Cholesterol Transfer from Serum Lipoproteins to Mycoplasma Membranes†

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ABSTRACT: The mechanism of cholesterol transfer from human serum lipoproteins to mycoplasma membranes was studied with growing cells and isolated membranes of *Acholeplasma laidlawii*. The low-density lipoproteins (LDL) were better cholesterol donors than were the high-density lipoproteins (HDL), providing about one-third more cholesterol to membranes of growing cells and nearly twice as much cholesterol to the isolated membranes at the highest lipoprotein concentration tested. All cholesterol taken up by cells and isolated membranes were unesterified. Cholesterol was taken up by isolated membranes from LDL and HDL at nearly the same rate. This rate was, however, somewhat slower than that for the uptake of cholesterol from cholesterol–lecithin liposomes. Nevertheless, the maximum amounts of cholesterol incorporated by the membranes from liposomes were less than from the lipoproteins, even when the cholesterol-to-phospholipid ratio of the liposomes greatly exceeded that of the lipoproteins. Up to 45% of the lipoprotein unesterified cholesterol was incorporated by the membranes with little or no concommitant uptake of lipoprotein protein or phospholipid. The depletion of the lipoprotein particles of their unesterified cholesterol was not accompanied by any significant changes in the electrophoretic mobility of the lipoprotein particles and in their protein and phospholipid composition. It is concluded that cholesterol is transferred from the lipoproteins to the membranes by a simple exchange process which does not involve the adherence or fusion of the particle with the membrane and does not result in any significant degradation of the lipoprotein particle.

Mammalian cells are required to maintain a delicate metabolic balance between the level of exogenously incorporated cholesterol provided by serum lipoproteins and the endogenous synthesis of the sterol (Brown and Goldstein, 1976). This balance is critical to the life of the cell as too little cholesterol would affect membrane growth and replenishment, while too much will impair membrane function and may trigger the build-up of fatty material, which in arterial cells is the forerunner of atherosclerosis (Papahadjopoulos, 1974; Small and Shipley, 1974). It has been suggested that atherosclerosis may be caused by a malfunction in the way cholesterol is transferred from serum lipoproteins to the cells of the arterial intima. Studies on mammalian cells in culture indicated receptor-mediated binding and subsequent endocytosis of the lipoproteins by the cells, followed by the metabolic breakdown of the lipoprotein particles inside the cells (Brown and Goldstein, 1976; Stein and Stein, 1975). Obviously, by using these experimental systems, it is difficult if not impossible to study the transfer of cholesterol from the lipoprotein particle to the cell membrane itself by direct exchange. Hence, the contribution of this mode of cholesterol transfer to the overall pattern of cellular cholesterol metabolism has been difficult to evaluate with the cell culture systems.

We have recently suggested the use of mycoplasmas as model systems for studying the transfer of cholesterol from serum lipoproteins to cell membranes (Slutzky et al., 1976). The mycoplasmas are especially well suited for this purpose, as they are capable of incorporating large amounts of cholesterol into their cell membranes, and many of them require it for growth. All the mycoplasmas, including the *Acholeplasma* species, which do not require cholesterol for growth, are unable to synthesize cholesterol, nor are they able to esterify it or hydrolyze cholesteryl esters (Razin, 1975; Razin and Rottem, 1977). Furthermore, these minute organisms are not capable of pinocytosis, and as they have no cell wall, their interactions with serum lipoproteins can be studied as strictly a membrane phenomenon (Slutzky et al., 1976).

In the current study, *Acholeplasma laidlawii* cells and their isolated cell membranes were used. This sterol-nonrequiring mycoplasma was chosen because it enabled the experiments to be started with cholesterol-free membranes, so that the equilibrium of the cholesterol exchange process between the membranes and serum lipoproteins was in favor of the membranes. *A. laidlawii* also differs from several sterol-requiring mycoplasmas in its total inability to incorporate cholesteryl esters, thus limiting the exchange process to unesterified cholesterol only (Argaman and Razin, 1965; Slutzky et al., 1976). Moreover, the isolated membranes of *A. laidlawii*, unlike those of some other mycoplasmas, form very stable suspensions, facilitating cholesterol exchange studies for extended periods of time (Gershfeld et al., 1974).

The specific aims of the present study were to elucidate the mechanism of cholesterol transfer from serum lipoproteins to the membrane and the factors which determine the effectiveness of the different lipoproteins as cholesterol donors. The major question investigated was whether cholesterol is transferred to the membrane by a simple exchange process during a transient contact between the lipoprotein particle and the membrane, or whether the cholesterol uptake process involves the adherence and fusion of the particle with the membrane, and the subsequent degradation of the particle components.

The results reported in this communication indicate that cholesterol is transferred from the lipoprotein particle to the mycoplasma membrane by an exchange process, which leaves the lipoprotein particle intact, though highly depleted in unesterified cholesterol.

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Materials and Methods

Preparation of Lipoproteins. Human low- and high-density lipoproteins were isolated by ultracentrifugation according to the method of Havel et al. (1955) in a Spinco ultracentrifuge, Model L2 65B, from fasting human serum containing 1 mg/mL of EDTA. Low-density lipoprotein and high-density lipoprotein were isolated at densities 1.019–1.063 g/cm^3 and densities 1.063–1.21 g/cm^3, respectively. The lipoproteins were washed twice at their respective densities and then dialyzed exhaustively against 0.9% NaCl containing 0.01% EDTA (pH 7.4). All procedures were performed at 4 °C. The lipoproteins were shown to be free of plasma proteins and other lipoproteins by immunodiffusion techniques and lipoprotein electrophoresis as described previously (Fainam et al., 1975). Before use, the lipoprotein solutions were centrifuged at 48 000*g* for 1 h and the supernatant fluids were filtered through a 0.45-μm pore size Millipore filter. All the chemical analyses were performed on the filtered lipoprotein solutions.

In some experiments LDL and HDL[1] were iodinated with ^{125}I employing the procedure of MacFarlane (1958) as modified by Bilheimer et al. (1972). About 95% of the ^{125}I label was found to be associated with the lipoprotein apoprotein moieties while 3 to 4% of the label was found in the lipid moieties.

Liposome Preparation. Liposomes were prepared from egg lecithin (Makor Chemicals, Jerusalem) and [^{3}H]cholesterol (New England Nuclear) as follows: 75 mg of lecithin and either 3, 15, or 30 mg of [^{3}H]cholesterol (0.2 μCi/mg) in chloroform were dried in a conical plastic sonication vessel. Ten milliliters of 0.25 M NaCl was added and the mixture was sonicated in a Branson Model 185 W sonicator equipped with a standard tip at an output of 85 W. To guard against oxidation of the egg lecithin the sonication was carried out under a nitrogen atmosphere in the cold for three 10-min periods with 2-min interruptions for temperature equilibration. After sonication the liposomes were centrifuged at 48 000*g* for 1 h at 4 °C to remove metallic particles shed by the probe. The amount of lipid material sedimented after this procedure was negligible. Aliquots of the supernatant fluids were assayed for radioactivity to determine the cholesterol content. An additional aliquot was applied to a column (45 × 2.5 cm) of Sepharose 4B equilibrated with 0.25 M NaCl. Samples were eluted with the same buffer and the column fractions were assayed for radioactivity and lipid phosphorus. About 25% of the radioactivity and lipid phosphorus were excluded in the void volume, indicating that the remaining 75% of the lipid dispersion, which was retained by the column, consisted primarily of unilamellar vesicles (Newman and Huang, 1975).

Organisms and Membrane Preparation. Acholeplasma laidlawii (oral strain) was grown in a modified Edward medium which contained no source of cholesterol (Razin and Rottem, 1976). Cholesterol when needed was added as a component of human LDL or HDL. The cells were harvested after 18 h of incubation at 37 °C and washed once in the cold with 0.25 M NaCl. Cell membranes were isolated by osmotic lysis of the organisms, washed once in deionized water, and then in dilute β buffer (0.15 M NaCl–0.05 M Tris–0.01 M 2-mercaptoethanol, in deionized water adjusted to pH 7.4 with HCl and then diluted 1:20 with deionized water), and again in deionized water (Razin and Rottem, 1976). The washed membranes were resuspended in 0.05 M phosphate buffer, pH 7.0, and kept at −70 °C until used.

[1]Abbreviations used: LDL, low-density lipoproteins; HDL, high-density lipoproteins; UC/PL, unesterified cholesterol/phospholipid molar ratio; EDTA, ethylenediaminetetraacetic acid.

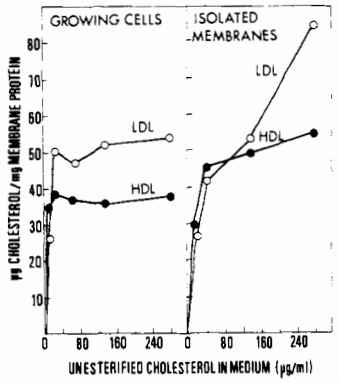

FIGURE 1: Uptake of cholesterol from LDL and HDL by growing cells and isolated membranes of *A. laidlawii*. Cells were grown for 18 h at 37 °C in 50-mL volumes of serum-free Edward medium supplemented with the isolated lipoproteins to provide the indicated levels of unesterified cholesterol. Membranes isolated from *A. laidlawii* grown without cholesterol (2.5 mg of membrane protein) were incubated at 37 °C for 18 h in 10 mL of 0.05 M phosphate buffer, pH 7.0, containing various concentrations of the isolated lipoproteins.

Uptake of Cholesterol by Isolated Membranes. Isolated membranes were incubated with either LDL, HDL, or liposomes in 0.05 M phosphate buffer, pH 7.0, at 37 °C with constant shaking. In all experiments lasting over 5 h, thallium acetate (0.025%, w/v) and penicillin (300 U/mL) were added to the medium to prevent bacterial contamination. At the end of the incubation period the membranes were collected by centrifugation at 48 000*g* for 15 min at 4 °C and washed once in cold 0.05 M phosphate buffer. In experiments where liposomes were used the membranes were solubilized with 10% sodium dodecyl sulfate and assayed for radioactivity using toluene–Triton X-100 scintillation liquor (Kahane and Razin, 1977). In experiments where lipoproteins were used the membranes were extracted with chloroform:methanol (2:1) for 2 h at 45 °C and the amounts of cholesterol and phospholipid were determined as described in the following section.

In some experiments ^{125}I-labeled lipoproteins were used to test the effects of prolonged incubation with membranes on the apoprotein moieties. In this case cold trichloroacetic acid, to a final concentration of 5%, was added to the supernatant fluids obtained after removal of the membranes by centrifugation. The Cl_3CCOOH-precipitable material was collected by filtration of the suspension through a 0.45-μm Millipore filter. The distribution of radioactivity in the precipitable and nonprecipitable fractions was then determined by counting the radioactivity of the filters and samples of the filtrates in a Packard autogamma spectrometer.

In other experiments the lipoproteins in the incubation mixtures after removal of the membranes were concentrated by ultrafiltration, using an Amicon XM100 (100 000 molecular weight exclusion) membrane. The concentrated lipoproteins were electrophoresed on cellulose acetate or solubilized in 5% sodium dodecyl sulfate and electrophoresed on gels containing 2.8% polyacrylamide, 0.5% agarose, and 0.1% sodium dodecyl sulfate (Bourque and Naylor, 1971). The gels were stained with Coomassie brilliant blue and destained in 7% acetic acid (Fairbanks et al., 1971).

Analytical Procedures. Protein was determined by the Lowry procedure (Lowry et al., 1951). The lipid extracts of the membranes and lipoproteins obtained by chloroform: methanol (2:1) extraction were analyzed for total, unesterified, and esterified cholesterol by a modification of the procedure of Argaman and Razin (1965) and Rudel and Morris (1973). The lipid extracts were chromatographed on silica gel G plates

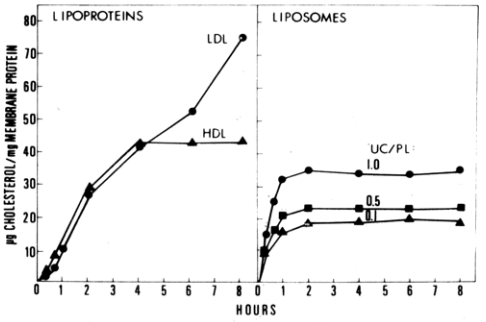

FIGURE 2: Kinetics of cholesterol uptake by *A. laidlawii* membranes from serum lipoproteins and cholesterol–lecithin liposomes. The membranes (2.5 mg of membrane protein/mL) were incubated in 0.05 M phosphate buffer, pH 7.0, containing the isolated lipoproteins in a concentration providing 40 μg of unesterified cholesterol/mL, or cholesterol–lecithin liposomes of various molar ratios (UC/PL) also providing 40 μg of cholesterol/mL.

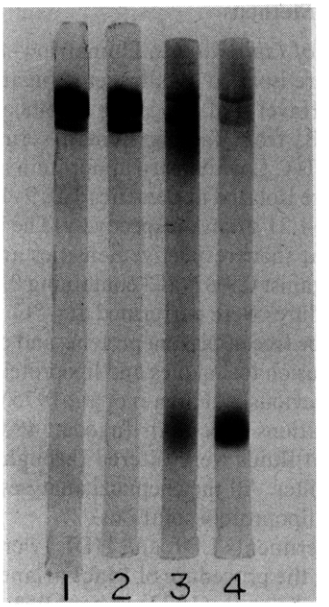

FIGURE 3: Electrophoretic patterns in gels containing sodium dodecyl sulfate of LDL and HDL before and after incubation for 18 h at 37 °C with *A. laidlawii* membranes. (Gel 1) LDL before incubation; (gel 2) LDL after incubation; (gel 3) HDL before incubation; (gel 4) HDL after incubation.

using benzene:ethyl acetate (5:1) as the solvent. The cholesterol and cholesteryl ester zones were scraped into test tubes and the lipid was eluted into 1 mL of glacial acetic acid by incubation for 10 min at 45 °C. The silica gel was sedimented by centrifugation at 2000*g* for 10 min and the supernatant fluids were transferred to cuvettes containing 1 mL of *o*-phthaldehyde (1 mg/mL) in glacial acetic acid. After incubation for 10 min at room temperature, 1 mL of concentrated sulfuric acid was added to each tube. After an additional 10 min incubation, the optical densities were read at 550 nm. Total lipid phosphorus in the lipid extracts was determined by the method of Ames (1966). Analysis of the lipid species in the extracts was done by thin-layer chromatography using silica gel G plates and two solvent systems. Plates were developed to half their heights with a polar system to separate the phospholipids (chloroform–methanol–acetic acid–water, 25:15:4:2, by volume) and after drying rechromatographed in an apolar system (benzene:ethyl acetate, 5:1) to separate the neutral lipids. Lipid spots were identified with iodine vapor by comparison with known standards.

Results

Cholesterol was transferred from LDL and HDL to growing *A. laidlawii* cells and to membranes isolated from cells grown in the absence of cholesterol. Figure 1 shows that, whereas the uptake of cholesterol by membranes of growing cells reached a maximum at a relatively low cholesterol concentration in the growth medium, uptake of cholesterol by isolated membranes continued to rise above the level reached by the membranes of the growing cells. This was particularly apparent with high concentrations of LDL, where a maximum level of cholesterol uptake was not reached even after 18 h of incubation. It is also seen from Figure 1 that LDL was a better donor of cholesterol to both the growing cells and isolated membranes than was HDL, providing about one-third more cholesterol to the growing cells and nearly twice as much cholesterol to the isolated membranes at the highest lipoprotein concentration tested. Despite the fact that the lipoproteins contained high concentrations of esterified cholesterol (from three to four times the concentration of unesterified cholesterol), esterified cholesterol could not be detected in either the membranes of cells grown with the lipoproteins or in the isolated membranes incubated with the lipoproteins.

Figure 2 shows the kinetics of cholesterol uptake by isolated membranes from LDL and HDL compared with that from cholesterol–lecithin liposomes. The results show that the rate of cholesterol uptake from liposomes was somewhat faster than that from the lipoproteins, but the level of cholesterol reached in the membranes was definitely lower, even with the liposomes having the higher cholesterol to phospholipid ratio.

Tables I and II show that the unesterified cholesterol was the only component of the lipoproteins which was transferred to the membranes in significant quantities. Up to 45% of the unesterified cholesterol component of the lipoproteins was found to have been transferred to the membranes. The use of ^{125}I-labeled lipoproteins enabled us to estimate the amount of the lipoprotein protein bound to the membranes. As can be seen in Table II, the amounts of lipoprotein protein bound to the membranes were very small in comparison with those of cholesterol bound. Table II also shows that the amount of cholesterol actually bound to the membranes was much higher than that calculated according to the amount of lipoprotein bound, assuming that the bound protein represents intact lipoprotein particles adhering to or fusing with the membranes.

The effects of the prolonged incubation of the lipoproteins with membranes on the lipoproteins themselves were evaluated by several procedures. The prolonged incubation did not cause the lipoproteins to precipitate, as was shown by the absence of a visible pellet upon centrifugation of lipoproteins incubated without membranes. This observation was confirmed by the almost complete recovery of ^{125}I-labeled lipoproteins in the supernatant fluid. Table III shows that the inclusion of membranes in the incubation system did not cause degradation of lipoprotein protein. A decrease in Cl_3CCOOH-precipitable counts would have been indicative of proteolysis of the lipoproteins, but this was not found. This finding was supported by cellulose acetate electrophoresis of LDL and HDL, which showed that lipoproteins before and after incubation with the membranes yielded identical electrophoretic patterns. In addition, the polyacrylamide gel electrophoretic patterns of sodium dodecyl sulfate solubilized LDL and HDL protein did not show any significant changes in their apoprotein moieties following prolonged incubation with the membranes (Figure 3).

The apparent stability of the lipoprotein protein component

TABLE I: Uptake of Cholesterol and Phospholipid by Isolated *A. laidlawii* Membranes from LDL and HDL.[a]

Lipoprotein added	Unesterified cholesterol in medium (μg/mL)	% lipoprotein unesterified cholesterol incorp by membranes[b]	μg of cholesterol/mg of membrane protein	μmol of phospholipid/ mg of membrane protein	μmol of cholesterol/ μmol of phospholipid
None	0		0	0.29	0
LDL	20	41	27.3	0.30	0.24
	40	32	42.9	0.30	0.37
	140	12	53.7	0.32	0.43
	280	9	85.5	0.34	0.65
HDL	20	45	30.1	0.28	0.28
	40	34	45.8	0.29	0.42
	140	11	50.1	0.28	0.46
	280	6	56.7	0.30	0.49

[a] Isolated membranes incubated for 18 h at 37 °C with LDL or HDL (those shown in Figure 1) were extracted for 2 h at 45 °C with chloroform–methanol (2:1). Cholesterol and phospholipid content were determined as described in Materials and Methods. Results of a representative experiment. [b] All cholesterol in membranes was unesterified.

TABLE II: Uptake of Lipoprotein Protein by Isolated *A. laidlawii* Membranes as Compared with Uptake of Lipoprotein Cholesterol.[a]

Lipoprotein added[b]	Lipoprotein protein in medium (μg/mL)	Lipoprotein protein bound (μg/mg of membrane protein)[c]	% lipoprotein protein bound	Calcd cholesterol (μg/mg of membrane protein)[d]	Actual cholesterol taken up (μg/mg of membrane protein)
LDL	30	2.0	2.0	1.3	27.3
	60	3.2	1.6	2.1	42.9
	210	11.7	1.7	7.8	53.7
	420	17.1	1.2	11.4	85.5
HDL	160	5.0	0.9	0.6	30.1
	320	6.3	0.6	0.8	45.8
	1120	13.8	0.4	1.7	50.1
	2240	17.2	0.2	2.2	56.7

[a] Results of a representative experiment. [b] The lipoprotein proteins were labeled with ^{125}I. The specific activities of the LDL and HDL added were 3.2×10^5 cpm/mg and 1.2×10^5 cpm/mg, respectively. [c] The amount of protein bound was calculated from the radioactivity data according to the specific radioactivity of the proteins. [d] The numbers indicate how much cholesterol would have been taken up as a component of lipoprotein particles if the protein bound by the membranes was all contained in intact lipoprotein particles.

on interaction with the mycoplasma membranes was paralleled by a similar stability of the lipoprotein phospholipid component. Thin-layer chromatography of lipid extracts from HDL and LDL incubated with membranes for 18 h at 37 °C showed no differences in lipid composition, aside from decreased unesterified cholesterol content. Simultaneous incubation of the lipoproteins without membranes did not cause any significant increase in the esterified cholesterol and lysophospholipid content of the lipoproteins, indicating the absence of any significant lecithin:cholesterol acyltransferase activity in the lipoprotein preparations.

Discussion

In contrast to the findings with animal cells in culture, where net transfer of cholesterol from serum lipoproteins involves the endocytosis of the lipoprotein particles, the hydrolysis of their cholesteryl esters, and the degradation of their apoprotein moieties (Brown and Goldstein, 1976), the transfer of cholesterol from serum lipoproteins to mycoplasma membranes was not accompanied by any significant degradation of the lipoprotein particles, apart from their significant depletion of unesterified cholesterol. In fact, upon exposure to a cholesterol-rich source, such as membranes of *Mycoplasma gallisepticum*, the cholesterol-depleted lipoproteins could be "re-charged" with unesterified cholesterol, and serve again as cholesterol donors to *A. laidlawii* membranes (Slutzky, unpublished). Our conclusion is based on the absence of any evidence for proteolytic or lipolytic degradation of the protein, phospholipid or esterified cholesterol components of the lipo-

TABLE III: Effect of Incubation of ^{125}I-labeled LDL and HDL with *A. laidlawii* Membranes on the Percentage of the Cl_3CCOOH-Precipitable Moieties of the Labeled Lipoproteins.

	% of total radioactivity precipitated by Cl_3CCOOH[a]			
	LDL		HDL	
Incubation time	+ membranes	− membranes	+ membranes	− membranes
0	90	90	91	91
20 min	88	91	90	90
40 min	87	88	86	89
60 min	86	90	88	88
3 h	85	ND	87	ND
18 h	81	77	88	88

[a] LDL (60 μg of protein) or HDL (320 μg of protein) were precipitated from the incubation medium (after the experiments described in Tables I and II) by the addition of cold Cl_3CCOOH to a final concentration of 5%. The precipitate was collected by filtration through 0.45-μm Millipore filters and the radioactivity bound to the filters was determined. Results of a representative experiment.

protein particles, even after prolonged incubation of the lipoproteins with *A. laidlawii* membranes.

Our data clearly indicate that cholesterol is transferred from the lipoprotein particles to the membrane, apparently by an exchange process, during a transient contact of the particles with the membrane. The results presented in this paper exclude the adherence or fusion of intact lipoprotein particles with the membrane to any significant degree. This conclusion is based on the absence of any significant binding of the ^{125}I-labeled protein moieties of the lipoproteins to the membranes, on the complete absence of cholesteryl ester uptake, and on the very small or negligible binding of lipoprotein phospholipids to the membranes. The finding of negligible binding of ^{125}I-labeled lipoproteins to growing mycoplasma cells (Slutzky et al., 1976) can be taken to suggest that the conclusions obtained from studies with isolated mycoplasma membranes are also applicable to growing cells.

The isolated *A. laidlawii* membranes differed, however, from growing *A. laidlawii* cells in that the restrictions on the amount of cholesterol incorporated into the membranes of growing cells (Gershfeld et al., 1974; Razin et al., 1974; Razin, 1974) had been alleviated once the membranes were isolated from the cells. Several possible explanations may be advanced to explain this: (1) the exposure of the cytoplasmic surface of the isolated membrane has made available additional sites into which exogenous cholesterol may be incorporated; (2) after cell lysis the membrane may become more "relaxed" and allow for additional cholesterol to be packed into it; and (3) some sort of a "restrictive mechanism" controlling cholesterol uptake, active in growing or resting cells, is destroyed or rendered less effective by cell lysis (Gershfeld et al., 1974). Yet, the maximum level of cholesterol reached in isolated *A. laidlawii* membranes after 18 h incubation with LDL was 85 μg of cholesterol per mg of membrane protein, a value not higher than that attained in membranes of growing sterol-requiring mycoplasmas (Slutzky et al., 1976; Razin and Rottem, 1977). The molar ratio of unesterified cholesterol to membrane phospholipids (UC/PL) in the *A. laidlawii* membranes reached the value of 0.65 (Table I) which is still lower than that in the cholesterol donor, LDL (Arbogast et al., 1976).

Our previous studies, with growing mycoplasmas, showed that the levels of cholesterol in membranes attained with LDL markedly exceeded those attained with HDL as a cholesterol donor (Slutzky et al., 1976). The present results, obtained with isolated membranes, indicate that the final level of cholesterol in membranes reached with LDL is again much higher than with HDL. Why then do the cell membranes incorporate more cholesterol from LDL than from HDL: is it due to the preferential binding of LDL to the mycoplasma membranes, as was found for fibroblasts in culture (Brown and Goldstein, 1976)? As can be seen in Table II, the answer is no, since we could not find any significant binding of either HDL or LDL to the membranes, as measured by the binding of ^{125}I-labeled protein moieties of the lipoproteins. Furthermore, the complete absence of cholesteryl ester uptake and the very small or negligible binding of lipoprotein phospholipids to the membranes exclude the adherence or fusion of intact lipoprotein particles with the membranes to any significant degree. Hence, it is clear that the higher cholesterol uptake from LDL does not depend on the preferential binding of these lipoproteins to the membranes.

Another possible explanation for the differences between HDL and LDL as cholesterol donors to mycoplasmas may be based on differences in the rate of cholesterol transfer from the various lipoproteins. However, our findings (Figure 2) indicate that the kinetics of cholesterol transfer from HDL to the membranes are not different from those obtained with LDL, but again the final level of cholesterol in membranes incubated with LDL is much higher than in those incubated with HDL. We would like to propose that the greater capacity of LDL to donate cholesterol depends on its high molar ratio of unesterified cholesterol to phospholipid (UC/PL), about 0.7 to 0.8 compared with a value of only 0.1 to 0.2 for HDL (Arbogast et al., 1976). Studies with mammalian cells and cholesterol–lecithin dispersions as cholesterol donors agree with this, as the higher the UC/PL ratio of the cholesterol donor relative to that of the membrane, the more effective it is in donating cholesterol to the membrane (Cooper et al., 1975; Shattil et al., 1975). Our results with isolated *A. laidlawii* membranes incubated with lecithin–cholesterol liposomes point in the same direction by showing that the higher the UC/PL of the liposomes the more effective they are in transferring cholesterol to the membranes. Similar results were obtained when cholesterol–lecithin liposomes served as cholesterol donors to growing mycoplasmas (Kahane and Razin, 1977). Hence, the lower UC/PL in HDL as compared with LDL may very well explain its inferior performance as a cholesterol donor. Yet our experiments also show that simple comparison of lipoproteins and liposomes based on their UC/PL values might be misleading, as HDL donated much more cholesterol to the mycoplasma membranes than liposomes having the equivalent UC/PL value (Figure 2). One explanation for the higher effectiveness of HDL as compared with liposomes may be based on the current models for HDL structure (Verdrey and Nichols, 1975; Morrisett et al., 1975). In these models all the unesterified cholesterol is placed close to the particle surface, making it available for exchange. In the liposomes, on the other hand, about one-half of the cholesterol content may be located in the inner half of the lipid bilayers, and, due to its very slow trans-bilayer movement (flip-flop), will be unavailable for exchange (Poznansky and Lange, 1976).

In conclusion, our data indicate that a significant portion of the unesterified cholesterol content of serum lipoproteins can be transferred to the mycoplasma cell membrane by a simple exchange process which does not involve the adherence, fusion, or degradation of the lipoprotein particle. That a similar process may be operative in eukaryotic cell systems as well has been suggested by the studies of Cooper et al. (1975) with erythrocytes and serum lipoproteins from patients suffering from spur cell anemia. The contribution of the direct transfer of unesterified cholesterol from serum lipoproteins to the plasma membrane of mammalian cells should, therefore, be included in any discussion of the overall balance of cellular cholesterol following interaction of the cells with serum lipoproteins.

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References

Ames, B. N. (1966), *Methods Enzymol. 8,* 115.

Arbogast, L. Y., Rothblat, G. H., Leslie, M. H., and Cooper, R. A. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3680.

Argaman, M., and Razin, S. (1965), *J. Gen. Microbiol. 38,* 153.

Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972), *Biochim. Biophys. Acta 260,* 212.

Bourque, D. P., and Naylor, A. W. (1971), *J. Chromatogr. 56,* 79.
Brown, M. S., and Goldstein, J. L. (1976), *Science 191,* 150.
Cooper, R. A., Arner, E. C., Wiley, J. S., and Shattil, S. J. (1975), *J. Clin. Invest. 55,* 115.
Fainam, M., Glangeaud, M. C., and Eisenberg, S. (1975), *Biochim. Biophys. Acta 386,* 432.
Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.
Gershfeld, N. L., Wormser, M., and Razin, S. (1974), *Biochim. Biophys. Acta 352,* 371.
Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955), *J. Clin. Invest. 34,* 1345.
Kahane, I., and Razin, S. (1977), *Biochim. Biophys. Acta* (in press).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
MacFarlane, A. S. (1958), *Nature (London) 182,* 53.
Morrisett, J. D., Jackson, R. L., and Gotto, A. M. (1975), *Annu. Rev. Biochem. 44,* 183.
Newman, G. C., and Huang, C. (1975), *Biochemistry 14,* 3363.
Papahadjopoulos, D. (1974), *J. Theor. Biol. 43,* 329.
Poznansky, M., and Lange, Y. (1976), *Nature (London) 259,* 420.
Razin, S. (1974), *FEBS Lett. 47,* 81.
Razin, S. (1975), in Progress in Surface and Membrane Science, Danielli, J. F., Rosenberg, M. D., and Cadenhead, D. A., Ed., New York, N.Y., Academic Press, p 257.
Razin, S., and Rottem, S. (1976), in Biochemical Analysis of Membranes, Maddy, A. H., Ed., London, Chapman and Hall, Ltd., p 3.
Razin, S., and Rottem, S. (1977), *Trends Biochem. Sci.* (in press).
Razin, S., Wormser, M., and Gershfeld, N. L. (1974), *Biochim. Biophys. Acta 352,* 385.
Rudel, L. L., and Morris, M. D. (1973), *J. Lipid Res. 14,* 364.
Shattil, S. J., Anaya-Galindo, R., Bennett, J., Coleman, R. W., and Cooper, R. A. (1975), *J. Clin. Invest. 55,* 636.
Slutzky, G. M., Razin, S., Kahane, I., and Eisenberg, S. (1976), *Biochem. Biophys. Res. Commun. 68,* 529.
Small, D. M., and Shipley, G. G. (1974), *Science 185,* 222.
Stein, O., and Stein, Y. (1975), *Biochim. Biophys. Acta 398,* 377.
Verdrey, R. B., and Nichols, A. V. (1975), *Chem. Phys. Lipids 14,* 123.

(Na, K)ATPase Activity in Membrane Preparations of Ouabain-Resistant HeLa Cells†

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ABSTRACT: Membrane preparations from two independent ouabain-resistant HeLa cell clones, HI-B1 and HI-C1, each appear to contain two species of (Na, K)ATPase. Two-thirds of the total (Na, K)ATPase in each mutant is indistinguishable from the enzyme in preparations of wild type cells with respect to ouabain binding, ouabain inhibition of (Na, K)ATPase activity, and dependence of ATP hydrolysis on Na, Mg, K, and ATP concentration. The remaining (Na, K)ATPase activity in the mutants is up to 1000 and 10 000 times, respectively, more resistant to ouabain than wild type enzyme. Resistance results from a lower affinity of the mutant enzymes for the inhibitor. The presence of Na, K, or Mg has little or no effect on the degree of resistance expressed by the mutant enzymes, although the resistance of the wild type enzyme varies 400-fold in the presence of different ligands. Incubation with 5×10^{-8} M ouabain abolishes the activity of the wild type enzyme without affecting the activity of the resistant enzymes. Using this procedure we compared the parameters of ATP hydrolysis via the resistant and wild type enzymes. Ouabain-resistant (Na, K)ATPase of HI-C1 has an apparent $K_{0.5}$ for potassium 3-4 times higher than that of either wild type enzyme or the resistant enzyme of HI-B1.

Mutants resistant to ouabain, a specific inhibitor of the plasma membrane (Na, K)ATPase, can be selected in a single step from HeLa cell lines (Baker, 1976). Dose-response curves describing ouabain inhibition of K influx suggest that mutant clones contain two types of K transport site which differ in their sensitivity to the inhibitor. The pattern of inhibition observed with wild type cells is consistent with the presence of a single class of transport site. The results of binding studies with the resistant HeLa cells also suggest that they contain two classes of ouabain binding sites, one similar to that in wild type cells and one with less affinity for the inhibitor. For the single-step mutants that have been examined, there appear to be two wild type sites for each ouabain-resistant one.

In this paper we describe the characteristics of the (Na, K)ATPase in membrane preparations of the wild type and two different ouabain-resistant clones of HeLa cells.

Experimental Procedure

Cell Lines, Media, and Growth Conditions. "Wild type HeLa" designates a clone, isolated in this laboratory, from HeLa cells (CCL 2) obtained from the American Type Culture

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