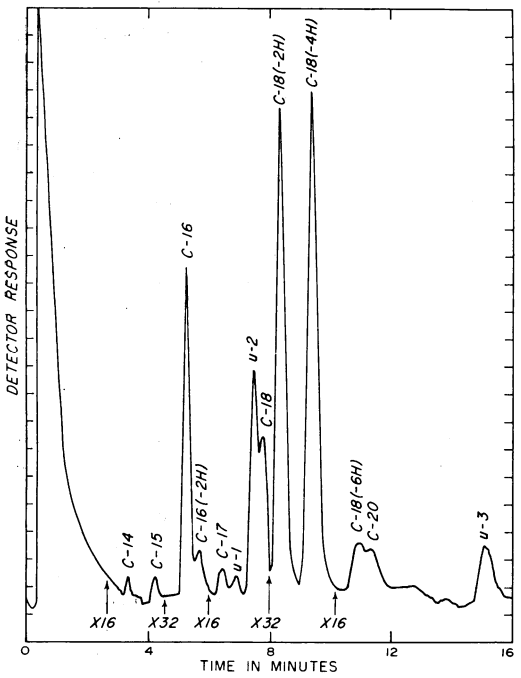

FIG. 2. *Gas chromatography of the methyl ester of the fatty acids of Penicillium atrovenetum at the 24-hr growth stage on a diethylene glycol adipate column. The range was 100 and the attenuation setting is indicated by the arrows. The abbreviation system used is that of Dole et al. (1959); U-1 is probably a heptadecenoic acid.*

The percentage of the fatty acids per unit dry weight of mycelium increased during the first 24 hr from 2 to 5.5, and then rose again during the log phase of growth. A slight decrease occurred as the fungus entered the decline and death phase of growth (Table 1).

Linoleic acid was the major fatty acid in the spores of *P. atrovenetum*, 66% of the total fatty acids (Table 2). Oleic and palmitic acids each made up about 13%. The remaining fatty acids in the spores, pentadecanoic, palmitoleic, heptadecanoic, stearic, and arachidic, were present in about equal quantities and comprised the remaining 8%.

A very striking change in the relative composition of the fatty acids occurred when the spores germinated and formed hyphae (Table 2). Linoleic acid decreased from 66 to 36% on the basis of total fatty acids, whereas oleic acid inincreased from 12 to 31%. However, the decrease in the percentage of linoleic acid was not due to its degradation. The percentage of linoleic acid per unit dry weight of mycelium increased slightly, whereas, at the same time, there was a

TABLE 2. *Percentage of the individual fatty acids in the total fatty acid fraction of Penicillium atrovenetum during growth and development*

Fatty acid	Growth cycle (hr)									
	0*	24	36	48	60	72	84	108	132	156
Myristic	0	0.4	0.4	0.2	0.3	0.4	0.4	0.5	0.5	0.5
Pentadecanoic	1.4	0.6	0.5	0.6	0.7	0.9	1.1	1.3	1.2	1.1
Palmitic	13.9	13.8	13.0	14.5	15.2	17.6	19.7	20.9	21.0	19.5
Palmitoleic	1.4	2.7	1.7	1.2	0	0	0	0	0	0
Heptadecanoic	1.2	0.8	0.9	0.9	1.4	1.2	0.7	0.9	0.8	0.8
U-1†	0	0.3	0.5	0.4	0	0	0	0	0	0
U-2	0	4.6	5.7	1.1	0	0	0	0	0	0
Stearic	1.5	3.6	3.4	4.6	6.1	10.0	12.0	13.3	12.9	12.8
Oleic	13.2	30.9	31.4	30.5	30.6	31.9	32.2	30.0	31.0	30.5
Linoleic	65.7	35.3	33.8	43.0	42.8	35.4	31.5	31.6	30.9	31.5
Linolenic	0	2.0	1.9	0.8	0	0	0	0	0	0
Arachidic	1.9	1.9	2.1	1.7	2.9	2.4	2.4	1.8	2.9	3.4
U-3	0	3.1	4.9	0.6	0	0	0	0	0	0

* The zero time value is for the spores that were used as inoculum.

† U-1 is probably a heptadecenoic acid.

ninefold increase in the percentage of oleic acid (Table 1).

Another interesting change during this period was the appearance of U-2, U-3, myristic, heptadecenoic, and linolenic acids. In this lag phase following spore germination, palmitoleic, heptadecenoic, linolenic, U-2, and U-3 reached a maximum, but later disappeared when the organism reached the log phase of growth (Table 2). With the disappearance of these five fatty acids in the log phase, there was a corresponding increase in the percentage of linoleic acid.

A second decrease in linoleic acid at 72 hr could be explained by the continued increase of the saturated fatty acids, palmitic and stearic. Stearic acid, for example, on the basis of total fatty acids, increased steadily during the entire growth cycle from 1.5% in the spores to 13% at 108 hr. The percentages of the remaining fatty acids showed no appreciable changes during this period.

DISCUSSION

Except for the compound tentatively identified as heptadecenoic acid, all the identifiable fatty acids present in the spores and mycelium have been reported in at least several other fungi (Cochrane, 1958; Tulloch, Craig, and Ledingham, 1959; Tulloch and Ledingham, 1960, 1962; Hughes, 1962; Molitoris, 1963).

The increase in the fatty acids during the period of spore germination and early lag phase is very similar to that occurring during the germination of *Penicillium oxalicum* spores (Caltrider, 1962). This process differs from the obligate parasites, *Puccinia graminis tritici* and *Uromyces phaseoli*, in which the fatty acid content decreased during spore germination (Shu, Tanner, and Ledingham, 1954; Caltrider et al., 1963). However, these two *Penicillium* species require an exogenous carbon source for germination, whereas the rust spores germinate in distilled water. Furthermore, the composition of these *Penicillium* spores differs from that of the rust spores: the rusts have an initially high fatty acid content, approximately 15%, whereas in *P. atrovenetum* and *P. oxalicum*, it is less than 2%.

Nonsaponifiable lipids may be important in the early development of fungi. They increased rapidly during spore germination in both the saprophytes *P. atrovenetum* and *P. oxalicum*, as well as in the obligate parasites *P. graminis tritici* and *U. phaseoli* (Caltrider et al., 1963). With *P. atrovenetum*, moreover, these compounds decreased even while the fungus was in the lag phase of growth. Though the changes in ergosterol content follow this general pattern, the presence of other unknown nonsaponifiable lipids is also associated with early development. The peak concentration of total nonsaponifiable lipid in *P. atrovenetum* occurred even earlier than ergosterol.

A change in the fatty acid composition from more unsaturated to less unsaturated compounds also seems to be related to fungal development. During the germination of *P. atrovenetum* spores, linoleic acid decreased, whereas oleic acid increased. Later, during mycelial growth, linoleic acid again decreased, but this time there was a corresponding increase in palmitic and stearic acids. Similar changes from more unsaturated to less unsaturated fatty acids have been reported during fungal growth. In *P. javanicum*, the change was from linoleic to oleic acid (Coots, 1962); in *T. nudum*, it was from linolenic to palmitic and oleic acids (Leegwater et al., 1962); and in *P. blakesleeanus* (Bernhard et al., 1957), the percentage of linolenic acid decreased with the growth of the fungus.

The function of the changes in the fatty acid pattern which accompany the growth of *P. atrovenetum* are not understood at the present time. Nevertheless, it is evident that a shift in synthesis from an unsaturated fatty acid to ones more saturated occurs with the development of the fungus, and that such changes with age seem common in all fungi that have been studied. Generally, the percentage of enzyme-forming compounds, such as amino acids, nucleotides, RNA, and protein, together with ergosterol and other nonsaponifiable lipids, are highest when fungi are preparing for their rapid growth phase. On the other hand, storage compounds, such as carbohydrates and fatty acids, increase to a maximum near the end of the log phase when the cells are no longer dividing. Cell metabolism can then be used to make materials needed to aid in the survival of the microbe. Potential energy sources, such as fats and carbohydrates, as well as cell-wall material, would enable the spores to withstand unfavorable environmental conditions and long periods of dormancy.

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