Biochimica et Biophysica Acta, 352 (1974) 371–384

BBA 76667

CHOLESTEROL IN MYCOPLASMA MEMBRANES*
I. KINETICS AND EQUILIBRIUM STUDIES OF CHOLESTEROL UPTAKE BY THE CELL MEMBRANE OF *ACHOLEPLASMA LAIDLAWII*

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(Received January 25th, 1974)

SUMMARY

1. An experimental system consisting of isolated cell membranes, labeled cholesterol, Tween 80 and buffer was devised for the study of the factors controlling the amount of cholesterol taken up by mycoplasma membranes.

2. Cholesterol uptake by *Acholeplasma laidlawii* membranes and by polystyrene beads obeyed first order kinetics. The rate constant was a linear function of the amount of membrane in suspension.

3. Tween 80 decreased the rate constant for cholesterol uptake, but above the critical micelle concentration of Tween 80 the rate constant was independent of the detergent concentration.

4. The energy of activation for cholesterol uptake was about 6 kcal/mole with membranes, and about 3 kcal/mole with polystyrene beads.

5. The results indicate that cholesterol in the presence of Tween 80 is adsorbed as part of a complex with Tween 80 and is then incorporated into the cell membrane.

6. No apparent limit to the amount of cholesterol taken up by the membranes could be reached in the experimental system, suggesting that the mechanism controlling cholesterol uptake in growing cells does not operate or operates faultily in isolated membranes.

INTRODUCTION

Mycoplasmas may serve as excellent models for the study of cholesterol incorporation and function in biomembranes. Cholesterol is an essential membrane component in all *Mycoplasma* species. Since these organisms are unable to synthesize cholesterol, they depend on the growth medium for its supply. This enables us, within limits, to regulate the amount of cholesterol in the membrane and to analyze the effects of such variations on membrane properties [1]. The closely related *Achole-*

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

plasma species do not require cholesterol for growth, but are nevertheless able to incorporate it into their membrane [2, 3], a fact which has recently been utilized to test the effects of cholesterol on the permeability of the membranes [4, 5].

The cholesterol content of mycoplasma membranes varies from about 4 % of the total membrane lipid in *Acholeplasma laidlawii* [2] to nearly 40 % in *Mycoplasma hominis* [6] when the cells are grown under similar conditions. The factors causing this pronounced variation have not been delineated. Earlier studies [7, 8] suggest that cholesterol uptake by mycoplasmas does not depend on the metabolic activity of the cell and is better characterized as a physical adsorption process. In view of the apparent physical nature of the uptake process, we have examined a number of physical factors which may be responsible for the wide range of the cholesterol content in membranes of different mycoplasmas.

In this report we describe the kinetics of cholesterol uptake and washout from *A. laidlawii* membranes and compare these processes with those taking place on polystyrene beads suspended in the same medium. The results obtained with polystyrene beads, which have a structurally simpler surface than the membranes, allow us to assess the membrane's contribution to cholesterol uptake and washout since the processes in solution are the same for both systems. An effort has been made to define our experimental system by controlling the cholesterol and membrane concentrations. To extend the solubility of cholesterol in the aqueous medium Tween 80 was chosen as a carrier instead of the usual lipoprotein [7–9]. This was necessary since the form of the cholesterol in the lipoprotein complex may be poorly defined.

It will be shown in this report that cholesterol uptake by mycoplasma membranes may be treated as a simple adsorption process in that it obeys the same kinetics as uptake by polystyrene beads. However, there are fundamental differences between the two systems, and these will be discussed.

MATERIALS AND METHODS

Organism and membrane preparation

A. laidlawii (oral strain) was grown statically in a modified Edward medium [10]. The organisms were harvested after 18–20 h incubation at 37 °C and were washed twice in the cold with 0.25 M NaCl. Cell membranes were isolated by osmotic lysis of the organisms [11], washed once with deionized water, then in 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.5, and again in deionized water. The washed membranes were resuspended in 0.05 M phosphate buffer, pH 7.0, and kept at −20 °C until used.

Measurement of cholesterol uptake by membranes and by polystyrene beads

Cholesterol (99 % pure, Applied Science Laboratories, State College, Pa., U.S.A.) was dissolved in ethanol to give a 1 % (w/v) solution. [4-^{14}C]Cholesterol (0.05 mCi per 0.351 mg, New England Nuclear, Boston, Mass.) was added to 2 ml of this stock solution. The specific activity of the labeled cholesterol stock solution used throughout the study was $4.5 \cdot 10^9$ cpm/g of cholesterol.

Various dilutions in ethanol of the labeled cholesterol stock solution were added to small volumes of a 1 % Tween 80 solution, warmed if necessary for clearing

and then added to Erlenmeyer flasks containing 50-ml quantities of 0.05 M phosphate buffer, pH 7.0, prewarmed to 37 °C. The final cholesterol concentration could be varied from $5 \cdot 10^{-7}$ to 10^{-5} M with the Tween 80 concentration 0.01 %. By increasing the Tween 80 concentration to 0.1 % the concentration of cholesterol in solution could be raised to 10^{-4} M. The flasks were pre-warmed in 37 °C shaking water bath (90 strokes per min, 4 cm amplitude of each stroke) for 15 min. Membrane suspension (containing 5 mg membrane protein per ml) was then added to each flask to give a final concentration of membranes equivalent to 50 μg of membrane protein per ml. At zero time and at various time intervals thereafter 5-ml samples were withdrawn and centrifuged immediately at $32\,000 \times g$ for 10 min at 22 °C. For the kinetic analysis 10 min was added to the elapsed time at each interval to allow for the cholesterol which had adsorbed during centrifugation. When the actual amounts of cholesterol and protein in the membranes were determined in additon to radioactivity the experiments were scaled up so that at each time interval 50-ml samples of the suspension could be taken for analysis. The supernatant fluids were separated and the pellets were resuspended in 5 ml of 0.05 M phosphate buffer, pH 7.0, and centrifuged as above. The pellets were resuspended in 0.9 ml of the buffer, and 0.1 ml of 0.2 M sodium dodecylsulfate was added to solubilize the membranes. Radioactivity was determined in duplicate 0.2-ml samples of the solubilized membranes and in duplicate 0.4-ml samples of the supernatant fluids separated after the first centrifugation. In all experiments lasting over 5 h thallium acetate (0.025 %, w/v) was added to the suspending medium to prevent bacterial contamination.

Cholesterol uptake by polystyrene beads was carried out as described for the mycoplasma membranes. Each flask containing 50 ml of cholesterol plus Tween 80 solution in buffer received 1 ml of a 1 % (w/v) suspension of polystyrene beads (0.35 μm diameter). The centrifugation period was 30 min to assure the complete sedimentation of the beads after cholesterol adsorption. As with the membranes the centrifugation time (30 min) was added to the elapsed time to allow for cholesterol adsorption during centrifugation. Washing of the beads and radioactivity determinations were as described for the membranes, with the exception that sodium dodecylsulfate was not added to the sedimented beads.

Measurement of cholesterol washout

A. laidlawii membranes were incubated in phosphate buffer containing 10^{-6} M labeled cholesterol and 0.01 % Tween 80 for 5 h at 37 °C. The membranes were collected by centrifugation and washed once with 0.05 M phosphate buffer, pH 7.0. The membranes containing radioactive cholesterol were resuspended in 50 ml volumes of 0.05 M phosphate buffer, pH 7.0, with or without cholesterol or Tween 80. The flasks were shaken in a 37 °C water bath and 5-ml samples were withdrawn at various time intervals. The amount of labeled cholesterol bound to the membranes or liberated into the medium was determined by the same method used for determination of cholesterol uptake. To study the washout of cholesterol from polystyrene beads, the beads were loaded with cholesterol by incubating them for 20 h at 37 °C in 10^{-5} M cholesterol in phosphate buffer containing 0.01 % Tween 80. The beads were collected by centrifugation and were washed in phosphate buffer. Cholesterol washout from the beads was measured as described above for the membranes.

Analytical procedures

Protein was determined by the Folin-phenol method of Lowry et al. [12] using bovine serum albumin as standard. Cholesterol in membrane lipids extracted with chloroform–methanol (2:1, v/v) was determined by the $FeCl_3$ method [13]. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer using a dioxane–toluene scintillation liquor [14].

Calculations

Kinetics. The experimental system consists of a fixed volume of solution containing C_s^0 moles of cholesterol, specific activity σ (cpm/mole), to which is added as a suspension a given amount of cell membranes containing a fixed number of cholesterol binding sites which is proportional to the weight M of membrane protein. The rate of adsorption of cholesterol to the membrane is measured by counting the radioactivity of an aliquot of the suspension for the supernatant R_s and the sedimented membrane R_m as a function of time. The process may be represented as

$$M + C_s \underset{k_w}{\overset{k_u}{\rightleftharpoons}} C_m \tag{1}$$

where at time t, C_s is the amount of cholesterol in solution and C_m the amount adsorbed to the membrane; k_u and k_w are the rate constants for uptake and washout, respectively. Let α represent the fraction of sites occupied by cholesterol, and $(1-\alpha)$ is therefore the fraction of sites unoccupied.

The net rate of adsorption is given by

$$\frac{-dC_s}{dt} = \frac{dC_m}{dt} = k_u C_s M(1-\alpha) - k_w C_m . \tag{2}$$

At equilibrium

$$\frac{dC_m}{dt} = 0$$

and, from Eqn 2

$$k_w = k_u \frac{C_s^{eq} M(1-\alpha)}{C_m^{eq}} \tag{3}$$

where the superscript eq denotes equilibrium amounts.

Since $C_s^0 = C_s + C_m = C_s^{eq} + C_m^{eq}$ Eqn 3 may be written

$$k_w = k_u \frac{(C_s^0 - C_m^{eq}) M(1-\alpha)}{C_m^{eq}} \tag{4}$$

Substituting for k_w in Eqn 2 yields

$$\frac{dC_m}{dt} = k_u M(1-\alpha) \frac{C_s^0}{C_m^{eq}} (C_m^{eq} - C_m) \tag{5}$$

Assuming $\alpha \ll 1$, integration of Eqn 5, evaluating the integration constant for the condition that at $t = 0$, $C_m = 0$, yields

$$\ln\left(\frac{C_m^{eq}}{C_m^{eq}-C_m}\right) = \frac{C_s^0}{C_m^{eq}} k_u M \cdot t \tag{6}$$

for a given isotope

$$C_m = \frac{R_m}{\sigma}, \qquad C_s = \frac{R_s}{\sigma}$$

where σ is the specific activity of the labeled cholesterol and R_s and R_m are the radioactivities of the supernatant and membranes obtained for a given volume of suspension sampled at time t. Thus Eqn 6 may be written in the alternative form using radioactivities:

$$\ln\left(\frac{R_m^{eq}}{R_m^{eq}-R_m}\right) = \frac{R_s^0}{R_m^{eq}} k_u M \cdot t \tag{6a}$$

In this experiment each of the values of R in Eqn 6a is measured; thus for any fixed value of M, the plot of $\ln (R_m^{eq}/(R_m^{eq}-R_m))$ against t will give k_u for the value of M.

To find k_W, Eqn 3 is transformed, using the definition for C_s^0, into

$$(k_w/k_u M)+1 = C_s^0/C_m^{eq}$$

This relation is then introduced into Eqn 6 to yield finally

$$\ln\left(\frac{C_m^{eq}}{C_m^{eq}-C_m}\right) = (k_w+k_u M)t \tag{7}$$

Alternatively, k_w may be evaluated directly from washout studies. In this case the same conditions prevail as for the uptake kinetics in that the system is maintained as a closed system. Thus, any cholesterol which has desorbed may also readsorb with the system ultimately reaching an equilibrium distribution of radioactive cholesterol between the surface and solution. The relation

$$-\frac{d \ln C_m}{dt} = k_w \tag{8}$$

can be deduced directly from Eqn. 2. Integration of Eqn 8, recognizing that at $t = 0$, $C_m = C_m^0$ and $C_m/C_m^0 = R_m/R_m^0$ we obtain

$$\ln \frac{R_m}{R_m^0} = -k_w t \tag{9}$$

The initial slope of semilogarithmic plots of R_m/R_m^0 against t yields k_w.

Adsorption equilibrium. To obtain the equilibrium distribution of cholesterol between the solution and the membrane, cholesterol uptake was followed as long as 48 h. However, membrane fragmentation was detected after about 5 h and was accompanied by erratic results. Since no significant change in the distribution of radioactivity was detected after about 3 h the values of membrane and solution radioactivity obtained 3–5 h after the start of the experiment were taken to calculate the amount of cholesterol in the membranes in equilibrium with the measured amount of cholesterol in solution. The amount of cholesterol per g of membrane protein was then calculated from the specific activity of the cholesterol.

RESULTS

Cholesterol uptake

The kinetics of cholesterol uptake by *A. laidlawii* membranes and by polystyrene beads is shown in Fig. 1A where R_m, the radioactivity of an aliquot of membrane or beads, is plotted against time; analysis of these curves according to Eqn 6 or 6a is shown in Fig. 1B. The figure shows only the data obtained with 10^{-6} M cholesterol, but similar curves were obtained with all the tested cholesterol concentrations, ranging from $5 \cdot 10^{-7}$ to 10^{-5} M. The constant slope of the curves in Fig. 1B is in accordance with our model, and indicates that the kinetics is first order with respect to the cholesterol concentration in solution. The rate constants for cholesterol uptake by the membranes and by the polystyrene beads are given in Table I. It can be seen that on increasing the cholesterol concentration in the medium the rate constants calculated for the membranes and for the polystyrene beads did not change significantly.

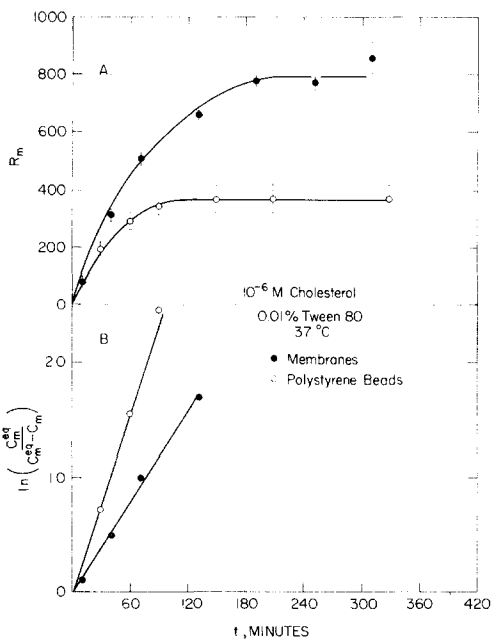


Fig. 1. Kinetics of cholesterol uptake by *A. laidlawii* membranes and by polystyrene beads. The uptake medium contained 10^{-6} M labeled cholesterol, 0.01 % Tween 80, 25 μg/ml of membranes (●) or 0.2 mg/ml of 0.35-μm diameter polystyrene beads (○). Temperature = 37 °C. A. Radioactivity of sedimented particles (R_m) as a function of time. The vertical limits of each point represent the S.E. B. Plot, according to Eqn 6, of $\ln(C_m^{eq}/(C_m^{eq}-C_m))$ as a function of time.

To test the dependence of cholesterol uptake on the surface area of the membranes, experiments were carried out in which different amounts of membranes were incubated with a given concentration of cholesterol and Tween 80 in the same volume. Fig. 2 shows that k_u increases linearly with M (μg membrane protein) up to

TABLE I

INFLUENCE OF CHOLESTEROL CONCENTRATION ON k_u, THE RATE CONSTANT FOR UPTAKE

Membrane suspension: 50 μg protein/ml; polystyrene bead suspension: 0.2 mg/ml; suspending medium: 0.01 % Tween 80 in buffer solution. Temp. 37 °C.

Concentration cholesterol (moles/l)	k_u (min^{-1})	
	Polystyrene beads	Membranes
$5 \cdot 10^{-7}$	$3.1 \cdot 10^{-3}$	$0.9 \cdot 10^{-2}$
$1 \cdot 10^{-6}$	$3.5 \cdot 10^{-3}$	$1.2 \cdot 10^{-2}$
$5 \cdot 10^{-6}$	$2.5 \cdot 10^{-3}$	$1.0 \cdot 10^{-2}$
$1 \cdot 10^{-5}$	$2.2 \cdot 10^{-3}$	$1.0 \cdot 10^{-2}$
Mean:	$2.8 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$1.0 \cdot 10^{-2} \pm 0.1 \cdot 10^{-2}$

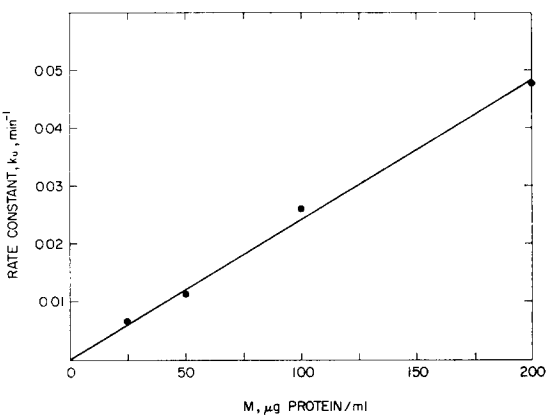


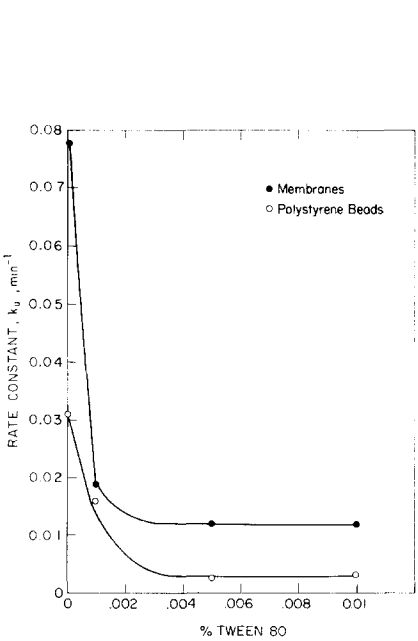
Fig. 2. Effect of cell membrane concentration on the rate constants of cholesterol uptake. The suspension medium contained 10^{-6} M cholesterol, 0.01 % Tween 80 and various amounts of cell membranes (M). k_u was calculated according to Eqn 6.

200 μg of membrane protein per ml. It was therefore decided to use a concentration of 50 μg/ml of membrane protein in all subsequent experiments.

The effect of Tween 80 on the rate constants of cholesterol uptake by *A. laidlawii* membranes and by polystyrene beads is shown in Fig. 3. Increase in Tween 80 concentration decreased the rate of uptake. In the absence of Tween 80 cholesterol uptake is virtually complete within the time of the first sampling. Moreover, a significant amount of cholesterol (about 30 % of the total added initially) is taken up by the glass walls of the flasks as may be deduced by subtracting the sum of cholesterol in the membrane and in solution from the initial total. It should be noted that the presence of 0.01 % Tween 80 eliminates cholesterol adsorption to the glass.

Cholesterol washout

Experiments were designed to test whether there is a difference in cholesterol washout from membranes and from polystyrene beads. Fig. 4 shows that essentially there was no cholesterol washout from the polystyrene beads when suspended in



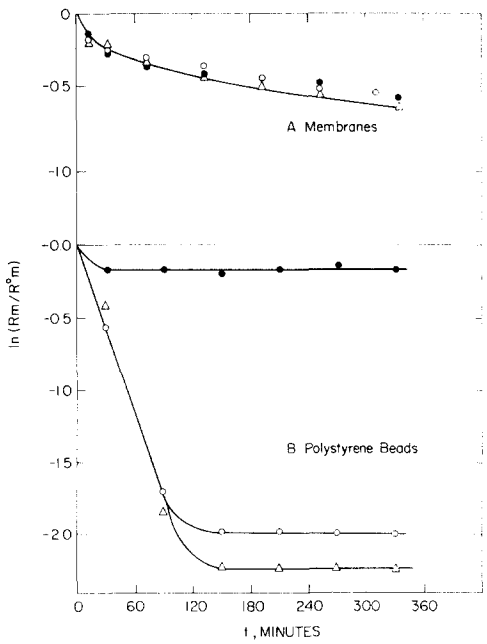


Fig. 3. Effect of Tween 80 on rate constants of cholesterol uptake k_u by *A. laidlawii* membranes and polystyrene beads. The suspension medium contained $5 \cdot 10^{-7}$ M cholesterol and 50 μg of membrane protein per ml (●) or 0.2 mg/ml of 0.35 μm diameter polystyrene beads (○).

Fig. 4. Washout kinetics of cholesterol from *A. laidlawii* membranes (A) and from polystyrene beads (B). Plot of $\ln(R_m/R_m^0)$ as a function of time, according to Eqn 9, where R_m^0 and R_m are the radioactivities in a given aliquot of sedimented particles at $t = 0$, and $t = t$, respectively. k_w is estimated from the initial slopes. Washout was tested in 0.05 M phosphate buffer, pH 7.0 (●); in the same buffer containing 0.01 % Tween 80 (△), or in the buffer containing 0.01 % Tween 80 and 10^{-5} M cholesterol (○), temperature = 37 °C. Each point represents the mean from at least 3 experiments.

phosphate buffer with no additives. When the membranes were suspended in the same buffer, some release of labeled cholesterol occurred. The addition of Tween 80 to the buffer increased most markedly the rate of cholesterol washout from polystyrene beads, but only slightly that from membranes. Further addition of unlabeled cholesterol to the Tween 80 did not alter the rate of washout to any marked extent (Fig. 4). The rate constants for cholesterol washout from membranes and polystyrene beads under the different experimental conditions listed in Table II were obtained from Eqn 9 by using the initial slope of the curves in Fig. 4. For comparison, values of k_w obtained from Eqn 7 are also listed. The agreement between the two methods is reasonable.

To examine whether cholesterol taken up during cell growth differs from the cholesterol taken up by the cell membrane preparation, k_w (rate constant for cholesterol washout) was measured for membranes prepared from cells which had taken up cholesterol during cell growth and compared with k_w values obtained with membranes which had adsorbed cholesterol directly. The values for k_w are listed in Table III; k_w is essentially the same for both preparations.

TABLE II

RATE CONSTANTS FOR CHOLESTEROL WASHOUT k_w FROM *A. LAIDLAWII* MEMBRANES AND POLYSTYRENE BEADS

Washout medium*	k_w (min^{-1})			
	A. laidlawii membranes		Polystyrene beads	
	Eqn 7	Eqn 9**	Eqn 7	Eqn 9**
1. Buffer alone	$1.4 \cdot 10^{-2}$	$1.4 \cdot 10^{-2}$	–	$0.5 \cdot 10^{-2}$
2. Buffer+0.01 % Tween 80	$1.3 \cdot 10^{-2} \pm 0.1 \cdot 10^{-2}$	$1.5 \cdot 10^{-2}$	$2.4 \cdot 10^{-2} \pm 0.4 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$
3. Buffer+0.01 % Tween 80+ 10^{-5} M cholesterol	$1.3 \cdot 10^{-2} \pm 0.1 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$	$2.4 \cdot 10^{-2} \pm 0.4 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$

* Temp. 37 °C.

** Experimental error is approximately 20 %.

TABLE III

RATE CONSTANT FOR CHOLESTEROL WASHOUT, k_w, FROM *A. LAIDLAWII* MEMBRANES; COMPARISON OF METHOD OF PREPARATION FOR CHOLESTEROL–MEMBRANE SYSTEM

Method of preparation	k_w (min^{-1})*
A. Cells grown in radioactive cholesterol	$2.2 \cdot 10^{-2} \pm 0.4 \cdot 10^{-2}$ (4)
B. Membranes adsorbed cholesterol directly	$1.5 \cdot 10^{-2} \pm 0.3 \cdot 10^{-2}$ (5)

* S.D. values, and number of samples used in the calculations. Eqn 9 used for calculating k_w.

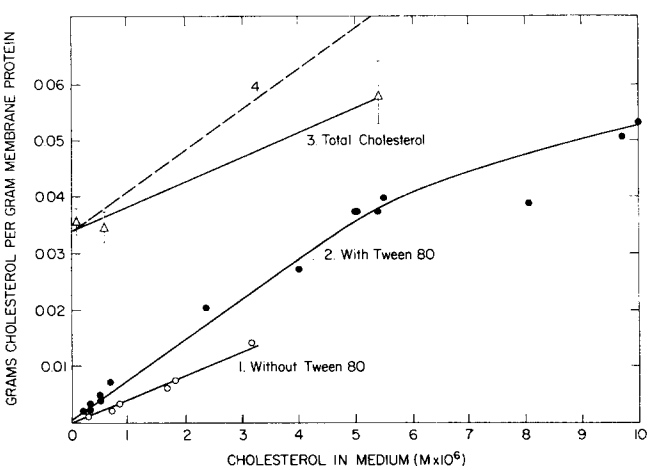


Fig. 5. Equilibrium distribution of cholesterol between the membranes and solution. 1. [^{14}C]-Cholesterol distribution in the absence of Tween 80 (○); 2. [^{14}C]Cholesterol distribution in the presence of 0.01 % Tween 80 (●); 3. Total cholesterol in membranes (initial unlabeled cholesterol + adsorbed radioactive cholesterol) (△). 4. Total cholesterol content of membranes calculated on the assumption that no cholesterol is exchanged (– – –).

Equilibrium distribution of cholesterol

The distribution of cholesterol between the membranes and solution at equilibrium is given in Fig. 5, where the weight of cholesterol per gram membrane protein is plotted against the concentration of cholesterol in the equilibrium solution. It should be noted that the amount of cholesterol which is removed from solution by the membranes in many instances significantly lowers the original concentration of the solution. The concentrations given in Fig. 5 are the equilibrium values, not the initial values. In the absence of Tween 80 the amount of cholesterol bound will be limited by the solubility of cholesterol in water which is about $5 \cdot 10^{-6}$ M [15]. Two results may be deduced from Fig. 5: Tween 80 increases the amount of cholesterol taken up by the membranes, and there does not appear to be a limit to the amount of cholesterol which may be taken up. The total amount of cholesterol in the membrane (the initial amount+the adsorbed amount) is increased on incubation with the labeled cholesterol and Tween 80.

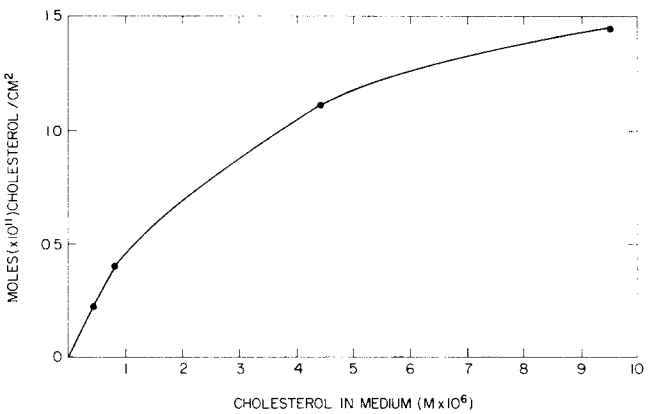


Fig. 6. Equilibrium adsorption of cholesterol to polystyrene beads. Calculation of the surface available for adsorption was based on a density of 1.04 for polystyrene [15] and a uniform particle size (0.35 μm diameter).

Fig. 6 shows the number of moles of cholesterol adsorbed per cm^2 of the polystyrene bead surface at equilibrium; the Tween 80 concentration was 0.01 %. The calculations were made by assuming a uniform particle size (diameter = 0.35 μm) and a density of 1.04 for the beads [15]. The figure shows that maximum adsorption appears to be leveling off at $1.5 \cdot 10^{-11}$ moles cholesterol per cm^2, a value which is equivalent to 1100 $Å^2$ per molecule. In comparison the area per molecule of cholesterol in a condensed monolayer is only about 40 Å per molecule [16]. In the absence of Tween 80 almost all the cholesterol (approx. 95 %) in the solution ($5 \cdot 10^{-7}$ M) was taken up by the polystyrene beads (data not shown).

DISCUSSION

There are three major points which we wish to establish in this study: (a) the nature of the adsorption process, (b) the function of Tween 80 in the adsorption pro-

cess, and (c) the final configuration of cholesterol in the membrane, i.e. does adsorbed cholesterol actually enter the membrane. We shall examine each of these questions in detail.

The process of cholesterol uptake by *A. laidlawii* membranes and polystyrene beads in the simplest formulation includes the following steps: (1) Diffusion of cholesterol through a relatively thin layer adjacent to the surface. (2) Adsorption to the surface. (3) Desorption from the surface. (4) Diffusion away from the surface. These four processes are implicitly included in the model for adsorption represented by Eqn 1. It may be assumed that all processes which occur in solution, namely steps (1) and (4), will be similar for both membranes and polystyrene beads, and if any differences in the cholesterol uptake process exist they will reflect differences only in the properties of the two surfaces. The kinetic analysis of the process indicates that there are both similarities and differences between the membranes and the polystyrene beads.

With respect to the overall kinetics of the cholesterol uptake processes represented by the equilibrium model of Eqn 1 both membranes and polystyrene beads obey first order kinetics as shown in Fig. 1B; there is a linear relation between the ordinate and the abscissa as predicted by Eqn 6. Also in keeping with first order kinetics, the rate constant for uptake k_u is independent of cholesterol concentration, as seen in Table I. Moreover, k_w, the rate constant for cholesterol washout, may be calculated directly from the uptake studies (see Eqn 7). Comparison of k_w values obtained from Eqn 7 and from direct measurement of cholesterol washout are in agreement, as seen in Table II.

In principle, the adsorption isotherms for both membranes and polystyrene beads may be obtained from the relative values of k_u and k_w in Eqn 3. Since k_w/k_u is a constant, Eqn 3 predicts that the isotherms will be straight line plots passing through the origin. Figs 5 (Curves 1 and 2) and 6 are in agreement with this prediction with a tendency for the slope to decrease at high concentrations. Moreover, a simple calculation indicates that the slope of the curves in Figs 5 and 6 are, within experimental error, as predicted by the values of k_w/k_u given in Tables I and II. For example, taking the value for k_u as 10^{-2} min^{-1} for 50 μg protein/ml solution and for $k_w = 1.3 \cdot 10^{-2}$ min^{-1} the slope predicted for Curve 2 (Fig. 5) is 0.8; the actual slope is 0.9.

All our data appear to conform within reasonable limits to the model for cholesterol adsorption represented by Eqn 1. Thus we conclude that for polystyrene beads and membranes cholesterol uptake is governed by diffusion, adsorption and desorption processes.

Since Fig. 1B is linear almost to the point where uptake is complete (compare with Fig. 1A), our assumption that α is much less than 1 is valid, i.e. that the fraction of cholesterol sites filled is still very small even with the cholesterol solution concentration as high as 10^{-5} M.

It should also be noted that the relative values of k_u for membranes and polystyrene beads are not of great significance since the rate constants are also a linear function of the surface areas (see Fig. 2). Thus the differences in k_u for membranes and polystyrene beads seen in Table I also include differences in total surface area.

The rate constant of cholesterol uptake is markedly reduced upon addition of Tween 80, but is constant at concentrations exceeding 0.005 % (Fig. 3). This effect is found with both membranes and polystyrene beads. Since the critical micelle

concentration for Tween 80 at 25 °C is 0.0033 % and is not expected to change drastically at 37 °C (Becher, P., personal communication), we may assume that in solutions in which the critical micelle concentration is exceeded cholesterol is solubilized by the detergent micelle.

There are three possible mechanisms whereby Tween 80 may reduce k_u: (1) Cholesterol sites may be competitively occupied by Tween 80, i.e. α in Eqn 2 is large. (2) An equilibrium exists between cholesterol in the Tween 80 micelle and free cholesterol in solution, and only free cholesterol is adsorbed. (3) The cholesterol-Tween 80 micelle is adsorbed in toto. The first mechanism seems unlikely particularly since the uptake kinetics indicate that α remains small even in the presence of Tween 80. The second and third possibilities are difficult to distinguish on the basis of kinetics alone and without a direct analysis of the Tween 80 uptake. It may be argued that the reduced rate of uptake in the presence of Tween 80 may be due to either a slow rate of dissociation of cholesterol from the Tween 80 complex, or a slow rate of diffusion of the larger cholesterol–Tween 80 complex. However, the fact that k_u is independent of Tween 80 concentration above the critical micelle concentration (Fig. 3) suggests that all uptake above the critical micelle concentration is of the Tween 80–cholesterol micelle. Tween 80 may thus act similarly to serum proteins or phospholipids which were shown to slow down cholesterol uptake by mycoplasmas [7] and animal cells [9, 17] in buffer systems in vitro.

The final question remaining in this study is whether the adsorbed cholesterol is incorporated within the domain of the membrane lipids or remains as a separate, distinct pool in close proximity with the membrane. In Fig. 5, Curve 3 represents the total cholesterol associated with the membranes (adsorbed cholesterol plus cholesterol present initially in the membrane), while Curve 4 (dashed line) represents the total cholesterol that would be present if the adsorbed cholesterol (Curve 2) was simply added to the membrane without exchanging for membrane cholesterol. Curve 3 is the actual analysis of total membrane cholesterol; it is less than the amount expected if no cholesterol exchange occurred. Thus, a significant proportion of adsorbed cholesterol, approximately one-half, has been exchanged with the cholesterol originally present in the membrane.

The incorporation of cholesterol into the membrane is also supported by the values of the activation energies for adsorption, E_a; these values for polystyrene beads and membranes are given in Table IV. In the presence of Tween 80 E_a is about 6 kcal/

TABLE IV

INFLUENCE OF TEMPERATURE ON k_u FOR *A. LAIDLAWII* MEMBRANES AND POLYSTYRENE BEADS

Membrane suspension: 50 μg/ml; polystyrene bead suspension: 0.2 mg/ml; suspending medium: 10^{-6} M cholesterol and 0.01 % Tween 80 in buffer solution. E_a = activation energy.

T (°K)	k_u (min^{-1})	
	Membranes	Polystyrene beads
273	$3.2 \cdot 10^{-3}$	$1.8 \cdot 10^{-3}$
310	$1.1 \cdot 10^{-2}$	$3.2 \cdot 10^{-3}$
E_a (cal/mole):	5700	2600

mole for the membranes, while it is only about 3 kcal/mole for the polystyrene beads. The small value of 3 kcal/mole suggests that the major energy barrier for adsorption to polystyrene beads is diffusion, while adsorption to the membranes has an additional energy barrier of about 3 kcal/mole which we believe represents the process of incorporation of cholesterol into the membrane.

Finally, k_w values are the same whether cholesterol is incorporated during growth or adsorbed by the membrane preparation directly (see Table III).

The fate of the cholesterol–Tween 80 micelle after its attachment to the membrane is not known. However, the fact that more cholesterol is adsorbed in the presence than in the absence of Tween 80 (which indicates that the Tween 80–cholesterol micelle is more strongly bound than free cholesterol, see Curves 1 and 2, Fig. 5) suggests that the entire complex is incorporated into the membrane. Additional studies are in progress to test this hypothesis. It is of interest to note that 0.01 % Tween 80 has been successfully used as a carrier for cholesterol in growth experiments with most *Mycoplasma* species [18] suggesting that even when associated with the membrane, Tween 80 at the 0.01 % level does not cause any radical alteration in the organization of membrane lipids.

Previous claims that cholesterol uptake by mycoplasma cells [7, 19] or animal cells in culture [9] is an irreversible adsorption process seems not to be true for our system. Our results are consistent with experiments with erythrocyte or animal cell membranes in which cholesterol exchange was demonstrated [20].

Under our experimental conditions, complete saturation of the cholesterol binding sites was never achieved (see also refs 7 and 9). Moreover, the amount of cholesterol in *A. laidlawii* membranes could be raised above the usual 30–40 μg cholesterol per mg protein as shown in Fig. 5, Curve 3. However, this increase in cholesterol content could not be achieved with growing cells. The maximal cholesterol concentration in membranes isolated from *A. laidlawii* cells grown with excessive amounts of cholesterol does not exceed 40 μg/mg of membrane protein or about 8–10 % of the total membrane lipid [5]. Hence, the mechanism controlling cholesterol uptake by growing cells does not seem to operate, or operates faultily in isolated membranes.

ACKNOWLEDGMENT

This work was supported by grant FG-Is-286 from the U.S. Department of Agriculture under Public Law 480.

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