Biochimica et Biophysica Acta, 598 (1980) 628—640

BBA 78739

PHOSPHOLIPID AND CHOLESTEROL UPTAKE BY MYCOPLASMA CELLS AND MEMBRANES

SHMUEL RAZIN, SHIRLEY KUTNER, HAVA EFRATI and SHLOMO ROTTEM

Biomembrane Research Laboratory, The Hebrew University-Hadassah Medical School, Jerusalem (Israel)

(Received October 9th, 1979)

Key words: Phospholipid uptake; Cholesterol uptake; Growth; (Mycoplasma)

Summary

The ability of growing mycoplasma cells and their isolated membranes to take up exogenous phospholipids was correlated with their ability to take up cholesterol. Horse serum or vesicles made of phosphatidylcholine and cholesterol served as lipid donors. Growing cells of five *Mycoplasma* species took up significant quantities of phosphatidylcholine and sphingomyelin as well as free and esterified cholesterol. In contrast, growing cells of three *Acholeplasma* species failed to take up any of the exogenous phospholipids, and only incorporated low amounts of free cholesterol and no esterified cholesterol. Hence, the ability of mycoplasmas to take up large quantities of cholesterol appears to be correlated with an ability to take up exogenous phospholipids. Isolated membranes of *Mycoplasma capricolum* and *Acholeplasma laidlawii* took up lower amounts of cholesterol than did membranes of growing cells and did not take up phospholipids. Inhibition of *M. capricolum* growth decreased the ability of the cells to take up exogenous phospholipids and cholesterol. The possibility that the contact between the lipid donors and the membrane involves specific receptors best exposed in actively growing cells is discussed.

Introduction

Mycoplasmas offer several unique advantages for investigating the factors influencing the incorporation of exogenous lipids into biomembranes. The plasma membrane of these procaryotes is not covered by a cell wall, which enables it to interact directly with exogenous lipid donors. In addition, the majority of the mycoplasmas require exogenous cholesterol for growth and incorporate large quantities of it into the plasma membrane. The inability of

mycoplasmas to synthesize or esterify cholesterol, to hydrolyze cholesteryl esters, and to pinocytose lipoprotein particles facilitates their use in investigation of the factors controlling the transfer of cholesterol from an exogenous cholesterol source to the plasma membrane [1—8]. In addition, the mycoplasmas included in the genus *Acholeplasma* do not require cholesterol and incorporate much less of it into their membranes [3,5,9]. Hence, elucidation of the factors which restrict cholesterol uptake by *Acholeplasma* may lead to the better understanding of the mechanisms controlling cholesterol uptake.

Serum lipoproteins, the natural donors of cholesterol to the parasitic mycoplasmas, contain significant quantities of phospholipids. Therefore it seemed of interest to test whether these phospholipids are incorporated into the membranes together with cholesterol. This question acquired particular importance in light of the finding [3] that the amount of cholesterol incorporated into the membrane of any specific mycoplasma is largely determined by its phospholipid content. The phospholipids synthesized by mycoplasmas (phosphatidylglycerol, diphosphatidylglycerol, phosphoglycolipids and their derivatives; Ref. 10) can be easily distinguished from the choline-containing sphingomyelin and phosphatidylcholine of serum lipoproteins. Information on the uptake of serum phospholipids by mycoplasmas is meager. Sphingomyelin and phosphatidylcholine were detected in *Mycoplasma pneumoniae* [11—13] and *Spiroplasma citri* [14,15] grown with serum. *Mycoplasma hominis* grown with phosphatidylcholine-cholesterol dispersions incorporated both lipids into its cell membrane [5] *Mycoplasma gallisepticum* was also found to take up phosphatidylcholine and sphingomyelin from serum, but the incorporated phosphatidylcholine was modified by deacylation and reacylation with saturated fatty acids [16]. This fragmentary information prompted us to investigate more systematically the ability of mycoplasmas to incorporate exogenous phospholipids, and to correlate this with cholesterol uptake. The results reported here show that *Acholeplasma* species, which only incorporate low amounts of free cholesterol and no esterified cholesterol, are unable to incorporate serum phospholipids. In contrast, *Mycoplasma* species incorporate both free and esterified cholesterol as well as significant quantities of phosphatidylcholine and sphingomyelin. The incorporated phospholipids become part of the lipid domain of the membrane, providing additional sites for cholesterol incorporation.

Materials and Methods

Organisms and growth conditions. Acholeplasma laidlawii (oral strain), *Acholeplasma axanthum* (S-410), *Acholeplasma granularum* (BTS 39, ATCC 19168), *M. gallisepticum* (A 5969) and *Mycoplasma capricolum* (California kid, ATCC 27343) were grown in a modified Edward medium [17] supplemented with 0.5% (w/v) glucose and 5% (v/v) horse serum, and adjusted to pH 8.0. *M. hominis* (ATCC 15056 and ATCC 23114) and *Mycoplasma arginini* (G 230) were grown in the same medium, but with 20 mM L-arginine replacing the glucose supplement, and the initial pH of the medium was adjusted to 6.5. The organisms were harvested after 20—24 h of incubation at 37°C when the absorbance of the cultures reached values between 0.2 and 0.3 at 640 nm. The sedimented organisms were washed twice and resuspended in 0.25 M NaCl. *M.*

pneumoniae (FH, ATCC 15531) was grown in Hayflick's medium [18] supplemented with 10% horse serum and dispensed in 70-ml aliquots in Roux bottles. The bottles were incubated in the horizontal position for 4 days at 37°C. The medium was removed by decantation and the thin sheet of organisms sticking to the glass surface was washed twice with 0.25 M NaCl, scraped off the glass with a rubber policeman, and suspended in a small volume of 0.25 M NaCl.

Cell membranes were isolated by osmotic lysis of the mycoplasmas, as described in detail by Razin and Rottem [17]. To facilitate the isolation of *M. capricolum* membranes free of DNA, 20 μg/ml pancreatic deoxyribonuclease was added to the lysed cell preparation [17].

Phospholipid and cholesterol donors. Horse serum or phosphatidylcholine-cholesterol vesicles were used as donors. The horse serum was filtered through a 0.45 μm pore Millipore membrane filter before use, to remove any precipitable material present in the serum. Lipid vesicles were prepared from egg phosphatidylcholine (egg lecithin, Makor Chemicals, Jerusalem) and cholesterol (Sigma) as follows: a solution of 22 mg phosphatidylcholine and 10 mg cholesterol in $CHCl_3$ (phosphatidylcholine/cholesterol molar ratio 1.0 : 0.9) was dried under N_2 in a sonication vessel. 10 ml of 0.4 M sucrose was added and the mixture was sonicated in the cold under N_2 in a Heat Systems model W350 sonicator (Heat Systems Ultrasonics Inc., Plainview, NY) at an output of 130 W, 20 kHz, for three 15-min periods with 5-min interruptions for temperature equilibration. The resulting vesicle suspension was centrifuged at 38 000 × *g* for 30 min at 4°C to remove metallic particles shed by the probe. The amount of lipid material sedimented during centrifugation was negligible. Over 95% of the lipid in the vesicle suspension was retained on filtration through a Sepharose 2B column [19], indicating that it consisted primarily of unilamellar vesicles. The vesicle suspension was kept at 4°C and used within 2—3 days of its preparation.

Measurement of phospholipid and cholesterol uptake. M. capricolum was grown in Edward medium supplemented with 0.2% (w/v) bovine serum albumin, 25 μg/ml elaidic acid, 0.3% (v/v) horse serum, or phosphatidylcholine-cholesterol vesicles sufficient to provide 1 μg cholesterol/ml of growth medium. *A. laidlawii* was grown in the same medium, but without serum or lipid vesicles. After 20 h of incubation at 37°C, the absorbance of the *M. capricolum* culture reached 0.12—0.16 at 640 nm, while that of *A. laidlawii* reached 0.22—0.26. At this point the cultures were supplemented with either horse serum to raise its concentration in the culture medium to 5%, or with lipid vesicles to raise the cholesterol concentration to 20 μg/ml. Incubation was continued for another 4—6 h at 37°C and samples were withdrawn at various time intervals. The organisms in the samples were harvested, washed and their membranes were isolated and analyzed for protein, cholesterol and phospholipid as described under Analytical procedures.

Phospholipid and cholesterol uptake from serum and vesicles was also tested with isolated membranes of *M. capricolum* grown with 0.3% horse serum, and of *A. laidlawii* grown with no serum. The uptake mixture consisted of 0.05 M phosphate buffer, pH 7.0, containing 5% horse serum, or phosphatidylcholine-cholesterol vesicles (to yield 20 μg cholesterol/ml) and membranes (0.1 mg protein/ml). The suspension was incubated statically at 37°C for 4—6 h, and 25-ml samples were withdrawn at various time intervals. Shaking of the serum-

containing suspensions is undesirable as it may cause surface denaturation and precipitation of serum proteins. The membranes were collected by centrifugation at 38 000 × *g* for 25 min and were analyzed for their protein and lipid content.

The effect of growth inhibition on phospholipid and cholesterol uptake was tested with *M. capricolum*. The organisms were grown in Edward medium supplemented with 0.15% (v/v) horse serum, 0.2% (w/v) bovine serum albumin and 25 μg/ml elaidic acid. When the culture reached an absorbance of 0.10 at 640 nm, it was divided into four portions. One was centrifuged and the sedimented cells were resuspended in fresh medium at one-sixth of the original culture volume. 100 μg/ml chloramphenicol and 10^{-3} M iodoacetate were added to the second and third portions, respectively. The fourth portion was left untreated and served as a control. After 30 min of incubation at 37°C, horse serum (5%) was added to all the culture portions, and incubation was continued for 4 h at 37°C. Samples were withdrawn at various time intervals as described above.

Analytical procedures. Protein was determined according to Lowry et al. [20]. Lipids were extracted from cells or membranes with $CHCl_3/CH_3OH$ (2 : 1, v/v; Ref. 21). The lipid extracts were analyzed for cholesterol by the colorimetric technique of Rudel and Morris [22] and for lipid phosphorus by the method of Ames [23] after digestion of the sample with an ethanolic solution of $Mg(NO_3)_2$. Thin-layer chromatography of polar lipids was done on Silica gel HR (Merck) plates. The plates were first developed at room temperature with petroleum ether (b.p. 40—60°C)/acetone (3 : 1, v/v), dried and developed at 4°C with $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, by vol.). Lipid spots were detected by iodine vapor, and phospholipid spots were identified by the molybdate spray reagent [24] by comparison with known standards. For quantitation of the phospholipids, the area of the spots was scraped off the plate and phosphorus was determined directly on the collected silica gel material by the Ames technique [23]. Neutral lipids were separated on Silica gel G (Merck) plates, using benzene/ethyl acetate (5 : 1, v/v) as the developing solvent. The free and esterified cholesterol spots were detected by iodine vapor and identified by a sulfuric acid/acetic anhydride spray reagent (5 ml sulfuric acid and 5 ml acetic anhydride in 80 ml ethanol; free and esterified cholesterol spots stain red-violet upon heating of the plates at 110°C for 5 min). For quantitation of the free and esterified cholesterol, the areas of the corresponding spots were scraped off the plates and the silica gel was extracted with 2 ml of $CHCl_3$ at 45°C for 30 min. The silica gel was then sedimented by centrifugation, and washed with another portion of 2 ml $CHCl_3$. The two $CHCl_3$ supernatants were combined, dried under N_2, and their cholesterol content was determined colorimetrically [22].

Results

Uptake of cholesterol and phospholipid from serum during growth

A series of representative *Acholeplasma* and *Mycoplasma* species was tested for their capacity to incorporate exogenous cholesterol and phospholipids during growth in a serum-containing medium. Table I shows that the *Myco-*

TABLE I

CHOLESTEROL AND PHOSPHOLIPID UPTAKE FROM SERUM BY GROWING *ACHOLEPLASMA* AND *MYCOPLASMA* SPECIES

The organisms were grown with 5% (v/v) horse serum for 20—24 h at 37°C. *M. pneumoniae* was grown with 10% (v/v) horse serum for 4 days at 37°C. The data represent the mean values and range (in brackets) of 3—4 experiments with different batches of organisms.

Organism	Lipids in cells (μmol/g cell protein)			
	Free cholesterol	Esterified cholesterol	Sphingomyelin	Phosphatidylcholine
A. laidlawii	10.2 (9.2—11.3)	0 *	0	0
A. axanthum	3.7 (3.2—4.0)	0 *	0	0
A. granularum	28.5 (27.9—29.0)	0 *	0	0
M. gallisepticum	76.0 (54.3—108.0)	4.7 (3.3—7.5)	15.5 (11.3—20.6)	37.8 *** (30.2—45.3)
M. hominis (ATCC 23114)	88.8 (87.6—89.9)	15.2 (14.1—16.4)	24.8 (23.7—26.0)	14.5 (12.5—16.5)
M. hominis (ATCC 15056)	76.4 (65.9—91.6)	30.6 (25.2—39.2)	32.7 (20.6—40.3)	20.3 (10.3—26.9)
M. arginini	58.2 (55.4—61.1)	27.5 (23.6—31.5)	37.8 (37.5—38.1)	31.9 (26.9—36.9)
M. pneumoniae **	85.9	58.0	40.3	16.6
M. capricolum	67.2 (56.0—76.0)	67.7 (57.4—77.0)	20.8 (14.4—25.9)	40.1 (34.1—48.4)

* A weak spot, with an R_F close to that of esterified cholesterol, could be detected by iodine vapour, but as it did not stain red-violet with the sulfuric acid/acetic anhydride spray, it does not represent esterified cholesterol.

** Data of one experiment only.

*** Phosphatidylcholine taken up by *M. gallisepticum* was modified by reacylation with saturated fatty acids [16].

plasma species incorporated significant quantities of cholesterol and phospholipids from the serum component of the growth medium. Although serum lipoproteins are much richer in esterified than in free cholesterol [25] the mycoplasmas incorporated more of the latter. *M. capricolum* was the exception, in which free and esterified cholesterol were taken up in about equimolar amounts. The *Acholeplasma* species differed radically from the *Mycoplasma* species by their inability to incorporate serum phospholipids. Moreover, the acholeplasmas did not incorporate any esterified cholesterol and incorporated significantly less free cholesterol than the *Mycoplasma* species (Table I).

Kinetics of cholesterol and phospholipid uptake by growing cells and isolated membranes

M. capricolum and *A. laidlawii* were selected for a detailed study of cholesterol and phospholipid uptake, as these mycoplasmas can be grown with little or no serum, respectively [9,26]. Therefore uptake experiments can be started with isolated membranes suspended in growth medium instead of buffer yielded lipids. Fig. 1 shows the kinetics of free cholesterol uptake from serum by growing cells and isolated membranes of *A. laidlawii*. The rate of cholesterol uptake

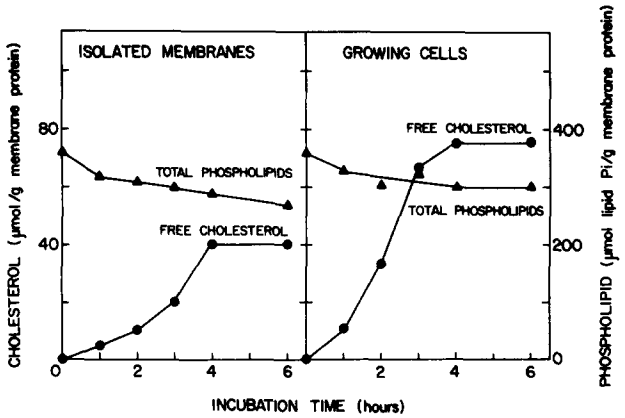

Fig. 1. Kinetics of cholesterol and phospholipid uptake from serum by growing cells and isolated membranes of *A. laidlawii*. *A. laidlawii* was grown without serum for 20 h at 37°C. The culture (absorbance of 0.22 at 640 nm) was divided into two portions. One received 5% horse serum and was incubated for another 6 h at 37°C (absorbance at the end of the incubation period reached 0.33). Cell membranes were isolated from the other portion of culture and were incubated in phosphate buffer containing 5% horse serum for 6 h. Zero time marks the time of addition of the serum to the medium or buffer.

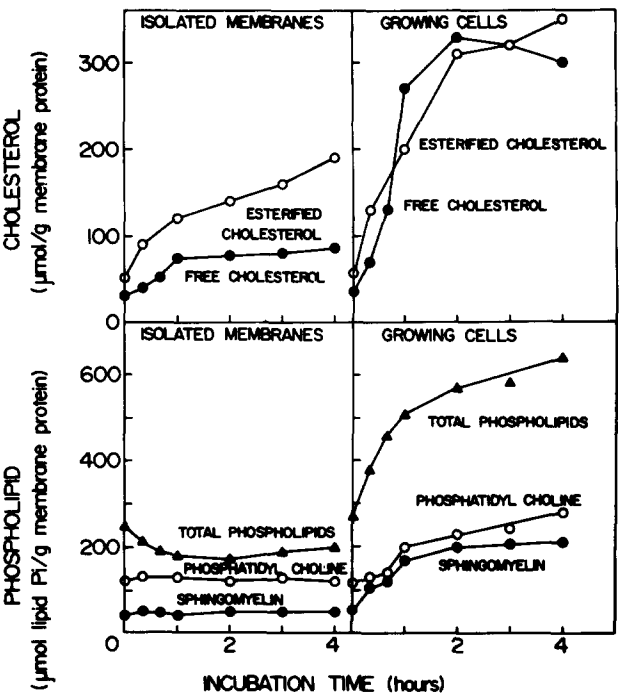

Fig. 2. Kinetics of cholesterol and phospholipid uptake from serum by growing cells and isolated membranes of *M. capricolum*. Experimental details as in legend to Fig. 1, but the organisms were grown with 0.3% serum (reaching an absorbance of 0.14) before the addition of 5% serum (at zero time in the figure). At the end of the experiment, the absorbance of the culture reached 0.35.

and the maximal amount taken up at equilibrium were higher in membranes of growing cells than in isolated membranes. Esterified cholesterol and phospholipids were not taken up by either the growing cells or the isolated membranes. In fact, a decline of about 10—20% in total membrane phospholipids was noted (Fig. 1).

The finding that isolated *A. laidlawii* membranes differ from membranes of growing cells in exogenous lipid uptake was even more pronounced with *M. capricolum* (Fig. 2). As with *A. laidlawii*, the isolated *M. capricolum* membranes incorporated significantly less free and esterified cholesterol than did membranes of growing cells. Moreover, whereas uptake of free cholesterol by the isolated membranes reached an equilibrium state after about 1 h, uptake of esterified cholesterol continued. It reached a level about twice as high as that of free cholesterol after 4 h, in contrast to the essentially equivalent uptake of free and esterified cholesterol by membranes of growing cells. The difference between isolated membranes and membranes of growing cells was even more striking with respect to phospholipid uptake. The low levels of sphingomyelin and phosphatidylcholine in the isolated membranes remained essentially unchanged during the loading period, and total membrane phospholipid values even decreased (Fig. 2). On the other hand, membranes of growing cells incorporated large quantities of sphingomyelin and phosphatidylcholine. Uptake of exogenous phospholipids accounted for essentially all the significant increase in the membrane phospholipid/protein ratio during the 4 h period (Fig. 2).

Replacing serum with phosphatidylcholine-cholesterol vesicles simplified the uptake systems by limiting the species of exogenous lipids available to the membranes. As can be seen in Figs. 3 and 4, the major findings obtained with serum as the cholesterol and phospholipid donor were repeated with vesicles as donors of exogenous lipids. Thus, as with serum, the rate and extent of free

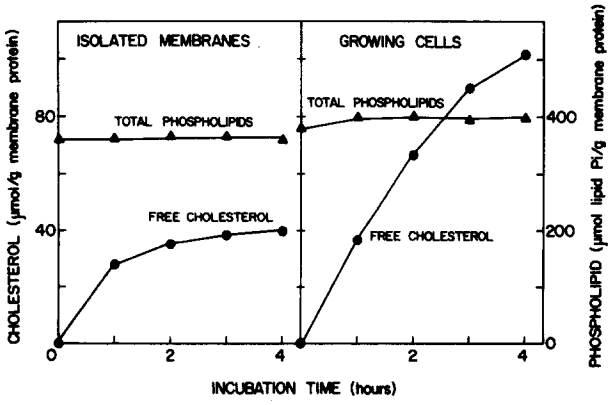

Fig. 3. Kinetics of cholesterol and phospholipid uptake from phosphatidylcholine-cholesterol vesicles by growing cells and isolated membranes of *A. laidlawii*. Experimental details as in legend to Fig. 1, but phosphatidylcholine-cholesterol vesicles (molar ratio 1.0 : 0.9; final cholesterol concentration in medium 20 μg/ml) replaced the 5% horse serum. Absorbance of the culture before the addition of liposomes was 0.28, and at the end of the experiment it reached a value of 0.33.

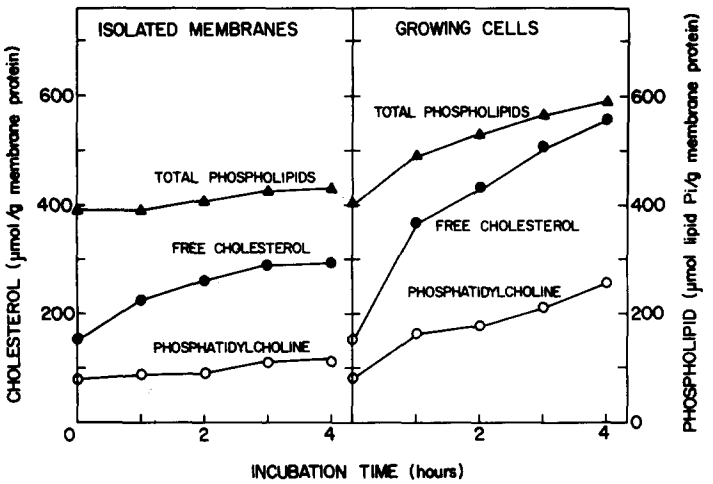

Fig. 4. Kinetics of cholesterol and phospholipid uptake from phosphatidylcholine-cholesterol vesicles by growing cells and isolated membranes of *M. capricolum*. Experimental details as in legend to Fig. 1, but the initial culture was grown with 1 μg/ml of cholesterol provided by the phosphatidylcholine-cholesterol vesicles. Zero time on the figure marks the time of addition of the high vesicle concentration (20 μg/ml cholesterol) to either the culture or the membrane suspension. Absorbance of the culture at zero time was 0.16, increasing to 0.28 at the end of the experiment.

cholesterol uptake was significantly higher with membranes of growing *A. laidlawii* and *M. capricolum* cells than with isolated membranes. Likewise, as with serum, significant quantities of phosphatidylcholine were taken up by membranes of growing *M. capricolum* cells (Fig. 4) but not by membranes of growing *A. laidlawii* cells (Fig. 3). However, differing from the findings with serum, some phosphatidylcholine was taken up by isolated *M. capricolum* membranes (Fig. 4).

The experimental system for testing uptake of lipids by isolated membranes contained at least five times as much membrane protein/ml as the system for testing uptake by growing cells. It can be argued that the lower amount of cholesterol taken up by isolated membranes was the result of a limited supply of exogenous cholesterol. This possibility was ruled out by showing that isolated membranes of *A. laidlawii* and *M. capricolum* failed to incorporate more cholesterol when the serum concentration in the uptake system was raised from 5% to 20%, or when the concentration of the lipid vesicles was increased to provide 100 μg cholesterol/ml. In addition, uptake experiments with isolated membranes suspended in growth medium instead of buffer yielded similar membrane cholesterol levels. This indicates that lower cholesterol uptake by isolated membranes was not due to the difference in the medium in which uptake was tested.

Table II compares the effectiveness of serum and lipid vesicles as cholesterol donors by expressing the data as molar ratios of cholesterol to total membrane phospholipids. The table shows that the rate of cholesterol uptake by growing *A. laidlawii* cells or by isolated membranes was higher with vesicles than with serum, though the maximal level attained was essentially the same with both

TABLE II

CHOLESTEROL/PHOSPHOLIPID RATIO IN *A. LAIDLAWII* AND *M. CAPRICOLUM* MEMBRANES

Calculated from data of experiments presented in Figs. 1—4, in which growing cells or isolated membranes were exposed to 5% horse serum, or to phosphatidylcholine-cholesterol vesicles (molar ratio 1.0:0.9; final cholesterol concentration 20 μg/ml). FC, free cholesterol; EC, esterified cholesterol; PL, total membrane phospholipids.

Organism	Time (h)	Horse serum				Vesicles	
		Isolated membranes		Growing cells		Isolated membranes	Growing cells
		FC/PL	EC/PL	FC/PL	EC/PL	FC/PL	FC/PL
A. laidlawii *	2	0.03	0	0.12	0	0.09	0.16
	4	0.14	0	0.25	0	0.11	0.26
M. capricolum	0	0.12	0.30	0.12	0.29	0.38	0.37
	2	0.42	0.93	0.50	0.51	0.64	0.80
	4	0.44	1.33	0.45	0.53	0.68	0.94

* Zero time values are not given as there was no cholesterol in membranes at that time.

donors. As for *M. capricolum*, the molar ratio of free cholesterol to total membrane phospholipids reached about the same level in membranes of growing cells by their decreased ability to take up free and esterified cholesterol, and vesicles as donors, this ratio was much higher in membranes of growing cells than in isolated membranes. Furthermore, the free cholesterol/phospholipid ratio attained with vesicles was definitely higher than that obtained with serum (Table II). It should be pointed out here that with horse serum as donor, a significant amount of esterified cholesterol was taken up by the membranes, a lipid not included in the vesicles.

Effect of growth inhibition on cholesterol and phospholipid uptake

The isolated *M. capricolum* membranes differed from membranes of growing cells by their decreased ability to take up free and esterified cholesterol, and by their inability to take up exogenous phospholipids (Fig. 2). This may suggest that uptake of the exogenous lipids depends on metabolic activity and growth. To test this possibility, growth of the organisms used in the uptake experiments was inhibited by either chloramphenicol or iodoacetate, or by simply transferring the organisms to a small volume of fresh growth medium before the addition of 5% serum, so that the high cell concentration prevented further growth. Thus, the absorbance of the concentrated culture increased only from 0.60 to 0.68 during the 4 h experimental period, whereas that of the untreated control culture increased from 0.10 to 0.23. No significant increase in absorbance was noted in the chloramphenicol- and iodoacetate-treated cultures. Table III shows marked increase in the phospholipid content in membranes of the untreated cells, following their exposure to the higher serum concentration. This increase could be accounted almost entirely to the incorporation of phosphatidylcholine and sphingomyelin. Inhibition of growth by iodoacetate or by increasing the cell concentration decreased phospholipid uptake markedly. The decrease in uptake in the chloramphenicol-treated cells was less

TABLE III

EFFECT OF GROWTH INHIBITION ON PHOSPHOLIPID AND CHOLESTEROL UPTAKE BY *M. CAPRICOLUM* CELLS

For experimental details see Materials and Methods. Data represent the mean values of three experiments. At zero time the serum concentration was raised from 0.15% to 5%. PC, phosphatidylcholine; SPM, sphingomyelin; PL, total membrane phospholipids; FC, free cholesterol; EC, esterified cholesterol.

Treatment	Incubation time (h)	Phospholipids (μmol lipid P_i/g membrane protein)			Cholesterol (μmol/g membrane protein)		Cholesterol/phospholipid (molar ratio)	
		PC	SPM	PL	FC	EC	FC/PL	EC/PL
Untreated cells	0	39	0	233	25	54	0.11	0.23
	4	205	136	599	271	265	0.46	0.44
Iodoacetate-treated cells	4	94	51	365	215	226	0.59	0.62
Concentrated cells	4	120	45	348	151	187	0.44	0.54
Chloramphenicol-treated cells	4	146	88	541	279	256	0.50	0.47

pronounced. The decreased phospholipid uptake in the inhibited cells was accompanied by a decrease in cholesterol uptake, so that the molar ratios of cholesterol/phospholipid were not very different in the inhibited and the control cells (Table III).

Discussion

Our findings indicate that the restricted ability of *A. laidlawii* to take up exogenous lipids [3—5,9] is a property shared by other *Acholeplasma* species. Sterol-requiring *Mycoplasma* and *Spiroplasma* species are capable of incorporating significant quantities of phosphatidylcholine and sphingomyelin from serum lipoproteins, in addition to free and esterified cholesterol. The inability of isolated *M. capricolum* membranes to take up phospholipids is an interesting and somewhat unexpected finding. A possible explanation can be based on the assumption that receptors for serum lipoproteins or lipid vesicles are lost during membrane isolation. The evidence available so far neither supports nor discounts the presence of specific receptors for lipoproteins and lipid vesicles on mycoplasma membranes. The finding that phospholipid uptake by *M. capricolum* cells was affected by inhibiting cell growth (Table III) suggests that phospholipid uptake is enhanced when the cell membrane is in a dynamic growing state. The exposure of proteins on the mycoplasma cell surface was recently shown to be affected by changes in the electrochemical ion gradient across the cell membrane. Ionophores and growth inhibitors markedly decreased the exposure of proteins on the mycoplasma cell surface [27,28]. Hence, if proteins on the mycoplasma cell surface participate in the binding of lipoproteins and lipid vesicles, as was found for various eucaryotic cells [29,30], then the higher degree of exposure of these proteins in metabolically active cells may facilitate contact with the lipid donors and enhance phospholipid transfer. It seems that the contact between the lipid donors and *A. laidlawii* membranes is of a transitory nature, and does not involve prolonged adsorption or fusion of

the donor with the membrane [4,5]. The fact that isolated *M. capricolum* membranes retained their ability to take up cholesterol from serum or lipid vesicles indicates contact between lipid donors and isolated membranes. However, it is possible that this contact suffices for cholesterol transfer, but is not close or prolonged enough for phospholipid transfer. The observation of Bloj and Zilversmit [31], that cholesterol exchange between vesicles and erythrocytes is 13-fold faster than phosphatidylcholine exchange, is in line with the above suggestion.

The present data also contribute to our knowledge of the factors influencing cholesterol uptake by mycoplasmas. Our finding, that the marked difference in cholesterol-binding capacity between *A. laidlawii* and *M. capricolum* cells is retained in their isolated membranes, indicates that the mechanism restricting cholesterol uptake in *A. laidlawii* is a physicochemical one. This mechanism probably depends on a specific composition and organization of membrane components, rather than on active cholesterol exclusion dependent on metabolic energy, as we previously suggested [2]. On the other hand, the dependence of phospholipid uptake on growth in *M. capricolum* influences cholesterol uptake indirectly. The exposure of *M. capricolum*, grown with low concentrations of cholesterol and phospholipids, to high concentrations of these lipids, increased the membrane phospholipid/protein ratio within 4 h by about 50—100% (Figs. 2 and 4). This increase was almost entirely due to exogenous phospholipid uptake. The phospholipids taken up are apparently integrated into the membrane, indicated by their resemblance to the other membrane lipids in extractibility by ether and susceptibility to phospholipase A_2 digestion and by their marked effect on the endogenous phospholipid synthesis (Gross, Z. and Rottem, S., unpublished results). Thus, the marked increase in the phospholipid/protein ratio resulting from phospholipid uptake by the growing *M. capricolum* cells was accompanied by a fast increase in the membrane cholesterol content.

The lower incorporation of cholesterol by isolated *A. laidlawii* membranes than by membranes of growing cells appears contradictory to our previous results [2], in which isolated *A. laidlawii* membrane preparations were found to take up about twice as much cholesterol as membranes of intact, non-growing cells and about six times as much cholesterol as membranes of growing cells. This discrepancy is apparently due to the use of Tween 80-cholesterol complexes as donors in the earlier studies [1,2]. It is highly plausible that the relatively high concentration of the detergent Tween 80 in the experimental system (0.01—0.1%) could cause serious perturbations in the membrane lipid bilayer, influencing its cholesterol-binding capacity. The finding that cholesterol uptake by isolated *A. laidlawii* cells and membranes in the presence of 0.1% Tween 80 reached extremely high levels with no sign of saturation of the cholesterol-binding sites (Fig. 6 in Ref. 2) supports the above suggestion. It also appears that the perturbations caused by the detergent are more pronounced in isolated membranes, probably due to the better accessibility of the lipid bilayer to the detergent in this case. Although data to substantiate the above assumptions are still lacking, it is clear that the use of Tween 80 as a carrier for cholesterol suffers from serious deficiencies.

Our results support the principle stated by Cooper [32] that the amount of

cholesterol incorporated into a membrane depends on the amount of phospholipid available for interaction with it. Yet, it is clear that the inability of *A. laidlawii* to incorporate exogenous phospholipids does not suffice to explain its low cholesterol content, as the free cholesterol/phospholipid ratio in its membrane is much lower than in *M. capricolum* (Table II). Moreover, *A. laidlawii* membranes contain large amounts of glycolipids [10] which may provide additional sites for cholesterol binding, and these are unaccounted for in the cholesterol/phospholipid ratio.

The finding of significant quantities of esterified cholesterol in *Mycoplasma* membranes poses a serious problem. Generally, biological membranes contain little or no esterified cholesterol, and it is hard to incorporate cholesteryl esters into artificial phospholipid bilayers [33,34]. We still have no idea where the esterified cholesterol is localized in mycoplasma membranes. Does it form oily droplets within the hydrophobic core of the membrane, as in serum lipoproteins? Or is it located outside the lipid bilayer? Our observation that *M. capricolum* incorporates more free cholesterol in the absence of esterified cholesterol than in its presence (vesicles versus serum as donors, Table II) suggests that esterified cholesterol may compete with free cholesterol on the same binding sites. This observation may concur with the recent report by Odriozola et al. [35] that the sterol requirement of *M. capricolum* can be met by cholesterol methyl ether almost as well as by cholesterol.

Acknowledgements

This work was supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel. We thank N.L. Gershfeld for helpful discussions and suggestions.

References

1 Gershfeld, N.L., Wormser, M. and Razin, S. (1974) Biochim. Biophys. Acta 352, 371—384
2 Razin, S., Wormser, M. and Gershfeld, N.L. (1974) Biochim. Biophys. Acta 352, 385—396
3 Razin, S. (1974) FEBS Lett. 47, 81—85
4 Slutzky, G.M., Razin, S., Kahane, I. and Eisenberg, S. (1977) Biochemistry 16, 5158—5163
5 Kahane, I. and Razin, S. (1977) Biochim. Biophys. Acta 471, 32—38
6 Clejan, S., Bittman, R. and Rottem, S. (1978) Biochemistry 17, 4579—4583
7 Razin, S. (1978) Biochim. Biophys. Acta 513, 401—404
8 Razin, S. and Rottem, S. (1978) Trends Biochem. Sci. 3, 51—55
9 Argaman, M. and Razin, S. (1965) J. Gen. Microbiol. 38, 153—160
10 Smith, P.F. (1979) in The Mycoplasmas (Barile, M.F., Razin, S., Tully, J.G. and Whitcomb, R.F., eds.), Vol. I, pp. 231—257, Academic Press, New York
11 Beckman, B.L. and Kenny, G.E. (1968) J. Bacteriol. 96, 1171—1180
12 Plackett, P., Marmion, B.P., Shaw, E.J. and Lemcke, R.M. (1969) Aust. J. Exp. Biol. Med. Sci. 47, 171—195
13 Razin, S., Prescott, B., Caldes, G., James, W.D. and Chanock, R.M. (1970) Infect. Immun. 1, 408—416
14 Razin, S., Hasin, M., Ne'eman, Z. and Rottem, S. (1973) J. Bacteriol. 116, 1421—1435
15 Freeman, B.A., Sissenstein, R., McManus, T.T., Woodward, J.E., Lee, I.M. and Mudd, J.B. (1976) J. Bacteriol. 125, 946—954
16 Rottem, S. and Markowitz, O. (1979) Biochemistry 18, 2930—2935
17 Razin, S. and Rottem, S. (1976) in Biochemical Analysis of Membranes (Maddy, A.H., ed.), pp. 3—26, Chapman and Hall, London
18 Hayflick, L. (1965) Tex. Rep. Biol. Med. 23 (Suppl. 1), 285—305
19 Huang, C.-H. and Thompson, T.E. (1974) Methods Enzymol. 32, 485—489

20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Rottem, S. and Razin, S. (1973) J. Bacteriol. 113, 565—571
22 Rudel, L.L. and Morris, M.D. (1973) J. Lipid Res. 14, 364—366
23 Ames, B.N. (1966) Methods Enzymol. 8, 115—118
24 Dittmer, J.C. and Lester, R.L. (1964) J. Lipid Res. 5, 126—127
25 Eisenberg, S. and Levy, R.I. (1975) Adv. Lipid Res. 13, 2—90
26 Rottem, S., Yashouv, J., Ne'eman, Z. and Razin, S. (1973) Biochim. Biophys. Acta 323, 495—508
27 Amar, A., Rottem, S. and Razin, S. (1978) Biochem. Biophys. Res. Commun. 84, 306—312
28 Amar, A., Rottem, S. and Razin, S. (1979) Biochim. Biophys. Acta 552, 457—467
29 Basu, S.K., Goldstein, J.L. and Brown, M.S. (1978) J. Biol. Chem. 253, 3852—3856
30 Pagano, R.E., Sandra, A. and Takeichi, M. (1978) Ann. N.Y. Acad. Sci. 308, 185—199
31 Bloj, B. and Zilversmit, D.B. (1977) Biochemistry 16, 3943—3948
32 Cooper, R.A. (1978) J. Supramol. Struct. 8, 413—430
33 Kleinig, H. (1970) J. Cell Biol. 46, 396—402
34 Janiak, M.J., Loomis, C.R., Shipley, G.G. and Small, D. (1974) J. Mol. Biol. 86, 325—339
35 Odriozola, J.M., Waitzkin, E., Smith, T.L. and Bloch, K. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4107—4109