Lipid Catabolism of Relapsing Fever Borreliae

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Relapsing fever borreliae require lipid compounds for growth in vitro. In this study, the major pathways of lipid catabolism in three species of tick-borne relapsing fever borreliae were investigated. Thin-layer chromatography was used to compare chloroform-methanol extracts of fresh culture media with extracts of exhausted culture media after organisms were removed by centrifugation. The chromatographic data demonstrated that lysolecithin was removed from the culture media during growth of the spirochetes, whereas lecithin, sphingomyelin, triglycerides, and cholesterol esters were not affected by growth of the organisms. Sonic extracts of the organism were tested for the presence of specific enzymes of lipid catabolism. Lysolecithinase, glycerophosphorylcholine diesterase, and acid phosphatase activities were demonstrated. Thus, these organisms can sequentially dissimilate lysolecithin to fatty acids, choline, inorganic phosphate, and glycerol. Assays for phospholipases A, C, and D, α-glycerophosphate dehydrogenase, alkaline phosphatase, and lipase were negative.

Lipids are essential nutrients for all cultivable, pathogenic spirochetes (17, 18). Recently, a culture medium was described which permitted, for the first time, sustained in vitro growth of relapsing fever borreliae (21). When the medium was rendered lipid free, the borreliae did not grow. This suggested that lipids were essential nutrients for borreliae also. This study is a report of lipid utilization and catabolism in three species of tick-borne relapsing fever borreliae.

MATERIALS AND METHODS

Organisms. Borrelia hermsi, isolated from a patient who contracted relapsing fever at Lake Tahoe, Calif., was supplied in frozen mouse tissues by Edith Coffey of the California Department of Public Health in Berkeley. Homogenates of thawed infected tissue were injected into CF-1 mice, and infection was maintained by serial passage of blood into additional mice.

B. parkeri and B. turicatae were obtained in their tick vectors from Willy Burgdorfer of the Rocky Mountain Laboratory, Hamilton, Mont. Organisms were isolated by feeding the ticks on CF-1 mice and maintained by serial passage of blood into additional mice.

Blood of infected mice was used to inoculate borreliae of each species into the culture medium developed by Kelly (21). Cultures were maintained at 35 C with weekly transfers into fresh media. After 2 years of serial passages, borreliae grown in the medium retained infectivity for mice.

Thin-layer chromatography. Uninoculated media and media from a 10-day culture with borreliae removed by centrifugation were adjusted to neutral pH for extraction of total lipids by the method of Kasarov and Addamiano (19), modified by use of dry nitrogen gas to evaporate solvent from extracted lipids. Commercially prepared plates of silica gel G were activated at 110 C for 1 h before use.

For chromatography of neutral lipids, the solvent mixture used was n-hexane-diethyl ether-glacial acetic acid (73:25:2, vol/vol/vol) (28), and the solvent mixture for separating phospholipids was chloroform-methanol-water (65:50:10, vol/vol/vol). Separated lipid compounds were located by exposing silica gel plates to iodine vapors or by spraying them with rhodamine B (35).

Enzyme preparation. Enzyme assays were performed with either twice-washed whole cells suspended in 0.15 N saline or with sonic extracts of cell suspensions. Cells were washed by using a Sorvall RC2-B centrifuge to rapidly sediment suspended borreliae (20,000 x g for 5 min). Each milliliter of washed cell suspension contained borreliae from 16 ml of fully grown culture (8 x 10⁹ cells).

Sonic extracts of borrelial suspensions were prepared at 0 C by using a Bronwill Biosonic sonic oscillator (model B10-11) with probe intensity set at 80% for 1 min. Complete disintegration of cells in 5 ml of suspension was obtained under these conditions. Sonic extracts frozen and stored at −20 C had enzyme activity comparable with that of fresh sonic extracts.

Chemicals and materials. Silica gel G plates (20 by 20 cm) were obtained from E. Merck, Darmstadt, Germany. Cholesterol was purchased from Fisher Scientific Co., St. Louis, Mo. Phospholipases A and C and all other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.
Enzyme assays. Phosphatidic acid-lyase (EC 2.1.1.4), phospholipase A activity was measured spectrophotometrically by using the hydroxide acid method of Magee and Thompson (27) for determining ester content. Venom of the cottonmouth moccasin, Ancistrodon piscivorus leucostoma, was used as a positive control in the assay.

Lysolecithin acyl-hydrolase (EC 3.1.1.5, phospholipase B) activity was detected by using the modified hydroxide acid method as described by Shapiro (34). Phosphate buffer (0.04 M) was used in the assay except at pH 5.0, where phthalate buffer (0.04 M) was used.

Phosphatidylcholine cholinephophohydrolase (EC 3.1.4.3, phospholipase C) activity was measured by using the phosphorus release assay of Ottolenghi (30). The Lowry reagent (25) was used to measure inorganic phosphate. Phospholipase C from Clostridium welchii was used as a positive control.

Phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4, phospholipase D) activity was measured by a modification of the procedure used by Davidson and Long (6). Ovolecithin (16 µmol) was dispersed in 1.25 ml of 0.1 M sodium acetate buffer (pH 5.6). To this was added 0.25 ml of diethyl ether and finally 1.0 ml of enzyme in 0.85% saline. Incubation was at 25 C for 2 h. Cold 6% perchloric acid (3 ml) was used to terminate each enzyme reaction. Phosphatidic acid and ovolecithin were extracted from inactivated reaction mixtures with 1 ml of diethyl ether which was removed after centrifugation (1,000 x g, 10 min). Inactivated reaction mixtures were then filtered through Whatman no. 1 filter paper, and 1-ml volumes of reaction mixture were placed in vacuo for 1 h to remove traces of ether. Choline content of each 1-ml portion was then determined by the method of Haayaishi and Kornberg (13). Phospholipase D was prepared from fresh cabbage (6) for use as a positive control.

L-3-Glycerophosphorylcholine glycerophosphohydrolase (EC 3.1.4.2, glycerophosphorylcholine diesterase) activity was measured by the method of Haayaishi and Kornberg (13), except that glycine buffer (0.05 M) rather than glycyglycine buffer was used. Cadmium was removed from commercial glycerophosphorylcholine by the method of Tattrie and McArthur (38) before it was used as substrate for the enzyme.

L-Glycerol-3-phosphate nicotinamide adenine dinucleotide (NAD) oxidoreductase (EC 1.1.1.8, L-α-glycerophosphate dehydrogenase) activity was measured by the assay of Lee et al. (23) and also by the method of Beisenherz et al. (4). L-α-Glycerophosphate dehydrogenase was prepared from sonic extracts of Saccharomyces cerevisiae for use as a positive control in both assay methods (12).

Orthophosphoric monoester phosphohydrolase (EC 3.1.3.1, alkaline phosphatase; and EC 3.1.3.2, acid phosphatase) activity of B. hermsi sonic extracts was measured by a slight modification of the method of Heppel (15). Sodium acetate (pH 5.0) and glycerol (pH 9.0) buffers were used in place of ethanolamine buffer; Whatman no. 1 filter paper was used for filtration after enzyme inactivation. Inorganic phosphate was determined by using the Lowry reagent (25). Alkaline and acid phosphatases were prepared from sonic extracts of S. cerevisiae for use as positive controls in the assays (37). Additional assays of alkaline and acid phosphatase activities were performed by using p-nitrophenylphosphate (11) and o-carboxyphenylphosphate (5, 16), respectively, as substrates.

Glycerol ester hydrolase (EC 3.1.1.3, "lipase") activity was determined by the method of Patel et al. (31).

RESULTS

Lipids present in culture media. Lipids were extracted from un inoculated growth media and an equivalent amount of media from a 10-day culture from which B. hermsi had been removed by centrifugation. Thin-layer chromatography of phospholipids present in extracts of uninoculated growth media demonstrated the presence of lecithin, sphingomyelin, and lysolecithin. Similar chromatograms of phospholipid extracts from exhausted media showed a total absence of lysolecithin. The levels of sphingomyelin and lecithin, however, did not differ when compared to extracts from uninoculated media (Fig. 1).

When neutral and acidic lipids in extracts of fresh growth media were studied by thin-layer chromatography, cholesterol, cholesterol esters, fatty acids, and triglycerides were identified on the chromatograms. In comparison, similar chromatograms of lipids extracted from exhausted growth media (10-day cultures) indicated decreased amounts of cholesterol and fatty acids. Cholesterol ester and triglyceride concentrations did not differ when compared with control chromatograms.

Identical chromatographic findings were observed in lipid extracts of media in which B. parkeri and B. turicatae had been propagated.

Lysolecithin acyl-hydrolase. The absence of lysolecithin in exhausted culture media suggested that the compound was metabolized by borreliae during growth. Accordingly, the organisms were studied to determine whether enzymes for the dissimilation of lysolecithin were present.

Sonic extracts of B. hermsi were found to contain lysolecithin acyl-hydrolase (EC 3.1.1.5, phospholipase B), which hydrolyzes lysolecithin to a fatty acid and glycerophosphoryl choline. Crude enzyme preparations had maximal activity at pH 37 and 40 C (Fig. 2) and a pH optimum of 6.5 at 37 C in 30-min assays (Fig. 3). Enzyme activity was stimulated slightly by ethylenediaminetetraacetate (EDTA) (10<sup>-5</sup> M) and moderately by Triton X-100 (1.6 x 10<sup>-3</sup> M). Maximal enzyme activity was obtained with...
EDTA and Triton X-100 together at the above concentrations; however, the individual effects of the two compounds were not totally additive (Table 1).

The effects of other compounds on the activity of crude lysolecithin acyl-hydrolase are shown in Table 1. Calcium, magnesium, or zinc ions and p-chloromercuribenzoic acid (PCMB) had no effect or inhibited enzyme activity. Mercuric chloride interfered with color development in the assay, so its effect could not be determined.

TABLE 1. Effect of various compounds on lysolecithin acyl-hydrolase activity of B. hermsi

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (mol/liter)</th>
<th>Enzyme activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
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<td>85</td>
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<tr>
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<td>MgCl₂</td>
<td>10⁻²</td>
<td>100</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>10⁻⁴</td>
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</tr>
<tr>
<td>HgCl₂</td>
<td>5 x 10⁻³</td>
<td>-b</td>
</tr>
<tr>
<td>PCMB</td>
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<td>29</td>
</tr>
<tr>
<td>EDTA</td>
<td>10⁻⁵</td>
<td>121</td>
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<tr>
<td>Triton X-100</td>
<td>1.6 x 10⁻³c</td>
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<td>EDTA and</td>
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<tr>
<td>Triton X-100</td>
<td>1.6 x 10⁻³c</td>
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bHgCl₂ interfered with color development in the assay. cBased on average molecular weight of 646 for Triton X-100.

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**FIG. 1.** Thin-layer chromatogram of known phospholipid compounds with phospholipids from fresh culture media and media from a 10-day culture of B. hermsi. The lipids are lecithin (A), sphingomyelin (B), lysolecithin (C), lipids from fresh culture media (D), and lipids from exhausted culture media (E). The lipids above lecithin were not identified on this chromatogram.

**FIG. 2.** Effect of temperature on activity of lysolecithin acyl-hydrolase from B. hermsi at pH 6.5 in a 30-min assay.

**FIG. 3.** Effect of pH on activity of lysolecithin acylhydrolase from B. hermsi at 37 C in a 30-min assay.
Both *B. parkeri* and *B. turicatae* were also found to have lysolceithin acyl-hydrolase activity.

**L-3-Glycerophosphorylcholine glycerophosphohydrolase.** Additional enzyme studies were performed to determine the fate of glycerophosphoryl cholines (GPC), one of the products of lysolceithin acyl-hydrolase activity. Sonic extracts of *B. hermsi* were found to hydrolyze GPC, with the production of choline and α-glycerophosphate. The enzyme L-3-glycerophosphorylcholine glycerophosphohydrolase (EC 3.4.1.2, glycerophosphorylcholine diesterase) had maximal activity at 50°C in 30-min assays (Fig. 4). The pH optimum at 50°C with glycine buffers was 8.4 (Fig. 5); however, when assays were performed at 25°C, the pH optimum was 9.0.

Table 2 shows the effects of various compounds on glycerophosphorylcholine diesterase activity. The individual stimulatory effects of calcium chloride (10^{-2} M) and Triton X-100 (1.6 x 10^{-4} M) were not completely additive when the compounds were placed in the enzyme reaction together; however, the compounds together produced greater stimulation of enzyme activity than either compound alone. Magnesium chloride and zinc chloride were either inhibitory or had no effect at the concentrations tested. Mercuro chloride and PCMB had no effect on enzyme activity at the concentrations tested. EDTA had little effect on enzyme activity at 2 x 10^{-5} M, but produced complete inhibition at 5 x 10^{-4} M (Fig. 6). EDTA inhibition of enzyme activity at 10^{-4} M was completely reversed by calcium chloride at 2 x 10^{-4} M.

**Orthophosphoric monoester phosphohydrolase.** The further metabolism of choline, one of the products of glycerophosphorylcholine diesterase action on glycerophosphoryl choline, was not investigated. α-Glycerophosphate, the other product of the diesterase activity, was studied to determine whether the compound was hydrolyzed by the organisms. Sonic extracts of *B. hermsi* were found to contain orthophosphoric monoester phosphohydrolase activity.

**Table 2. Effect of various compounds on L-3-glycerophosphorylcholine glycerophosphohydrolase activity of *B. hermsi*.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc (mol/liter)</th>
<th>Enzyme activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
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<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
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<tr>
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<td>3 x 10^{-5}</td>
<td>9</td>
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<tr>
<td>EDTA</td>
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</tr>
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<tr>
<td>CaCl₂ and Triton X-100</td>
<td>10^{-4} (CaCl₂)</td>
<td>158</td>
</tr>
</tbody>
</table>

*Reaction mixtures contained 0.4 ml of glycerophosphoryl choline solution (0.02 M, pH 7.0); 0.2 ml of glycine buffer (0.2 M, pH 8.4); 0.2 ml of a 1:20 dilution of crude enzyme; 0.2 ml of test compounds in water to give the final concentrations indicated in this table. Assays were incubated at 50°C for 30 min.  
*Based on average molecular weight of 646 for Triton X-100.*
drolase (EC 3.1.3.2, acid phosphatase) which hydrolyzes α-glycerophosphate to glycerol and inorganic phosphate. Enzyme activity was demonstrated with either L-α-glycerophosphate or o-carboxyphenylphosphate as substrates. A pH optimum of 5.0 was obtained in 37 C assays. Since the enzyme appeared to be rather nonspecific, as are acid phosphatases from other sources (22, 39), it was not characterized further.

The rates of hydrolysis in this and the other positive enzyme assays were linear for at least 0.5 hour.

**Negative enzyme assays.** Specific enzyme assays were performed to determine whether other enzymes important in lipid catabolism were present in relapsing fever borreliae. A particular effort was made to determine whether L-α-glycerophosphate dehydrogenase activity was present. This enzyme permits α-glycerophosphate produced from lipid catabolism to enter the glycolytic pathway, or dihydroxyacetone phosphate produced during glycolysis to be converted into α-glycerophosphate for lipid synthesis.

In attempts to demonstrate L-glycerol-3-phosphate:NAD oxidoreductase activity (EC 1.1.1.8, L-α-glycerophosphate dehydrogenase), two different assay procedures were employed. Enzyme activity was not detected with either procedure using sonic extracts or washed intact cells, although positive assays were obtained with both procedures with enzyme preparations from *S. cerevisiae*, which were used as positive controls.

Orthophosphoric monoester phosphohydrolase (EC 3.1.3.1, alkaline phosphatase) was not demonstrable in sonic extracts of *B. hermsi* by either of two different assay procedures.

Negative enzyme assays with *B. hermsi* sonic extracts or intact cells as a source of enzyme were also obtained for phosphatidyl acyl-hydrolase (EC 3.1.1.4, phospholipase A), phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3, phospholipase C), phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4, phospholipase D), and glycerol ester hydrolase (EC 3.1.1.3, lipase). Data from thin-layer chromatographic studies were consistent with results from specific enzyme assays.

Figure 7 summarizes the findings from this investigation of lipid catabolism in borreliae.

**DISCUSSION**

Lipids have been shown to be essential nutrients for pathogenic spirochetes as well as for closely related, nonpathogenic species (17, 18). Their function in the metabolism of these organisms, however, varies widely in the different genera.

Members of the genus *Leptospira* derive most of their energy from β-oxidation of long-chain fatty acids (3, 14). In contrast, neither the cultivable treponemes nor borreliae use fatty acids as a major source of energy. Energy metabolism in borreliae is achieved primarily through glycolysis, whereas the cultivable treponemes ferment amino acids or carbohydrates (1, 10).

The requirement by leptospires for free fatty acids is satisfied by enzymes present in the organisms which hydrolyze triglycerides and phospholipids (19, 20). Members in the genus *Treponema* may be able to cleave fatty acids from monoglycerides (33), but evidence of phospholipid catabolism is lacking. Borreliae hydrolyze fatty acids from lysolecithin but cannot metabolize other major phospholipids or triglycerides.

Cultivable treponemes and members of the genus *Leptospira* require long-chain fatty acids for growth (17, 18). Nutritional studies demonstrate that fatty acids can serve as the sole lipid nutrient for *B. hermsi* in culture media lacking lysolecithin or rabbit serum (unpublished data). Thus, members of all three genera have in common a requirement for exogenous long-chain fatty acids, apparently for use as cell structural elements.

Borreliae have a restricted ability to catabolize lipids, as indicated by their inability to obtain fatty acids from most of the complex lipids present in rabbit serum. This limitation may be related to the nutritional conditions present in ticks where relapsing fever organisms must adapt for extended coexistence (8).

Cholesterol has been shown to be a constituent of relapsing fever borreliae (9), and in the present investigation it was selectively removed from the culture media during growth of the organisms. It serves an important nutritional role in the Nogouchi strain of *Treponema*.
pallidum (32) and may be important in borreliae. Cholesterol has been shown to have a role in regulating membrane permeability in Mycoplasma laidlawii B and could serve a similar function in membranes of borreliae (26). Alternatively, the presence of cholesterol in borreliae may be incidental rather than essential. Removal of cholesterol from culture media during growth of the organisms might be due to non-specific adsorption to the cells.

In chemical analyses of relapsing fever organisms, Felsenfeld et al. (9) detected the presence of lecithin. If this phospholipid is a significant component of borrelial cells, it is likely the organisms have the ability to synthesize lecithin, since in this study there was no evidence of removal of the compound from the media during growth.

The thin-layer chromatographic method used in this study is a semi-quantitative technique, and its limitation in detecting extremely small changes in certain substrate concentrations is recognized.

The borreliae could have used minute amounts of lecithin (several micrograms) without detection by thin-layer chromatography; however, enzyme studies revealed no catabolism of lecithin, and nutritional studies (unpublished data) have shown that borreliae will not grow when lecithin is provided as the sole lipid substrate in an otherwise complete culture medium.

Smith reported the presence of α-glycerophosphate dehydrogenase activity in Borrelia duttoni (36); however, it was not detected in B. hermsi by either of two assay techniques using intact cells or sonic extracts. The absence of this essential enzyme for exchange of three-carbon compounds between pathways of carbohydrate and lipid metabolism in effect makes B. hermsi more dependent on its host for provision of certain specific nutrients than is the case with B. duttoni.

Lyssolecithin acyl-hydrolase from B. hermsi has characteristics similar to the same enzyme from other sources as to pH optimum and the effects of several inhibitors (24, 29); however, the crude borrelial enzyme is stimulated by EDTA and Triton X-100, whereas this is not reported for the enzyme from other sources.
In respect to pH optimum and the effect of inhibitors, GPC diesterase from B. henselae is similar to the same enzyme prepared from other sources (2, 7).

ACKNOWLEDGMENTS

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LITERATURE CITED