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CHOLESTEROL IN MYTOPLASMA MEMBRANES*

II COMPONENTS OF *ACHOLEPLASMA LAIDLAWII* CELL MEMBRANES RESPONSIBLE FOR CHOLESTEROL BINDING

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SUMMARY

1. Cholesterol uptake by *Acholeplasma laidlawii* membranes was unaffected by the removal of 75 % of the membrane protein by pronase digestion. Lipid depletion of the membranes by aqueous acetone extraction greatly reduced the amount of cholesterol taken up, indicating that the major portion of the cholesterol bound to the mycoplasma membrane is incorporated into its lipid domain.

2. The influence of the fatty acid composition of membrane lipids on cholesterol uptake was tested using for this purpose oleate- and palmitate-enriched *A. laidlawii* membranes at various temperatures. The activation energy for cholesterol uptake was about the same for all the membranes tested, but the rate constant for uptake was significantly higher for the oleate-enriched membranes. The amount of cholesterol in the membrane at equilibrium was unaffected by the fatty acid composition.

3. The isolated membrane preparations took up about twice as much cholesterol as membranes of intact, non-growing *A. laidlawii* cells and about six times as much cholesterol as membranes of growing cells.

4. It is proposed that growing cells utilize some mechanism for excluding cholesterol from the membrane during growth.

INTRODUCTION

The major goal of this study is elucidation of the factors which control the amount of cholesterol in mycoplasma membranes. The initial aspects of the study entailed the description of the formal kinetics of uptake and washout of cholesterol from *Acholeplasma laidlawii* membranes in a defined in vitro system consisting of buffer, cholesterol, and Tween 80 [1]. Summarizing some of those results, we demon-

* Experiments were performed at the Hebrew University-Hadassah Medical School.

strated that (a) cholesterol taken up directly by membrane preparations is experimentally indistinguishable from that taken up by growing cells, (b) the amount of cholesterol taken up by membrane preparations greatly exceeds the amount of cholesterol present in the membranes of growing cells.

The present report deals specifically with membrane components of *A. laidlawii* membranes responsible for cholesterol uptake. Membranes either lipid-depleted, pronase-digested, or differing in lipid composition were used to separate the lipid and protein contributions to the cholesterol uptake process. The results indicate that most of the cholesterol is taken up by the lipid domain of the membrane; however, the amount of cholesterol taken up is not sensitive to the fatty acid composition of membrane lipids. The significance of these results in relation to factors which control the cholesterol content of mycoplasma membranes will be discussed.

MATERIALS AND METHODS

Membrane preparations

Membranes were prepared from *A. laidlawii* (oral strain) as described in the accompanying paper [1]. Variations in the fatty acid composition of membrane lipids were induced by growing the organisms in Edward medium [2] without PPLO Serum Fraction but with 0.5 % (w/v) of bovine serum albumin fraction V (Sigma, St. Louis, Mo., U.S.A.) treated with charcoal to remove fatty acids [3] and with 20 μg/ml of either oleic or palmitic acid added from an ethanolic stock solution. In some experiments the membranes isolated from cells grown with added palmitate and oleate were further purified by layering the membrane suspension on a 45 % sucrose solution and centrifuging it at 35 000 rev./min in the SW 41 rotor of a Spinco Model L-2 ultracentrifuge for 2 h at 4 °C. The membranes banded on top of the sucrose solution while a black sediment resulting from precipitated medium components sedimented to the bottom.

Preparation of lipid-depleted membranes

Membranes were depleted of lipids by two successive extractions with acetone–water–ammonia (90 : 10 : 0.03, by vol.) at room temperature [4]. Since the lipid-depleted membranes showed a tendency to aggregate, their suspension was treated in an M.S.E. ultrasonic oscillator for 15–30 s at 20 kcycles prior to its use in the cholesterol binding experiments.

Digestion of membranes by pronase

Digestion of *A. laidlawii* membranes (2 mg membrane protein per ml of 0.05 M phosphate buffer, pH 7.0) by pronase (200 μg/ml) was carried out at 37 °C for 2 h. The treated membranes were sedimented by centrifugation at 34 000 × *g* for 60 min, washed once and resuspended in the phosphate buffer. This treatment resulted in the removal of about 75 % of the membrane protein as determined by direct analysis of the remaining membrane protein. Electrophoretic analysis of the digested membranes in polyacrylamide gels containing sodium dodecylsulfate showed the disappearance of all the polypeptide bands which characterize the native membranes and the accumulation of fast-moving, low molecular weight peptides. Membrane lipids were not affected by the pronase treatment [5].

Measurement of cholesterol uptake and washout

The preparation of the labeled cholesterol solution in Tween 80, the techniques used to measure cholesterol binding and washout, and the calculations were the same as described previously [1]. Either [4-^{14}C]cholesterol (0.14 Ci/g, New England Nuclear, Boston, Mass.) or uniformly labeled [^{3}H]cholesterol (4.6 Ci/mmole or 12 Ci/g, The Radiochemical Centre, Amersham, England) were used.

Preparation of non-growing, intact A. laidlawii cell suspensions

Since mycoplasmas are very sensitive to lysis when suspended in non-nutrient media at 37 °C [6, 7] the cholesterol suspension medium had to be changed so that we could measure cholesterol uptake by *A. laidlawii* intact cells. The addition of 0.25 M NaCl to the phosphate buffer binding mixture increased the tonicity of the medium but failed to provide satisfactory protection against cell lysis. Good protection against lysis could be achieved by replacing the buffer solution with the growth medium from which the cholesterol source, the PPLO Serum Fraction, was omitted. Incubation of *A. laidlawii* cells in this medium for 5 h at 37 °C did not cause any significant loss of protein or nucleic acids from the cells. Moreover, the high cell density employed in the binding experiments prevented the cells from multiplying.

Analytical procedures

Protein was determined by the Folin–phenol method of Lowry et al. [8] using bovine serum albumin as standard. Cholesterol in membrane lipids extracted with chloroform–methanol (2 : 1, by vol.) was determined by the $FeCl_3$ method [9]. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer using a dioxane–toluene scintillation liquor [10].

Gas–liquid chromatography

Methyl esters of the fatty acids were prepared by 5 min heating to boiling of the dried lipid samples in a methanolic solution of BF_3 (Supelco, Inc., Bellefonte, Pa.). The resultant methyl esters were extracted with light petroleum ether (B.P. 40–60 °C) and subjected to gas–liquid chromatography in a Packard model 840 instrument equipped with a polar column (200 cm × 0.3 cm, 15 % of diethylene glycol adipate on chromosorb W). Fatty acids were identified by their retention time relative to that of standard methyl ester mixtures (Supelco, Inc.).

RESULTS AND DISCUSSION

The influence of lipid depletion and pronase treatment of membranes on cholesterol uptake

Lipid extraction of *A. laidlawii* membranes removes about 95 % of the lipids [4], while we have found that pronase removes about 75 % of the membrane protein. Thus a comparison of cholesterol uptake kinetics for treated membranes allows us to assess the relative importance of protein and lipid components of the membrane in cholesterol uptake.

Fig. 1 gives the results of a typical experiment in which lipid-depleted and pronase-digested membranes are compared to the untreated, native membranes. The amount of lipid in the pronase treated membrane suspension corresponds to the

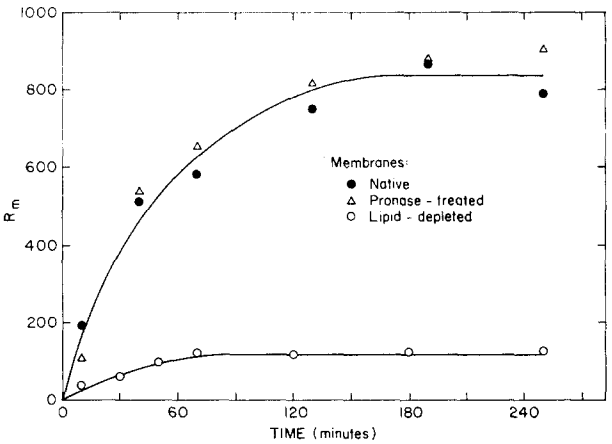

Fig. 1. Cholesterol uptake by native (●), lipid-depleted (○), and pronase-digested (△) *A. laidlawii* membranes; radioactivity of the membrane R_m (cpm) against time. Lipid depletion by aqueous acetone removed over 95 % of the total membrane lipids, while pronase digestion removed about 75 % of the protein. The suspension of lipid-depleted membranes was sonicated for 15 s to disperse aggregates. The uptake mixture contained 10^{-6} M [4-^{14}C]cholesterol, 0.01 % Tween 80 and equivalent quantities of treated or untreated membranes.

amount in the untreated membrane system, while the amount of protein in the lipid-depleted system corresponds to the protein content of the untreated control suspension. The results indicate that removal of the membrane lipids greatly reduces the amount of cholesterol subsequently taken up, but partial removal of the protein (about 75 % is removed) does not significantly change either the amount of cholesterol taken up or the kinetics of the process as compared with the untreated system. The values for the rate constant of cholesterol uptake, k_u, as calculated by Eqn 6 (preceding paper [1]) are presented in Table I. The values for the pronase-treated and untreated systems are the same while that for the lipid-depleted system is significantly smaller. Since the lipid domain of the membranes appears to be the main element controlling cholesterol uptake in the membrane preparations, we examined the influence of the fatty acid composition of membrane lipids on cholesterol uptake.

TABLE I

INFLUENCE OF ALTERED LIPID AND PROTEIN CONTENT OF *A. LAIDLAWII* MEMBRANES ON THE RATE CONSTANT FOR CHOLESTEROL UPTAKE k_u

Suspending medium contains 10^{-6} M cholesterol, 0.01 % Tween 80; temp. 37 °C.

Membrane	k_u (min^{-1})*
Untreated (native)	$1.1 \cdot 10^{-2} \pm 0.1 \cdot 10^{-2}$ (6)
Lipid-depleted (95 % of lipid removed)	$2.2 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$ (4)
Pronase-treated (75 % of protein removed)	$0.9 \cdot 10^{-2} \pm 0.2 \cdot 10^{-2}$ (3)

* S.D. values, and number of samples (in parentheses) in each study.

Effect of temperature on cholesterol uptake by oleate- and palmitate-enriched membranes

To study the influence of the fatty acid composition of the membrane lipids on cholesterol uptake we prepared oleate- and palmitate-enriched membranes (see Methods) and compared the kinetics of cholesterol uptake of these systems with that for untreated membranes and for polystyrene beads. Table II shows the marked difference in the fatty acid composition of the total membrane lipids extracted from *A. laidlawii* cells grown with palmitate or oleate. It can be seen that the lipids of the palmitate-grown cells are highly saturated, palmitic and stearic acids constituting about 60 % of the total fatty acids. The lipids of the oleate-grown cells, on the other hand, contain a high percentage (about 60 %) of the unsaturated oleic acid. The native cell membranes have compositions of these acids intermediate between the oleate- and palmitate-enriched membranes.

The results of typical cholesterol loading studies are presented in Figs 2–5. These figures show that increasing the temperature increases the rate of cholesterol uptake by all the systems. This can be seen by comparing the values of k_u as a function of temperature listed in Tables III–VI.

TABLE II

FATTY ACID COMPOSITION OF *A. LAIDLAWII* MEMBRANE LIPIDS

The fatty acid-enriched organisms were grown in the serum-free basal Edward medium supplemented with 0.5 % (w/v) of lipid-extracted bovine serum albumin fraction V and 20 μg/ml of either palmitic or oleic acid.

Fatty acid*	Percent of fatty acid in membrane lipids		
	Native	Oleate-enriched	Palmitate-enriched
10:0	4.2	2.3	14
12:0	5.7	0.5	7
14:0	10.5	3.0	3.5
16:0	25	5.8	54
18:0	18	9.0	7
18:1	12	56.6	7.5
others	25.2	22.6	7

* The first number indicates the number of carbon atoms and the second the number of double bonds in the fatty acid.

TABLE III

INFLUENCE OF TEMPERATURE ON THE RATE CONSTANT FOR CHOLESTEROL UPTAKE, k_u, BY POLYSTYRENE BEADS

The suspending medium contains 10^{-6} M cholesterol and 0.01 % Tween 80.

Temp. (°C)	$10^3 \times k_u$ (min^{-1})
0	1.8
22	2.7
37	3.2
50	4.1

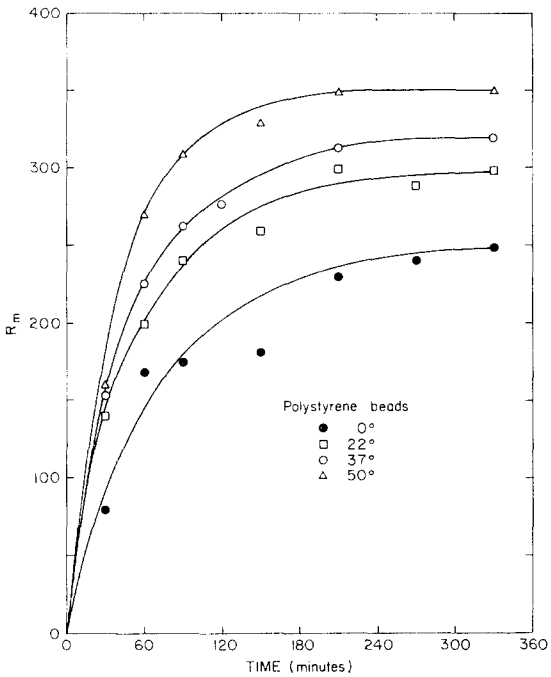

Fig. 2. Influence of temperature on cholesterol uptake by polystyrene beads; R_m (radioactivity in the beads, cpm) as a function of time. The suspending medium contained 10^{-6} M [^{3}H]cholesterol and 0.01 % Tween 80.

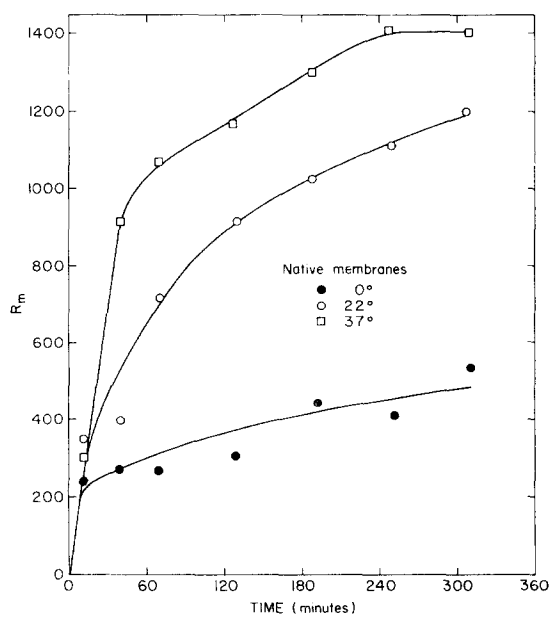

Fig. 3. Effect of temperature on cholesterol uptake by native *A. laidlawii* membranes; R_m (radioactivity in the membrane, cpm) as a function of time. The suspending medium contained 10^{-6} M

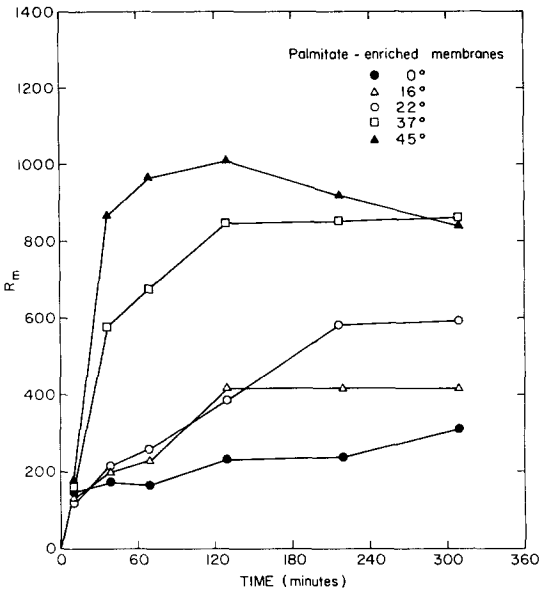

Fig. 4. Effect of temperature on cholesterol uptake by *A. laidlawii* membranes enriched with palmitic acid; R_m (radioactivity in the membrane, cpm) as a function of time. Membranes were isolated from cells grown in the basal Edward medium supplemented with 0.5 % (w/v) fatty acid-poor albumin and 20 μg/ml of palmitic acid and were tested for uptake in the usual suspending medium containing 10^{-6} M [^{3}H]cholesterol and 0.01 % Tween 80.

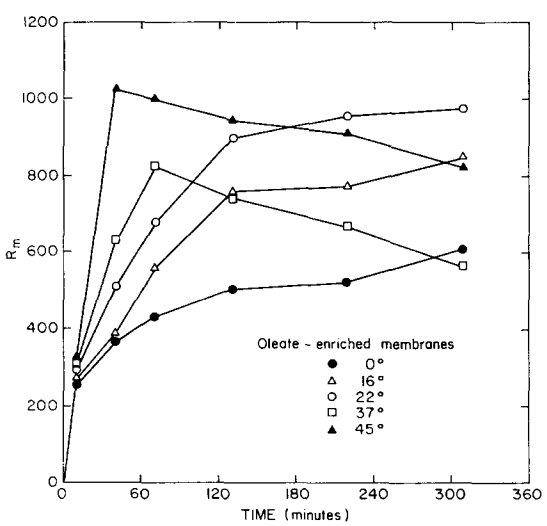

Fig. 5. Effect of temperature on cholesterol uptake by oleic acid-enriched *A. laidlawii* membranes; R_m (radioactivity in membranes (cpm) vs time). Membranes were isolated from cells grown in the basal Edward medium supplemented with 0.5 % (w/v) fatty acid-poor albumin and 20 μg/ml of oleic acid and were tested for uptake in the usual suspending medium containing 10^{-6} M [^{3}H]cholesterol 0.01 % Tween 80.

TABLE IV

INFLUENCE OF TEMPERATURE ON k_u AND k_w BY NATIVE (UNALTERED) *A. LAIDLAWII* MEMBRANES

Comparison between values of k_u calculated by Eqns 1 and 7. The suspending medium contains 10^{-6} M cholesterol and 0.01 % Tween 80.

Temp. (°C)	$10^3 \times k_u$ (min^{-1})*		k_w (min^{-1})
	Eqn 1	Eqn 7**	
0	2.9±0.3 (3)	3.2±0.3 (3)	$9.6 \cdot 10^{-3}$
10	4.3±0.2 (3)	3.9±0.1 (3)	$1.3 \cdot 10^{-2}$
22	10.0±1.5 (3)	8.7±0.2 (3)	$1.5 \cdot 10^{-2}$
37	13.4±0.6 (3)	12.0±0.2 (3)	$1.6 \cdot 10^{-2}$

* Number of samples used for calculating S.D. values are given in parentheses.
** Eqn 7 from preceding paper (ref. 1).

TABLE V

INFLUENCE OF TEMPERATURE ON k_u AND k_w BY OLEATE-ENRICHED *A. LAIDLAWII* MEMBRANES

Suspending medium contains 10^{-6} M cholesterol, 0.01 % Tween 80; k_u calculated by Eqn 1.

Temp. (°C)	$10^3 \times k_u$ (min^{-1})*	$10^2 \times k_w$ (min^{-1}) (10-min values)
0	7.3±1.0 (4)	2.9
10	9.9±3.4 (6)	2.9
22	16.4±3.8 (5)	3.5
37	23.6±5.5 (5)	4.0

* Number of samples used for calculating S.D. values are given in parentheses.

TABLE VI

INFLUENCE OF TEMPERATURE ON THE RATE CONSTANT FOR CHOLESTEROL UPTAKE, k_u, BY PALMITATE-ENRICHED *A. LAIDLAWII* MEMBRANES

Suspending medium 10^{-6} M cholesterol, 0.01 % Tween 80.

Temp. (°C)	$10^3 \times k_u$ (min^{-1})*
0	2.6±1.2 (4)
10	4.1±1.9 (4)
22	5.5±2.5 (4)
37	9.0±2.7 (4)

* Number of samples used for calculating S.D. values are given in parentheses.

For the oleate-enriched membranes, the R_m vs time curves (Fig. 5) show a maximum which becomes more pronounced at higher temperatures. This was usually not observed with native and palmitate-enriched membranes (except at temperatures > 45 °C), or with polystyrene beads. Protein determinations from sedimented membranes indicated that this effect results from the marked tendency of the oleate enriched membranes to fragment at higher temperatures. Normalizing the R_m data for the membrane protein loss generally eliminates the maximum in the R_m vs time curve. The important point is that the apparent loss of cholesterol shown by the oleate-enriched systems does not allow us to estimate C_m^{eq}, the amount of cholesterol taken up at equilibrium, and hence the rate constant k_u. To obviate this difficulty, and to avoid the tedium of measuring protein composition at each point of the R_m vs time curve, we shall use Eqn 2 (Gershfeld et al.) [1] in the form

$$k_u \cong k_w \cdot \frac{R_m}{R_s} - \frac{\Delta \ln R_s}{\Delta t} \tag{1}$$

From Eqn 1, measuring k_w, the rate constant for cholesterol washout, and using the initial values of R_m, we can calculate k_u. To assure that our calculations are reasonable we tested Eqn 1 by using data obtained for the native membranes calculating k_u from Eqn 7 of Gershfeld et al. [1].

The values for k_u and k_w for both native and oleate-enriched membranes as a function of temperature are given in Tables IV and V respectively. The values for k_u for the native membranes calculated by Eqn 7 of Gershfeld et al. [1] and by the present Eqn 1 are in reasonable agreement.

Arrhenius plots of the rate constants k_u for all four systems are linear for the 0–37 °C range of temperatures. The calculated activation energies are given in Table VII. The values for E_a are about 6 kcal/mole for the membranes and about 3 kcal/mole for the polystyrene beads. Thus, despite a radical change in the fatty acid composition of the membranes, there appears to be no significant difference in the energetics of the cholesterol uptake process. In contrast, for the polystyrene beads the value of E_a is significantly lower than for the membranes, reflecting the different character of the surfaces.

It is of interest that while the values of E_a are the same for all the membranes there is a significant difference in the values of the rate constants k_u. One might

TABLE VII

INFLUENCE OF FATTY ACID COMPOSITION OF *A. LAIDLAWII* MEMBRANES ON THE ENERGY OF ACTIVATION E_a FOR CHOLESTEROL UPTAKE; POLYSTYRENE BEADS ANALYSIS INCLUDED FOR COMPARISON

$E_a = \Delta \ln k_u / \Delta(1/T)$ evaluated from least squares analysis; values listed with S.D. values and the number of independent runs (in parentheses).

Membrane	E_a (kcal/mole)
Unaltered (native)	6.6±0.8 (12)
Oleate-enriched	5.6±0.7 (20)
Palmitate-enriched	5.8±0.7 (15)
Polystyrene beads	2.9±0.3 (4)

TABLE VIII

COMPARISON OF RELATIVE AMOUNT OF CHOLESTEROL TAKEN UP C_m^{eq} BY OLEATE-ENRICHED AND NATIVE (UNALTERED) MEMBRANES

Temperature: 37 °C.

Membrane	$10^2 \times k_u$ (min^{-1})	$10^2 \times k_w$ (min^{-1})	$k_u/k_w = C_m^{eq}/C_s^{eq}$
Oleate-enriched	2.4	4.0	0.60±0.1
Native	1.2	1.8	0.70±0.1

have expected these values to be the same and no explanation for these differences can be given at this time. More important, however, is whether the differences in k_u also reflect differences in the amounts of cholesterol that would be present under equilibrium conditions. To examine this point more critically we must compare the ratio $k_w/k_u = C_m^{eq}/C_s^{eq}$ (see Eqn 3, Gershfeld et al. [1]) for each of the systems in question. These values are presented in Table VIII for oleate-enriched and native membranes, for which the values of k_u differ most. Table VIII indicates that the ratios k_u/k_w are the same within experimental error, and thus for a given value of C_s^{eq} the amount of cholesterol taken up by each membrane at equilibrium is also the same. Hence, while the values of k_u differ for oleate-enriched and native membranes, they are compensated by the values of k_w.

Cholesterol control in mycoplasma membranes

On the basis of our kinetic arguments, the lipid component of *A. laidlawii* membranes is responsible for most of the cholesterol uptake while the protein component's contribution is relatively small. However, the nature of the fatty acids in the lipids does not appear to be a significant factor in determining the amount of cholesterol taken up, though the rate processes may be strongly dependent on the fatty acid composition of the membrane. It is relevant to ask how general these results are with respect to whole cells of *A. laidlawii*. To examine the difference between whole intact cells and the isolated membranes used in the present study, we measured the uptake of cholesterol by a suspension of non-growing *A. laidlawii* cells and measured the total cholesterol content as a function of time.

Our results show that cholesterol uptake by isolated *A. laidlawii* membranes exceeds that of membranes of intact cells by about a factor of two when tested under the same conditions (Fig. 6). An obvious explanation for this difference is that in the isolated membrane preparation, shown to consist of unsealed vesicles with very large holes [11], both the inner and outer membrane surfaces are available for cholesterol binding. The fact that throughout the experimental period (over 5 h) the amount of cholesterol taken up by membranes of intact cells was only one half of that taken up by the isolated membranes may be taken to suggest that the cholesterol bound to the outer membrane surface is restricted to the outer half of the lipid bilayer core without being able to move to the inner half of the lipid bilayer. If true, this behavior of cholesterol resembles the very slow "flip-flop" of phospholipids in artificial lipid bilayers [12] and in biomembranes [13, 14] and suggests that cholesterol is not normally distributed symmetrically in the membrane. Evidence for asymmetric

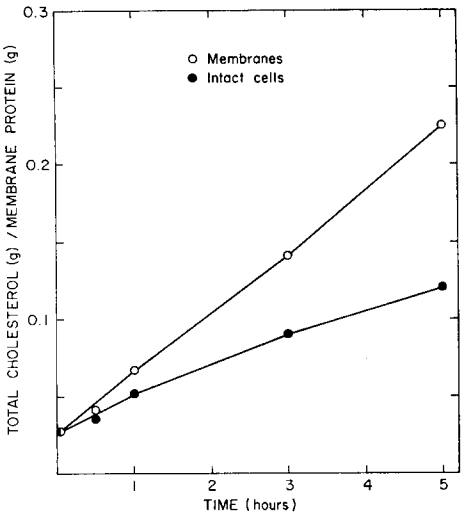

Fig. 6. Cholesterol uptake by isolated membranes and by membranes of intact *A. laidlawii* cells; total amount of cholesterol per mg membrane protein vs time. The cells and membranes were incubated at 37 °C in Edward basal medium containing 10^{-4} M [^{3}H]cholesterol and 0.1 % Tween 80. Samples were withdrawn at various time intervals and the cells were lysed by osmotic shock. The membranes were washed in phosphate buffer and their total cholesterol content was determined as described in Materials and Methods.

chlolesterol distribution between the two halves of the lipid bilayer of the myelin membrane has recently been obtained by X-ray diffraction [15].

We may summarize the physical factors responsible for cholesterol uptake by stating that in general membrane lipids account for most of the uptake. Therefore, a possible mechanism to account for the differences in cholesterol content is that the amount of lipid in the membranes varies in each strain of mycoplasma. However, Table IX indicates that the ratio of non-cholesterol lipid to protein varies little with each strain (0.45–0.55). Given the results that large amounts of cholesterol are taken up by membranes and non-growing cells, one must conclude that the distinc-

TABLE IX

PROTEIN AND LIPID COMPOSITION OF MYCOPLASMA MEMBRANES (W/W)

Membranes	Cholesterol / Total lipid	Total lipid / Protein	Total lipid —cholesterol	Reference
A. laidlawii B	0.04	0.56	0.54	16
Mycoplasma bovigenitalium	0.24	0.63	0.48	16
Mycoplasma mycordes var. capri	0.30	0.80	0.56	16
Mycoplasma hominis	0.37	0.72	0.45	17

tion between each strain of growing mycoplasma lies in its mechanism of excluding cholesterol from the membranes, and that the restriction of cholesterol uptake is applied during the growth process. These concepts will be examined in future studies.

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