[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF DUKE UNIVERSITY]

Chemical Studies of Certain Pathogenic Fungi. II. The Lipids of *Monilia albicans*[1]

BY ROBERT L. PECK AND CHARLES R. HAUSER

In the first paper of this series was reported a chemical study of the lipids of *Blastomyces dermatiditis*.[2] The present paper is concerned with the investigation of the lipids of a somewhat similar pathogenic fungus, *Monilia albicans*.

Kappes[3] in 1890 extracted the dried cells of *Monilia albicans* with ether and obtained 4.28% of lipids. He did not, however, establish their chemical composition. In our experiments, using an alcohol–ether mixture and chloroform as extracting agents, we isolated 5.3% lipid material. This was separated into phosphatide and acetone-soluble fractions. The data on these fractions are summarized in Tables I–IV, which also include data on the lipids of *Blastomyces dermatiditis* for comparison. The methods of extraction and analysis employed in the present study are the same as those previously described.[2]

TABLE I

PERCENTAGES OF LIPIDS EXTRACTED

Organism and wt., g. of extracted cells (dried *in vacuo*)		Alc.–ether	$CHCl_3$	Total
Monilia albicans 698.0 g.	Grams	37.6	1.2	38.8
	Extd., %	5.1	0.2	5.3[a]
Blastomyces dermatiditis 818.5 g.	Grams	81.7	4.6	86.3
	Extd., %	9.0	0.5	9.5[a]

[a] Per cent. of wt. of dried extracted cells plus wt. of total extracted lipids.

TABLE II

PROPORTIONS OF PHOSPHATIDE AND ACETONE-SOLUBLE FRACTIONS

Organism		Phosphatide	Acetone-soluble
Monilia albicans	Grams	1.3	37.5
	% of whole cell[a]	0.2	5.1
	% of lipids[b]	3.4	96.6
Blastomyces dermatiditis	Grams	21.0	65.3
	% of whole cell[a]	2.3	7.2
	% of lipids[b]	24.3	75.7

[a] Per cent. of wt. of dried extracted cells plus wt. of total extracted lipids. [b] The lipids are the ether-soluble lipids extracted as in our experiments.

(1) By the term *Monilia albicans*, we are referring to the yeast-like fungus in the sense of Benham [*J. Infectious Diseases*, **49**, 183 (1931)], and Martin [*J. Bact.*, **34**, 99 (1937)]. This organism has been known to cause thrush, moniliasis, perlèche, meningitis, vulvovaginitis, and certain dermatoses [J. G. Hopkins, *Arch. Dermatol. Syphilol.*, **25**, 599 (1932)], as well as fatal systemic disease.

(2) Peck and Hauser, THIS JOURNAL, **60**, 2599 (1938).

(3) Kappes, Dissertation, Leipzig, 1890. Quoted by Flügge, "Die Mikroorganismen," 1896, pp. 96, 99.

The phosphatide fraction of *Monilia albicans* formed only a small percentage of the total lipids extracted (Table II), the main part consisting of the acetone-soluble fraction. The phosphatide was a cream-colored, solid, rather hygroscopic substance. It had a nitrogen:phosphorus ratio of 1.2:1. On saponification of the phosphatide, glycerophosphoric acid was isolated and identified. A mixture of fatty acids also was isolated, but because of the small amount obtained these were not identified. The water-soluble portion gave a positive Molisch test, indicating the presence of carbohydrate in the phosphatide. The nitrogenous constituents were not identified.

TABLE III

CONSTANTS OF THE ACETONE-SOLUBLE FRACTION

	Monilia albicans	*Blastomyces dermatiditis*
Iodine number	81.0	106.1
Acid value	96.3	45.3
Saponification value	183.9	191.5
Ester value	87.6	146.2

TABLE IV

PERCENTAGE COMPOSITION OF THE ACETONE-SOLUBLE FRACTION

	Monilia albicans	*Blastomyces dermatiditis*
Water soluble	6.9	4
Unsaponifiable	13.6	8
Fatty acids	79.5	88
Solid (saturated) acids	20.6	14.5
Liquid (unsaturated) acids	58.9	73.5
Iodine number	106	116
Palmitic acid[a]	15.5	9.7
Stearic acid[a]	5.1	4.8
Oleic acid[b]	48.6	55
Linoleic acid[b]	10.3	18.5
Glycerol	Present	Present
Ergosterol[c]	7	4

[a] Calcd. from wt. and neutral eq. of solid acids. [b] Calcd. from wt. and iodine no. of liquid acids. [c] Colorimetric analysis.

The acetone-soluble fraction was a brown oil consisting probably of a mixture of glycerides, free fatty acids and free and bound sterols. The data on this fraction are summarized in Tables III and IV. On saponification, the acetone-soluble fraction gave a mixture from which ergosterol, glycerol, and palmitic, stearic, oleic and linoleic acids were isolated and identified.

It may be observed from Table I that *Blastomyces dermatiditis* yielded nearly twice as much lipid material soluble in neutral solvents as did *Monilia albicans*. This might have been due to the differences in composition of the culture media and temperature of growth. From Table II, which concerns the partition of the lipids extracted by neutral solvents, it is evident that *Monilia albicans* yielded a considerably smaller percentage of phosphatide and a correspondingly greater percentage of acetone-soluble fat than did *Blastomyces dermatiditis*.

Judging from the data thus far obtained, the phosphatides of *Monilia albicans* and *Blastomyces dermatiditis* are similar. The nitrogen:phosphorus ratio was approximately 1:1 in both. On hydrolysis, both phosphatides gave fatty acids, glycerophosphoric acid and nitrogenous compounds. The water-soluble portion obtained in each case gave a positive test for carbohydrates.

The acetone-soluble fractions of the two organisms are also similar, as can be seen from Tables III and IV. The acetone-soluble fraction from *Blastomyces dermatiditis*, however, was more highly unsaturated and contained a smaller percentage of free fatty acids and a higher percentage of esters than the corresponding fraction from *Monilia albicans* (Table III). Saponification of these acetone-soluble fractions gave the same compounds in each case (Table IV). Although the qualitative composition of the acetone-soluble fractions of the two organisms appears to be the same, certain quantitative differences are found. These may have been the result of differences in culture media and growth conditions.

In our experiments we wished to obtain the lipid material as nearly unchanged from the native condition as possible and therefore employed mild extraction procedures. It has been shown by Turpeinen[4] and others[5] that the lipids present in microörganisms are not entirely extracted by neutral solvents and that a more drastic treatment is necessary if the total lipids are to be secured. Preliminary experiments[6] in this Laboratory have indicated that on treatment with hot alcoholic hydrochloric acid *Monilia albicans* yields much more bound phosphatide than *Blastomyces dermatiditis*. This difference in availability of phosphatide in the two organisms may be of significance.

(4) Turpeinen, *Ann. Acad. Sci. Fennicae*, irr., Series **A**, 46 (1936).

(5) (a) Crowder, Stodola, Pangborn and Anderson, THIS JOURNAL, **58**, 636 (1936); (b) Dawson, *J. Bact.*, **4**, 133 (1919).

(6) These experiments were carried out on cells previously extracted by alcohol–ether mixtures and by chloroform.

Experimental

Materials.—Sabauroud's medium[7] was used for the cultures in the present experiments. Bacto-dextrose, bacto-peptone and agar-agar (all Difco Laboratories brand) were used in preparing the medium. Control experiments showed that negligible amounts of ether-soluble substances were removed from the medium during harvesting of the organisms.

Anhydrous ether, acetone and chloroform dried over drierite were freshly distilled before using. Alcohol was freshly distilled from potassium hydroxide. The solvents were saturated with nitrogen or carbon dioxide before use. Air was displaced with nitrogen or carbon dioxide from all vessels used to hold unsaturated lipids.

Growth and Extraction.—A virulent strain of *Monilia albicans* was grown on Sabauroud's medium at room temperature. The four days growth was washed from the surface of the cultures with $1/8$ *N* sodium chloride solution, centrifuged, resuspended in fresh salt solution and again centrifuged. The washing with salt solution was repeated once more and the cells were then placed in liter flasks with about ten times their volume of a mixture of alcohol and ether (1:1).

The flasks were kept in the dark in the cold-room for four weeks. After filtration and washing with ether, the cells were resuspended in alcohol–ether mixture and allowed to stand for another four weeks. The cells were then filtered off, washed with ether and extracted with chloroform for four weeks. The extracted cells after washing with chloroform and drying *in vacuo* were a buff-colored powder weighing 698 g.

Concentration of Extracts.—The alcohol–ether extracts and washings were concentrated in an all-glass apparatus under reduced pressure until an aqueous emulsion of the lipids remained. This was extracted thoroughly with ether. After drying over sodium sulfate, the ether extract was evaporated yielding 37.6 g. of red brown oil. The aqueous solution remaining after the extraction of the lipids with ether was saved for future examination.

The chloroform extract on concentration yielded 1.20 g. of dark brown oil. Since examination showed it to be similar to the alcohol–ether extract, it was combined with the latter.

Separation of the Phosphatide and Acetone-Soluble Fractions.—Treatment of the total extracted lipids (38.8 g.) with acetone yielded 1.3 g. of acetone-insoluble crude phosphatide. A few drops of the acetone solution and washings from the precipitation of the phosphatide fraction were tested with saturated alcoholic strontium chloride, but only a negligible amount of insoluble material was obtained. The acetone solution and washings were therefore concentrated, yielding 37.5 g. of acetone-soluble fat.

Phosphatide Fraction.—Repeated precipitation of the crude phosphatide from ether solution by means of acetone yielded 0.508 g. of rather hygroscopic cream-colored powder. When heated in a capillary tube, this purified phos-

(7) Sabauroud's medium consists of 40 g. of glucose, 36 g. of agar, 10 g. of peptone and 1000 cc. of water.

phatide softened at 105°, was fully liquid at 125° and began to decompose at about 130°.

Micro-anal. Found: N, 2.18; P, 3.97. Ratio: Nitrogen:Phosphorus, 1.2:1.

The purified phosphatide was saponified in essentially the same manner as described by Anderson[8] and coworkers. To a benzene solution of the phosphatide was added a slight excess of potassium hydroxide dissolved in absolute alcohol. After standing for twenty-four hours at room temperature, the mixture was centrifuged and the residue was washed with benzene and ether. The residue was then dissolved in water and acidified with acetic acid. The fatty acids which precipitated were extracted with ether but were not identified. A Molisch test on a portion of the aqueous solution gave a positive test for carbohydrates. The rest of the aqueous solution was treated with neutral lead acetate. The precipitate was centrifuged off, suspended in water and decomposed with hydrogen sulfide. The lead sulfide was filtered off and the filtrate neutralized with barium hydroxide. To this solution two volumes of alcohol were added and a precipitate was obtained which was evidently the barium salt of glycerophosphoric acid. This was purified by reprecipitation from water solution by alcohol.

Micro-anal. Calcd. for $C_3H_7O_6PBa \cdot 2H_2O$: Ba, 39.99; P, 9.03. Found: Ba, 39.96; P, 8.62.

Acetone-Soluble Fraction.—This fraction was a brown oil with an iodine number[9] of 104. Only traces of phosphorus and nitrogen were present. The saponification value was 183.9, the acid value 96.3, and the ester value 87.6.[10]

The acetone-soluble fraction was saponified with 4% alcoholic potassium hydroxide in an atmosphere of nitrogen. After distilling off most of the alcohol, the saponification mixture was diluted with water and the unsaponifiable material was extracted with ether. The ether solution, dried over sodium sulfate, was evaporated, yielding a brown semi-crystalline soft mass. This on crystallization from a mixture of two parts of alcohol to one of benzene, gave white crystals melting at 159–161°, and a brown oil which was not studied. The crystals were proved to be ergosterol by the mixed melting point method and by the preparation of the acetate. The melting point of the latter, 172–174°, was not lowered when mixed with a sample of the acetate made from authentic ergosterol.

The alkaline aqueous solution remaining after extraction of the unsaponifiable material was acidified with hydrochloric acid and the fatty acids were extracted with ether. The acid aqueous solution was neutralized and concentrated under reduced pressure to dryness. Extraction of the residue with a mixture of equal parts of alcohol and ether yielded on concentration of the extract a small amount of thick brown sirup. Benzoylation of a portion of this sirup yielded a very small amount of crystals which melted at 75–76° after recrystallization. These were proved by the mixed melting point method to be glycerol tribenzoate. This establishes the presence of glycerol in the lipids.

The ether solution containing the fatty acids was dried over sodium sulfate and on evaporation of the ether yielded a brown solid semi-crystalline mass of fatty acids.

Separation by the Twitchell[11] lead salt procedure gave 26% solid acids and 74% liquid acids. The solid acids melted at 51° and had a mean molecular weight of 263.

A portion of the solid acids was converted to the methyl esters and distilled at 0.5 mm. pressure. The lowest boiling fraction was saponified and the acid recovered and recrystallized. This acid melted at 62.5–63°. It was proved to be palmitic acid by the mixed melting point method.

Micro-anal. Calcd. for $C_{16}H_{32}O_2$: C, 74.91; H, 12.58. Found: C, 75.26; H, 12.65.

The highest boiling fraction was saponified and the acid isolated. This melted at 69–70° and was proved by the mixed melting point method to be stearic acid.

Micro-anal. Calcd. for $C_{18}H_{36}O_2$: C, 75.95; H, 12.76. Found: C, 75.75; H, 12.65.

A portion of the liquid acids was reduced in alcohol solution with hydrogen and platinum oxide catalyst.[12] The reduced acid was recovered as a pale buff crystalline solid melting at 69–70° and having a neutral equivalent of 284.4. The mixed melting point using pure stearic acid (melting at 70–71°) showed no depression. The reduced acid was thus almost pure stearic acid. Since no liquid acid was isolated on reduction, it appears that no liquid saturated acid was present in the fat. The reduced acid was recrystallized for analysis.

Micro-anal. Calcd. for $C_{18}H_{36}O_2$: C, 75.95; H, 12.76. Found: C, 76.13; H, 12.90.

Another portion of the liquid acids was oxidized with alkaline permanganate as described by Lapworth and Mottram.[13] On extraction of the crude oxidized acids with boiling chloroform, an acid remained undissolved. This melted at 171–172° after crystallization from alcohol, and was shown by the mixed melting point method to be tetrahydroxystearic acid.

Micro-anal. Calcd. for $C_{18}H_{36}O_6$: C, 61.99; H, 10.42. Found: C, 62.13; H, 10.59.

The acid soluble in chloroform was extracted with petroleum ether after removing the chloroform. The residue was crystallized from alcohol and melted at 129–130°. This was dihydroxystearic acid, since no depression was observed in the melting point on admixture with authentic dihydroxystearic acid.

Micro-anal. Calcd. for $C_{18}H_{36}O_4$: C, 68.26; H, 11.47. Found: C, 68.40; H, 11.26.

A further portion of the liquid acids was brominated in cold light petroleum ether (b. p. 30–60°) solution. The product was completely soluble in ethyl ether in the cold, hence acids more unsaturated than linoleic were probably not present. The insoluble bromides were crystallized from petroleum ether (b. p. 70–90°). The crystals

(8) Anderson, Lothrop and Creighton, *J. Biol. Chem.*, **125**, 299 (1938).

(9) All iodine numbers were determined according to Rosenmund and Kuhnhenn, *Z. Nahr. Genussm.*, **46**, 154 (1923).

(10) Saponification value, acid value and ester value were determined according to Assoc. Off. Agric. Chem., "Methods of Analysis," Washington, D. C., 1936, 4th ed.

(11) Twitchell, *Ind. Eng. Chem.*, **13**, 806 (1921).

(12) Voorhees and Adams, THIS JOURNAL, **44**, 1397 (1922).

(13) Lapworth and Mottram, *J. Chem. Soc.*, **127**, 1628 (1925).

melted at 113–114° and were shown to be tetrabromostearic acid by the mixed melting point method.

Micro-anal. Calcd. for $C_{18}H_{32}O_2Br_4$: Br, 53.29. Found: Br, 53.08.

The bromides which were soluble in the brominating solution were evidently largely dibromostearic acid.

Micro-anal. Calcd. for $C_{18}H_{34}O_2Br_2$: Br, 36.15. Found: Br, 37.27.

The above data on the liquid acids establish the presence of oleic and linoleic acids in the acetone-soluble fat and indicate that no higher unsaturated acids occur.

Acknowledgment.—The present work was supported in part by grants from the Duke University Research Council and the John and Mary R. Markle Foundation. We wish also to express our gratitude to Drs. D. T. Smith and Donald S. Martin of the Duke University Medical School for advice on growing the fungus.

Summary

1. The lipids of *Monilia albicans* soluble in alcohol and ether and in chloroform have been investigated. These lipids make up 5.3% of the weight of the cells.

2. The lipids were separated into 3% phosphatide fraction and 97% acetone-soluble fraction.

3. The phosphatide had a nitrogen:phosphorus ratio of 1.2:1. On saponification, glycerophosphoric acid was isolated and identified. A mixture of fatty acids was isolated but the individual acids were not identified. The water-soluble portion gave a positive Molisch test, indicating the presence of carbohydrates.

4. The acetone soluble fraction probably consisted of a mixture of glycerides, free fatty acids, and free and bound sterols. After saponification of this fraction, ergosterol, glycerol, and palmitic, stearic, oleic and linoleic acids were isolated from the resulting mixture and identified.

5. The data obtained were compared with the lipids of *Blastomyces dermatiditis*.

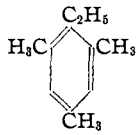
DURHAM, N. C. RECEIVED NOVEMBER 25, 1938

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

Polymethylbenzenes. XXIII. The Preparation and Physical Properties of 3- and 5-Ethylpseudocumenes and of Ethylmesitylene[1]

BY LEE IRVIN SMITH AND MATTHEW A. KIESS[2,3]

In connection with the studies on the Jacobsen rearrangement, three of the six ethyltrimethylbenzenes have been prepared in quantity and certain of their physical properties have been determined. The three hydrocarbons investigated are ethylmesitylene (I), 3-ethylpseudocumene (II) and 5-ethylpseudocumene (III).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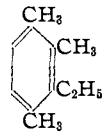
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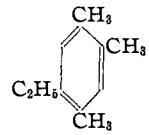
III

Ethylmesitylene (I).—This hydrocarbon was first synthesized by Jannasch and Wigner[4] in 20% yields from bromomesitylene, ethyl bromide and sodium. A short time later, Töhl[5] repeated this synthesis, and he also varied it by using iodomesitylene and ethyl bromide, as well as bromomesitylene and ethyl iodide. Klages[6] obtained a mixture of mesitylene and ethylmesitylene by reducing mesitylmethylcarbinol with hydriodic acid, while Klages and Keil[7] obtained the hydrocarbon in good yield by reduction of vinylmesitylene. Von Auwers[8] reduced acetomesitylene with zinc and hydrochloric acid, and Willgerodt and Scholz[9] reduced the ketone with ammonium sulfide, both reductions giving the hydrocarbon.

In the present work, the hydrocarbon I was synthesized by two methods. Conversion of bromomesitylene to the Grignard reagent and ethylation of the latter produced the hydrocarbon in over-all yields of slightly better than 40% (based upon the mesitylene used). Conversion

(1) Paper XXII: Nightingale and Smith, THIS JOURNAL, **61**, 101 (1939).

(2) Abstracted from a thesis by Matthew A. Kiess, presented to the Graduate Faculty of the University of Minnesota, in partial fulfilment of the requirements for the Ph.D. degree, November, 1938.

(3) Present address, Department of Chemistry, St. Johns College, Collegeville, Minn.

(4) Jannasch and Wigner, *Ber.*, **28**, 2028 (1895).

(5) Töhl, *ibid.*, **28**, 2459 (1895).

(6) Klages, *ibid.*, **35**, 2256 (1902).

(7) Klages and Keil, *ibid.*, **36**, 1644 (1903).

(8) Von Auwers, *Ann.*, **419**, 120 (1919).

(9) Willgerodt and Scholz, *J. prakt. Chem.*, [2] **81**, 386 (1910).