Cholesterol Binding in *Schistocerca gregaria* and Insect Cell Lines

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Cholesterol binding affinities in different tissue homogenates of *Schistocerca gregaria* (Forskal) and insect cell line are reported. In the first set of experiment, binding could be observed in haemolymph, ovary, testis, fat body, midgut, mucin and serum proteins of both sexes of *Schistocerca gregaria*. The binding assays were conducted in the presence of labelled and unlabelled cholesterol. The total number of binding sites (Rt) are found to be $4.77 \times 10^{8}$ mole/L while extracted proteins from 1st and 2nd peaks show $7.10 \times 10^{9}$ and $12.8 \times 10^{9}$ mole/L. The binding sites in delipidated mucin have shown some higher binding ($2.97 \times 10^{8}$ mole/L) than the non-lipidated mucin ($1.38 \times 10^{8}$ mole/L). Insect cell lines Sf-9 and BmN cells have kd value of $1.494 \times 10^{8}$ and $4.08 \times 10^{8}$ mole/L in crude homogenate.

**Introduction**

After ingestion it is essential that sterols are absorbed by the gut wall to fulfill the sterol requirement of an insect. In isolated as well as in intact tissues, these sterols are certainly absorbed. In sterol absorption, it is essential that these sterols have a specific binding affinity with the tissue proteins. However, relatively little information is available on cholesterol binding affinity in insects.

The cholesterol binding specificity has been studied by many researchers in mammalian proteins. Nemeez and Schroeder studied the affinity of the recombinant fatty acid binding proteins from the rat liver. A fatty acid binding protein was isolated by Veerkamp *et al.*. However, binding specificity of JHBP *Manduca sexta* by monoclonal antibodies has been studied by Goodman *et al.*. Until now, four intracellular proteins which bind with lipophilic agents have been found in insects. Recently, from the midgut cytosol of *Manduca sexta* revealed the presence of two fatty acid binding proteins; MFB1 and MFB2. MFB1 has a single affinity binding site, with a dissociation constant < 100 mM. Recheri *et al.* studied, equilibrium constants for the binding of fatty acids with fatty acid binding proteins from adipocyte, intestine, heart and liver using a fluorescent probe. In the present investigation cholesterol binding affinity was calculated in the crude midgut homogenates as well as in the purified proteins. Affinity was also calculated for the ovary, testis, haemolymph, serum, fat body and insect cell line.

**Materials and Methods**

**Maintenance of Insect Cultures**

Insects used for this study were the locust, *Locusta migratoria* Linn and *Schistocerca gregaria*. Insects were reared under crowded conditions in aluminium cages (approximately $37 \times 37 \times 37$cm$^3$). The cages were provided with a 25 watt bulb and a photoperiod regime of 12L:12D was maintained. The temperature in the cages varied from 32 to 36 °C and the relative humidity was between 60-80 per cent. The insects were fed on a diet of green grass, leaves of maize (*Zea mays*), jai (*Avena sativa*) or jowar (*Sorghum vulgare*) in the laboratory. Adult locusts, of 8-15 days after the last moult were used for experiments.

**Chemicals**

Cholesterol $^3$H (Sp. Act. 7.45 Ci/mmol) was obtained from the Radiochemical Centre Amersham,
England. N'-2-hydroxymethylpariprazine-N'-2 hydroxethylpariprazine N'-2 ethane sulphonic acid (Hepes); tris (hydroxymethyl aminomethane (Tris)-HCl, N,N,N,N-dimethylaminoctetraacetic acid (EDTA), 2,5-diphenyloxazole (PPO), 1,4-bis(5-phenylloxazole-2YL) benzene (POPOP), N,N-methylene-bis-acrylamide, N,N-tetramethylenediamine (TEMED) and Sepharose CL 6B-200 were from Sigma Chemical Co, USA.

Culture Media and Cell Lines

Sterile TNMFH (Grace’s medium), RL 86 medium, cell lines SF-9 (Spodoptera frugiperda ovarian follicle cells), and BmN (Bombyx mori cells), cell culture flasks T-25, petridish 60mm and 90mm diameter (Corning, USA) were used for the cultures.

Seeding the Cells

Suspended cells were cultured in Grace’s medium supplemented with 5% of fetal calf serum (Sigma Co. USA) and a physiological dose of antibiotics from a gentamycin stock solution of 10 μg/mL and 10 μL of streptomycin 20 units/mL were made and added in culture. Cells were supplemented regularly with medium after every third day and half of the medium was changed for every subculture.

Assay for Radioactivity

The radioactivity was estimated by a Packard Tricarb Liquid Scintillation Spectrophotometer. The samples in scintillation vials were taken in triplicate in aliquots of fixed volume and added 5 mL toluene based scintillation fluid (5 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis(2-5-phenyloxazoyl) benzene (POPOP) and counted for 5 minutes each. Appropriate background corrections were made and the quenching correction was also done in the individual samples by channel ratio method.

Protein Estimation

Samples were prepared for protein estimation using ultracentrifugation and TCA precipitation method. For protein estimation, Lowry’s method was used.

Cholesterol Binding Assay

Cholesterol binding affinity was calculated using Scatchard Analysis. The affinity binding para-

meters included (i) R = Total receptor concentration, (ii) KD = Dissociation constant, (iii) KA = Association constant, (iv) N = Non-specific binding and (v) SB = Specific binding.

(I) Cholesterol binding assay with Spodoptera frugiperda ovarian follicle cells (SF-9) and Bombyx mori ovarian nuclear cells (BmN)

In vitro experiments were conducted with SF-9 cells and BmN cell homogenates. Cells were cultured in Grace’s medium supplemented with 5% FCS and physiological dose of antibiotics, 20 μL gentamycin (10 μg/μL) and amphotericin-B 10 μL (20 μg/μL) in T-25 flasks. Cell proliferation and confluency were observed routinely in every sub-culture and cells were harvested at the time of S-phase. Freshly harvested cells were centrifuged at 4°C at 1000 × g (Hermle BHG 360K) for 15 minutes and pellet was sonicated at 45 Hz for 3 min in ice chamber (Ultrasonic Instrument Co.). The freshly prepared crude cell homogenate was used for cholesterol binding assay.

Ligand Preparation for Cholesterol Binding Assay

(a) 3H-cholesterol (Sp. activity 7.45 Ci/mmol) was diluted by propylene glycol to prepare a solution of 36.2 × 10^6 mole/L concentration.

(b) The stock solution of unlabelled ligand was prepared by dissolving crystalline cholesterol in absolute alcohol to give concentrations of 1.81 × 10^6 mole/L, 1.81 × 10^6 mole/L and 1.81 × 10^6 mole/L. The working unlabelled ligand was prepared by further dilution of the stock solution to give 1.81 × 10^6 mole/L and 1.81 × 10^6 mole/L solutions. The following experiments were conducted:

(a) In first experiment, binding sites were saturated by incubating 100 μL of cell homogenate with increasing concentrations (36.2 × 10^6 to 36.2 × 10^6 mole/L) of labelled ligand in 1.5 mL Eppendorf tubes (Tarson) at 30°C for 2h. Total assay volume was maintained at 200 μL with PBS (pH 7.4) in each tube. After incubation tubes were centrifuged at 4°C for 30 minutes (Plasto Crafts Superspin R). Supernatant was removed and pellet was washed and rewarshed and centrifuged. Scintillation counting was done on a Beckman Model for 1 minute for each vial.

(b) For competitive binding unlabelled ligand was used in excess for saturating the 50% of the total
binding sites and tubes were preincubated for 30 minutes. In other experiments, unlabelled and labelled ligands were used in increasing and decreasing concentrations of ligand to determine the competitive binding.

**Gel Filtration Chromatography**

*Elution of the Gel Filtration Column and Estimation of Radioactivity*

Sepharose CL-6B-200 Column was used for the purification of cholesterol binding proteins. PBS buffer (pH 7.6, 50mM) was used for all the subsequent elutions. The column was held erect with a stand tightly holding with the clips. Flow rate of 22-24 mL/h, was maintained by using a peristaltic pump for a continuous buffer supply. Accessories were connected with a fraction collector (Pharmacia FRAC-100) and fractions were collected at fixed interval of time at a constant flow rate of 20-24 mL/h.

Single aliquot was taken for radioactive determination from each fraction and samples were mixed with 5 mL of scintillation cocktail. Samples were counted in a liquid scintillation counter (Aloka Model in USIC) for one minute each.

(II) In vivo and in vitro Binding of ³H-Cholesterol with Midgut Cholesterol Binding Proteins

**Preparation of Midgut Tissue Homogenate**

Adult male and female *Schistocerca* were maintained on cotton soaked with water for 24 h the locusts were dissected under cold PBS (pH 7.4, 50 mM) within 30 minutes after 24 h for collecting the tissue. Midgut tissue was washed thoroughly with cold buffer. The tissue was meshed and vortexed. The tissue was homogenized in Kontes hand homogenizer. The homogenate was sonicated in an ice chamber 50 Hz for 10 minutes (Ultrasonic Co.). Sonicated tissue was centrifuged at 1,20,000 x g for 45 minutes in a Sorvall A 1256 Rotor at 4°C. Supernatant was removed very carefully without disturbing the pellet and the volume was measured.

A known quantity of radiolabelled cholesterol was added to the supernatant and mixed. Supernatant was incubated for 1 h at 30°C with constant shaking. Proteins were estimated according to Lowry's method. From supernatant three aliquots of 10 μ each were taken for estimation of radioactivity.

**Study of ³H-Cholesterol Binding Affinity in Tissue Proteins in vitro**

Cytosolic preparations from midgut, ovary, testis and haemolymph were examined for characterization of binding sites using ³H-cholesterol. The binding sites were saturated in presence of increasing concentration of labelled ligand (36.2 x 10⁻¹⁰ mol/L - 3.62 x 10⁻⁸ mole/L). ³H cholesterol was incubated with 50 μL of cytosolic homogenate in a total volume of 200 μL. An incubation time of 60 min was used in all subsequent determinations of binding. The bound cholesterol was separated from the free cholesterol by using cold centrifuge at 25,000 g for 45 min.

The saturability of binding ³H cholesterol was explored by adding increasing concentrations of ³H-cholesterol to cytosolic preparations with an excess of unlabelled cholesterol added to the incubation tube. The specific binding was calculated by subtracting non-specific binding from total binding. Transformation of these data to a Scatchard plot yielded a straight line (Figure 1), demonstrating a single type of high affinity binding site. Dissociation
The dissociation constant was estimated using midgut cytosol preparations from adult Schistocerca gregaria (Table 1). An estimate from the slope of curve obtained from the binding data yielded an dissociation constant of about 1.64 x 10⁻⁷ (Figure 2a), thereby suggesting that protein present in the cytosol functions as a high affinity carrier in in vivo. Purified cholesterol binding proteins revealed two types of binding sites (a) high affinity binding site and (b) one low affinity binding site (Figures 2b and 2c). The receptor concentration was measured for both of the affinities by using the x-axis intercept. All affinity binding parameters calculated from the purified proteins are depicted in Table 1.

In order to study the saturation the mucin preincubated in excess of cholesterol and bile salts. There was higher saturation of the binding sites was observed in presence of cholesterol (1.38 x 10⁸ mole/L) than in presence the bile salts (1.24 x 10⁹ mole/L). Mucin showed a lesser affinity than the
Table 1 — Cholesterol binding affinity constants determined from the Scatchard plot analysis in tissues of Schistocerca gregaria, Sf-9 and BmN cells

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Total binding sites (Rt) × 10^9 mole/L</th>
<th>Dissociation constant (Kd) × 10^7 mole/L</th>
<th>Association constant (Ka) × 10^-7 mole/L</th>
<th>Non-specific binding (NB) × 10^-7 mole/L</th>
<th>Specific Binding (SB) × 10^-9 Mole/L</th>
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<tbody>
<tr>
<td>Midgut</td>
<td>4.77</td>
<td>1.64</td>
<td>0.60</td>
<td>203</td>
<td>2.52</td>
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<tr>
<td>Pool</td>
<td>0.71</td>
<td>15.6</td>
<td>6.4</td>
<td>0.166</td>
<td>0.47</td>
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<td>Pool++</td>
<td>1.28</td>
<td>44.8</td>
<td>2.23</td>
<td>0.292</td>
<td>0.854</td>
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<tr>
<td>Mucin*</td>
<td>2.97</td>
<td>32.8</td>
<td>0.30</td>
<td>0.121</td>
<td>1.71</td>
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<tr>
<td>Mucin**</td>
<td>1.38</td>
<td>62</td>
<td>1.61</td>
<td>1.38</td>
<td>2.79</td>
</tr>
<tr>
<td>Mucin***</td>
<td>0.124</td>
<td>44.0</td>
<td>2.27</td>
<td>1.06</td>
<td>1.86</td>
</tr>
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<td>Serum (M)</td>
<td>1.45</td>
<td>31.3</td>
<td>3.19</td>
<td>5.28</td>
<td>5.122</td>
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<tr>
<td>Serum (F)</td>
<td>0.83</td>
<td>33.0</td>
<td>3.02</td>
<td>3.493</td>
<td>3.540</td>
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<td>Fat body (M)</td>
<td>1.99</td>
<td>31.2</td>
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<td>Fat body (F)</td>
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<td>2.0</td>
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<td>Haem (M)</td>
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<td>8.3</td>
<td>12.1</td>
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<tr>
<td>Haem (F)</td>
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<td>4.750</td>
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<td>Testis</td>
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<tr>
<td>Ovary</td>
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<td>62.8</td>
<td>1.58</td>
<td>0.189</td>
<td>0.03</td>
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<td>Sf-9 cells</td>
<td>1.27</td>
<td>14.94</td>
<td>6.69</td>
<td>3.641</td>
<td>5.860</td>
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<tr>
<td>BmN cells</td>
<td>1.22</td>
<td>40.8</td>
<td>2.45</td>
<td>0.281</td>
<td>1.40</td>
</tr>
</tbody>
</table>

* Mucin was extracted in PBS, lyophilized, delipidated with acetone and dissolved in 5% NaCl.
** Mucin was extracted in presence of unlabelled cholesterol, lyophilized and dissolved in 1% Triton X-100.
*** Mucin was extracted in presence of bile salts 50µL sodium taurocholate (18.5 µmole), 50µL deoxycholate (24.0 µmole) and 50 µL glycylcholate (2 µmole).
M denotes Male, and F Female

midgut homogenate. Affinity difference against the association of cholesterol in midgut and midgut mucin was calculated from the assay.

Binding affinity and receptor concentration was also calculated for testis, ovary and haemolymph. The dissociation constant for all these tissues was estimated using cytosol preparations from the binding data. The concentration of cholesterol association with binding proteins obtained from the intercept Bmax (Rt) was estimated to be 0.60 × 10^7 L/mole.

Discussion

The dose dependent experiments (Table 1) showed that dissociation constant from the midgut supernatant and from the purified fractions gave similar incorporation in the presence of labelled ligand. This indirectly suggest that binding proteins are similar in both the assays. For the affinity binding assay, tissue proteins were saturated with increasing concentration of 3H cholesterol. Competitive binding of 3H cholesterol was studied in the presence of 100-fold excess of unlabelled ligand. Non specific binding amounted to about 20-35% of the total binding which also consists of a low affinity specific binding. When isolated midgut portions were incubated in the presence or absence of bile salts a thick mucous material was found secreted from the midgut wall. This insoluble material contained protein which probably function for the entrance of cholesterol in the epithelial cells¹. This was confirmed as a cholesterol carrier mucoprotein complex by Mayer et al.
The isolated midgut mucous protein when added to the cholesterol containing medium, a low affinity was obtained. This has been discussed here after. The delipidated mucin exhibited a greater binding of $^3$H-cholesterol than the mucin isolated in the presence of cholesterol as well as in the presence of bile salts. The $Kd$ value was calculated for the delipidated mucin (3.28 x 10$^4$ mole/L) showed a higher saturation of the binding sites in the presence of bile salts (1.24 x 10$^5$ mole/L) than the cholesterol (1.38 x 10$^4$ mole/L).

The dose dependent experiments showed that dissociation constant form supernatant and from purified fractions have similar values in the presence of radiolabel ligand. This indirectly suggests that the binding protein is same in both the assays. Competitive binding of $^3$H-cholesterol was studied in presence of 10-fold excess of unlabelled ligand. Non-specific binding was higher than the specific one, which is amount to about 40% of the total binding, consisting of both true and non-specific binding and a low affinity binding.

Recheri et al. studied binding affinity in mammalian system. equilibrium constants were measured at 37 °C for palmitate, stearate, oleate, linoleate, linolenate and arachidonic acid binding to six FABPs from intestine, heart and adipose and liver from different species. Equilibrium constants for each fatty acid were found to be extremely sensitive to the tissue of origin of the FABP but largely independent of species differences. The measured values of dissociation constants $Kd$ ranged from about 2 to 1000 nm, depending upon the tissue origin of the FABP and FA. L-FABP gave $Kd$ of 0.4 + 0.2) mm and 1.4 + 0.5 sites/monomer.

When purified unlabelled proteins (Figures 1a, 1b and 1c) of S. gregaria midgut were analyzed for affinity binding, the binding affinity from both of the regions gave dissociation constant ($Kd$) of 1.56 x 10$^8$-4.48 x 10$^8$ mole/L (Figures 2b and 2c). The presence of high molecular weight protein (= 32-50 kd) from the first region of protein elution gave higher binding than the second region. The association constant ($Ka$) from the first peak was higher 1.66 x 10$^9$ than the second peak 0.925 x 10$^9$ mole/L.

The affinity binding experiments may help in understanding the distinctive mechanism of luminal absorption of cholesterol and its loading at the cell surface for further transfer to other tissues as a bound ligand or a cell synthesized ligand protein. Present study provide evidence for a cytoplasmic binding protein in all the analysed tissues. In BmN cells the initial binding of cholesterol to membrane receptors has been ruled out. None the less, the BmN cell binding proteins can be presumed to be receptor because of its specific binding, and saturability. There are indications that cholesterol binding proteins serves as a specific carrier molecule in cells.

Cholesterol binding proteins in BmN cells have dissociation constant of 1.5 x 10$^8$ mole/L, association constant of 6.66 x 10$^7$ mole/L while the total receptor concentration is calculated 1.22 x 10$^8$ mole/L. Similarly cytosol of Drosophila Kc cells contains a protein that binds JH with specificity, and high affinity with a $Kd$ of 1.56 x 10$^8$ mole/L.

Affinity experiments showed that cholesterol bound in vitro to a low molecular weight protein. Other preliminary results have also demonstrated that in cholesterol fed insects in vivo also bind to a low molecular weight protein. It has already been shown that lipoproteins bind with lipids released by the fat body and these lipoproteins are same in different insects which has been addressed here and is the first report of its kind.

An analysis of Scatchard plot obtained for the binding of cholesterol to haemolymph proteins in the present study, showed that cholesterol bind specifically with haemolymph proteins. The affinity of haemolymph proteins was quite high. An apparent dissociation constant ($Kd$) of 1.428 x 10$^8$ mole/L was calculated for cholesterol binding. In plant feeding insects, conversion of phystosterol to cholesterol in the haemolymph cell before the release of sterol into the circulation medium may be necessary step in the transport of sterols as haemolymph proteins showed low affinity for cholesterol in the present study.

From the above facts it is clear that low affinity binding obtained in all the tissues in vitro which associated cholesterol is indeed due to the presence of cholesterol binding proteins. Essentially these proteins cause intracellular binding and subsequently deliver the cholesterol from the midgut lumen. In the midgut it is possible that the cholesterol is transferred spontaneously from the midgut to haemolymph as protein bound non-specifically. From cholesterol binding with the purified proteins of the midgut, and
insect cell lines receptors are present on the cell surface. Once cholesterol binds to the receptor with a weak affinity and the complex remains intact on solubilization and gets its way for transportation from one organ to another. This study needs to be carried further in order to show some other important facts.

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