Summary

1. Alkyl γ-oxalylcrotonates were prepared by the condensation of alkyl vinylacetate and alkyl oxalate in the presence of sodium.

2. γ-Oxalylcrotonic acid was prepared by the hydrolysis of the sodium derivative of ethyl γ-oxalylcrotonate. It was oxidized with 30% hydrogen peroxide to glutaconic acid.

3. Ethyl O-carbethoxy-γ-oxalylcrotonate, methyl O-carbomethoxy-γ-oxalylcrotonate and ethyl O-carbethoxy-γ-oxalylacetate were prepared by the action of ethyl chlorocarbonate on the corresponding sodium derivative. Diethyl adipate, dimethyl adipate and diethyl succinate, respectively, were formed by the reduction of these compounds.

4. Ethyl O-acetyl-γ-oxalylcrotonate was catalytically reduced to diethyl adipate.

5. The mechanism of these reductions has been discussed.

RAHWAY, N. J. RECEIVED DECEMBER 29, 1933

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

The Chemistry of Mold Tissue. IV. The Lipids of Aspergillus Sydowi[1,2]

BY F. M. STRONG AND W. H. PETERSON

In comparison with the large number of papers on the production of organic acids and other compounds by the common molds, relatively few publications deal with the composition of the mycelium itself. In previous investigations[3] the lipid and sterol contents of a large number of molds have been determined, and one of the lipid constituents, ergosterol, has been isolated and identified. In the present study the lipids of one of these molds, *Aspergillus sydowi*, have been examined in detail. This mold was selected because it can be grown readily in large quantities,[3d] and also contains a large percentage of lipoidal material.

Previous investigations of the lipids of fungus tissue have been limited mainly to yeasts and to the higher fungi. Most of the work dealing with molds has been concerned with variations in the amount of fat produced under different conditions of growth.[4] With the exception of linoleic acid, which Takata[5] has shown to be present in *A. oryzae*, and of ergosterol, which has been found in various molds,[3b] apparently no rigorous characterization of the constituents of any mold fat has ever been made. Palmitic, oleic and other fatty acids have been reported by certain investigators,[6,7] but the evidence in most cases rests merely on melting points without supporting analytical data. Likewise in most cases the mold studied was not definitely identified. Various other authors have determined some of the fat constants on the lipids obtained from certain molds, without claiming to have identified any particular constituent of the fat.[8]

The method of extraction employed in this investigation was in general similar to that followed by Anderson in his extensive study of the lipids of tubercle and other acid fast bacilli.[9]

Experimental Part

Extraction of the Crude Lipids.—The mold used was grown in large sterilized incubators as previously described.[3d] When ready to harvest, it was killed by steaming, separated from the culture medium and dried for several days at 65°. The dry material was then finely ground.

Five kilograms of the ground mycelium was thoroughly extracted at room temperature with successive portions of alcohol–ether (1:1) during a period of two weeks.[10] The combined extracts were concentrated to one liter, dissolved in ether, washed with water, and the solvent removed under reduced pressure. The residual oil was a clear, deep red liquid weighing 607 g., or 12% of the mycelium extracted. It dissolved readily in alcohol, ether, chloroform and acetone at room temperature. Titration revealed the presence of about 22% of free acid, calculated as oleic. On standing, crystals of ergosterol

(1) This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

(2) Presented in part at the Chicago Meeting, American Chemical Society, September, 1933.

(3) Peterson and co-workers, (a) *J. Biol. Chem.*, **90**, 369 (1931); (b) *ibid.*, **97**, 483 (1932); (c) *Biochem. Z.*, **246**, 401 (1932); (d) *Ind. Eng. Chem.*, **25**, 213 (1933); (e) *Zentr. Bakt. Parasitenk.*, II Abt., **89**, 370 (1934).

(4) Terroine and co-workers, *Bull. soc. chim. biol.*, **9**, 12, 588, 604, 678 (1927); Behlin, *ibid.*, **8**, 1081, 1120 (1926); Bohn, *Compt. rend.*, **193**, 441 (1931); Pontillon, *ibid.*, **191**, 1148, 1367 (1930); Pearson and Raper, *Biochem. J.*, **21**, 875 (1927); Porges, *Botan. Gazz.*, **94**, 197 (1932).

(5) R. Takata, *J. Soc. Chem. Ind., Japan*, **32**, 171B (1929).

(6) H. H. Barber, *Biochem. J.*, **23**, 1158 (1929).

(7) M. X. Sullivan, *Science*, **38**, 678 (1913).

(8) Browne, THIS JOURNAL, **28**, 453 (1906); Sumi, *Biochem. Z.*, **195**, 161 (1928); Pontillon, *Compt. rend.*, **191**, 1148 (1930).

(9) Anderson, *Physiol. Rev.*, **12**, 166 (1932).

(10) The extraction and working up of all unsaturated lipids was carried out under atmospheres of carbon dioxide or nitrogen.

slowly separated. Some of the more important chemical and physical characteristics of the oil are given in Table I.

TABLE I

CHEMICAL AND PHYSICAL CHARACTERISTICS OF *A. sydowi* LIPIDS

Density 25°/4°	0.9198	Ester no.	126.1
n_D^{20}	1.4682	Insoluble acids	80.8%
Iodine no.		Volatile acids	0.46%
(Hanus)	114.4	Unsaponifiable	8.18%
Saponification		Phosphorus	0.26%
no.	169.5	Nitrogen	0.39%
Acid no.	43.4	Ash	None

Additional lipoidal material was obtained by the extraction of the mycelial residue with chloroform, but has not yet been investigated.

Another batch of sixteen kilos of the mycelium was extracted with acetone and then with alcohol–ether, and the extracts worked up as before. The yield of crude lipids amounted to 1230 g., or 7.7% of the mold taken. Most of the work to be described was carried out with the material obtained in this batch.[11]

Separation of the Phospholipid.—1230 grams of the crude lipids dissolved in 7 liters of acetone was cooled to −4° and precipitated with 175 cc. of alcoholic magnesium chloride. The precipitate was filtered off, dissolved in chloroform, reprecipitated by acetone, and the supernatant liquid decanted and combined with the first acetone filtrate. When the solvent was removed, an oil remained, which contained only 0.028% phosphorus, and was designated as "simple lipids."

The phospholipid, assumed to be in the form of a magnesium chloride complex, was reprecipitated several times from chloroform by acetone, and then treated with a strong solution of ammonia in methyl alcohol.[12] An inorganic precipitate was filtered off, and the "free" phospholipid reprecipitated several times more as above described. It was finally obtained as a light brown, non-hygroscopic powder, soluble in chloroform and ether, but insoluble in acetone. Qualitative tests revealed the presence of magnesium, although chlorine was absent. Tentative analytical results indicate that the ratio P:N:Mg is 1:2:2. It was possible to remove nearly all the magnesium by one shaking with cold, dilute, hydrochloric acid.[13] The free phospholipid so obtained was found to be soluble in acetone. The investigation of this substance is being continued.

Saponification of the Fat and Isolation of Glycerol.—The simple lipids were saponified by refluxing with 5% alcoholic potassium hydroxide in an atmosphere of nitrogen. After distilling off most of the alcohol and diluting with water, the unsaponifiable material together with unchanged fat was extracted with ether, re-saponified, and the unsaponifiable portion obtained by again shaking out with ether.

The two soap solutions resulting from these operations were combined, acidified, and the fatty acids collected in ether. After drying over anhydrous sodium sulfate the ether was removed, and the acids further dried by heating under nitrogen at 110°. When cold the crude fatty acids formed a dark semi-solid mass.[14]

The acidified aqueous solution was evaporated to dryness, the residue taken up in alcohol–ether (1:1), and the insoluble salts filtered off. The filtrate was evaporated to a thick sirup, taken up in absolute alcohol, filtered and evaporated. To remove more water this treatment was repeated several times, and finally 71.6 g. of a thick red-brown sirup was obtained. Negative Fehling and Molisch tests indicated the absence of carbohydrates.

8.5 grams of the sirup was distilled, but because of excessive frothing only 2 g. of distillate of b. p. 130–140° (2 mm.) could be collected. The distillate was benzoylated by heating with pyridine and benzoyl chloride. Recrystallization of the ester from 95% alcohol gave snow white crystals, m. p. 76–77°. A mixed melting point with authentic glycerol tribenzoate showed no depression. Calcd. for $C_{24}H_{20}O_6$: C, 71.3; H, 4.99. Found: C, 71.4; H, 4.96.

A rough estimate of the amount of glycerol in the sirup was obtained by acetylation, which resulted in an 80% yield of triacetin, b. p. 100–105° (2 mm.). Calcd. for $C_9H_{14}O_6$: saponification equivalent, 72.4. Found: 74.2.

Examination of the Fatty Acids.—223.4 grams of the crude fatty acids was separated into saturated and unsaturated fractions by the lead soap–ether method.[15] The saturated acids were obtained as a colorless, waxy solid; wt. 64 g., I no. 2.8. The unsaturated acids consisted of a dark oil; wt. 144 g., I no. 124.4.

Unsaturated Acids.—An attempt to separate further the unsaturated fraction into mono- and poly-ethylenic acids by the lithium soap–ether method of Tsujimoto[16] was unsuccessful. The lithium soap was insoluble both in hot and cold acetone, and on acidification yielded acids having the original iodine number.

The methyl esters of 100 g. of the crude unsaturated acids were prepared,[17] and distilled at diminished pressure (yield 90%). The following fractions were collected.

Fraction	B. p., °C. (2 mm.)	Weight, g.	Corresponding acid Iodine no.	Neut. equiv.
I	163–168	91	122	282
II	165–190	3.3	110	304

Identification of the Acids in Fraction I. A. Bromination.—A sample of the acids obtained by saponification of Fraction I was brominated in cold petroleum ether solution, and the insoluble bromides carefully crystallized out according to the procedure recommended by Bloor.[18]

(11) We wish to acknowledge the assistance of Dr. L. M. Pruess in making this extraction, and in the identification of the sterol fraction.

(12) This procedure has been recommended by Levene, *J. Biol. Chem.*, **48**, 189 (1921), for regenerating free lecithin from its cadmium chloride salt.

(13) Rudy and Page, *Z. physiol. Chem.*, **193**, 266 (1930), were able to obtain cephalin from its barium salt by shaking with dilute hydrochloric acid.

(14) Preliminary experiments indicated that the free and combined fatty acids of the oil were similar in character. Consequently no effort was made to examine them separately.

(15) Association of Official Agricultural Chemists, "Methods of Analysis," Washington, D. C., 1930, 3d ed., p. 324.

(16) Tsujimoto and Kimura, *J. Chem. Ind. (Japan)*, **26**, 891 (1923).

(17) Reid and Cox, THIS JOURNAL, **54**, 224 (1932).

(18) Snyder and Bloor, *J. Biol. Chem.*, **99**, 562 (1933).

The precipitate was completely soluble in ether, indicating the absence of acids more unsaturated than linoleic. After two recrystallizations from hot ligroin (b. p. 60–80°) colorless needles were obtained, m. p. 113–114°. When mixed with tetrabromostearic acid there was no depression. Calcd. for $C_{18}H_{32}O_2Br_4$: C, 36.0; H, 5.38; Br, 53.28. Found: C, 36.3; H, 5.30; Br, 53.96.

The petroleum ether soluble portion of the brominated products was a dark oil presumably consisting largely of dibromostearic acid. Calcd. for $C_{18}H_{34}O_2Br_2$: Br, 36.15. Found: Br, 36.65, 37.02.

B. Oxidation.—Another sample of the acids from Fraction I was oxidized with alkaline permanganate.[19] The crude oxidized acids were extracted several times with boiling chloroform, and the insoluble portion recrystallized once from alcohol. The product melted at 172–173°, and showed no depression when mixed with tetrahydroxystearic acid. Calcd. for $C_{18}H_{36}O_6$: C, 62.02; H, 10.42. Found: C, 62.0; H, 10.36.

The chloroform filtrate was evaporated to dryness and the residue extracted with petroleum ether. The insoluble part after being recrystallized once from alcohol and again from Skellysolve "B" melted at 130–131°, and at 129–131° when mixed with known dihydroxystearic acid. Calcd. for $C_{18}H_{36}O_4$: C, 68.29; H, 11.47. Found: C, 68.3; H, 11.28.

C. Reduction.—The above results indicate that oleic and linoleic acids were present in Fraction I, but do not exclude the possible presence of rather large quantities of some other constituent, especially of branched chain acids such as were found by Anderson.[9] Accordingly a 10-g. sample was reduced catalytically with Adams platinum oxide catalyst, saponified, and the fatty acids isolated. The reduced acid without purification melted at 68–70°, had a neutral equivalent of 286.3 and showed no depression of the melting point when mixed with pure stearic acid. The yield was 97.4%. Consequently Fraction I very probably consisted entirely of methyl oleate and methyl linoleate.

Investigation of the Acids in Fraction II.—The free acids from Fraction II were reduced and recrystallized several times from alcohol. Although the product, which melted at 74–76° and had a neutral equivalent of 296, was apparently still a mixture, further purification with the small quantity at hand was not attempted. The results indicate that a small amount of some higher unsaturated acid was present in the fat.

Saturated Acids.—The methyl esters of 64 g. of the crude saturated acids were prepared,[17] and distilled at diminished pressure (yield 92%). Ten fractions of approximately equal weights were collected. Fractions of similar saponification equivalents were combined and redistilled with the results shown in Table II.

Isolation of Individual Acids.—Fraction II was redistilled and the free acids obtained in the usual manner. The acid once recrystallized from dilute alcohol melted at 63.5–64°. Mixed with pure palmitic acid there was no depression. Calcd. for $C_{16}H_{32}O_2$: C, 74.9; H, 12.58; neutral equivalent, 256.3. Found: C, 74.5; H, 12.44; neutral equivalent, 255.6.

Recrystallization of the acid from Fraction V yielded pure stearic acid melting at 68–70°. Calcd. for $C_{18}H_{36}O_2$: C, 75.98; H, 12.76; neutral equivalent, 284.3. Found: C, 76.2; H, 12.68; neutral equivalent 285.5.

TABLE II

DISTILLATION OF THE SATURATED ESTERS

Fraction	B. p., °C.	Weight, g.	Neut. equiv. of correspond-ing acid
I	125–130	3.75	258
II	130–135	7.60	...
III	135–140	5.77	262
IV	146–151	12.63	279
V	149–152	8.88	279
VI	152–156	7.25	280
VII	165–170	0.58	297
VIII	170–175	1.02	288[a]
IX	Residue	2.70	298
X[b]	175–200	2.57	361

[a] No explanation was found for this break in the steady increase of the neutral equivalent.

[b] This fraction was the highest boiling one obtained in the first distillation, and was not redistilled.

The acid from Fraction X after four recrystallizations from 95% alcohol melted at 83–84°. The melting point was not changed by two more recrystallizations. *n*-Tetracosanic acid melts at 84–85°.[20] Calcd. for $C_{24}H_{48}O_2$: C, 78.18; H, 13.12; neutral equivalent, 368.4. Found: C, 78.43; H, 13.11; neutral equivalent, 367.9.

A sample of this high melting acid was subjected to an x-ray examination.[21] Required x-ray spacings for n-C_{24} acid: B, 57.76 Å.; C, 52.62 Å. Found: B, 57.77; C, 52.4. These data indicate that the material was *n*-tetracosanic acid, containing traces of another acid.

Fraction nine was unsuccessfully examined by repeated crystallizations for C_{20} or C_{22} acids. It is probable that this fraction was a mixture of C_{18} and C_{24} acids.

Volatile Acids.—From five grams of the simple lipids volatile acid equivalent to 3.65 cc. of 0.0725 *N* KOH was obtained. The fat, therefore, contained 0.46% of volatile acids, calculated as butyric. No attempt was made further to characterize this fraction.

Unsaponifiable Matter.—Recrystallization of this dark semi-solid material from alcohol-benzene (2:1) yielded white needles, m. p. 160–162°; $[\alpha]_D^{30}$ −128.3° (a = −2.57°, c = 2 in chloroform, l = 1).[11] The melting point and rotation reported for ergosterol are 163° and −130°.[22]

The crystals were acetylated by heating with acetic anhydride and zinc chloride. The product recrystallized from ether-acetone melted at 173–174°; $[\alpha]_D^{29}$ −87.7° (a = −1.73°, c = 1.99 in chloroform, l = 1). Reported for ergosterol acetate:[23] m. p. 172–173°, rotation, −87.4°.

These results establish the presence of ergosterol in the fat. A quantitative sterol determination by the colorimetric method[3c] indicated that about one-third of the un-

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

(20) Francis, Piper and Malkin, *Proc. Roy. Soc.* (London), **128A**, 217 (1930); Bleyberg and Ulrich, *Ber.*, **64B**, 2504 (1931).

(21) This work was kindly done by Dr. S. H. Piper, University of Bristol, England, to whom the authors wish to express their sincere thanks.

(22) Windaus and Borgeaud, *Ann.*, **460**, 236 (1928).

(23) Reindel, Walter and Rauch, *ibid.*, **452**, 34 (1927).

saponifiable matter was of a non-sterol character. The nature of this fraction has not yet been determined.

Quantitative Composition of the Fat.—The approximate quantitative composition of the simple lipids of *A. sydowi* as calculated from neutral equivalents and iodine numbers is given in Table III. The percentages, except as otherwise indicated, are based on weights actually isolated.

TABLE III

COMPOSITION OF THE SIMPLE LIPIDS OF *A. sydowi*[a]

Fatty acids	80.8	Unsaturated acids	52.9
Volatile acids (calcd.		Oleic	29.6
as butyric)	0.46	Linoleic	16.3
Saturated acids	22.6	Higher acids	1.7
Palmitic	8.8	Unsaponifiable	8.18
Stearic	11.0	Total sterols	5.36[b]
n-Tetracosanic	0.9	Glycerol	4.2

[a] Figures indicate percentage of the original lipids.

[b] Based on the colorimetric sterol determination.

Summary

1. The alcohol–ether extract of *A. sydowi* has been shown to contain a phospholipid which appears to possess rather unusual properties.

2. The following fatty acids were isolated from the simple lipids and identified: oleic, linoleic, palmitic, stearic and *n*-tetracosanic.

3. The water soluble fraction was shown to consist largely of glycerol.

4. Ergosterol was isolated from the unsaponifiable material.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF ILLINOIS]

The Preparation of Anhydrous Ethylenediamine

BY JOHN C. BAILAR, JR.

In the course of an investigation undertaken some time ago, it was necessary to use considerable amounts of anhydrous ethylenediamine. Apparently only two methods of preparing this material have been suggested. A. W. Hofmann, who first reported the great stability of the hydrate,[1] obtained the anhydrous base by repeatedly distilling the material from metallic sodium.[2] This method has been used by subsequent investigators.[3] Kraut, Rhoussopoulos and Meyer[4] discovered that the base can be dehydrated by heating to 100° in a sealed tube for several hours with freshly melted sodium hydroxide. Since neither of these methods is convenient for the preparation of more than very small amounts of material, a new method was developed.

The method depends upon the fact that ethylenediamine reacts with zinc oxalate to give a compound which is stable at room temperatures, but decomposes readily on heating to 200°. This salt crystallizes from water in white needles, which analysis shows to be $[ZnC_2H_4(NH_2)_2]C_2O_4$.

Anal. Calcd.: Zn, 30.62; C, 22.49; H, 3.77; N, 13.14. Found: Zn, 30.44; C, 22.63; H, 3.82; N, 12.81.

Procedure.—Zinc oxalate is mixed with a little more than an equivalent weight of ethylenediamine hydrate and the compound so formed is dissolved in a minimum amount of boiling water (about 1 cc. for each gram of zinc oxalate used). The crystals that form on cooling are filtered off and washed with a little alcohol. More crystals are obtained by concentrating the mother liquor. When the crystalline material is thoroughly dry, it is heated *in vacuo* to 200°. The anhydrous ethylenediamine is liberated, and condenses to a colorless liquid. The zinc oxalate which remains after the distillation usually has a slight yellow color; the contamination is very slight, however, and the zinc oxalate may be used repeatedly.

The ethylenediamine so prepared boils between 116 and 117° and has a density of 0.907 at 17°. Redistillation from sodium will remove any trace of moisture which was not removed in the drying of the zinc oxalate complex salt. The yield is nearly quantitative.

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(1) Hofmann, *Jahresber. Fortschritte Chem.*, **11**, 343 (1858).

(2) Hofmann, *Proc. Roy. Soc.* (London), **10**, 229 (1859).

(3) (a) Michaelis and Grauz, *Ber.*, **30**, 1009 (1897); (b) Elgort, *J. Russ. Phys. Chem. Soc.*, **61**, 950 (1929).

(4) Kraut, Rhoussopoulos and Meyer, *Ann.*, **212**, 255 (1882).