



Pergamon

Journal of Insect Physiology 48 (2002) 609–618

Journal
of
Insect
Physiology

www.elsevier.com/locate/jinsphys

Cholesterol efflux from larval *Manduca sexta* fat body in vitro: high-density lipophorin as the acceptor

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Received 24 January 2002; received in revised form 1 April 2002; accepted 1 April 2002

Abstract

The objective of this study was to characterize the transfer of cholesterol from *Manduca sexta* larvae fat body to high-density lipophorin. [³H]-Cholesterol-labeled fat body was incubated with lipophorin under different conditions and cholesterol transfer was determined. Transfer rate exhibited a hyperbolic dependency on lipophorin concentration with an apparent K_m of 3.6 mg/ml, which is consistent with either an aqueous diffusion mechanism of cholesterol transfer or a receptor-mediated process. Several results, including the high K_m , the high activation energy, and the lack of Ca^{2+} dependence favor aqueous diffusion model. In addition, anti-lipid transfer particle antibodies had only a small inhibitory effect, suggesting it is not involved in cholesterol transfer. However, the transfer was inhibited in the presence of suramin, which would be consistent with a receptor-mediated process. The effects of suramin may be complex because it can change membrane properties when bound to the lipophorin receptor and affect the rate of cholesterol desorption. The preponderance of data suggests that the export of cholesterol from fat body to lipophorin follows a simple aqueous diffusion pathway. Although we cannot completely exclude some contribution from a receptor-mediated pathway, it seems that if such a pathway were present, it represents a minor route. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Lipophorin; Cholesterol transfer; *Manduca sexta*; Lipid transfer particle

1. Introduction

Rothblat et al. (1999) recently summarized several decades of work on the mechanism of cholesterol efflux from vertebrate cells. They propose a complex model for cholesterol export that depends on both the cell type and the extracellular acceptor. The components of this model for cholesterol transfer between membranes and extracellular phospholipid containing acceptors are: (1) aqueous diffusion, in which free cholesterol desorbs from the membrane and subsequently is incorporated into a phospholipid-containing acceptor (Johnson et al., 1991); (2) direct interaction of the acceptor with the membrane via a “microsolubilization” process by which cholesterol is removed from the membrane; (3) a “microsolubilization” process mediated by the interaction of a lipopro-

tein with a membrane receptor; and (4) via the scavenger receptor class B type I (SR-BI) (Rothblat et al., 1999).

In contrast to vertebrates and plants, insects cannot make cholesterol via de novo synthesis because they lack the enzyme(s) squalene synthase and/or lanosterol synthase (Beenackers et al., 1985). Consequently, for normal growth, development, and reproduction, insects require a dietary source of sterols. Little is known about the mechanism by which cholesterol is delivered from the gut to tissues, but it seems reasonable to assume that this is accomplished via the hemolymph lipoprotein, lipophorin (Chino et al., 1981; Jouni et al., 2002). Lipophorin functions as a reusable shuttle that carries and distributes many lipids from sites of absorption or synthesis to sites of storage and utilization. In larval *Manduca sexta* lipophorin has a density of 1.14–1.16 g/ml and contains about 60% proteins, which includes one copy each of apolipophorin I ($M_r \sim 250,000$) and apolipophorin II ($M_r \sim 80,000$) and 40% lipid. The major lipids associated with lipophorin are diacylglycerol (DAG), hydrocarbons, phospholipids, and cholesterol (Soulages and Wells, 1994).

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Recently, we have shown that the lipid transfer particle (LTP) facilitates the transfer of DAG from larval midgut to lipophorin (Canavoso and Wells, 2001) and the bi-directional transfer of DAG between larval fat body and lipophorin (Canavoso et al., 2002). LTP is a very high-density lipoprotein with a molecular mass of more than 670 kDa. It contains 14% lipids and three-glycosylated apoproteins. LTP is synthesized in the fat body and secreted into the hemolymph (Van Heusden et al., 1996). In addition to its role in DAG transfer in larvae, LTP is also involved in the mobilization of DAG from adult fat body (Van Heusden and Law, 1989).

Although available data clearly demonstrate the essential role LTP plays in promoting DAG transfer from cells to lipophorin, the mechanism is still unknown. One possibility is that LTP plays a role in the formation of a lipophorin–receptor complex. Alternatively, LTP might act to enhance the function of the tissue-specific lipid transfer factors, which could determine the direction of the lipid transfer (reviewed in Arrese et al., 2001). However, the role of LTP in the transport of other lipids is unknown.

The present study was undertaken to investigate the mechanism(s) by which cholesterol is exported from larval *M. sexta* fat body and to ascertain if the lipophorin receptor and LTP are involved in this process.

2. Materials and methods

2.1. Materials

Glutathione, dithiothreitol, suramin, benzamidine hydrochloride, cholesterol oxidase and Grace's medium were purchased from Sigma (St Louis, MO). (1,2n) – ^3H – Cholesterol in 99.9% ethanol (specific activity 52 Ci/mmol) and Affi-gel Protein A were purchased from Amersham Pharmacia Inc. (Piscataway, NJ). Centricon filters were from Amicon (Beverly, MA).

2.2. Insects

M. sexta were reared at 25–27 °C on an artificial diet prepared from wheat germ (Fernando-Warnakulasuriya et al., 1988). Second day fifth instar larvae (3.1–3.2 g) were fasted for 10 min, and then fed a small piece of diet (0.3 cm × 0.3 cm × 0.3 cm) containing 4 μCi ^3H –cholesterol. Following consumption of the diet, the insects were placed on unlabeled diet. Twenty-four hours later, the fat body was dissected under cold phosphate buffered saline (PBS buffer) (50 mM sodium phosphate, 150 mM NaCl, pH 6.5) containing 1 mM ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM glutathione, 0.1 mM diisopropylfluorophosphate and 0.5 mM benzamidine, and then washed twice with Grace's medium.

2.3. Lipophorin isolation

High-density lipoproteins were isolated from the hemolymph of third and fourth day fifth instar larvae by density gradient ultracentrifugation as previously described (Prasad et al., 1986). Fractions containing lipophorin were dialyzed against 20 mM Tris–HCl buffer containing 150 mM NaCl, 2 mM EDTA and 0.5 mM benzamidine hydrochloride (pH 6.5), concentrated using YM-100 Centricon filters and washed with Grace's medium. ^3H –Cholesterol–labeled lipophorin was prepared from hemolymph of *M. sexta* fed 1 μCi ^3H –cholesterol for 24 h, following the same procedure. The purity of the lipophorin preparations was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, using either linear (7.5%) or gradient (4–15%) SDS-PAGE prepared as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250.

2.4. Measurements of cholesterol transfer

^3H –Cholesterol–labeled fat bodies were incubated in Grace's medium in the presence of different cholesterol acceptors. At the indicated time intervals, a 50 μl aliquot of the incubation medium was mixed with 100 μl of PBS containing 2 mg/ml of bovine serum albumin (BSA) and centrifuged at 10,000g for 10 min to remove any detached cells. At the end of the incubation, the tissues were separated from the incubation medium by filtration using a nylon mesh. The tissues were washed thoroughly with PBS and lysed in 2 ml of 0.1 N NaOH. Aliquots were used for the determination of protein concentration and for radioactivity counting using a scintillation counter. All experimental procedures were carried out in triplicate, at room temperature, and on an orbital shaker at 50 rev/min unless otherwise stated.

2.5. Effect of temperature and pH on cholesterol transfer

For these studies, 3.3 mg/ml of lipophorin was used. To study the effect of pH, lipophorin was dialyzed into a buffer containing MES (0.01 M), morpholinopropanesulfonic acid (MOPS) (0.01 M), Trizma base (0.5 mM) and NaCl (0.15 M) of different pH values between 5 and 7. Changes in pH of the medium containing the tissues were introduced gradually over a period of 30 min. For the effect of temperature on transfer studies, ^3H –cholesterol fat bodies and lipophorin were pre-equilibrated to temperatures ranging from 4 to 42 °C prior to starting the transfer studies.

2.6. Effect of EDTA and suramin on cholesterol transfer

To determine the effect of EDTA on cholesterol efflux, ^3H –cholesterol fat bodies were pre-incubated with

increasing concentration of EDTA (5 mM to 40 mM) on a shaker at 50 rev/min and 26 °C. After 30 min, tissues were transferred to a medium containing 3.3 mg/ml lipophorin with the corresponding concentration of EDTA. The amount of radioactivity transferred to the medium was measured for 2 h. The effect of suramin (a lipophorin receptor inhibitor) was determined by pre-incubating labeled fat bodies with 4 mM suramin for 30 min, then the tissues were washed with Grace's medium and transfer studies were conducted as described above. For control studies, fat bodies were incubated with the same metabolic inhibitors and transfer studies were carried out on ice to ensure that the inhibitors did not affect membrane structure and cause any leakage of cholesterol.

2.7. Anti-LTP antibody

Antiserum against purified LTP was obtained from a New Zealand white rabbit as described by Ryan et al. (1988). The IgG fraction was then purified using Affigel Protein A, and stored at –80 °C. To elucidate the role of LTP in the transfer of cholesterol to lipophorin, labeled fat body tissues were pre-incubated with anti-LTP antibodies (2 mg IgG/ml) washed extensively, incubated in the presence or absence of lipophorin (3.3 mg/ml) and transfer studies were carried as described above.

2.8. Treatment of labeled fat body with cholesterol oxidase

³H-Cholesterol-labeled fat bodies were incubated with cholesterol oxidase (2 units) for 1 h at 34 °C, following the method described by Fukasawa et al. (2000), except that Triton was replaced by 100 mg/ml of BSA. After the incubation, the tissue was washed extensively with buffer and incubated with or without lipophorin (3.0 mg/ml) for 2 h, and transfer studies were conducted as described above. At the end of the incubation, the fat body was washed three times with PBS, homogenized in 1 ml NaOH, and 100 µl were used for counting radioactivity. Another 100 µl of the homogenized fat body and separately a 200 µl aliquot of the incubation medium containing lipophorin were extracted with methanol:chloroform (1:2) (Folch et al., 1957). Solvents were evaporated under N₂, lipids were redissolved in hexane and fractionated by thin-layer chromatography (TLC) using hexane:diethyl ether:acetic acid (120:20:1) (Jouni et al., 1995). Spots corresponding to cholesterol and cholestenone were assayed for radioactivity by liquid scintillation counting.

2.9. ³H-Cholesterol-labeled fat body

To study the transfer of [³H] – cholesterol from fat body to lipophorin, first day fifth instar *M. sexta* larvae

were fed on a piece of diet labeled with [³H] – cholesterol (2 µCi), then switched to unlabeled diet until the fat body was used in the transfer studies. This technique produced [³H] – cholesterol – labeled fat body with the same specific activity at days 4 and 5 of the fifth instar and in all wandering stages. [³H] – Cholesterol – labeled fat body was obtained from *M. sexta* at the indicated developmental stages. Labeled fat body was dissected out, washed, incubated in Grace's medium containing unlabeled larval lipophorin (1.5 mg/ml) for 2 h, and transfer studies were carried out as described above.

2.10. Protein determination

Protein concentration was determined using a modified Lowry method with BSA as a standard (Markwell et al., 1978).

2.11. Analyses of data

Efflux studies are reported as dpm of ³H-cholesterol transferred to lipophorin per mg of fat body protein, or are calculated as the percentage of initial ³H-cholesterol in the fat body. For the time course transfer studies, data were fit to a linear regression equation using Graph Pad (Graph Pad Software, Inc.). Slopes from the time course studies were analyzed by a non-linear regression using the following equation: $Y = V_{\max} \times X / (K_m + X)$, where Y is the slope, X is the lipophorin concentration, V_{\max} is the maximal velocity, and K_m is the concentration of lipophorin required to reach half-maximal velocity. For statistical analysis, Student's unpaired *t*-tests were used to determine the significance of differences between means.

3. Results

3.1. Specificity of cholesterol transfer to lipophorin

Specificity of ³H-cholesterol transfer from fat body to lipophorin was determined in Grace's medium with or without bovine serum albumin, gelatin (a matrix protein), or lipophorin (Fig. 1). Neither Grace's medium alone, nor in the presence of albumin or gelatin induced cellular cholesterol transfer. However, lipophorin specifically and significantly induced a $12.9 \pm 0.6\%$ transfer of the initial ³H-cholesterol from the fat body ($P < 0.001$). Increasing fat body concentration from one to four-fold was accompanied by a linear increase in the amount of ³H-cholesterol transferred to lipophorin (data not shown).

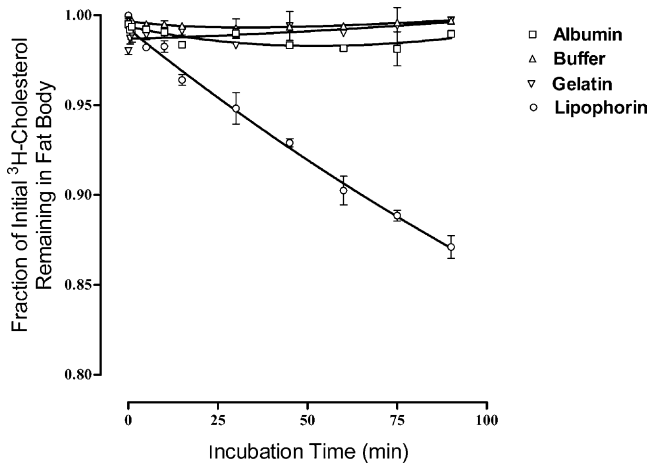


Fig. 1. Specificity of cholesterol transfer from ^3H -cholesterol-labeled fat body to lipophorin. ^3H -cholesterol-labeled fat body tissues were incubated in Grace's medium alone (Δ) or with bovine serum albumin (2 mg/ml; \square), gelatin (2 mg/ml; ∇) or lipophorin (3.3 mg/ml; \circ). Transfer of [^3H] - cholesterol from tissues to different medium was determined as described in the Materials and methods section. Data represent the average of three determinations \pm the standard error (SE).

3.2. Time course and concentration dependence studies

At several different concentrations of lipophorin (0.1 mg/ml to 9.0 mg/ml) the amount of cholesterol transferred from fat body to lipophorin was linearly dependent on time (Fig. 2A). Analysis of the time course data by nonlinear least squares demonstrated a saturation behavior for the amount of cholesterol transferred to increasing concentrations of lipophorin with an apparent $K_m = 3.6 \pm 0.9$ mg/ml (Fig. 2B). To confirm that the plateau observed at the highest concentration of lipophorin was a real saturation and not due to depletion of cholesterol available for release, a control study was carried out by incubating labeled fat body with 9.0 mg/ml lipophorin for a longer period of time (3 h). The transfer was significantly higher (25%) than observed in a 2 h incubation (20%), indicating that the observed plateau was a reflection of saturation and not depletion of the cholesterol pool available for release.

3.3. Effect of temperature and pH

The effects of temperature and pH on transfer of cholesterol from *M. sexta* larvae fat body to 3.0 mg/ml lipophorin are depicted in Fig. 3. Cholesterol transfer to lipophorin was strongly temperature-dependent. At 4 °C, minimal cholesterol transfer from fat body was observed (Fig. 3A). Increasing the temperature to 26 °C induced an eight-fold increase in cholesterol transfer. Further increases in temperature to 37 °C and 42 °C induced a 11.6-fold and a 15.8-fold increase in the radioactive cholesterol transferred to lipophorin, respectively. Using

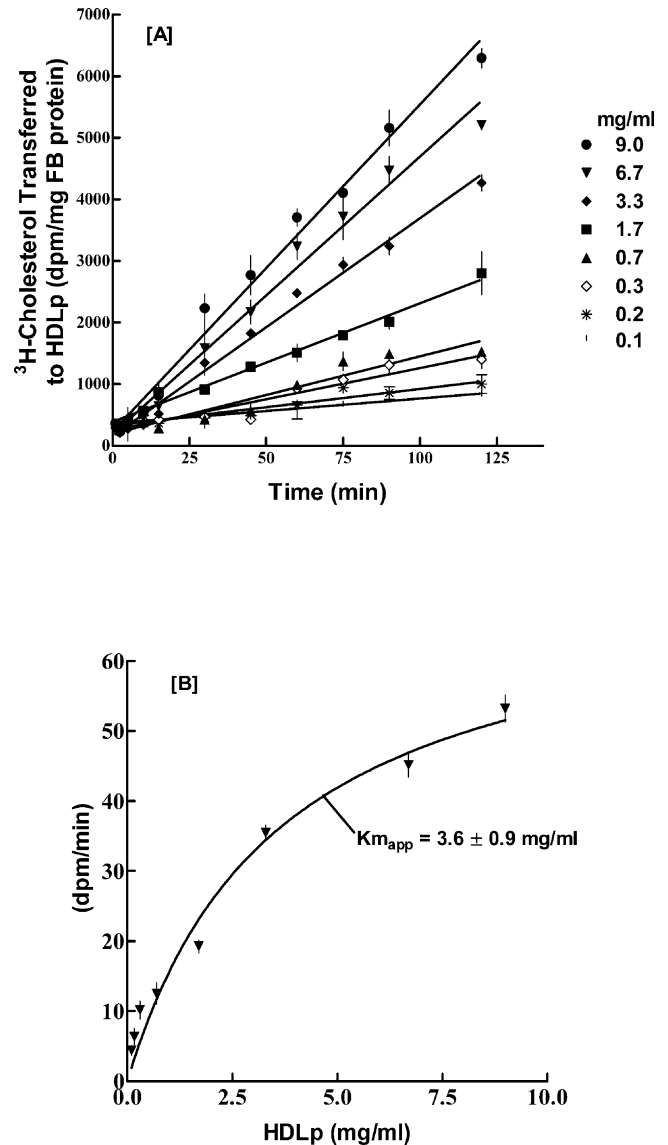


Fig. 2. Time course and concentration dependence of cholesterol transfer. Labeled fat body tissues were incubated with different concentrations of lipophorin ranging from 0.1 mg/ml to 9 mg/ml. At the indicated time intervals, the radioactivity was determined (refer to Materials and methods section for more details). (A) Dpm of ^3H -cholesterol transferred to lipophorin per mg protein fat body versus time (min). (B) Rate of cholesterol transferred to lipophorin (dpm/min) versus concentrations of lipophorin. Values represent averages of four determinations \pm SE.

the Arrhenius equation, we calculated an activation energy of 61.9 ± 7.5 kJ/mol (Fig. 3B).

The pH of the incubation medium exhibited a large effect on cholesterol transfer from labeled fat body (Fig. 3C). Cholesterol transfer data demonstrated a classic bell-shaped curve with an optimum pH at 6.2, where the maximum amount of cholesterol was transferred to lipophorin. After 2 h and at pH 6.2, a 1.3-fold to 3.7-fold increase in cholesterol transfer was observed compared to other pHs tested.

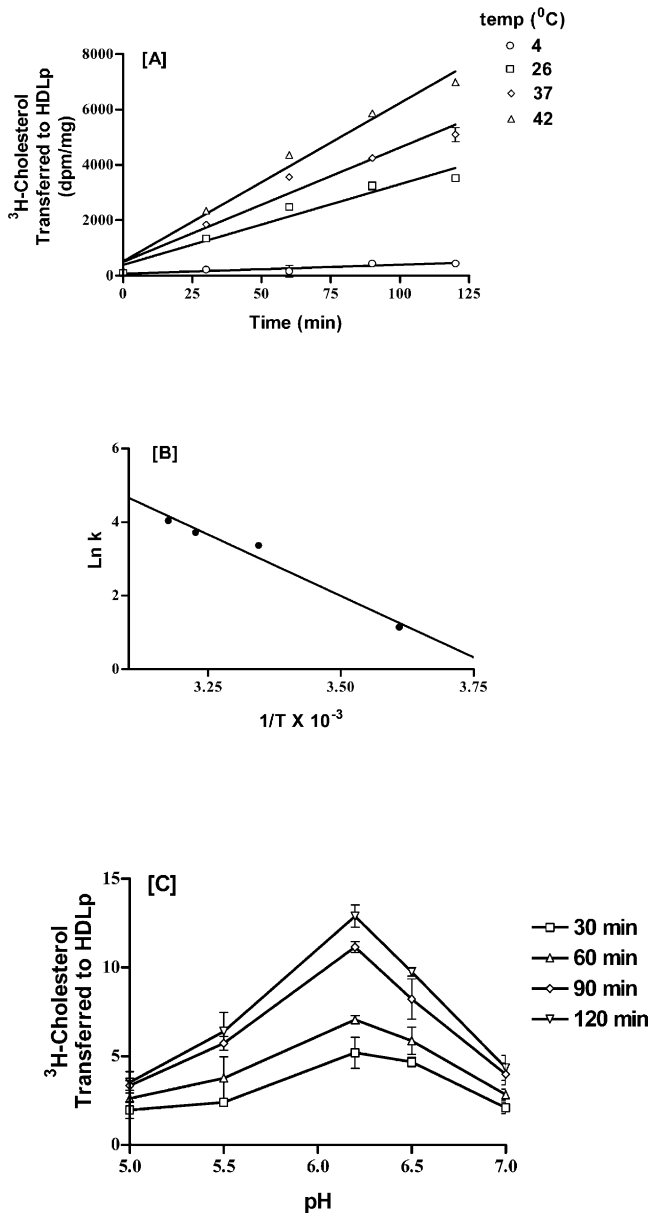


Fig. 3. Effect of temperature and pH on radiolabeled cholesterol transferred to lipophorin. (A) ^3H -Cholesterol-labeled fat body tissues were incubated in Grace's medium containing lipophorin (3.3 mg/ml) at temperatures ranging from 4 to 42 °C. (B) Arrhenius plot of transfer data. (C) The effect of pH (5 and 7) on cholesterol transfer to 3.3 mg/ml lipophorin. At the indicated time intervals, aliquots were used for radioactivity counting (refer to Materials and methods section for more details). Values represent averages \pm SE of three or four determinations.

3.4. Effect of EDTA, anti-LTP antibodies and suramin on cholesterol transfer

Previously, Tsuchida and Wells (1988) have shown that receptor binding of lipophorin is calcium-dependent and that EDTA is able to abolish this binding process. To test whether cholesterol transfer is mediated by the characterized lipophorin receptor of fat body, we carried

out transfer studies in the presence of increasing concentrations of EDTA (5 mM to 40 mM) (Fig. 4A). The results demonstrated that EDTA had little or no effect on the transfer of cholesterol from fat body, suggesting that binding of lipophorin to its receptor, which is Ca^{2+} -dependent, is not required for cholesterol transfer.

In larval *M. sexta*, we have recently shown that LTP

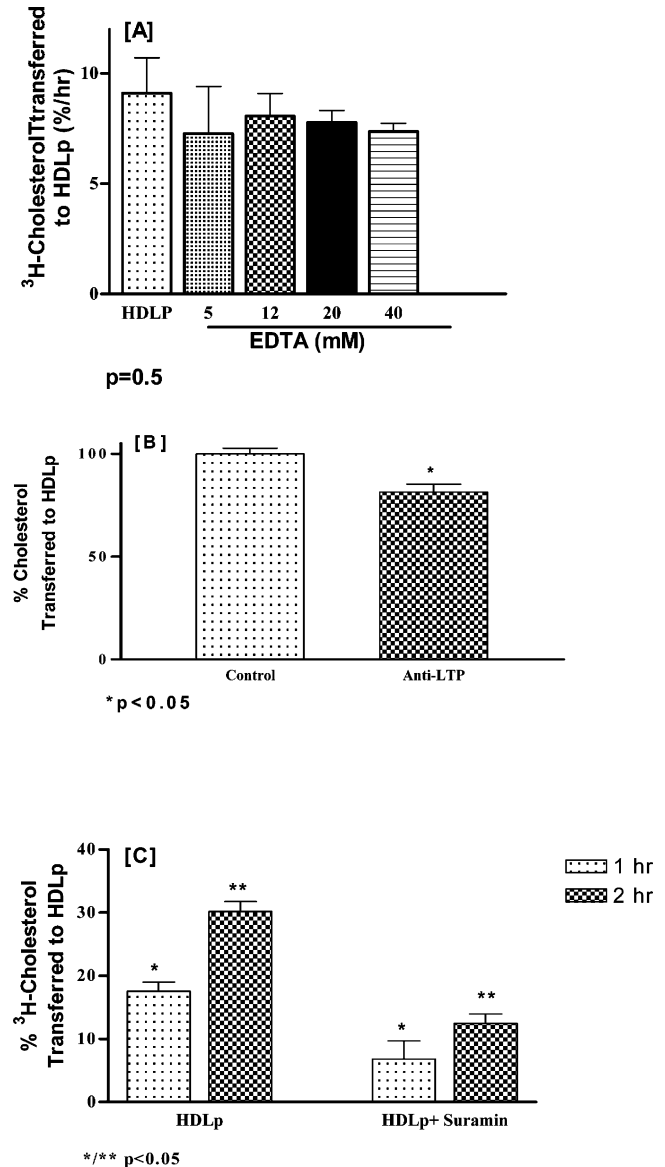


Fig. 4. Effect of EDTA, anti-LTP antibody and suramin on cholesterol transfer to lipophorin. (A) To determine the effect of EDTA on cholesterol transfer, ^3H -cholesterol-labeled fat bodies were pre-incubated for 30 min with EDTA (5–40 mM), transferred to medium containing 3.3 mg/ml lipophorin with the corresponding concentration of EDTA, and the radioactivity released to lipophorin was determined after 2 h. ^3H -Cholesterol-labeled fat bodies were pre-incubated for 30 min with 2 mg/ml anti-LTP-IgG (B) or 4 mM suramin (C), the tissues were washed and incubated in Grace's medium containing 3.3 mg/ml of lipophorin, and transfer studies were determined as described in the Materials and methods section. For control studies, fat body tissues were incubated with anti-LTP alone. Values represent averages \pm SE of four determinations.

plays a significant role in the transfer of DAG from midgut to lipophorin (Canavoso and Wells, 2001), and from lipophorin to fat body (Canavoso et al., 2002). It was therefore important to determine whether LTP plays a role in cholesterol transfer from fat body. Treatment of fat body with anti-LTP antibodies had only a small (20%) inhibitory effect on the transfer of cholesterol to lipophorin (Fig. 4B), suggesting that LTP does not facilitate cholesterol transfer. However, suramin (4 mM), a polysulfated polycyclic hydrocarbon known to compete with lipophorin for receptor binding (Tsuchida and Wells, 1988), significantly impaired export of cholesterol (50%; $P < 0.05$) from fat body to lipophorin (Fig. 4C), suggesting the contribution of the lipophorin receptor in this transfer process.

3.5. Effect of cholesterol oxidase on cholesterol transfer

In vertebrate systems, more than one pool of exchangeable cholesterol is present (Rothblat et al., 1999). In order to determine whether this was the case for *M. sexta* fat body, the tissues were treated with cholesterol oxidase, which will only react with cholesterol in the outer half of the membrane bilayer. After 1 h of incubation, the fat body was washed, transferred to Grace's medium in the presence or absence (control) of lipophorin and transfer studies were carried out (Fig. 5). Cholesterol oxidase oxidized less than 10% of total cholesterol present in fat body (Fig. 5A), demonstrating that only a small portion of cholesterol is accessible to the enzyme. The location of the unoxidized cholesterol (90%) might be in the inner leaflet of the plasma membrane, in internal membranes of the cell, or sequestered in an inaccessible form in the outer leaflet of the plasma membrane.

Treatment with cholesterol oxidase had no effect on the amount of radioactivity transferred to lipophorin (data not shown). TLC analysis of the radioactivity transferred to lipophorin showed that approximately 70% was in the cholesterol form, whereas cholestenone accounted for about 30% (Fig. 5B), demonstrating that cholestenone is more readily available for export than unmodified cholesterol. In addition, these data strongly suggest the presence of more than one cholesterol pool in the fat body, and show that cholesterol in both pools is available for export to lipophorin.

3.6. Developmental changes in cholesterol transfer

Previous reports from our laboratory (Prasad et al., 1986; Fernando-Warnakulasuriya et al., 1988) have suggested a fundamental change in *M. sexta* fat body lipid metabolism during the wandering stage, where the fat body is converted from a lipid-storing to a lipid-mobilizing organ. To determine whether these changes affect

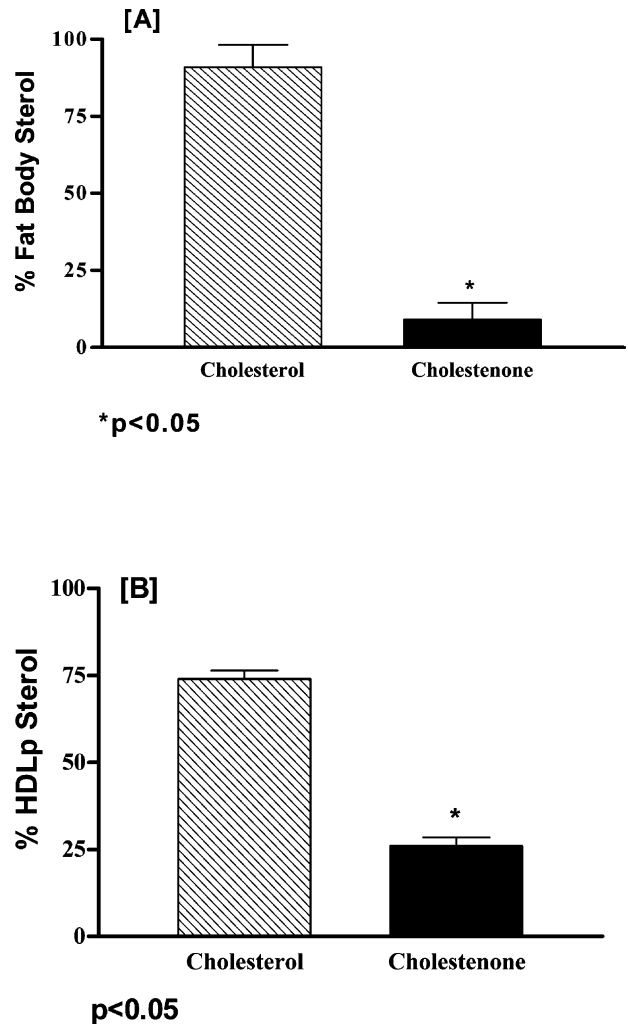


Fig. 5. Effect of cholesterol oxidase on cholesterol transfer. Fat bodies were preincubated with 2 units of cholesterol oxidase for 1 h, then washed extensively and incubated in Grace's medium with or without lipophorin (3.0 mg/ml) for 2 h, and transfer studies were determined as mentioned in the Materials and methods section. (A) The relative amount of cholesterol and cholestenone in the fat body after treatment with cholesterol oxidase. (B) The relative distribution of cholesterol and cholestenone transferred to lipophorin after treatment with cholesterol oxidase. Values represent averages \pm SE of three determinations.

cholesterol transfer in feeding and non-feeding stages of the larvae, transfer studies were determined using ^3H -cholesterol-labeled fat body from day 4 and day 5 of fifth instar, prewanderers (12 h after day 5) and wanderers (day 1–day 5). Cholesterol mobilization from and to lipophorin was significantly decreased from the larval stage (feeding larvae) until the second day of wandering (non-feeding larvae) (Fig. 6A, B). The direction of cholesterol transfer in this *in vitro* system favors incorporation into fat body rather than transfer in the reverse direction (from fat body to lipophorin) except for day 5 of fifth instar (Fig. 6A).

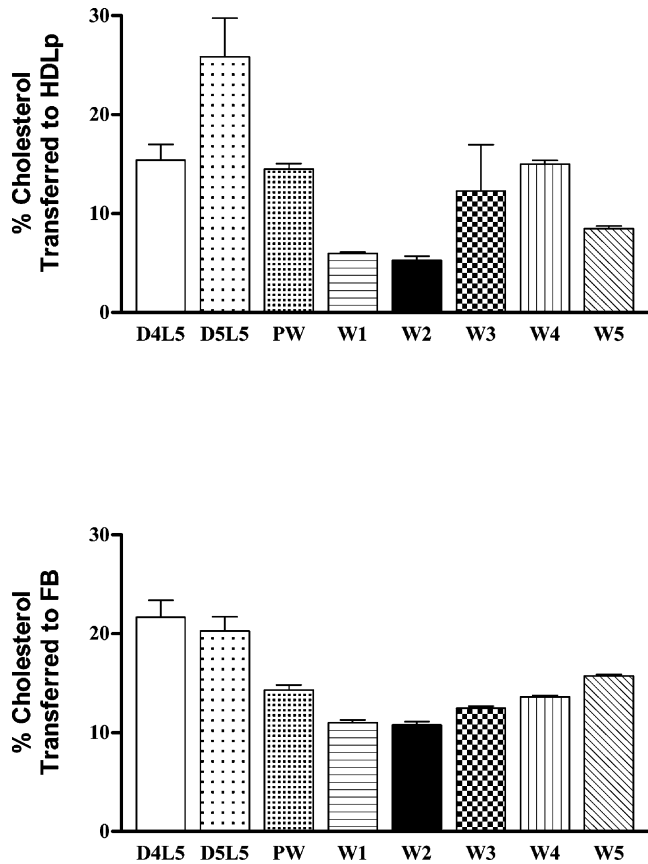


Fig. 6. Cholesterol transfer in feeding and non-feeding stages of *M. sexta*: The nomenclature used to describe the stages is: D4 and D5 refer to the 4th and 5th day of the feeding fifth instar larvae; PW is 12 h after D5 and represents the beginning of the wandering stage; W1 through W5 represent days 1 to 5 after D5. (A) Transfer of cholesterol from fat body to lipophorin was determined by incubating ^3H -cholesterol-labeled fat body from different stages with 1.5 mg/ml larval lipophorin for 2 h at room temperature. (B) Transfer of cholesterol from ^3H -cholesterol-labeled lipophorin (1.5 mg/ml) to fat body of different stages of *M. sexta* larvae. Cholesterol transfer was determined as described in the Materials and methods section. Results are expressed as a percentage of $\text{dpm} \pm$ standard error of the mean SEM ($n=3-4$) found in the initial amount of cholesterol in fat body or lipophorin.

4. Discussion

Lipophorin, the major lipid transport vehicle in insects, carries and distributes many lipids from sites of absorption or synthesis to sites of storage and utilization. *M. sexta* lipophorin shares some functional features with the mammalian high-density lipoprotein. Both lipoproteins are synthesized as nascent particles that acquire some of their lipids after secretion into the hemolymph or blood. Both bind to receptors and selectively transfer lipids without being internalized or degraded. The fact that *M. sexta* cannot synthesize cholesterol by the de novo pathway and that lipophorin binds its receptor on fat body without being internalized or degraded, makes it an ideal model to study cholesterol transfer without

interference from the newly synthesized cholesterol or by lipophorin uptake or secretion. Recently, we have documented that lipophorin is the only means by which absorbed cholesterol is transported in the hemolymph of *M. sexta* (Jouni et al., 2002). The mechanism by which lipophorin acquires its cholesterol from fat body and delivers it to other tissues is unknown and to our knowledge has never been investigated. In this report we carried out several studies to understand and characterize this pathway.

Cholesterol transfer from *M. sexta* fat body was specific for lipophorin. The transfer did not occur to albumin, which has been shown to play a significant role in cholesterol transfer between vertebrate cells and lipoproteins (Zhao and Marcel, 1996). Of course, there is no albumin-like protein in insect hemolymph that could play this role.

When fat body was incubated with increasing concentrations of lipophorin, saturation was achieved with an apparent K_m value of 3.6 ± 0.9 mg/ml. The hyperbolic dependence of cholesterol transfer rate on lipophorin concentration would be consistent with the binding of lipophorin to a membrane receptor. However, the apparent K_m derived from these kinetic experiments is more than 100 times higher than the K_d (25 $\mu\text{g}/\text{ml}$) determined for the dissociation of the lipophorin–receptor complex from larval *M. sexta* fat body membranes (Tsuchida and Wells, 1988). Thus cholesterol transfer does not reach maximum within lipophorin concentrations that normally allow maximal specific binding, suggesting that lipophorin binding to its receptor, although it may mediate cholesterol transfer at low lipophorin concentrations, cannot account for the bulk of cholesterol transfer from fat body to lipophorin. Hyperbolic kinetics can also be observed, when the mechanism of cholesterol transfer involves spontaneous desorption of cholesterol from the membrane into the aqueous solution followed by a second-order interaction between the desorbed cholesterol and lipophorin (Davidson et al., 1995). This aqueous diffusion process of cholesterol from the cell membrane to lipophorin does not require lipophorin binding to the membrane. We conducted several studies in order to distinguish between these two mechanisms, namely receptor-mediated and aqueous diffusion pathways.

4.1. Receptor-mediated versus aqueous diffusion mechanisms

Cholesterol transfer to lipophorin was strongly temperature-dependent. The amount of cholesterol transferred to lipophorin increased linearly with increasing temperature of the incubation medium. An increase in temperature is associated with a destabilizing effect on the membrane lipid components and thus favors aqueous diffusion mechanism. The activation energy for chole-

terol efflux from cells depends on the extent to which cholesterol must leave the membrane and move through the aqueous solution before encountering an acceptor, in our case lipophorin (Yancey et al., 1996). At one extreme, the aqueous diffusion transfer of cholesterol from the membrane to a lipophorin probably requires complete desorption of cholesterol from the membrane and is predicted to have an activation energy of about 75–100 kJ/mol (Yancey et al., 1996). At the other extreme, transfer of cholesterol to lipophorin bound to a membrane receptor does not require cholesterol to leave the membrane entirely before interacting with lipophorin and is predicted to have an activation energy of about 10 kJ/mol (Yancey et al., 1996). The observed activation energy (61.9 ± 7.5 kJ/mol) supports the predicted value for an aqueous diffusion mechanism rather than that predicted for a receptor-mediated process.

Cholesterol transfer was pH-dependent with an optimum pH at 6.2. Changes in pH are usually associated with changes in polarity of the lipid/protein membrane components and consequently are able to affect desorption rate of cholesterol from fat body membrane, as well as the binding of lipophorin to its receptor. The observed optimum pH for cholesterol transfer is in agreement with the optimum pH for lipophorin binding to its receptor (Tsuchida and Wells, 1988) and with the pH of *M. sexta* hemolymph. Thus the effect of pH was not able to distinguish between the two processes.

The *M. sexta* lipophorin receptor has been purified from larval fat body membranes (Tsuchida and Wells, 1988). Binding of lipophorin to its receptors on fat body membranes exhibited an absolute requirement for Ca^{2+} and addition of 2 mM EDTA completely abolished this binding process. In contrast, EDTA, even at very high concentrations (40 mM), had little or no effect on cholesterol transfer to lipophorin, suggesting that cholesterol transfer is not calcium-dependent. This result would favor the aqueous diffusion mechanism.

Suramin, a polycyclic hydrocarbon, which competes with lipophorin for receptor binding (Tsuchida and Wells, 1988), inhibited transfer of cholesterol from fat body to lipophorin (Fig. 5B) ($P < 0.4$). While the inhibitory effect of suramin (4 mM) might indicate that a receptor-mediated process plays a role in cholesterol transfer from fat body to lipophorin, these results need to be interpreted cautiously, because at a concentration of 1 mM suramin had no effect on cholesterol transfer to lipophorin (data not shown), although it has been shown to abolish binding of lipophorin to its membrane receptor (Tsuchida and Wells, 1988). Furthermore, the effect of suramin is complex. Suramin has been shown to bind and precipitate lipophorin (Gondim and Wells, 2000). Although the fat body was treated with suramin in the absence of lipophorin, and the suramin washed away before adding lipophorin, we cannot eliminate the possibility that suramin may have affected the properties

of lipophorin. In addition, suramin is a negatively charged molecule and when bound to the lipophorin receptor could alter the properties of the membrane. This, in turn, would affect the ability of cholesterol to desorb from the membrane (Rothblat et al., 1999). Therefore, the effect of suramin does not clearly prove the contribution of a receptor.

We have shown recently that in larval *M. sexta* LTP plays a significant role in the transfer of DAG from midgut to lipophorin (Canavoso and Wells, 2001) and from lipophorin to fat body (Canavoso et al., 2002). Therefore, it was of interest to determine if LTP mediates cholesterol transfer from fat body. Treatment of fat body with anti-LTP antibody resulted in a small inhibition of cholesterol release from fat body. Such a small effect is difficult to interpret; however, it appears that the majority of cholesterol transfer is LTP-independent. While this does not eliminate the possibility of receptor-mediated transfer of cholesterol, at the very least it shows that the mechanism must be different from that for DAG transfer (Canavoso et al., 2002). In fact, these data can be interpreted as favoring an aqueous diffusion model for the transfer of cholesterol from fat body to lipophorin.

Taken together, our results indicate that cholesterol transfer from fat body to lipophorin takes place mainly by an aqueous diffusion process. However, the results do not conclusively eliminate the minor contributions of the lipophorin receptor and LTP in this transfer pathway.

4.2. Cholesterol pools of fat body

Cholesterol transfer studies from mammalian cells demonstrated the presence of at least two pools of exchangeable cholesterol (Yancey et al., 1996; Rothblat et al., 1999). Mahlberg and Rothblat (1992) were able to verify that the two pools are not a reflection of bidirectional flux between donor and acceptor, but rather, cholesterol is located in two kinetically distinct pools on the cell membrane. Treatment of fat body with cholesterol oxidase, which can only react with cholesterol in the outer half of the membrane bilayer, had no effect on the amount of radioactivity transferred to lipophorin (data not shown). However, only 10% of the fat body cholesterol pool was accessible to cholesterol oxidase, indicating, as in mammalian systems, that more than one cholesterol pool is present on the fat body. Furthermore, our results demonstrate that the accessible sterol pool, as measured by oxidation by cholesterol oxidase, is more readily transferred to lipophorin (30%) than the bulk of the cellular cholesterol.

4.3. Developmental changes in cholesterol mobilization

We also determined the effects of the developmental changes on cholesterol transfer from and to fat body.

The physiological and morphological changes that occur during the development of the insect are extraordinary. In *M. sexta* some of these changes are reflected in the mobilization of lipid; for example, more DAG from lipophorin is incorporated into the fat body of feeding larvae than into the fat body of the wandering stage (Tsuchida and Wells, 1988; Canavoso et al., 2002). Cholesterol transfer from fat body to lipophorin was significantly affected by the developmental changes of the *M. sexta*. At the feeding stage, similar to the mobilization of DAG, the direction of cholesterol in this in vitro system favors incorporation into the fat body rather than its transfer in the reverse direction (from fat body to lipophorin). At the non-feeding stage, more cholesterol is mobilized from fat body to lipophorin. Previous reports from our laboratory (Prasad et al., 1986; Fernando-Warnakulasuriya et al., 1988) have suggested a fundamental change in *M. sexta* fat body lipid metabolism during the wandering stage, where the fat body is converted from a lipid-storing to a lipid-mobilizing organ. The changes in cholesterol transfer reflect changes in the properties of the fat body during development, as the fat body of *M. sexta* is composed of only one cell type at each developmental stage (Willott et al., 1988).

5. Summary and conclusions

The preponderance of data suggests that the export of cholesterol from the fat body to lipophorin follows a simple aqueous diffusion pathway. This surface transfer occurs passively, without the direct input of metabolic energy or the uptake and degradation of cholesterol acceptors. In an aqueous diffusion mechanism, cholesterol movement from fat body to lipophorin follows its concentration gradient. This means that the fat body can act as a reservoir for total body cholesterol, releasing cholesterol to lipophorin as lipophorin is depleted of cholesterol by delivering it to other tissues. Although we cannot completely exclude some contribution from receptor-mediated and/or LTP-dependent pathways, it seems that if such pathways were present, they represent a minor route. The presence of two pools of cholesterol in the fat body, one in the outer leaflet of the plasma membrane and the other presumably in internal membranes, would be consistent with this homeostatic mechanism.

Acknowledgements

We thank Mary Hernandez for insect care. This work was supported by NIH grant GM50008.

References

- Arrese, E.L., Canavoso, L.E., Jouni, Z.E., Pennington, J.E., Tsuchida, K., Wells, M.A., 2001. Lipid storage and mobilization in insects: current status and future directions. *Insect Biochemistry and Molecular Biology* 31, 7–17.
- Beenackers, A.M.T., van der Horst, D.J., Van Marrewijk, W.J.A., 1985. Insect lipids and lipoproteins and their role in physiological processes. *Progress in Lipid Research* 24, 19–67.
- Canavoso, L.E., Wells, M.A., 2001. Role of lipid transfer particle in delivery of diacylglycerol from midgut to lipophorin in larval *Manduca sexta*. *Insect Biochemistry and Molecular Biology* 31, 783–790.
- Canavoso, L.E., Yun, H.K., Jouni, Z.E., Wells, M.A., 2002. Lipid transfer particle mediates the delivery of diacylglycerol from lipophorin to fat body in larval *Manduca sexta*. *Insect Biochemistry and Molecular Biology* (in press).
- Chino, H., Downer, R.G.H., Wyatt, G.R., Gilbert, L.I., 1981. Lipoprotein, a major class of lipoproteins of insect hemolymph. *Insect Biochemistry* 11, 491.
- Davidson, W.D., Gillote, K.L., Lund-Katz, S., Johnson, W.J., Rothblat, G.H., Phillips, M.C., 1995. The effect of high density lipoprotein phospholipid acyl chain composition on the efflux of cellular free cholesterol. *The Journal of Biological Chemistry* 270, 5882–5890.
- Fernando-Warnakulasuriya, G.J.P., Tsuchida, K., Wells, M.A., 1988. Effect of dietary lipid content on lipid transport and storage during larval development of *Manduca sexta*. *Insect Biochemistry* 18, 211–214.
- Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry* 226, 497–509.
- Fukasawa, M., Nishijima, M., Itabes, H., Takano, T., Hanada, K., 2000. Reduction of sphingomyelin level without accumulation of ceramide in Chinese hamster ovary cells affects detergent-resistant membrane domains and enhances cellular cholesterol efflux to methyl- β -cyclodextrin. *The Journal of Biological Chemistry* 275, 34028–34034.
- Gondim, K.C., Wells, M.A., 2000. Characterization of lipophorin binding to the midgut of larval *Manduca sexta*. *Insect Biochemistry and Molecular Biology* 30, 405–423.
- Johnson, W.J., Mahlberg, F.H., Rothblat, G.H., Phillips, M.C., 1991. Cholesterol transport between cells and high-density lipoproteins. *Biochimica et Biophysica Acta* 1085, 273–298.
- Jouni, Z.E., Winzerling, J.J., McNamara, D.J., 1995. 1,25-Dihydroxyvitamin D₃-induced HL-60 macrophages: regulation of cholesterol and LDL metabolism. *Atherosclerosis* 117, 125–138.
- Jouni, Z.E., Zamora, J., Wells, M.A., 2002. Absorption and tissue distribution of cholesterol in *Manduca sexta*. *Archives of Insect Biochemistry and Physiology* 49, 167–175.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 227, 680–685.
- Mahlberg, F.H., Rothblat, G.H., 1992. Cellular cholesterol efflux role of cell membrane kinetic pools and interaction with apolipoproteins AI, AII and Cs. *The Journal of Biological Chemistry* 267, 4541–4550.
- Markwell, M.A.K., Hass, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* 87, 206–210.
- Prasad, S.V., Fernando-Warnakulasuriya, G.J.P., Sumida, M., Law, J.H., Wells, M.A., 1986. Lipoprotein biosynthesis in the larvae of the tobacco hornworm, *Manduca sexta*. *The Journal of Biological Chemistry* 261, 17174–17176.
- Rothblat, G.H., de la Llera-Moya, M., Atger, V., Kellner-Weibel, G., Williams, D.L., Phillips, M.C., 1999. Cell cholesterol efflux: integration of old and new observations provides new insights. *Journal of Lipid Research* 40, 781–796.

- Ryan, R.O., Senthilathipan, K.R., Wells, M.A., Law, J.H., 1988. Facilitated diacylglycerol exchange between insect hemolymph lipophorins. *The Journal of Biological Chemistry* 263, 14140–14145.
- Soulages, J.L., Wells, M.A., 1994. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Advances in Protein Chemistry* 45, 371–415.
- Tsuchida, K., Wells, M.A., 1988. Isolation and characterization of a lipoprotein receptor from the fat body of an insect. *The Journal of Biological Chemistry* 265, 5761–5767.
- Van Heusden, M.C., Law, J.H., 1989. Characterization and identification of a lipoprotein lipase from *Manduca sexta* flight muscle. *Insect Biochemistry and Molecular Biology* 23, 785–792.
- Van Heusden, M.C., Yepiz-Plascencia, G.M., Walker, A.M., Law, J.H., 1996. *Manduca sexta* lipid transfer particle, synthesis by fat body and occurrence in hemolymph. *Archives of Insect Biochemistry and Physiology* 31, 39–51.
- Willott, E., Bew, L.K., Nagle, R.B., Wells, M.A., 1988. Sequential structural changes in the fat body of the tobacco hornworm, *Manduca sexta*, during the fifth larval stadium. *Tissue and Cell* 20, 635–643.
- Yancey, P.G., Rodriguez, W.V., Kilsdonk, E.P.C., Stoudt, G.W., Johnson, W.J., Phillips, M.C., Rothblat, G.H., 1996. Cellular cholesterol efflux mediated by cyclodextrins. Demonstration of kinetic pools and mechanism of efflux. *The Journal of Biological Chemistry* 271, 16026–16034.
- Zhao, Y., Marcel, Y.L., 1996. Serum albumin is a significant intermediate in cholesterol transfer between cells and lipoproteins. *Journal of Biochemistry* 35, 7174–7180.