COMPARISON OF LIPID COMPOSITION OF PLEUROPNEUMONIA-LIKE AND L-TYPE ORGANISMS

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ABSTRACT

SMITH, PAUL F. (University of Pennsylvania, Philadelphia) AND GEORGE H. ROTHBLAT. Comparison of pleuropneumonia-like and L-type organisms. J. Bacteriol. **83**:500–506. 1962.—The content of total lipid and nonsaponifiable lipid depended upon the type of organism; the greatest amounts were found in pleuropneumonia-like organisms that required cholesterol for growth and in L-1, the L-form of *Streptobacillus moniliformis*. Decreasing amounts were found in nonsterol-requiring strains of pleuropneumonia-like organisms, the stable L-form of *Proteus*, salt-requiring L-forms, and finally, the bacterial parents of the L-type organisms. Nonsterol-requiring pleuropneumonia-like organisms could synthesize their own nonsaponifiable lipid, but sterol-requiring strains could not. Ability or inability to synthesize nonsaponifiable lipid was often carried over to the L-type organisms from the bacterial parent. More nonsaponifiable lipid was found in the sedimentable fraction (cell membrane) than in the nonsedimentable fraction (protoplasm) of both pleuropneumonia-like and L-type organisms. None of the nonsaponifiable lipid synthesized by any organism tested appeared to be 3-β-hydroxy sterol. Differences in ability of various organisms to take up cholesterol and to be lysed by digitonin were established.

Distinction or identity of pleuropneumonia-like organisms (PPLO) and L-type organisms derived from bacteria is still unclarified. Morphological and serological approaches have thus far not been able to resolve this problem. An examination of the physiological aspects of the two types of organisms offers yet another approach which may prove fruitful. This is so particularly because PPLO possess a lipid physiology with certain aspects uniquely different from bacteria (Smith and Rothblat, 1960), thus presenting some distinguishing characteristics for study. A comparative examination of some general aspects of lipid metabolism of various representative strains of PPLO and L-type organisms and their parent bacteria was undertaken.

MATERIALS AND METHODS

Table 1 lists the organisms used, their nature and source, and the media in which they were grown. In the case of the saprophytic PPLO and the L-type organisms, the culture media were supplemented with 1% Difco PPLO serum fraction only if designated in the succeeding tables. Depending upon yield, 2 to 10 L cultures constituted a batch for one set of determinations. PPLO and L-type organisms were incubated for 48 hr and bacteria for 24 hr. Description of methods employed for harvesting and washing of cells, lipid extraction and saponification, carbon-14 counting, and chemical tests have been given previously (Smith and Rothblat, 1960).

In the studies on the incorporation of acetate and mevalonate into the nonsaponifiable lipids (NSL), 2 g of unlabeled carrier sodium acetate and 0.5 μc of sodium acetate-1-C^{14}, or 100 mg of mevalonic acid lactone and 0.6 μc of mevalonic acid-2-C^{14}, were added to each liter of medium.

Determination of the distribution of NSL between the sedimentable fraction (cell membrane) and supernatant fraction (protoplasm) of PPLO and L-type organisms was accomplished by either the gravimetric method or measurement of radioactivity or both. In the case of the radiometric method, cholesterol-4-C^{14} was added to the growth medium (Smith and Rothblat, 1960). Fractionation of the organisms was carried out by subjecting suspensions of the organisms

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TABLE 1. *Description of organisms and media*

Strain	Nature	Source	Growth medium
PPLO			
Parasitic			
07	Human genital	—	Difco PPLO broth + 1% PPLO serum fraction
Campo	Human genital	—	
J	Avian	—	
T-5	HeLa tissue cells	—	
Saprophytic-B-15	Bovine genital	D. G. Edward	Difco tryptose + 0.5% glucose + 0.5% acetate (±) 1% PPLO serum fraction
Laidlaw B	Sewage	D. G. Edward	
L-type organisms			
Salt requiring			
Campo	Derived from Campo D	L. Dienes	Albimi Brucella broth + 3% sodium chloride ± 1% PPLO serum fraction
D5L	Derived from D-5	L. Dienes	
ADA-L	Derived from ADA	L. Dienes	
GL8L	Derived from GL8	L. Dienes	
H-L	Derived from *S. aureus*-H	L. Dienes	
Nonsalt-requiring			
18-L	Derived from Proteus 18	L. Dienes	Difco tryptose + 0.5% glucose (±) PPLO serum fraction
L-1	Derived from *S. moniliformis*	M. Tourtellotte	Difco PPLO + 1% PPLO serum fraction
Bacteria			
Campo D	Diphtheroid from Campo PPLO	L. Dienes	Difco tryptose + 0.5% glucose
D-5	Diphtheroid from Campo PPLO	—	
D-17	Diphtheroid from Campo PPLO	—	
ADA	β-Hemolytic streptococcus	L. Dienes	
GL8	β-Hemolytic streptococcus	L. Dienes	
Proteus 18	—	L. Dienes	
S. moniliformis	—	M. Tourtellotte	

in distilled water to sonic vibrations in a Raytheon 9-kc magneto-constriction oscillator, PPLO being exposed for 15 min., L-type organisms for 25 min. After sonic treatment, the suspensions were centrifuged for 20 min at 30,000 × *g*. The sediment constituted the residue or membrane fraction, the supernatant fluid, the protoplasm. These fractions were then extracted for lipid determinations or counted. All chemical compounds were of commercial origin and possessed the highest purity available. Sodium acetate-1-C^{14} with a specific activity of 4 mc per mM and cholesterol-4-C^{14} with a specific activity of 24 mc per mM were obtained from the Nuclear-Chicago Corp., and mevalonic acid-2-C^{14} with a specific activity of 2 mc per mM from the California Corp. for Biochemical Research.

RESULTS

This study constituted a general survey of PPLO and L-type organisms and their parent bacteria, with respect to lipid content, lipid synthesis, distribution of lipid between the sedimentable fraction (cell membrane) and the supernatant fraction (protoplasm), and the general nature of the NSL. A growth requirement for cholesterol could be established only for parasitic PPLO. Saprophytic PPLO, L-type organisms, and bacteria could be readily cultivated in the absence of added sterol. However, if a very small inoculum was used to initiate liquid cultivation of saprophytic strains, 0.5% Difco PPLO serum fraction was required. Use of a 5% broth culture inoculum for liquid cultivation

precluded the necessity for the serum fraction. L-1, the L-phase of *Streptobacillus moniliformis*, (Levaditi, Nicolau, and Poincloux, 1925), grew well in complete Difco PPLO medium, but failed to grow in this medium devoid of lipids, even upon supplementation singly or with various combinations of lipid-free Difco PPLO serum fraction, cholesterol, and lecithin. It is possible that some required but unidentified lipid was removed by extraction, resulting in a deficient medium. Edward (1953) was unable to demonstrate a requirement by L-1 for cholesterol, but found acetone-insoluble lipid from egg yolk and bovine serum albumin necessary replacements for serum. However, complete absence of sterol was not assured, since his medium contained bovine albumin and an infusion of beef heart, shown to contain small amounts of cholesterol (Smith, Lecce, and Lynn, 1954) and yeast extract containing some ergosterol.

Table 2 presents the data on the lipid composition of the various organisms. Each result is an average of two to six determinations, and each determination was performed on a single batch of cells. Sterol was added to the growth medium of all parasitic strains of PPLO and of all L-type organisms except 18-L. No detectable sterol was present in the basal media not supplemented with sterol and used for the other organisms studied. Of the organisms tested, PPLO as a group possessed the highest total lipid and NSL content. The four strains of PPLO requiring sterol for growth contained more total lipid and NSL than the PPLO strains not requiring sterol for growth. Among the L-type organisms, those requiring high concentrations of sodium chloride for growth were intermediate between PPLO and bacteria with respect to content of both total lipid and NSL. The increased amount of lipid in the salt-requiring L-type organisms, representing about two to three times that of the parent organism, was probably a reflection of the absence of some cell-wall material, thereby decreasing the total solids content of an L-type organism compared to its parent bacterium.

L-type organisms not requiring high concentrations of salt for growth and stability were represented in the study by two strains. Proteus

TABLE 2. *Lipid content of various organisms*

Strain	Sample of whole cells, mg dry wt		Total lipid, % dry wt cells		Nonsaponifiable lipid, % dry wt cells	
	Average	Range	Average	Range	Average	Range
PPLO						
07	65.0	47.5–79.5	15.3	13.9–16.5	8.6	8.2–8.9
Campo	64.7	41.2–100.5	13.8	12.9–14.6	8.2	7.8–8.5
J	53.0	35.2–64.2	12.0	10.3–14.7	8.2	7.5–8.9
T-5	55.1	41.9–80.7	17.3	15.4–19.6	7.0	5.6–8.9
B-15	45.7	20.0–76.0	8.7	7.6–9.9	3.8	3.5–4.0
Laidlaw-B	80.6	16.5–298.8	8.3	7.4–10.3	5.1	4.2–6.7
L-type organisms						
Campo-L	276.2	22.3–530.1	3.3	—	1.7	0.7–1.6
D5L	159.1	145.6–172.6	3.7	3.2–4.1	1.1	0.6–1.6
ADAL	122.8	36.3–209.3	5.0	4.7–5.2	1.2	0.9–1.5
GL8L	202.2	85.9–318.5	4.8	—	1.3	1.2–1.3
HL	178.9	84.2–273.6	5.0	—	1.4	—
18-L	140.4	90.0–188.1	8.6	7.4–9.9	3.0	2.5–3.5
L-1	55.9	42.4–69.4	19.4	18.4–20.4	11.7	—
Bacteria						
Campo-D	76.2	46.1–106.2	2.4	2.1–2.6	0.6	0.4–0.7
D-5	86.4	57.6–115.1	1.9	1.1–2.7	1.3	0.8–1.8
D-17	61.2	61.0–61.3	2.9	2.3–3.4	1.6	1.1–2.0
ADA	96.2	86.6–105.7	1.3	1.2–1.4	0.5	0.4–0.5
GL 8	130.4	119.2–141.5	0.9	0.7–1.1	0.4	—
Proteus 18	77.6	72.5–82.7	4.4	3.9–4.8	0.5	—
S. moniliformis	214.4	125.9–341.4	2.6	2.5–2.7	2.1	2.0–2.1

18-L showed a greatly increased NSL content compared to its bacterial parent, yet only twice as much total lipid. The values for total lipid and NSL of this organism bear a similarity to the saprophytic PPLO. L-1, on the other hand, had at least five times more total lipid and NSL than its parent bacterium, *S. moniliformis*. The values for total lipid and NSL of L-1 were very much like those for the parasitic PPLO. The presence or absence of lipid in the culture medium had little effect on the amounts of lipid found in PPLO. For example, Laidlaw B contained an average of 8.9% total lipid and 4.1% NSL, when grown with sterol, and 8.3 and 5.1%, respectively, when grown without sterol. Corresponding values for strain B-15 were 8.6 and 4.0% and 8.7 and 5.1%.

Table 3 presents data on the capabilities of various organisms to synthesize their NSL from sodium acetate-1-C^{14} and mevalonic acid-2-C^{14}; 100 count/min (per mg NSL) or greater are considered significant. Parasitic PPLO, although by necessity grown in the presence of minimal cholesterol (0.003 mg per ml), were incapable of incorporating C^{14} into their NSL fractions. The two saprophytic strains, on the contrary, were capable of synthesizing labeled NSL from both C^{14}-labeled acetate and mevalonate. Salt-requiring L-type organisms derived from diphtheroids were incapable of NSL synthesis from the labeled precursors, in spite of the capability of one parent bacterium. Streptococcal L-type organisms behaved as their parent bacteria. Strains ADA and ADA-L could incorporate C^{14}-labeled acetate but not mevalonate. Such was the case with Proteus 18 and Proteus 18-L. Conversion of *S. moniliformis* to L-1 appeared to result in the loss of the ability to incorporate C^{14} into the NSL, similar to the case of the Campo diphtheroid and Campo L.

The distribution of dry wt and of NSL, determined as weight or radioactivity between the residue and the soluble fraction after sonic treatment, is shown in Table 4. About half of the dry wt of the cells is found in sedimentable material. With PPLO, 70 to 80% of the NSL is isolated from the residue; with the L-type organisms, the distribution varies with the strain. With L-1 and Proteus 18-L, however, the amount of NSL found in the residue exceeds half of the total. Variation of the distribution of NSL among the

TABLE 3. *Specific activity of nonsaponifiable lipids of various organisms grown in the presence of sodium acetate-1-C^{14} or mevalonic acid-2-C^{14}*

Strain	Sodium acetate-1-C^{14}	Mevalonic acid-2-C^{14}
PPLO		
07	21*	8
Campo	15	<1
J	32	10
T-5	32	4
B-15	111	108
Laidlaw-B	160	131
L-type organisms		
Campo	30	80
D5L	37	79
ADAL	244	99
GL8L	58	40
H-L	143	145
18-L	151	53
L-1	80	—
Bacteria		
Campo D	388	285
D-5	90	20
D-17	35	55
ADA	189	56
GL8	30	32
Proteus 18	529	50
S. moniliformis	169	126

* Count/min (per mg of nonsaponifiable lipids).

L-type organisms probably reflects the varying nature of the external layers of the different types used. Thus, with the osmotically labile forms, greater disintegration probably occurred.

A strictly qualitative analysis of the NSL of the various organisms is shown in Table 5. More complete analyses of the NSL fractions of representative PPLO is the subject of another report (Rothblat and Smith, 1961). If exogenous cholesterol is supplied to PPLO, all the strains contain digitonin-precipitable, slow-acting, Liebermann-Burchard-positive NSL. In no instance were fast acting Δ^7 sterols found. Saprophytic PPLO grown in the absence of sterol contained only ferric chloride-sulfuric acid-positive NSL. All L-type organisms contained slow-acting, Liebermann-Burchard-positive NSL when grown in the presence of cholesterol. This NSL was digitonin-precipitable only with D5L, Proteus 18-L, and L-1. When grown in the absence of cholesterol, ferric chloride-sulfuric acid-positive NSL only was found. Rebel et al. (1960) reported that the

TABLE 4. *Distribution of nonsaponifiable lipid between residue (cell membrane) and soluble fraction (protoplasm) of PPLO and L-type organisms*

Strain	Cell, dry wt*	Nonsaponifiable lipid				PPLO serum fraction (cholesterol) in growth medium
		Weight		Radioactivity		
		Residue*	Soluble fraction*	Residue†	Soluble fraction‡	
PPLO						
07	0.55 (0.48–0.60)	ND	ND	0.71 (0.63–0.84)	0.29 (0.15–0.36)	+
Campo	0.57	ND	ND	0.83	0.17 (0.16–0.18)	+
J	0.67	ND	ND	0.84	0.16 (0.16–0.17)	+
T-5	0.68	ND	ND	0.86	0.14 (0.13–0.15)	+
B-15	0.62	ND	ND	0.69	0.31	+
Laidlaw B	0.43 (0.40–0.48)	0.77 (0.76–0.81)	0.23 (0.20–0.24)	0.73 (0.70–0.75)	0.26 (0.25–0.30)	+
		0.81	0.19	ND	ND	0
L-type organisms						
D5L	0.55	ND	ND	0.88	0.12	+
GL8L	0.46 (0.46–0.53)	0.47	0.53	ND	ND	0
H-L	0.60 (0.50–0.85)	ND	ND	0.40 (0.26–0.45)	0.60 (0.60–0.71)	+
		0.26	0.74	ND	ND	0
18-L	0.39 (0.39–0.40)	0.56 (0.50–0.64)	0.44 (0.27–0.48)	ND	ND	0
L-1	0.50 (0.49–0.51)	ND	ND	0.65 (0.62–0.68)	0.29 (0.27–0.31)	+

* Expressed as the residue or soluble fraction (mg) divided by whole cells (mg); average values. Figures in parentheses represent the range of values. ND = not done.

† Residue (count/min) divided by whole cells (count/min).

‡ Soluble fraction (count/min) divided by whole cells (count/min).

lipid composition of Proteus 18-L differed from that of the parent bacterium, particularly by an increased level of cholesterol. Since their medium contained serum (Mandel, Terranova, and Sensenbrenner, 1957), and hence cholesterol, the cholesterol found in the organisms undoubtedly was adsorbed from the medium. Among the bacteria, ferric chloride-sulfuric acid-positive NSL was found in all but the β-hemolytic streptococcus, GL-8. *S. moniliformis* grown in the presence of cholesterol contained NSL of probable sterol nature. Fast-acting (Δ^7) Liebermann-Burchard-positive sterol was detected in this organism.

Uptake of C^{14}-labeled cholesterol and susceptibility to lysis by digitonin have already been reported for PPLO, some L-type organisms, and bacteria (Smith and Rothblat, 1960). Examination of these two phenomena was extended to *S. moniliformis* and L-1. In regard to uptake of cholesterol-4-C^{14}, L-1 behaved in a fashion typical of PPLO, i.e., a slow, regulated, and continuous uptake of cholesterol occurs. *S. moniliformis*, on the other hand, behaved as Proteus 18 (Smith and Rothblat, 1960), i.e., rapid adsorption occurred. A distinction was noted in that no uptake occurred until after a 1-hr lag period. In contrast to PPLO, neither L-1 nor *S. moniliformis* (grown with or without cholesterol) was lysed by the action of digitonin. Digitonin, however, did cause agglomeration and sedimentation of the L-1.

DISCUSSION

No clear-cut differentiation exists among the various organisms examined. Any given organism possesses some gradation of a given property. Such gradation is especially notable with composition of total lipid and NSL. Some L-type organisms possess a pattern similar to the two types of PPLO, i.e., some contain considerable quantities of lipid, others contain less, and some are similar to bacteria. In only two instances (L-1 and Campo L) was there any apparent change in lipid synthetic ability upon conversion of a bacterium to its L-type organism. The major difference between a stable L-type organism and its parent bacterium is the content of lipid.

A notable difference between PPLO and stable L-type organisms, on one hand, and the salt-requiring L-type organisms, on the other, is the preponderance of NSL in the sedimentable residue or cell membrane of the PPLO. This may be a reflection of the greater stability of the cell membranes of the PPLO and osmotically

TABLE 5. *Some properties of nonsaponifiable lipids of various organisms**

Strain	Digitonin precpitability	Ferric chloride reaction	Lieberman-Burchard		PPLO serum fraction (cholesterol) in growth medium
			Slow acting	Fast acting	
PPLO					
07	+	+	+	0	+
Campo	+	+	+	0	+
J	+	+	+	0	+
T-5	+	+	+	0	+
B-15	+	+	+	0	+
B-15	0	+	0	0	0
Laidlaw B	+	+	+	0	+
Laidlaw B	0	+	0	0	0
L-type organisms					
D-5L	+	+	+	0	+
D-5L	0	ND	0	0	0
GL8L	0	+	+	0	+
GL8L	0	+	0	0	0
H-L	0	+	+	0	+
H-L	0	ND	0	0	0
18-L	+	+	+	0	+
18-L	0	+	0	0	0
L-1	+	+	+	0	+
Bacteria					
D-5	0	+	0	0	0
D-17	0	+	0	0	0
GL8	0	0	0	0	0
Proteus 18	0	+	0	0	0
S. moniliformis	+	+	+	+	+
S. moniliformis	0	ND	0	0	0

* + = positive; 0 = negative; ND = not done.

stable L-type organisms. The assumption that the sedimentable sonic-treated residue represents cell membranes, in the case of the salt-requiring L-type organisms, is only partially justified, since Panos, Barkulis, and Hayashi (1960) have shown it to consist of debris from large bodies and small viable granular elements. However, since sonic treatment was carried out in distilled water, rupture of the granules probably occurred (Panos, *personal communication*). PPLO are nonviable after sonic treatment for 15 min (Smith and Sasaki, 1958), and electron micrographs show only cellular debris in the residue. Only a qualitative implication can be made that cell membranes and protoplasm were examined. The possibility exists that some intact cells were present in the sedimentable fraction, and some cell-membrane fragments were present in the supernatant fraction. However, it can be implied that the major portions of these two different fractions consisted of cell membranes and of protoplasm. The objects of this study did not necessitate quantitative separation of the component parts.

In both L-type organisms and PPLO, lipid appears to be a predominant component of the cell membrane. The specific nature of this lipid is variable even among PPLO, although when supplied exogenous cholesterol all PPLO and stable L-type organisms incorporate it. The ability to adsorb or incorporate sterol or other nonsaponifiable lipid into the cell membrane might explain the greater osmotic stability of these organisms. Conversely, the inability to incorporate sterol, in the case of salt-requiring L-type organisms, prevents stabilization in a physiologically osmotic environment. Stabilization of the latter organisms by high concentrations of salts may be a reflection of the condensation and solidification of monolayers of ionized long-chain lipids (e.g., phospholipids) by metal ions (Gurd, 1960).

Lipid is not the sole component imparting structure to the membrane. Further insight into

the nature of structural and morphological stability of L-type organisms and PPLO, and into the possible relation of these types of organisms, will depend in large part upon defining the components and complex nature of the cell membranes.

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