LIPIDS OF *BACILLUS STEAROTHERMOPHILUS*[1, 2]

STERLING K. LONG[3] AND O. B. WILLIAMS[4]

Department of Bacteriology, University of Texas, Austin, Texas

Received for publication September 8, 1959

The relationship of cellular lipids to thermophily and the heat resistance of bacterial spores has been studied but with occasional contradictory results. Explanation for differences may lie in attempts to compare the lipids of the same or different species of microorganism grown under widely varying cultural conditions. Evidence that cultural conditions alone may influence lipid content has been offered by Larson and Larson (1922), Heide (1939), and Starkey (1946).

Leathes and Raper (1925) were among the first to attempt correlation of growth temperature with degree of unsaturation of cellular lipids. Using lipids of plants and animals, they found that highly unsaturated lipids were produced at low environmental temperatures, whereas the most saturated lipids were obtained at high temperatures. The lipids extracted from species of *Aspergillus* and *Rhizopus* by Terroine *et al.* (1927) conformed to this principle but Prill *et al.* (1935) with *Aspergillus fischeri*, and Singh and Walker (1956) using *A. nidulans*, found an inverse correlation. Gaughran (1947) observed that the lipids of *Bacillus subtilis* decreased in quantity and unsaturation as the incubation temperature was raised above the optimum whereas under similar conditions the lipids of an unidentified thermophile were constant.

Early studies on the effect of lipids on heat resistance of bacterial spores have been reviewed by Gaughran (1947). Further evidence that lipids may be important in heat resistance was provided by Sugiyama (1951), who was able to increase the heat resistance of spores of *Clostridium botulinum* by adding long chain fatty acids to the medium. By contrast, Church *et al.* (1956) extracted intact spores, grown on a glycerol medium, with chloroform-methanol and obtained homogeneous sensitivity to ethylene oxide but no alteration of heat resistance. Bloor (1943) considers glycerol to be a wax progenitor, which suggests the possibility that the material extracted may have consisted of compounds other than lipids.

It was felt that analysis of the lipids of a thermophilic sporeforming organism grown under controlled conditions of medium and temperature might provide specific information regarding the type of lipids produced in response to temperature, and the importance of such substances in thermophily and heat resistance.

EXPERIMENTAL METHODS

Production of spores and vegetative cells. The 37 C spores and cells used in this study were produced by the batch method using the cultural conditions employed by Long and Williams (1960). Obtaining quantities of 55 C spores and vegetative cells from spore inocula presented a more difficult problem. As previously noted (Long and Williams, 1960), aeration prevented germination of spore inocula at this temperature. Preliminary studies indicated that sporulation approximating 50 per cent could be obtained at 55 C by culturing in shallow pans (23 by 38.5 by 5.5 cm) of tryptose basamin glucose broth. This method was used for spore and vegetative cell production at 55 C. The medium was dispensed in 1-L quantities into these pans (depth 13 mm), covered with sheets of aluminum foil, and autoclaved prior to use. Spore cultures were incubated for 5 days and vegetative cultures for 18 hr at 55 C. Spores and vegetative cells were harvested with a Sharples supercentrifuge at 50,000 rpm and washed thoroughly. Spore preparations were purified by the method of Long and Williams (1958).

Surface extraction of spores. Micro-Soxhlet extractors were employed for surface extraction of

[1] Supported in part by a research grant from Swift and Company, Chicago, Illinois.

[2] This work was taken from a dissertation submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of Texas, Austin.

[3] Present address: University of Florida Citrus Experiment Station, Lake Alfred, Florida.

[4] Deceased September 23, 1959.

lyophilized intact spores. Extraction thimbles were dried to constant weight at 70 C *in vacuo*, packed with lyophilized spores, dried to constant weight, and the weight of the spores determined by difference. The packed thimbles were extracted with the desired solvents for specified periods of time. The solvents were removed from the lipids under nitrogen. The solvent-free material thus obtained was dried to constant weight.

Preliminary solvent screening for extracting 37 C spore samples included methanol, chloroform-methanol (1:1), chloroform, carbon tetrachloride, acetone, petroleum ether, and diethyl ether. Diethyl ether seemed to better satisfy the desired requirements of good solvent action and specificity for lipids and was used for all further work. Increasing extraction time to 12 hr gave better results on succeeding samples.

Effect of surface extraction was determined microscopically on smears stained with the Ziehl-Neelsen acid-fast method, a malachite green spore stain, the Sudan black B method of Burdon

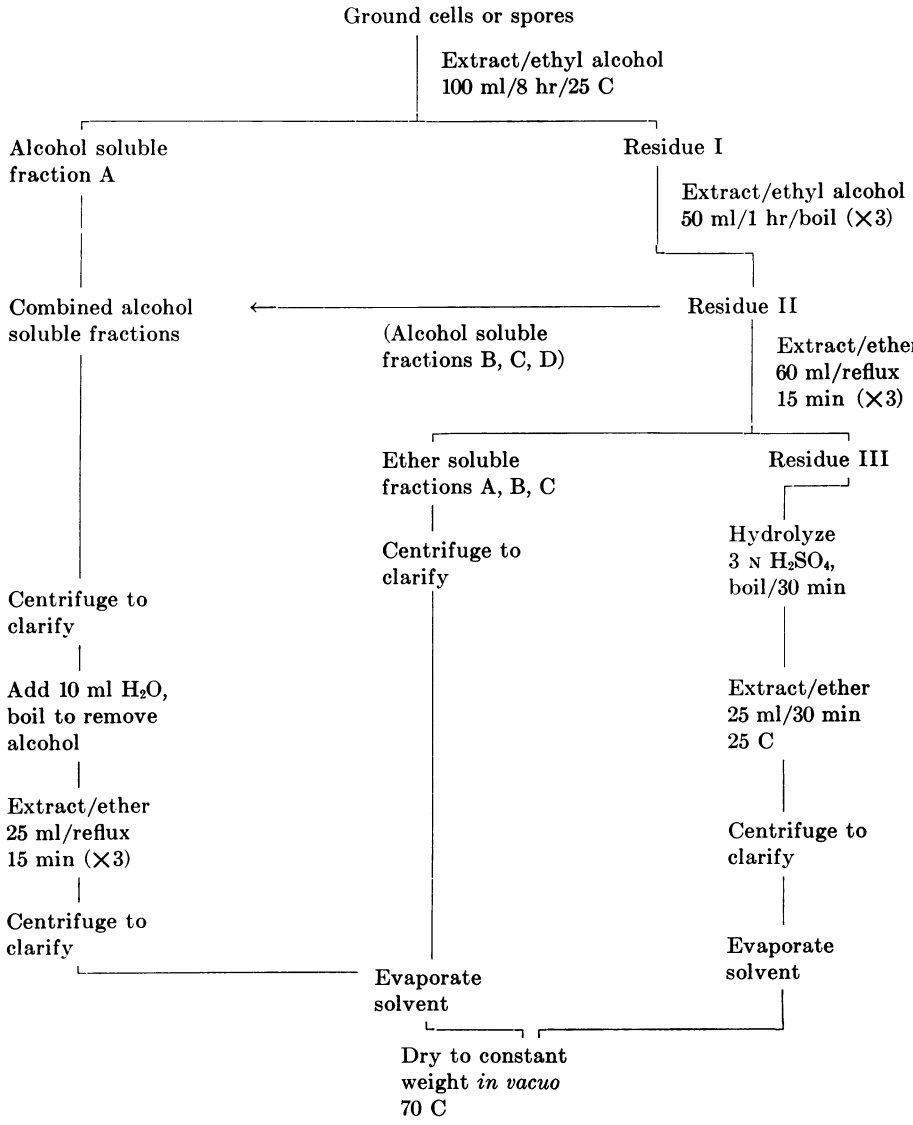

Figure 1. Lipid extraction

et al. (1942), and the Schuhardt and Young capsule stain. Comparison of heat resistance of extracted and unextracted spores was made by the method of Williams (1929). Quantitative determinations were made in a similar manner by total plate counts on tryptose-basamin-glucose agar.

Grinding of spores and vegetative cells. Satisfactory disruption of cells and spores was obtained by grinding with glass beads in a Waring Blendor (Lamanna and Mallette, 1954) using dry ice to maintain low temperature.

Complete lipid extraction. The procedure used for extraction of ground spores and cells was basically that of Bloor (1943). Details of extraction are indicated in figure 1. Nitrogen gas was used to prevent oxidation of extracted matter.

Separation of phospholipids. The method used was based upon the relative insolubility of these compounds in acetone. The procedure for separation of these materials (figure 2) was essentially that of Bloor (1943).

Iodine number determination. The micromethod determination of iodine number was that of Yasuda (1931). These determinations were made upon duplicate samples of spore and cell lipids and phospholipids. Iodine numbers of "reagent grade" oleic and linoleic acids were run as controls with all unknowns.

Chromatography. Attempts were made to identify the components of the respective lipid extracts by paper chromatography. The fractions analyzed included unhydrolized lipid, fatty acids, and nonsaponifiable fractions obtained on acid and alkali hydrolysis at elevated temperatures. Reference standards of highly purified fatty acids were run with all samples. Fractions were chromatographed for C_2–C_{10} fatty acids by the method of Brown and Hall (1950), and for C_{12}–C_{18} acids by the reversed phase method of Mangold *et al.* (1955). The procedures of Partridge (1948) and Davies (1956) were employed for detection of sugars in phospholipid fractions. Ninhydrin was used to test for free amino groups in hydrolized samples of this material. Spingosine

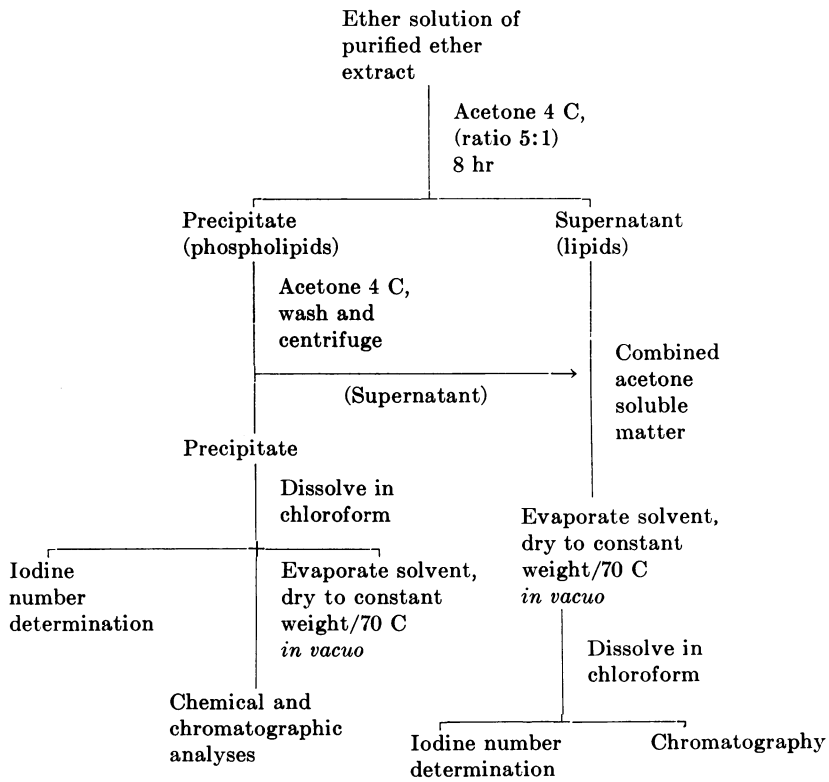

Figure 2. Separation of phospholipids

TABLE 1

Solvent screening; surface extraction of 37 C spores

Dry Wt Sample	Solvent	Soxhlet	Dry Wt Extract	Extractible Matter
g		*hr*	*g*	%
0.2883	Methanol	6	0.0472	16.37
0.2595	Chloroform-methanol, 1:1	6	0.0723	27.98
0.2979	Chloroform	6	0.0430	14.43
0.2686	Carbon tetrachloride	6	0.0463	17.24
0.1882	Acetone	6	0.0309	16.42
0.2135	Petroleum ether (b.p. 30–60 C)	6	0.0322	15.08
0.1846	Diethyl ether	6	0.0352	19.05

was determined by the method of Meredith and Sammons (1952).

Miscellaneous determinations. The Reinecke acid precipitation method of Thannhauser *et al.* (1939) was employed for detection of spingomyelin.

The Molisch reaction for carbohydrates was run on all fractions. Nonsaponifiable fractions were tested for the presence of cholesterol and ergosterol by the digitonin method of Schoenheimer and Sperry (1934), colorimetrically (Rosenheim, 1929), and by the Libermann-Burchard reaction.

RESULTS AND DISCUSSION

As would be expected, in view of differences in the properties of the extracting solvents, there was considerable variation in the quantities of material extracted from intact spores. The amount of surface extractible matter ranged from 14.43 to 27.98 per cent (table 1). Diethyl ether was adopted as the extracting solvent since it gave efficient extraction as well as greater specificity for lipids. Solvent screening (table 1) indicated somewhat greater quantities of ether extractible matter than was actually obtained in succeeding spore crops which may be attributable to insufficient washing of spores prior to lyophilization. The results obtained upon ether extraction of surface matter from a greater number of samples of spores are indicated in table 2. Each lot number represents a composite sample of spores obtained from 8 to 10 L of culture. The per cent extractible matter obtained from samples within a given spore lot, although subject to some variation, showed reasonable agreement. Comparison of values representing the average extractible matter from different spore lots, however, revealed considerable variation between the quantities obtained from individual lots. Average values for 37 C spores ranged from 6.90 to 13.11 per cent, whereas those from 55 C spores ranged from 7.42 to 10.13 per cent.

TABLE 2

*Surface extraction of spores**

Growth Temp	Lot No.	Dry Wt Sample	Soxhlet	Dry Wt Extract	Extractible Matter	Avg Extractible Matter
		g	*hr*	*g*	%	%
37 C	1	0.3000	12	0.0412	13.76	
		0.3000	12	0.0391	13.03	13.11
		0.3000	12	0.0376	12.53	
	2	0.2890	12	0.0232	8.03	
		0.3025	12	0.0220	7.27	6.90
		0.2835	12	0.0153	5.40	
	3	0.3000	12	0.0282	9.40	
		0.3000	12	0.0265	8.86	8.84
		0.2874	12	0.0247	8.63	
55 C	1	0.5000	12	0.0371	7.42	7.42
	2	0.3000	12	0.0294	9.73	9.51
		0.3000	12	0.0279	9.30	
	3	0.3000	12	0.0304	10.13	10.13

* Extracting solvent = diethyl ether.

Methods designed to determine the effect of surface extraction on 37 C and 55 C spores, e. g., staining, viability, and heat resistance, failed to detect any differences in these characteristics between extracted and unextracted samples. The peripheries of both types of spores were readily stainable by Sudan black B. Although this was indicative of lipid in the spore coats, its stainability was unaffected by ether extraction. No evidence was obtained to indicate the presence of any well-defined capsular material in 37 C or 55 C spores. Consideration of these results lends no support to early theories cited by Gaughran (1947) that lipid capsules may contribute to the heat resistance of bacterial spores.

The possibility that this "surface" lipid may have originated endogenously in the spore does not seem likely since the necessary disorgani-

zation of the spore contents would have resulted in altered properties of the spore. More likely explanations for the surface lipid of spores may be adsorption onto the spores of lipids present in components of the culture medium, or of the lipids released into the medium upon autolysis of vegetative cells. The observations of a number of workers lend indirect support to this hypothesis, e. g., Ley and Mueller (1946) and Dyar (1948).

The results obtained upon complete extraction of ground spores are indicated in table 3. The lipid content of 37 C spores ranged from 1.48 to 1.78 per cent, whereas those produced at 55 C varied between 1.34 and 1.88 per cent. In view of the numerous steps requisite to obtaining these lipids, the minor variations in lipid content between the two spore types are not considered significant. Complete acid hydrolysis of extracted spore residues and re-extraction with ether have indicated the efficiency of extraction to be approximately 95 per cent, therefore, the similarity in lipid content of these spores was not due to incomplete extraction.

TABLE 3

*Complete extraction of spores after surface extraction and grinding**

Growth Temp	Lot No.	Dry Wt Sample	Dry Wt Purified Extract	Extractible Matter
		g	*g*	%
37 C	1	0.6339	0.0113	1.78
	2	0.4268	0.0065	1.52
	3	0.5053	0.0075	1.48
55 C	1	0.4031	0.0054	1.34
	2	0.5151	0.0097	1.88
	3	0.2707	0.0045	1.66
	4	0.8197	0.0154	1.88

* Extracting solvents = 95 per cent ethyl alcohol and diethyl ether.

Extraction of the lipids from the two types of vegetative cells (table 4) reveals a considerably greater lipid content of the 37 C cells. The extractible lipid from these samples ranged from 3.3 to 5.4 per cent, with an average of 4.25 per cent. The lipid content of 55 C cells was 2.8 per cent. In this determination, allowance must be made for the fact that only one sample of 55 C cells was obtained.

Separation of the total extracts of 37 C and 55 C spores into lipid and phospholipid fractions gave the average values indicated in table 5. Comparison of these values shows an average true lipid content of 74.08 per cent for 37 C spores and 80.91 per cent for 55 C spores. The average phospholipid content of the total spore lipids was, respectively, 25.92 and 19.09 per cent.

A review of the literature on the subject of bacterial lipids has revealed no reported correlation between resistance to heat and observed ratios of lipid to phospholipid, although it is well established that phospholipids, in general, are more resistant to hydrolysis by heat than are true lipids. This line of reasoning, however, is not supported by the results shown in table 5 for the constituents of 37 C vegetative cells which were found to be 53.76 per cent lipid and 46.24 per cent phospholipid. If quantity of phospholipid alone was the determining factor in heat resistance, then these vegetative cells would have exhibited a high level of heat resistance. The results of iodine number determinations upon the lipids and phospholipids of spores and vegetative cells are recorded in table 6. These findings were not in agreement with those of workers already noted. Lipids of vegetative cells produced at 37 C were found to have lower iodine numbers than the lipids of the two types of

TABLE 4

Extraction of ground vegetative cells

Lot No.	Growth Temp, Vegetative Cells	Dry Wt Sample	Solvents	Dry Wt Extract	Extractible Matter
		g		*g*	%
CA	37 C	1.0000	Acetone-ether	0.0327	3.3
CB	37 C	1.0000	Alcohol-ether	0.0386	3.9
CC	37 C	0.9254	Alcohol-ether	0.0408	4.4
CD	37 C	1.1336	Alcohol-ether	0.0609	5.4
CE	55 C	0.2162	Alcohol-ether	0.0001	—
CF	55 C	0.6200	Alcohol-ether	0.0141	2.8

TABLE 5

Lipid and phospholipid content of spores and vegetative cells

Type Sample	Growth Temp	Avg Wt Extract	Avg Wt Lipid	Lipid	Avg Wt Phospholipid	Phospholipid
		g	*g*	%	*g*	%
Spores	37 C	0.0189	0.0140	74.08	0.0049	25.92
Cells	37 C	0.0372	0.0200	53.76	0.0172	46.24
Spores	55 C	0.0110	0.0089	80.91	0.0021	19.09

TABLE 6

*Iodine number determinations**

Type Sample	Growth Temp	Lipid Fraction Iodine No.	Phospholipid Fraction Iodine No.
Spores	37 C	47.1	0
Cells	37 C	40.5	7.9
Spores	55 C	189.7	13.1
Cells	55 C		
Oleic acid		73.2	
Linoleic acid		165.7	

* Average values.

Reported iodine values: Oleic acid = 89.9 to 90.0; linoleic acid = 181.0.

spores. A somewhat greater unsaturation was exhibited by 37 C spore lipids when compared with vegetative cells produced at the same temperature. The greatest unsaturation was found in the lipids of 55 C spores, which seems to be of the same order of magnitude as linoleic acid. Iodine values for all phospholipids indicated the absence of unsaturated lipids.

The results obtained upon chromatography of the fatty acids resulting from the hydrolysis of lipids of spores and vegetative cells provide no definite identification of these compounds. Although a number of methods were used, complete separation of constituent fatty acids was not demonstrable. In all tests, the presence of unknown constituents was detected moving with the solvent fronts. Observations by Deuel (1951) and Mangold *et al.* (1955) indicate that these are problems common to the chromatographic analysis of fatty acids, particularly those of high molecular weight (C_{18} and higher).

Some constituents of the samples produced isolated spots in certain solvents by reversed phase methods (table 7) but these showed no agreement with reference fatty acids run simultaneously. In general, the high molecular weight fatty acids employed as standards gave R_f values of 0.60 to 0.95 and all gave spots which strongly fluoresced in short wave length (2537 A) ultraviolet light. These acids were also readily detectable upon treatment of the test papers with α-dextrin and iodine vapors. With these reagents,

TABLE 7

Paper chromatography of hydrolyzed extracts

Type Sample	Solvent System*	R_f	Ultraviolet Light	α-Dextrin Iodine
37C vegetative cells	I	0.71	Fluoresce	White
		0.84	Absorb	Yellow
		0.96	Fluoresce	White
	II	0.33	Absorb	Yellow
		0.94	Fluoresce	White
	III	0.36	Fluoresce	White
		0.91	Fluoresce	White
	IV	0.88	Fluoresce	White
37 C spores	I	0.52	Fluoresce	White
		0.71	Absorb	Yellow
		0.83	Fluoresce	White
	IV	0.88	Fluoresce	White
	V	0.94	Fluoresce	White
55 C spores	I	0.73	Absorb	Yellow
		0.81	Fluoresce	White
	II	0.36	Fluoresce	White
		0.89	Fluoresce	White

* I = 85 per cent acetic acid, II = Butanol-acetic-water (4:1:5), III = Butanol-acetic-water (4:1:1), IV = Butanol-pyridine-water (4:1:1), and V = Butanol-ethylene glycol monoethyl ether-water-ethanol-NH_4OH (4:4:4:1.5:1.25).

saturated fatty acids gave yellow spots whereas unsaturated acids appeared as white spots on a dark brown background.

Although identification of the spots obtained was not possible, it seems likely that some conclusions may be drawn upon their general characteristics based on their chromatographic behavior. The average R_f values of fluorescing spots, with two exceptions, ranged from 0.52 to 0.96. These values are similar but not identical to those found for reference standards of 18 carbon chain length. The correlation of α-dextrin-iodine reactions with relative unsaturation suggests that the spots consisted entirely of unsaturated compounds. The absence of spots indicating saturated compounds, however, does not preclude their existence since it is possible that these acids were characteristically moving with the solvent front. Therefore, subject to the limits of interpretation, it appears that the fatty acids present in the hydrolyzed lipid samples were of high molecular weight, probably of C_{18}, or greater, chain length. The ultraviolet light-absorbing spots, R_f 0.33 to 0.84, were not considered to be fatty acids and may represent ether-soluble contaminants present in the acids.

No movement of starting spots was obtained upon attempts to determine the C_2–C_{10} fatty acid content by the method of Brown and Hall (1950) which indicates the absence of short chain fatty acids in these samples.

No evidence was obtained in the analysis of the phospholipid fractions to support the results of Dyer (1953) that the phospholipid of *B. stearothermophilus* consists primarily of sphingomyelin. Established procedures for the detection of certain of the components of sphingomyelin obtained upon acid hydrolysis (a fatty acid, two nitrogenous bases, choline and sphingosine, and phosphoric acid) were uniformly negative. Similarly, the sensitive and specific Reinecke acid method for precipitation of sphingomyelin yielded no detectable precipitate in unhydrolyzed samples. Dyer (1953) based his conclusions upon determinations of nitrogen to phosphorus ratios, which for sphingomyelin would approximate 2:1. Deuel (1951) questions the validity of such methods since monoaminophosphatides may be present in high concentrations without significantly altering this ratio.

Since sphingomyelin in the phospholipid fractions was not demonstrable, further tests were made for hydrolytic products of the remaining common phospholipids, cephalin and lecithin. The presence of cephalin phospholipids as major constituents of the lipids of spores and vegetative cells does not seem likely in view of the results obtained.

The lecithin phospholipids possess no functional groups of differential value and therefore specific tests for this type of material were not possible. The presence of another group of compounds generally classified as phospholipids, the phosphatidic acids, could not be determined for a similar reason. These compounds have a typical phosphatide structure except for the absence of a nitrogenous base.

Specific colorimetric tests upon all fractions of extracts from spores and vegetative cells were uniformly negative for the presence of the free sterols, ergosterol and cholesterol. These results are consistent with the observations of Deuel (1951).

SUMMARY

The presence of significant quantities of lipids on or near the surface of 37 C and 55 C spores of *Bacillus stearothermophilus* has been demonstrated. The average quantities of material extractible from intact 37 C spores ranged from 6.90 to 13.11 per cent and from 55 C spores, 7.42 to 10.13 per cent of dry spore weight. Extraction of this lipid had no demonstrable effect upon the staining, viability, or heat resistance of either spore type. It was suggested that the medium, the lytic products of the vegetative cells, or both, may be sources of this lipid, which becomes attached to the spores by adsorption. The presence of 1.48 to 1.78 per cent lipid in ground 37 C spores and 1.34 to 1.88 per cent in 55 C spores was demonstrated. The average lipid content of vegetative cells of *B. stearothermophilus* grown at 37 C was 4.25 per cent whereas those produced at 55 C contained only 2.80 per cent. The total lipid of spores and cells of this organism is composed of the following: 37 C spores, 74.08 per cent lipid, 25.92 per cent phospholipid; 55 C spores, 80.91 per cent lipid, 19.09 per cent phospholipid; 37 C vegetative cells, 53.76 per cent lipid and 46.24 per cent phospholipid. Iodine number determinations have shown the lipids of 55 C spores to be highly unsaturated whereas 37 C spore lipids were markedly less unsaturated. Vegetative cells produced at 37 C contained the

least unsaturated lipid. Phospholipid fractions gave low iodine numbers indicating relative saturation of these components. Attempts to identify the component fatty acids of the extracted lipids were unsuccessful. It was suggested that these fatty acids may be of 18 carbon, or greater, chain length. Analysis of the phospholipids of spores and vegetative cells indicated the absence of cephalin and sphingomyelin. It was suggested that the phospholipids of these organisms may be lecithins or phosphatidic acids. No sterols were found in the lipids of spores or vegetative cells.

REFERENCES

BLOOR, W. R. 1943 *The biochemistry of the fatty acids.* Reinhold Publishing Corp., New York.

BROWN, F. AND HALL, L. P. 1950 Separation of carboxylate ions on the filter paper chromatogram. Nature, **166,** 66–67.

BURDON, K. L., STOKES, J. C., AND KIMBROUGH, C. E. 1942 Studies of the common aerobic sporeforming bacilli. I. Staining for fat with Sudan black B-safranin. J. Bacteriol., **43,** 717–724.

CHURCH, B. D., HALVORSON, H., RAMSEY, D. S., AND HARTMAN, R. S. 1956 Population heterogeneity in the resistance of aerobic spores to ethylene oxide. J. Bacteriol., **72,** 242–247.

DAVIES, D. A. L. 1956 A specific polysaccharide of *Pasteurella pestis.* Biochem. J., **63,** 105–116.

DEUEL, H. J. 1951 *The Lipids, Vol. I, Chemistry.* Interscience Publishers, Inc., New York.

DYAR, M. E. 1948 Electrokinetical studies on bacterial surfaces. II. Studies on surface lipids, amphoteric material and some other surface properties. J. Bacteriol., **56,** 821–834.

DYER, D. 1953 The lipids of a thermophilic bacterium. Thesis for M.S. degree. University of Nebraska, Lincoln.

GAUGHRAN, E. R. L. 1947 The thermophilic microorganisms. Bacteriol. Rev., **11,** 189–225.

HEIDE, S. 1939 Zur Physiologie und Cytologie der Fettbildung beim *Endomyces vernalis.* Mit einen Beitrag zur Methodik der quantitativen Bestimmung kleinster Fettmengen. Arch. Mikrobiol., **10,** 125–188.

LAMANNA, C. AND MALLETTE, M. F. 1954 Use of glass beads for the mechanical rupture of microorganisms in concentrated suspensions. J. Bacteriol., **67,** 503–504.

LARSON, L. W. AND LARSON, W. P. 1922 Factors governing the fat content of bacteria and the influence of fat on pellicle formation. J. Infectious Diseases, **31,** 407–415.

LEATHES, J. B. AND RAPER, H. S. 1925 *The fats,* 2nd ed. Longmans, Green, and Company, London.

LEY, H. L. AND MUELLER, J. H. 1946 On the isolation from agar of an inhibitor for *Neisseria gonorrhoeae.* J. Bacteriol., **52,** 453–460.

LONG, S. K. AND WILLIAMS, O. B. 1958 Method for removal of vegetative cells from bacterial spore preparations. J. Bacteriol., **76,** 332.

LONG, S. K. AND WILLIAMS, O. B. 1960 Factors affecting growth and spore formation of *Bacillus stearothermophilus.* J. Bacteriol., **79,** 625–628.

MANGOLD, H. K., LAMP, B. G., AND SCHLENK, H. 1955 Indicators for the paper chromatography of lipids. J. Am. Chem. Soc., **77,** 6070–6072.

MEREDITH, P. AND SAMMONS, H. G. 1952 Horizontal paper chromatography. Analyst, **77,** 416–418.

PARTRIDGE, S. M. 1948 Filter paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg white, and foetal blood of sheep. Biochem. J., **42,** 238–248.

PRILL, E. A., WENCH, P. R., AND PETERSON, W. H. 1935 III. The chemistry of mould tissue. IV. Factors influencing the amount and nature of the fat produced by *Aspergillus fischeri.* Biochem. J., **29,** 21–33.

ROSENHEIM, O. 1929 VIII. A specific colour reaction for ergosterol. Biochem. J., **23,** 47–53.

SCHOENHEIMER, R. AND SPERRY, W. M. 1934 A micromethod for the determination of free and combined cholesterol. J. Biol. Chem., **106,** 745–760.

SINGH, J. AND WALKER, T. K. 1956 Effect of temperature of incubation on the chemical composition of *Aspergillus nidulans* fat. Research Bull. Panjab Univ., No. 92, 135–138. Hoshiarpur (Panjab, India).

STARKEY, R. L. 1946 Lipid production by a soil yeast. J. Bacteriol., **51,** 33–50.

SUGIYAMA, H. 1951 Studies on factors affecting the heat resistance of spores of *Clostridium botulinum.* J. Bacteriol., **62,** 81–95.

TERROINE, E. F., BONNET, R., KOPP, G., AND VECHOT, J. 1927 Sur la signification physiologique de liasons ethyleniques des acides gras. Bull. soc. chim. biol., **9,** 604.

THANNHAUSER, S. J., BENOTTI, J., AND REINSTEIN, H. 1939 Studies on animal lipids. XIV. The determination of lecithin, cephalin, and sphingomyelin in body fluids and tissues; with analyses of normal human sera. J. Biol. Chem., **129,** 709–716.

WILLIAMS, O. B. 1929 The heat resistance of bacterial spores. J. Infectious Diseases, **44,** 421–465.

YASUDA, M. 1931 The determination of iodine numbers of lipids. J. Biol. Chem., **94,** 401–409.