[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, DUKE UNIVERSITY]

Chemical Studies of Certain Pathogenic Fungi. I. The Lipids of *Blastomyces dermatiditis*[1]

BY ROBERT L. PECK AND CHARLES R. HAUSER

This series of investigations has been undertaken with the purpose of studying the constituents of certain pathogenic fungi with the hope that more light may be thrown on the chemical nature of the factors responsible for their pathogenicity. Data on nonpathogenic fungi are available to afford comparisons.

In this paper are presented the results obtained from a study of the lipid fraction of *Blastomyces dermatiditis*. No previous chemical study of the lipid fraction of *Blastomyces dermatiditis* in the yeast-like form could be found in the literature. The present investigation is concerned with the isolation and identification of the constituents of the lipids extracted from this organism by a mixture of alcohol and ether and by chloroform. The isolation and purification of the fractions were in general carried out according to the methods developed by Anderson.[2] In all operations involving unsaturated lipids, an atmosphere of nitrogen or carbon dioxide was maintained as far as possible. All solvents were purified and freshly distilled before use. During extractions and concentrations special precautions were taken in order to avoid contamination of the fungus lipids with extraneous fatty materials.

In Table I are given the percentages of lipids extracted from the cells. Table II gives the proportions of the phosphatide and the acetone-soluble fractions. In order to form an idea as to the precision of our methods of extraction and estimation, two small batches of organisms were grown and extracted for one month with a mixture of alcohol and ether. The extracts and washings were worked up as described in the experimental part. The results are given in Tables I and II, batches III and IV. These results indicate that the methods of extraction and of separation of the phosphatide and acetone-soluble fractions are reproducible within about five per cent.

TABLE I

PERCENTAGES OF LIPIDS EXTRACTED FROM *Blastomyces dermatiditis*

Batch and wt., g.		Lipids extracted Alc.–ether	CHCl₃	Total
I	Grams	81.7	4.6	86.3
818.5[a]	Extd., %[b]	9.0	0.5	9.5
II	Grams	18.8	1.6	20.4
209.0[a]	Extd., %[b]	8.2	0.7	8.9
III	Grams	6.1		6.1
62.7[a]	Extd., %[b]	8.9		8.9
IV	Grams	5.6		5.6
60.4[a]	Extd., %[b]	8.5		8.5

[a] Weight of extracted cells after drying *in vacuo*. [b] Per cent. of weight of dried extracted cells plus weight of total extracted lipids.

TABLE II

PROPORTIONS OF THE PHOSPHATIDE AND ACETONE-SOLUBLE FRACTIONS OF *Blastomyces dermatiditis*

Batch		Phosphatide	Acetone soluble
I	Grams	21.0	65.3
	% of whole cell[a]	2.3	7.2
	% of lipids[b]	24.3	75.7
II	Grams	1.7	18.8
	% of whole cell[a]	0.7	8.2
	% of lipids[b]	8.2	91.8
III	Grams	2.1	4.0
	% of whole cell[a]	3.0	5.9
	% of lipids[b]	34.1	65.9
IV	Grams	1.9	3.7
	% of whole cell[a]	2.9	5.6
	% of lipids[b]	34.3	65.7

[a] The whole cell weight is assumed as that of the dried extracted cells plus extracted lipids.

[b] The lipids are the ether-soluble lipids extracted as in our experiments.

Batch I was grown on a medium[3] containing a small amount of blood. Batches II, III, and IV were grown on the same medium without blood. It can be seen from Table II that the composition of the lipids of batch I is essentially the same as that of batches III and IV; hence it would appear that the presence of blood in the medium does not greatly influence the lipid composition.

Batch II was left for two weeks in a preliminary killing solution of 1% aqueous phenol before ex-

(1) *Blastomyces dermatiditis* [Gilchrist and Stokes (1898)] a yeast-like organism when grown at 37°, is the etiological agent of the human disease, blastomycosis. The disease may be either a skin infection or a fatal generalized condition. See Martin and Smith, *Am. Rev. T. B.*, in press.

(2) Anderson, *J. Biol. Chem.*, **74**, 525 (1927), and later papers.

(3) This medium was developed by Dr. Wm. H. Kelly of the Department of Medicine, Duke University. It consists of 10 g. of dextrose, 10 g. of peptone, 15 g. of agar-agar, 3 g. of beef extract, 0.5 g. of sodium chloride, and 1000 g. of distilled water. To this is added 20 cc. of laked blood (one part blood to three parts distilled water).

traction of the lipids, whereas the other batches were placed in alcohol and ether immediately after removal from the culture. It is evident from Table II that immediate extraction of the living organisms with alcohol and ether yields much more phosphatide than similar extraction of the organisms after allowing them to stand with aqueous phenol. It should be observed, however, that the total fat remains about the same in the two methods.

In Table III the data on the phosphatide fraction are presented. It can be seen that, although grown and extracted under somewhat different conditions, batches I and II yielded phosphatides of essentially the same composition. The phosphatide fraction is presumably a mixture containing substances resembling lecithin and cephalin. The nitrogen–phosphorus ratio is 1:1. On hydrolysis with aqueous sulfuric acid, glycerophosphoric acid, choline, ethanolamine and fatty acids were obtained. The first three compounds were isolated and identified. Evidence has been obtained which indicates that the following fatty acids were present: palmitic, stearic, oleic, and linoleic acids. It should be mentioned also that a small amount of carbohydrate was obtained on saponification of the phosphatide.

Tables IV and V give the data on the acetone-soluble fats. The acetone-soluble fraction ob tained from batch I was qualitatively the same as from batch II, but certain quantitative differences appear; these are probably due to the differences in treatment of the cells before extraction. The acetone-soluble fraction appears to be a mixture containing free fatty acids and sterols. On saponification it gave glycerol, ergosterol, and palmitic, oleic and linoleic acids. These compounds were isolated and identified. Stearic acid was probably also present.

TABLE III

PERCENTAGE COMPOSITION OF PHOSPHATIDE FRACTIONS

	Blastomyces dermatiditis Batch I	*Blastomyces dermatiditis* Batch II	Tubercle bacilli[a]
Water soluble	32	32	34
Ether soluble	68	68	64.2
Unsaponifiable	3	3	1.8
Total fatty acids	65	65	62.4
Solid acids	12.4		30.5
Liquid acids	52.6 (iodine no., 96)		33.7 (iodine no., 30)
Liquid satd. acids	0		20.9
Palmitic acid	8.2[b]		30.5
Stearic acid	4.2[b]		Very small amount
Oleic acid	49.1[c]		12.8
Linoleic acid	3.5[c]		0 (?)
Carbohydrates	Present	Present	30.4
Glycerophosphoric acid	3.1	Present	5.4
Choline	Present	Present	Absent
Ethanolamine	Present	Present	Absent
Nitrogen	1.78	1.71	0.4
Phosphorus	3.89	3.61	2.3
Nitrogen–phosphorus ratio	1:1	1:1	2:5

[a] See ref. 4. [b] Calculated on assumption that solid acids were mixture of palmitic and stearic acids. [c] Calculated from iodine number (96) and weight of liquid acids.

TABLE IV

CONSTANTS[a] OF ACETONE-SOLUBLE FAT

	Blastomyces dermatiditis Batch I	*Blastomyces dermatiditis* Batch II	Tubercle bacilli[b]
Iodine number	106.1	101.2	52.6
Melting point	Oil	Oil	33°C.
Acid value	45.3	60.3	60.3
Saponification value	191.5	170.0	203.6
Ester value	146.2	109.7	143.2
Reichert–Meissl value	2.7		3.9

[a] These constants with the exception of iodine number and melting point were determined in the case of *Blastomyces dermatiditis* according to Assoc. Official Agr. Chem. "Methods of Analysis," Washington, D. C., 1936, 4th ed. [b] See Anderson, *Physiol. Rev.*, 12, 166 (1932).

TABLE V

PERCENTAGE COMPOSITION OF THE ACETONE-SOLUBLE FATS

	Blastomyces dermatiditis Batch I	*Blastomyces dermatiditis* Batch II	Tubercle bacilli[c]
Unsaponifiable matter	8.0	15.4	10.3
Water soluble	4.0	4.8	6.3
Fatty acids	88.0	79.8	83.3
Solid acids (satd.)	14.5	11.9	30.3
Liquid acids	73.5	67.9	50.6
Iodine no.	116	126	31
Liquid saturated acids	0	0	38.0
Oleic acid[a]	55.0	37.7	10.6
Linoleic acid[a]	18.5	30.2	?
Palmitic acid[b]	9.7		30.3
Stearic acid[b]	4.8		Traces
Sterols[d]	4	6	0

[a] Calculated from the weight and iodine number of the unsaturated acid. [b] Calculated from weight and mean molecular weight of the saturated acids. [c] See ref. 4. [d] Colorimetric analysis.

In a later paper the lipids of *Blastomyces dermatiditis*, together with those of another pathogenic fungus, *Monilia albicans*, will be compared with the lipids of certain non-pathogenic fungi.

In this paper it seemed of interest to compare the lipids of *Blastomyces dermatiditis* with those of the tubercle bacillus,[4] since the clinical picture of the disease caused by the fungus resembles in certain respects that caused by the bacillus.

(4) R. J. Anderson, *Physiol. Rev.*, **12**, 166 (1932).

Data on the lipids of both organisms are listed in Tables III, IV and V.

It can be seen from Tables III and V that, while the lipids of *Blastomyces dermatiditis* and of tubercle bacilli have a number of similarities, certain striking differences appear. In the fungus are found a higher percentage of unsaturated acids and a lower percentage of saturated acids than are found in the tubercle bacillus, but, more significant, the latter organism contains a considerable amount of liquid saturated fatty acid, which appears to be entirely absent in the fungus. The phosphatide of the fungus (Table III) contains two nitrogenous constituents which apparently are not found in the tubercle bacillus, and the nitrogen–phosphorus ratio is different in the two organisms. Sterols (ergosterol) are present in the acetone-soluble fraction of the fungus (Table V) but this constituent apparently is not found in the tubercle bacillus. It is of interest that carbohydrates are present in the phosphatides of both organisms.

Experimental

Materials.—The culture medium was prepared from bacto-peptone, bacto-dextrose, and agar-agar purchased from Difco Laboratories, Detroit, Michigan, and beef extract from Armour and Company, reagent quality sodium chloride, and distilled water. In the media for batch I, laked sheep blood (one part blood to three parts distilled water) was used; no blood was in the media for batches II, III and IV. The medium was placed in liter prescription bottles which were then plugged with cotton and gauze and sterilized. The cultures were always checked for contamination; only pure cultures were used for the experiments.

Ether was dried over sodium; acetone and chloroform were dried over drierite. These were freshly distilled before use. Alcohol was freshly distilled from potassium hydroxide.

Growth and Extraction.—A virulent strain of *Blastomyces dermatiditis* (Strain F-2) was grown[5] for seven days on the solid media at 37° in the dark. The organisms (in the case of batches I, III, and IV) were washed from the media with 95% alcohol, collected in 5-gallon (19 liters) bottles, an equal volume of ether was added and the bottles were stoppered with corks covered with tin-foil and stored for one month at room temperature in the dark. They were shaken occasionally with a rotary motion. Control experiments have shown that no appreciable amounts of lipids were extracted from the media or the stoppers under the conditions used in this work. The cells were filtered off, washed with alcohol and ether, and reëxtracted for two weeks with a mixture of equal parts of alcohol and ether. After filtration and washing, the cells finally were extracted with chloroform for one month. The cells were filtered off, washed with chloroform, and dried *in vacuo* giving a cream-colored powder.

Batch II was washed from the culture with aqueous 1% phenol and allowed to stand under this solution for two weeks. The cells were then filtered off and placed in a mixture of equal volume of alcohol and ether. The procedure from this point was the same as in the extraction of the other batches.

Concentration of Extracts.—The alcohol–ether extracts and washings were concentrated under reduced pressure in an all-glass apparatus to a small volume. The resulting aqueous emulsion was thoroughly extracted with ether. The residual aqueous solution was treated as described in the next section. The ether extract, after drying over sodium sulfate, yielded on evaporation a clear red-brown oil (batch I, 81.7 g.; batch II, 18.9 g.). White crystals (batch I, 2.12 g.) appeared in this oil on standing overnight. These were separated and identified as ergosterol as described below.

The chloroform extract and washings, on concentration under reduced pressure, yielded a dark brown oil (batch I, 4.6 g.; batch II, 1.6 g.). This was similar to the alcohol–ether soluble material and was therefore combined with it.

The Residual Aqueous Solution.—The aqueous solution, remaining after the ether extraction of the lipids described in the previous section, was concentrated to small volume and treated with two volumes of 95% alcohol. A crude polysaccharide (batch I, 1.5 g.) was precipitated. The purified polysaccharide possessed biological activity and will be reported at another time. From the sirup obtained by concentrating the supernatant solution and washings, mannitol (batch I, 1.0 g.) melting at 164–165° was obtained. This was proved by the mixed melting point method, and by the preparation of the hexaacetate, melting at 121°, and of mannitol tribenzal[6] melting at 218–220°.

Separation of the Phosphatide and Acetone-Soluble Fractions.—On addition of acetone to the brown oil which formed the combined lipids, the phosphatide separated as a sticky brown residue on the bottom of the flask. This fraction was purified by repeated precipitation from ether solution by means of acetone. The phosphatide (batch I, 19.5 g., batch II, 1.5 g.) was a buff-colored hygroscopic powder. The acetone solutions and washing from the separation and purification of the phosphatide (about four liters) were concentrated under reduced pressure to a volume of approximately 300 cc. To this solution was added a small amount of saturated alcoholic strontium chloride. After standing overnight in the refrigerator, a further small amount of phosphatide had separated (batch I, 1.5 g., batch II, 0.2 g.).

The supernatant solution and washings from the separation of the phosphatide fraction yielded on concentration a clear red oil (batch I, 65.3 g., batch II, 18.8 g.). It contained only traces of phosphorus and nitrogen. This fraction was designated the acetone-soluble fat.

The Phosphatide Fraction.—The phosphatide, obtained as a pale buff-colored powder, softened at 100°, was fully liquid at 125°, and began to decompose at about 130°. It formed emulsions with water. The phosphorus–nitrogen ratio was 1–1.

Micro-Anal. Found: P, 3.89; N, 1.78.

(5) The organisms were grown in the laboratories of the Department of Medicine, Duke University, by one of us (R. L. P.).

(6) Preus, Peterson and Fred, *J. Biol. Chem.*, **97**, 483 (1932).

Hydrolysis was carried out by refluxing the phosphatide with 5% aqueous sulfuric acid for eight hours. After extraction of the fatty acids with ether, the water solution was freed quantitatively from sulfuric acid with barium hydroxide. The water-soluble constituents were separated essentially as described by Newman and Anderson[7] and by Peterson and co-workers.[8] Barium glycerophosphate was isolated as a white powder and was purified by means of its lead salt. This was decomposed with hydrogen sulfide, reconverted to the barium salt and dried in air.

Micro-Anal. Calcd. for $C_3H_7O_6PBa \cdot 2H_2O$: Ba, 39.99; P, 9.03. Found: Ba, 39.48; P, 8.91.

Choline was isolated as the chloroplatinate, melting point 235–236° with decomposition.

Micro-Anal. Calcd. for $(C_5H_{14}ON)_2PtCl_6$: Pt, 31.68. Found: Pt, 31.57.

Ethanolamine was isolated as the picrolonate, melting point 224–226° with decomposition.

Micro-Anal. Calcd. for $C_{12}H_{15}O_6N_5$: N, 21.55. Found: N, 21.30.

The residual sirup from the water-soluble fraction gave a positive acrolein test and a positive Molisch test. A small amount of a sticky residue appeared on the walls of the flask when a sample of the phosphatide was hydrolyzed with alcoholic potassium hydroxide. This was soluble in water and gave a positive Molisch test for carbohydrate. The small amount of this substance obtained has prevented further study at present.

The fatty acids made up 65% of the phosphatide. They were separated in the case of batch I by means of the lead salt treatment into 12.4% solid and 52.6% liquid acids. The solid acids were a white crystalline mass melting at 54–55° and having a mean molecular weight of 266. On the assumption that the mixture of solid acids consisted only of palmitic and stearic acids, the relative proportions of each may be calculated from the neutral equivalent and the weight of the mixed acids. This would indicate a mixture composed of about two-thirds palmitic and one-third stearic acid. A sample of the liquid acids was reduced with hydrogen and platinum oxide.[9] On treatment of the reduced acids by the lead salt method, a very small amount of liquid acid was obtained, but this was shown to be unsaturated. No liquid saturated acids were found. The solid reduced acid, crystallized once from alcohol, was a white crystalline mass, melting at 70–71°. Admixture with pure stearic acid (m. p. 70–71°) did not lower the melting point. The neutral equivalent was 284.8. This shows that the reduced acid was stearic acid (neut. equiv. 284.3).

Micro-Anal. Calcd. for $C_{18}H_{36}O_2$: C, 75.95; H, 12.76. Found: C, 76.14; H, 12.79.

On bromination of the unsaturated acids only a trace of petroleum ether insoluble acids was obtained. The main product was a brown oil, probably dibromostearic acid.

Micro-Anal. Calcd. for $C_{18}H_{34}Br_2O_2$: Br, 36.15. Found: Br, 37.20.

The iodine number (96) and the amount of stearic acid isolated on reduction indicate that the liquid acids consisted mainly of oleic acid with a small amount of linoleic acid.

Analysis of the Acetone Soluble Fat.—This fraction was a red-brown oil containing only traces of phosphorus and nitrogen. It possessed a peculiar musty smell. Some of its constants are given in Table III.

The oil (batch I, 33.7 g.; batch II, 15.1 g.) was saponified by refluxing with 4% alcoholic potassium hydroxide for eight hours. After most of the alcohol was distilled off, the soap solution was diluted with water and the unsaponifiable material thoroughly extracted with ether. The remaining solution was resaponified for three hours and again extracted with ether. The soap solution was then acidified with hydrochloric acid and the crude fatty acids were extracted with ether. The aqueous acid solution was concentrated to dryness under reduced pressure and extracted with alcohol–ether solution (1:1). This extract was concentrated to a sirup. The sirup was extracted with warm absolute alcohol. The alcohol extract was evaporated, yielding a pale brown sirup. The sirup, which was evidently crude glycerol, gave a positive acrolein test. A sample was benzoylated, giving a very small yield of a crystalline derivative melting at 75–76°. This melting point was not lowered by admixture with glycerol tribenzoate (m. p. 76°). The presence of glycerol is thus established.

The unsaponifiable material was a brown semi-solid mass (batch I, 8%; batch II, 15.4% of the acetone-soluble fat). Since a colorimetric determination indicated that about one-half of this unsaponifiable material consisted of sterols, it was recrystallized from a mixture of alcohol and benzene (2:1). The white crystals obtained melted at 158–160° and were identified as ergosterol by the mixed melting point method and by conversion to the acetate. The latter melted at 169–171° and when mixed with the acetate from authentic ergosterol showed no depression. The ergosterol mentioned under the heading "Concentration of Extracts" was identified in the same manner.

The fatty acids were separated by the lead salt method into solid and liquid acids. The crude solid acids melted at 55–56° and gave a neutral equivalent of 266. They were thus similar to the crude solid acids of the phosphatide fraction. The methyl esters of a portion of the solid acids were prepared and fractionally distilled under 0.5 mm. pressure. The top fraction was saponified and the free acid was isolated. After repeated crystallization from methyl alcohol and acetone, an acid melting at 62–63° was isolated. This was proved to be palmitic acid by the mixed melting point method and by analysis.

Micro-Anal. Calcd. for $C_{16}H_{32}O_2$: C, 74.91; H, 12.58. Found: C, 75.27; H, 12.59.

A sample (2.408 g.) of the liquid acids in alcohol solution was reduced catalytically with hydrogen and platinum oxide. The reduced acids, a semi-crystalline buff-colored mass, were separated by means of the lead salt treatment into a main fraction of white crystals and a very small amount of liquid acid. The recovery was 95% (2.291 g.). Since the small amount of liquid acid was shown to be unsaturated, it appears that no liquid saturated acids were present. The solid crystalline acid melted at 69.5–70.5°

(7) Newman and Anderson, *J. Biol. Chem.*, **102**, 229 (1933).

(8) Woolley, Strong, Peterson and Prill, THIS JOURNAL, **57**, 2589 (1935).

(9) Voorhees and Adams, *ibid.*, **44**, 1397 (1922).

and had a neutral equivalent of 283.8. Its melting point showed no depression on mixing with pure stearic acid (m. p. 70–71°, neut. equiv. 284.3); hence the reduced acid was stearic acid.

Anal. Calcd. for $C_{18}H_{36}O_2$: C, 75.95; H, 12.76. Found: C, 76.19; H, 12.82.

Oxidation of a small amount of the liquid acids with alkaline permanganate[10] yielded a mixture of dihydroxy- and tetrahydroxystearic acids. The dihydroxystearic acid was extracted from the crude mixed acids with chloroform. After crystallization from alcohol it melted at 129–130°. No depression was observed in the melting point on admixture with authentic 9,10-dihydroxystearic acid.

Anal. Calcd. for $C_{18}H_{36}O_4$: C, 68.26; H, 11.47. Found: C, 67.99; H, 11.48.

The acid insoluble in chloroform was crystallized from alcohol and melted at 171–172°. A mixed melting point with authentic tetrahydroxystearic acid showed no depression and established the substance as tetrahydroxystearic acid.

Anal. Calcd. for $C_{18}H_{36}O_6$: C, 61.99; H, 10.42. Found: C, 62.29; H, 10.21.

Another sample of the unsaturated acids was brominated in cold light petroleum ether. The precipitate which formed was crystallized from petroleum ether yielding a white crystalline acid melting at 113–114°. It was proved by the mixed melting point method to be tetrabromostearic acid.

Anal. Calcd. for $C_{18}H_{32}O_2Br_4$: Br, 53.29. Found: Br, 53.01.

The substance remaining in the petroleum ether after removal of the tetrabromostearic acid was a light brown oil. It was probably dibromostearic acid.

Anal. Calcd. for $C_{18}H_{34}O_2Br_2$: Br, 36.15. Found: Br, 38.21.

The above data show the presence in the acetone-soluble fat of both oleic and linoleic acids. Since no substance insoluble in ethyl ether was found on bromination, it appears that no higher unsaturated acids were present.

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

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Summary

1. The chemical composition of the lipids of the pathogenic fungus, *Blastomyces dermatiditis*, has been determined. The lipids make up about eight to ten per cent. of the weight of the whole dried cells.

2. The lipids were separated into approximately one-third phosphatide and two-thirds acetone-soluble fat.

3. The phosphatide on hydrolysis with aqueous sulfuric acid gave glycerophosphoric acid, choline, ethanolamine, and fatty acids. These substances were isolated and the first three identified. The fatty acids probably consisted of palmitic, stearic, oleic and linoleic acids. A small amount of carbohydrate was obtained on saponification.

4. The acetone soluble fat gave on saponification glycerol, ergosterol, and palmitic, oleic and linoleic acids. These were isolated and identified. Stearic acid probably was present also.

5. These results are compared with the data on tubercle bacilli and certain striking differences noted.

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The Reversibility of the Reaction between Triglycerides and Glycerol

BY HARLAND H. YOUNG, JR., AND HOWARD C. BLACK

The fact that triglycerides react with glycerol at high temperatures has been known for several years. The products obtained by such a reaction have been described in the literature[1] and more recently have become of commercial importance as emulsifying agents for water-in-oil emulsions. Many of the products reported have been prepared from natural fats and oils and necessarily have been complex mixtures. The designation of these as mixtures of mono- and diglycerides has been based upon saponification numbers, acetyl values and their marked solubility in the lower alcohols.[1a,c] Grün[1b] reported the formation of di- and triglycerides when monoglycerides were heated but did not identify the products completely. Fischer[2] prepared α-dibenzoylglycerol by the vacuum distillation of α-monobenzoylglycerol. More recently Hurd[3] has described the formation of glycerol triphenyl-

(1) (a) Bellucci, *Gass. chim. ital.*, **42**, II, 283 (1912); (b) Grün, *Chem. Umschau Fette, Öle, Wachse, Harze*, **32**, 225 (1925); (c) Tsuchiya and Akiyama, *J. Soc. Chem. Ind. Japan*, **36**, Suppl. binding, 233 (1933).

(2) Fischer, Bergmann and Bäwind, *Ber.*, **53**, 1589 (1920).

(3) Hurd, *et al.*, THIS JOURNAL, **59**, 1952 (1937).