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Biosynthesis of Phospholipids and Neutral Lipids of Monkey Kidney Cells (LLC-MK-2) Infected with *Chlamydia trachomatis* Strain Lymphogranuloma Venereum (38538)

V. S. C. FAN[1] AND H. M. JENKIN

The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

Studies on the lipid synthesis of isolated *Chlamydia psittaci* strain meningopneumonitis have been reported by Gaugler *et al.* (1). Radioactive carbon from aspartate, isoleucine and glucose-6-phosphate were found to be incorporated into lipids of the organisms. Radioactivity was found to be predominantly in the fatty acids. The labeled fatty acids were located mainly in the phosphatidyl ethanolamine (PE) and, to a lesser extent, in the phosphatidyl choline (PC) fraction. Fan and Jenkin (2) investigated the lipid metabolism of monkey kidney cells (LLC-MK-2) infected with *C. trachomatis* strain lymphogranuloma venereum 440 LN (LGV) and showed that lipid synthesis of the infected cells was reduced about 12–18 hr after infection. The present communication is a report of the results of phospholipid and neutral lipid metabolism of normal and LGV-infected monkey kidney cells.

Materials and Methods. Cultivation of LGV and monkey kidney cells (MK-2). The method for cultivation of MK-2 cells and propagation of LGV have been described by Fan and Jenkin (2).

Total lipids of normal cells and cells infected with LGV were extracted by the method of Bligh and Dyer (3) as modified by Fan and Jenkin (2). Neutral lipids were first separated from phospholipids by thin layer chromatography (TLC), silica gel H (Merck, A.G., Darmstadt, Germany) employing a solvent system consisting of petroleum ether:diethyl ether, 90:10. Neutral lipids were then separated on silica gel H plates (0.25 mm thick) using the procedure described by Freeman and West (4).

Lipid separation and quantification. Phospholipids were separated by thin layer chromatography as described by Rouser *et al.* (5). Methyl esters of fatty acids were prepared by direct deacylation of the lipid and esterified in 5% methanolic HCl as modified according to Kates (6).

The procedure for use of internal standards for separation and quantification of fatty acids, using gas liquid chromatography (GLC), has been described by Townsend *et al.* (7). Individual phospholipid classes were quantified based on the phosphorus content of the lipid.

Phosphorus determination of individual phospholipid classes was performed according to the procedure described by Rouser *et al.* (5) with the following modification: direct perchloric acid digestion was carried out in teflon lined screw-cap test tubes. The tubes were heated to 180° in a heating block (Warner–Chilcott, Richmond, CA) for 45 min. The tubes were initially raised so that half of the tube was outside of the heating block to aid in refluxing the acid and to reduce internal pressure.

Sodium 1-^{14}C-acetate (1 mCi, 59 mCi/m*M* was purchased from Amersham/Searle, Arlington Heights, IL. DL-3-^{14}C-serine (0.1 mCi, 6 mCi/m*M*) was purchased from Calbiochem, Los Angeles, CA. The radioactivity of total lipid and fatty acid methyl esters was measured in 15 ml of toluene based liquid scintillation fluid prepared from Permablend I (Packard Instrument Co., Inc., Downers Grove, IL). A 0.2 ml portion of the aqueous deacylated phospholipid fraction was transferred to duplicate counting vials from each of three independent experiments. The acidity of the aqueous solution was neutralized with Hyamine (Packard Instrument Co., Inc.). Fifteen milliliters of Bray's scintillation fluid (8) was added to the sample vial. The radioisotope counting was performed in a Packard liquid scintillation spectrophotometer (model

[1] Present Address: Department of Biological Sciences, Florida State University, Tallahassee, FL 32306.

314EX). The specific activity of the lipid samples was calculated from the amount of radioactivity of the lipid sample and either the total lipid weight, total fatty acid weight or the quantity of lipid phosphorus.

The incorporation of 1-^{14}C-acetate into fatty acids of phospholipids of normal and LGV-infected MK-2 cells. Normal and LGV-infected cells (1.5×10^7 cells/32 oz prescription bottle) were labeled with 10 ml of Eagle's minimum essential medium supplemented with 5% calf serum (MEM CS_5) and 1-^{14}C-acetate (0.5 μCi/ml) for 12-hr intervals of time. Bottles containing the radioactive medium were placed on a platform rocker at 37° during the incubation period. Lipids were extracted at the end of the incubation period and separated by TLC. Individual purified phospholipid classes were deacylated. The radioactivity was measured as described above.

The incorporation of 1-^{14}C-acetate into triglycerides, diglycerides and monoglycerides. Normal and LGV-infected MK-2 cells (3×10^7 cells/32 oz prescription bottle) were labeled with 5 ml of 1-^{14}C-acetate (1 μCi/ml) in MEM CS_5 24 hr after infection for periods of 15, 30, 60 and 120 min. Triglycerides, diglycerides and monoglycerides were separated as described above. Fatty acids obtained by deacylation from these lipids were measured for radioactivity. Fatty acids were quantified by GLC using heneicosanoic acid (21:0) as an internal standard.

The incorporation of DL-3-^{14}C-serine into phosphatidyl ethanolamine. Separate bottles of normal and LGV-infected MK-2 cells (2×10^5 cells/2 oz prescription bottle) were incubated with DL-3-^{14}C-serine (0.1 μCi/ml) in growth medium for 6-hr intervals and sampled. Samples were pulse-labeled for 6 hr and then held in radioactive-free medium for another 6 hr and sampled. Phosphatidyl ethanolamine was isolated after each pulse and each chase period. The deacylated portion of the lipid was measured for its radioactivity and phosphorus content. The specific activity was expressed as counts/min/μg of inorganic phosphorus.

Results. Phospholipid synthesis and phospholipid composition of normal and LGV-infected MK-2 cells. The synthesis of phospholipids was examined in normal and LGV-infected cells using ^{14}C-acetate as a lipid precursor. The results of the acetate incorporation into fatty acids of phospholipids of normal and LGV-infected cells are shown in Table I.

A major difference in phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) synthesis was observed in LGV-infected cells 24–36 hr after infection. The infected cells showed an increase of synthesis of PE whereas there was a decrease in PC synthesis. Synthesis of phosphatidyl serine (PS) plus phosphatidyl inositol (PI) was reduced after 36 hr of infection. Sphingomyelin synthesis in the infected cells did not appear to be altered by the infection in the 60-hr period. Synthesis of cardiolipin was inhibited after initiation of the infection. A small increase in the amount of lysophosphatidyl choline radioactivity was observed. No major changes in phospholipid synthesis of normal MK-2 cells were observed over the 60-hr period except for an increase in the activity of cardiolipin.

Phosphorus analyses of phospholipids from normal and LGV-infected MK-2 cells are shown in Table II. A twofold increase of PE occurred during 36 hr of infection. No change was observed in the PE of normal cells in the same period of observation. A small increase in PC was also detected during the infection. The PC of normal cells was found to increase gradually during the infection. A slight increase of sphingomyelin was observed in normal cells during the first 24 hr of infection, but no further increase was found after 36 hr. Sphingomyelin changes were not found in the infected cells except after 48 hr of infection when a three- to fourfold decrease was observed. No change was found in cardiolipin content of normal or infected cells during the 60-hr period of observation. The PS + PI fraction was found to decrease within 36 hr of infection. An increase in the amount of lysophosphatidyl choline (LPC) was found 12 hr after infection. As the infection proceeded, the amount of LPC declined. Some reduction of LPC was observed in normal cells.

Incorporation of DL-3-^{14}C-serine into phos-

TABLE I. THE INCORPORATION OF 1-^{14}C-ACETATE INTO FATTY ACIDS OF PHOSPHOLIPIDS OF NORMAL AND LGV-INFECTED MONKEY KIDNEY (ML-2) CELLS.

		Pulse time—hours after infection				
		0–12	12–24	24–36	36–48	48–60
Cardiolipin	LGV:MK-2	0.9 ± 0.1[a]	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1.4 ± 0.3
	MK-2	0.9 ± 0.1	1.5 ± 0.2	1.6 ± 0.4	1.9 ± 0.4	3.8 ± 0.9
Phosphatidyl ethanolamine	LGV:MK-2	25.2 ± 3.5	25.1 ± 6.9	40.1 ± 9.0	19.9 ± 3.6	22.0 ± 5.0
	MK-2	25.9 ± 7.8	25.3 ± 6.6	22.2 ± 5.4	29.1 ± 2.3	20.5 ± 5.0
Phosphatidyl choline	LGV:MK-2	45.8 ± 4.6	41.6 ± 2.6	23.7 ± 7.1	50.3 ± 2.0	48.5 ± 5.0
	MK-2	45.1 ± 2.2	45.0 ± 5.4	46.4 ± 4.7	49.2 ± 1.5	51.5 ± 5.8
Sphingomyelin	LGV:MK-2	11.5 ± 2.3	14.5 ± 1.8	13.0 ± 1.3	17.9 ± 5.8	14.9 ± 0.8
	MK-2	9.9 ± 1.6	9.4 ± 2.3	10.6 ± 2.5	9.5 ± 8.0	9.3 ± 3.9
Phosphatidyl serine + phosphatidyl inositol	LGV:MK-2	16.4 ± 3.3	17.2 ± 8.8	19.9 ± 9.3	9.8 ± 0.9	9.4 ± 1.8
	MK-2	17.4 ± 3.6	18.5 ± 7.8	18.7 ± 6.2	10.0 ± 2.2	17.0 ± 11.7
Lysophosphatidyl choline	LGV:MK-2	1.1 ± 0.4	2.2 ± 0.6	2.4 ± 1.5	3.2 ± 0.4	4.4 ± 0.4
	MK-2	0.9 ± 0.6	0.8 ± 0.5	1.0 ± 0.7	0.6 ± 0.3	0.5 ± 0.2

[a] Relative percentage of radioactivity (cpm)-mean values ± the standard deviation from four independent experiments. The results of phosphatidyl serine and phosphatidyl inositol were combined due to incomplete separation of these two lipids in some of the samples.

TABLE II. PHOSPHOLIPID COMPOSITION OF NORMAL AND LGV-INFECTED MK-2 CELLS.

		Hours after infection				
		12	24	36	48	60
Cardiolipin	LGV:MK-2	6.3 ± 0.3[a]	4.4 ± 0.4	4.1 ± 0.4	5.5 ± 1.5	4.3 ± 1.9
	MK-2	4.4 ± 0.4	3.9 ± 0.8	4.7 ± 0.3	4.7 ± 0.2	4.6 ± 0.2
Phosphatidyl ethanolamine	LGV:MK-2	15.9 ± 1.5	28.0 ± 0.5	31.6 ± 3.6	27.4 ± 1.1	24.9 ± 1.6
	MK-2	32.5 ± 1.0	29.4 ± 6.2	26.9 ± 2.0	26.6 ± 1.4	28.5 ± 2.7
Phosphatidyl choline	LGV:MK-2	31.6 ± 6.8	39.2 ± 1.6	44.1 ± 2.0	40.0 ± 3.8	49.7 ± 2.3
	MK-2	38.5 ± 1.2	40.9 ± 3.7	44.6 ± 1.3	45.3 ± 1.3	44.1 ± 2.9
Sphingomyelin	LGV:MK-2	7.0 ± 1.3	7.2 ± 0.4	7.0 ± 2.3	11.8 ± 4.1	3.2 ± 0.7
	MK-2	6.5 ± 0.3	5.4 ± 1.0	9.6 ± 1.3	9.7 ± 1.3	9.6 ± 0.7
Phosphatidyl serine + phosphatidyl inositol	LGV:MK-2	24.8 ± 0.2	16.4 ± 1.5	10.2 ± 2.6	14.1 ± 1.9	13.1 ± 3.8
	MK-2	12.1 ± 0.9	18.1 ± 1.5	13.1 ± 0.8	12.6 ± 0.9	11.9 ± 3.2
Lysophosphatidyl choline	LGV:MK-2	9.3 ± 0.6	2.9 ± 0.2	1.8 ± 1.6	0.8 ± 0.2	0.9 ± 0.2
	MK-2	3.4 ± 0.3	3.5 ± 0.7	0.8 ± 0.4	0.8 ± 0.5	0.9 ± 0.4

[a] Relative percentage of lipid phosphorus: mean values ± the standard deviation from four independent experiments. The results of phosphatidyl serine and phosphatidyl inositol were combined due to incomplete separation of these two lipids in some of the samples.

phatidyl ethanolamine of normal and LGV infected MK-2 cells. There was a slight increase of serine into PE 6–12 hr after infection (Fig. 1). Labeled serine was incorporated at an increasing rate 18 hr after infection. Maximum incorporation was reached about 24 hr after infection and was followed by a decrease in the PE by 36 hr. No increase of serine incorporation into PE was observed in normal cells.

Accumulation of the PE synthesized from serine was observed in a pulse-chase experiment (Fig. 2). Normal and LGV-infected cells that were incubated in the presence of labeled serine for 6 hr were further incubated in the absence of labeled serine for another 6 hr. The result of this study showed that there was an increase in the accumulation of serine radioactivity in PE about 24 hr after infection. The activity reached a maximum 30–36 hr after infection. No increase of accumulation of serine radioactivity in PE was observed in normal cells during the 36-hr observation period.

Neutral glycerolipid synthesis in normal and LGV-infected MK-2 cells. Neutral glycerolipid synthesis was examined 24 hr after infection when overall lipid synthesis was low (2). The results of the triglyceride, diglyceride and monoglyceride syntheses (Table III) showed that LGV-infected cells incorporated half as much ^{14}C-acetate into acyl groups as did the normal cells. Triglycerides and monoglycerides of normal MK-2 cells continued

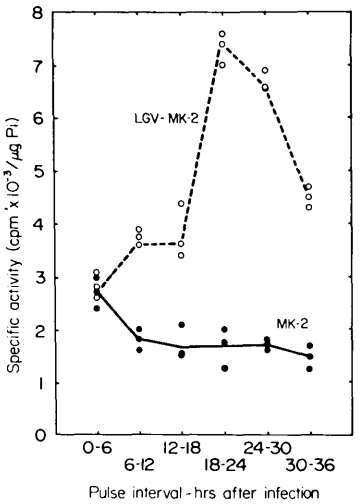

FIG. 1. The incorporation of DL-3-^{14}C-serine into phosphatidyl ethanolamine of normal and LGV-infected monkey kidney cells. Normal and LGV infected MK-2 cells (2×10^5 cells/2 oz prescription bottle) were incubated with DL-3-^{14}C-serine in MEM CS_5 (0.1 μCi/ml) every 6 hr after infection. Phosphatidyl ethanolamine was isolated after incubation. The deacylated portion of the lipid was measured for its radioactivity and phosphorus content. The specific activity is expressed as cpm/μg of inorganic phosphorus.

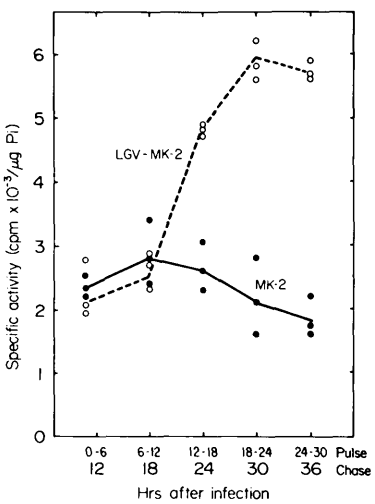

FIG. 2. The accumulation of DL-3-^{14}C-serine in phosphatidyl ethanolamine (PE) of normal and LGV-infected monkey kidney cells. Normal and LGV-infected cells were prepared and incubated with DL-3-^{14}C-serine as described in Fig. 1. Labeled cells were further incubated in the absence of labeled serine for another 6 hr. PE samples were isolated and deacylated. The deacylated portion of the lipid was measured for its radioactivity and phosphorus content. The specific activity is expressed as cpm/μg of inorganic phosphorus.

to incorporate ^{14}C-acetate into fatty acyl groups throughout the 120-min experimental period, whereas triglycerides and monoglycerides of the LGV infected cells reached a maximum after 60 min of incubation. The specific activity of diglycerides of normal and LGV infected cells reached a maximum after 60 min of incubation. A decrease in the specific activity was observed in the diglyceride fraction of normal and LGV-infected cells at 120 min. However, the specific activity of the diglyceride from LGV-infected cells was one-half of the normal cells after 60 and 120 min of incubation.

Discussion. Phospholipid synthesis and phospholipid composition of normal and LGV-infected MK-2 cells. In general, the patterns of phospholipid synthesis of normal and LGV-infected cells are similar for most of the 60-hr observation period. As has been stated earlier, host specific lipid synthesis continues after the infection, but at a reduced level. The amount of lipid synthesized by LGV is relatively small as compared to that of the MK-2 cell (7). Only when host cell lipid synthesis is reduced can the differences between normal and infected cell lipid synthesis be detected. This, rationally, can explain the similar patterns of acetate incorporation observed in 0–24 hr and 36–60 hr in normal and infected cells.

The synthesis of PE was increased 36 hr after infection. On the other hand, the PS + PI fraction of the infected cells decreased during the first 36 hr of infection. The inverse relationship between PE and PS plus PI fraction can be explained by the accumulation of ^{14}C-serine radioactivity in PE in the infected cells with a concomitant decrease in the PS fraction. The causes for continuous acetate incorporation into sphingomyelin (Table I) of the infected cells and the decrease of this lipid after 48 hr of infection are not known. A high amount of lysophosphatidyl choline (lyso PC) appeared in cells infected for 12 hr (Table II), but gradually decreased, suggesting that this may be due to breakdown of the inoculum material.

TABLE III. THE INCORPORATION OF 1-^{14}C-ACETATE INTO TRIGLYCERIDES, DIGLYCERIDES, AND MONOGLYCERIDES OF NORMAL AND LGV-INFECTED MK-2 CELLS.[a]

Time	Triglycerides		Diglycerides		Monoglycerides	
Minutes	MK-2	LGV:MK-2	MK-2	LGV:MK-2	MK-2	LGV:MK-2
			specific activity (CPM/μg of fatty acids)			
15	97	42	458	655	155	68
30	331	132	904	737	237	90
60	726	367	2362	1362	480	156
120	1241	398	1974	971	652	143

[a] The procedure is described in Materials and Methods.

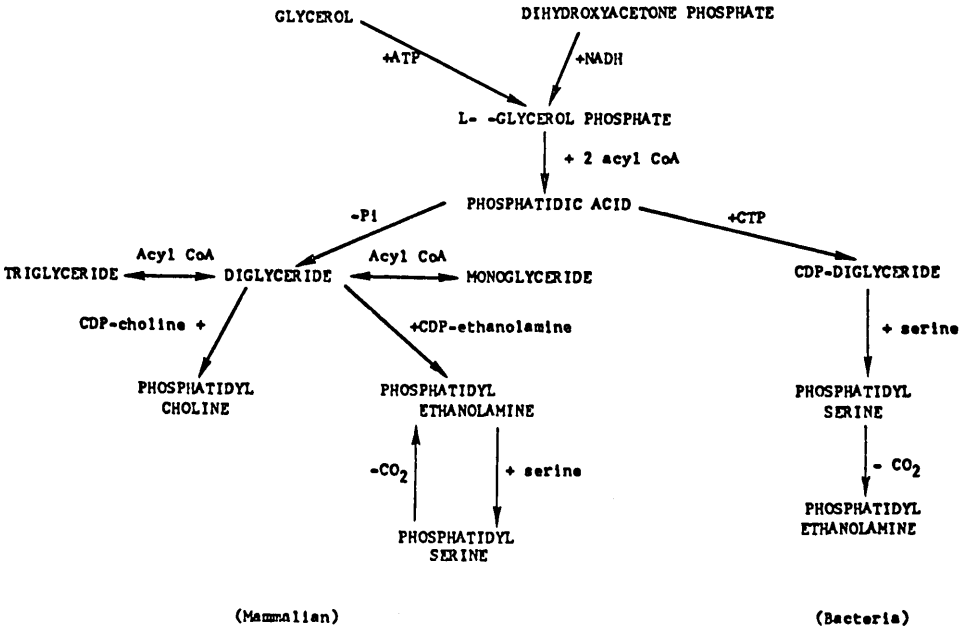

FIG. 3. Biosynthetic pathways of lipids in mammalian tissue and bacteria.

It is not known if lyso PC is turned over rapidly towards the end of the LGV infection. A higher amount of radioactivity was found in this lipid about 48–60 hr after infection (Table I). Lyso PC has been known to be associated with membrane lysis (9) and would be responsible for the pathogenic effects observed in cell culture or in the natural infection. The increase of radioactivity in lyso PC of the infected cells may suggest a breakdown of labeled PC to the lyso form. Further work is needed to study the metabolism of lyso PC in the LGV infection. This information may provide a clue to the release mechanism of LGV and perhaps lack of release of other *C. trachomatis* strains.

The *de novo* synthesis of phosphatidyl ethanolamine (PE) in bacteria from cytidine diphosphate–diglyceride (CDP–diglyceride) was reported by Kanfer and Kennedy (10). Phosphatidyl serine formed from CDP–diglyceride and serine was decarboxylated to yield PE (Fig. 3). Formation of PE in mammalian cells follows a pathway which differs from the bacterial system in that diglyceride reacts with CDP–choline or CDP–ethanolamine to yield PC or PE (11).

The incorporation of labeled acetate into phospholipids of the LGV-infected cells showed that PE synthesis was increased 24–36 hr after infection. DL-3-^{14}C-Serine was used to label PE with the assumption that if PE was actually synthesized by LGV

organisms, it would involve a decarboxylation of PS. The labeled carbon would appear in the ethanolamine moiety of the PE. The study of labeled serine incorporation (Figs. 1 and 2) indicated a nearly fourfold increase of serine incorporation into the deacylated portion of the PE (glycerol–phosphoethanolamine) in the infected cells. The PE synthesized in this manner was accumulated. Normal cells showed a steady low level of PE formation via this pathway. Formation of PE from serine in mammalian tissue has been reported (12, 13). The PE so formed was found to be mitochondria mediated. The observed PE formation from the labeled serine in normal MK-2 cells may be due to the activity of the mitochondria. The time-dependent nature of the PE synthesis suggested that this is a lipid synthetic event specific for LGV. The time of the occurrence of this event has been discussed earlier (2).

Diglycerides are an active class of lipids, since it undergoes rapid turnover in many biological systems (14, 15). The rapid turnover of diglycerides in LGV-infected cells suggested that the synthesis of this lipid was not completely inhibited by the infection. The specific activity of the diglycerides of LGV-infected cells indicated that the pool size of the diglycerides was only half the size of the normal cell. LGV infection reduced the amount and biosynthesis of diglycerides, triglycerides and monoglycerides in the cell.

The acylation of glycerides has been shown to play an important role in lipid synthesis in bovine mammary tissue (16). In the present study, the incorporation of radioactive acetate into triglycerides and monoglycerides reached a maximum after 60 min of incubation in LGV infected cells. The results of these experiments indicated that the acylation and deacylation reactions of MK-2 cells were inhibited by the LGV infection.

The studies in the formation of neutral glycerolipid synthesis (Table III) and phospholipid synthesis (Tables I and II) suggest that LGV inhibited MK-2 lipid synthesis in the infected cell.

Figure 3 shows the known pathways leading to the formation of some of the major lipid classes in bacteria and mammalian cells. The utilization of diglyceride in the formation of triglyceride and in the formation of the two major phospholipids, namely PC and PE, indicates the importance of the role of diglyceride in lipid biosynthesis of mammalian cells. By reducing the formation of diglyceride, the chlamydial agent could divert phosphatidic acid for the synthesis of CDP–diglyceride leading to the formation of the major phospholipid (PE) of the chlamydial agent. Since the chlamydial agent was found to lack aldolases in glucose utilization (17), it is necessary for the chlamydial agent to derive three carbon units for the required glycerol moiety of lipids from the host cell. Phosphatidic acid and its lyso form, glycerol phosphate and dihydroxyacetone phosphate could be used to satisfy the requirement for the three carbon units. Furthermore, by reducing the host lipid synthesis, cofactors for lipid synthesis could be made accessible to the chlamydial agent for specific chlamydial lipid synthesis. Moulder (18) reviewed the parasitic nature of the chlamydial agents in infected cells. The present studies add to his theory that the chlamydial agent not only exploits host "capacities" for DNA, RNA and protein synthesis (19), but also for lipid synthesis.

Summary. The biosynthesis of phospholipids and neutral lipids in normal and monkey kidney cells infected with lymphogranuloma venereum were compared using ^{14}C-acetate and ^{14}C-serine in pulse-chase experiments. Synthesis of phospholipids and neutral glycerolipids were inhibited in infected cells. Phosphatidyl ethanolamine (PE) synthesis increased while phosphatidyl choline, phosphatidyl serine and cardiolipin synthesis decreased in infected cells within 36 hr after infection. Sphingomyelin synthesis decreased after 48 hr of infection. The synthesis of PE in the infected cell followed a similar pathway found in bacteria and could be distinguished from the normal host cell. An explanation of the parasitic nature of chlamydial infection based on the requirement for lipid precursors has been proposed.

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