The midgut epithelium is capable of basolateral absorption of radio-labelled diacylglycerol from the alimentary canal. However, other parts of the alimentary canal are also involved, e.g., the foregut larvae are able to absorb a large variety of fatty acids for transport of lipoprotein. It has been also reported that the anterior part of the midgut in *Diatraea grandiosella* transports cholesterol more rapidly than the middle and posterior regions of the midgut. The first evidence of cholesterol uptake by a lipophorin was observed in the silkworm *H. cynthia*7. It has been also reported that the anterior part of the midgut in *D. grandiosella* transports cholesterol more rapidly than the middle and posterior regions of the midgut. The midgut epithelium of *Aeshna cyanea* larvae is able to absorb a large variety of fatty acids which are mainly incorporated as triacylglycerols and temporarily deposited in the form of lipid droplets11-13. The midgut epithelium is capable of basolateral absorption of radio-labeled diacylglycerol from the haemolymph.14,15.

Cholesterol is primarily absorbed by the midgut in *Locusta migratoria*. However, as to how the ingested sterols enter into the hemolymph from the midgut lumen and get associated with HDLp is largely unknown. It has been suggested that in higher animals, sterols are synthesized by the gut and get incorporated into HDLp. Certain reports suggest that DNA binding proteins regulate cholesterol uptake.16. Dantuma et al.17 suggested a receptor mediated uptake of lipoprotein ligand without requirement of internalization for transport of lipoprotein. Inspite of the extensive information available on fatty acid binding proteins in insects and mammals, cholesterol binding by a protein in insects is comparatively unknown.

In midgut tissue, the labelled fatty acids get incorporated into phosphatidic acid, diacylglycerol and triacylglycerol which subsequently get incorporated in the hemolymph lipophorin.18. Two models of lipolysis of triacylglycerol (TAG) have been suggested to occur in the midgut.19,20

The present study has been undertaken to answer the (i) transport of cholesterol from midgut to hemolymph and reproductive organs (ii) involvement of protein(s), its/their biosynthesis in the midgut tissue and role in cholesterol transport. Transport of radioactive cholesterol from midgut to hemolymph, synthesis of proteins in the gut and their role in chole-

### Protein mediated cholesterol absorption in locusts

*Schistocerca gregaria* (Forskal) and *Locusta migratoria* (Linn)

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Absorption and transport of 3H cholesterol from the midgut to hemolymph and other tissues was studied in the locusts *Schistocerca gregaria* and *Locusta migratoria*. *S. gregaria* are able to absorb dietary cholesterol in the midgut and release into the hemolymph *in vivo* and into the incubation medium *in vitro*. Certain proteins of midgut origin are involved in the absorption and release of cholesterol. The proteins designated as cholesterol binding proteins (CBP s) were fractionated by gel filtration chromatography using Sepharose CL-6B-200 column. Presence of a protein and its binding with cholesterol is confirmed by TCA precipitation after subsequent incubation of midgut in the incubation medium. Cholesterol binding with the proteins was also confirmed in native polyacrylamide gel electrophoresis. Biosynthesis of this protein takes place in the midgut which is inhibited by a protein synthesis inhibitor, cycloheximide. It also inhibits absorption and release of cholesterol from the midgut. The cholesterol binding activity was associated with a peak containing proteins ranging from molecular weights of 17-32 kDa in SDS-PAGE gels. Treatment of midgut with cycloheximide resulted in reduced cholesterol binding activity. Dilipidation of mucin and transport in presence of bile salts yielded a higher cholesterol binding activity. Although the absorption and release of cholesterol was observed in the hemolymph of both sexes, the ovary exhibited higher cholesterol binding as compared to testes.

Insects are unable to synthesize sterols, especially cholesterol *de novo* and rely exclusively on the diet for this compound.1-3. The main site of cholesterol absorption is the midgut, although other parts of the alimentary canal are also involved, e.g., the foregut (especially the crop) of *Gryllodes sigillatus, Camponotus compressus* and *Cylbister* sp., and the midgut caeca of *HeiroglYPHus nigrorepleTus*. It has been also reported that the anterior part of the midgut in *Diatraea grandiosella* transports cholesterol more rapidly than the middle and posterior regions of the midgut. The first evidence of cholesterol uptake by a lipophorin was observed in the silkworm *Philosamia cynthia*. In insects, dietary lipids are processed and absorbed in the midgut.8,10. The midgut epithelium of *Aeshna cyanea* larvae is able to absorb a large variety of fatty acids which are mainly incorporated as triacylglycerols and temporarily deposited in the form of lipid droplets.11-13. The midgut epithelium is capable of basolateral absorption of radio-labeled diacylglycerol from the haemolymph.14,15.
sterol binding was studied, in vitro. Cholesterol binding sites were characterized by assaying the tissue preparations from *Schistocerca gregaria* by gel filtration chromatography and direct centrifugation binding assays.

**Materials and Methods**

*Insects*—Locusts (*Schistocerca gregaria* (Forskål) and *Locusta migratoria* (Linn)) fed on leaves of maize (*Zea mays*) and/or *jai* (*Avena sativa*) or *jowar* (*Sorghum vulgare*) at 27±3°C, 40-50% RH with a photoperiod of 12L:12D were used. Adult male and female locusts (10-14 days old) were used. Experimental insects were starved for 24 hr and provided only water from wet cotton swabs in order to empty their gut.

*Chemicals*—*3H* Cholesterol (Sp. act. 7.45 Ci/mmol), and *35*Cl (Sp. act. 5Ci/mmol), *L*-phenyl (*2,3-*3H) alanine were obtained from Radiochemical Centre, Amersham, England while cycloheximide, N-(2-hydroxyethyl) piperazine-N'-2 ethanesulphonic acid (Hepes), Tris (hydroxy methyl amino methane, (Tris) HCl, ethylenediamine tetra acetic acid (EDTA), 2,5-diphenyloxazole (POPOP), 1,4 bis-2-(5-phenyl oxazolyl)-benzene (POPOP), N.N, methylbisacrylamide, N,N,N,N Tetramethylenediamine (TEMED), and Sepharose CL-6B-200 were procured from Sigma Chemical Co. U.S.A.

*Feeding procedure*—Each locust was individually placed under a funnel at room temperature and fed on small pieces (10mm×5mm) of maize leaves coated with a known quantity of *3H* cholesterol for 15 min. Unfed insects i.e. those not eating the whole piece of leaf were excluded from the observations. Corresponding control insects were provided fresh leaves without any treatment.

*Collection of hemolymph*—Hemolymph was collected using a Hamilton microsyringe (50 µl) by piercing ventral membrane between the head and thorax following the method described by Van der Horst et al. Hemolymph from individual insects was pooled and an equal volume of ice cold buffer (130 mM NaCl, 5 mM Tris HCl, 1.9 mM Na2HPO4, 10 mM EDTA, pH 7.5) was added and centrifuged for 2 min at 15,000 g to remove the hemocytes.

*Incubation of midgut*—Incubations were carried out in glass tubes containing 2 ml Heps at 30°C in a water bath. The rectum from the alimentary canal preparation was pulled slightly through the eye of the preparation hook. The head was placed above the eye of the hook, and the rectum hanged on the hook. This method facilitated the exposure of the entire midgut and gastric caeca to the incubation medium while the rectum and head were kept out. After a 2 hr incubation period, the medium was replaced by 2 ml fresh Heps supplemented with 5 mM EDTA and incubated for another 2 hr. At the end of incubation, the alimentary canal was removed and the medium centrifuged for 2 min at 9,000 g. Data were used only from those midgut incubations which the midgut remained intact as examined microscopically.

*Ultrasound centrifugation of midgut incubation medium and hemolymph*—The hemolymph and concentrated incubation medium were centrifuged in a 37 ml tube bearing 15 ml KBr (SD:1.3000) and overlaid with 0.9% NaCl. After centrifuging for 3.6 hrs at 49,000 rpm at 4°C, the samples were fractionated from bottom to top into 0.5 ml aliquots by an Ultratec fraction collector (LKB). Density and radioactivity of each samples were recorded.

*Preparation of midgut homogenate*—Adult locusts (*S. gregaria*) kept on water were dissected in cold PBS (pH 7.4) and their midgut isolated after dorsal incision on the exoskeleton just above the abdomen under conditions of sterility. The malpighian tubules were carefully removed and the midgut separated from hindguts. The midgut was incised in cold PBS along its length to clean out the lumen by repetitive rinsing (5 times) with buffer in cold PBS.

Membrane preparations were made at 4°C. Weighed tissue was homogenized in a Vertis glass-glass homogenizer. Homogenate was suspended in a known quantity of cold PBS and sonicated in the cold chamber at 50 Hz for 10 min and then centrifuged at 100,000 g for 45 min at 4°C. The membrane preparations thus obtained had 500-1000 µg/g protein and were used immediately for affinity binding experiments. A similar procedure was followed for gel filtration chromatography. Protein estimation was carried out according to Lowry et al. using BSA as standard and by BCA method. Incubation media from the locusts not treated with cycloheximide that showed incorporation of *3H* amino acids were pooled and dialysed to remove free amino acids and concentrated by dialysing against 1M sucrose. The samples were analyzed for presence of radioactivity on 3-30% PAGE gel.

*Electrophoresis*—Samples were analyzed on native PAGE and SDS PAGE gels and stained with Coomassie Blue. Molecular weights were calculated in relation to the migration by the proteins.
Determination of radioactivity—Radioactivity was estimated according to Tishler and Epstein. Aliquots of various fractions were counted for radioactivity on a Liquid Scintillation Spectrophotometer using a Packard Tri-Carb Model 4550.

Uptake of $^3$H cholesterol in vivo—Three adult locusts L. migratoria of each sex were given access to wet cotton swabs for 24 hr and then fed on leaves of A. sativa coated with 5 μCi of $^3$H cholesterol. Subsequently they were fed on plant diet for the next 24 hr. Hemolymph (5 μl) was withdrawn by a Hamilton microsyringe and used for estimation of radioactivity. Hemolymph proteins of adult females were analyzed on a 4-15% native PAGE to study the distribution of radioactivity.

Uptake of $^3$H cholesterol in vitro—Weighed tissue from L. migratoria was kept in 1000 μl of incubation medium (50 μl haemolymph: 950 μl PBS, pH 7.65) at 30°C for 2 hr with constant shaking. Subsequently the tissue was taken out and washed twice with cold PBS and homogenized in 200 μl buffer. Three aliquots of 10 μl each were assayed for radioactivity after incubation.

Aqueous samples of tissues analyzed were added to the scintillation fluid in a 5 ml vial. Radioactivity was estimated by counting these samples for 5 min in a Packard Tricarb Liquid Scintillation Spectrophotometer (Model 4550) using channel ratio method.

Absorption of cholesterol and its release in hemolymph—The following experiments involving $^3$H cholesterol were conducted: (a) 10 locusts S. gregaria were fed grass, coated with 3 μCi of radioactive cholesterol. Locusts were bled 16 hr later and 500 μl of hemolymph collected. The hemolymph was subjected to potassium bromide (1:3000) density gradient centrifugation. A yellow band was observed below the upper surface. The supernatant was withdrawn from the bottom of the tube by a peristaltic pump at 5 min intervals. Each fraction was analyzed for radioactivity and protein content; (b) 6 locusts S. gregaria were fed grass coated with 3 μCi of $^3$H cholesterol. The alimentary canal was removed 2 hr later and the midgut incubated at 37°C in 2 ml of Heps medium (pH 7). Radioactivity was recovered in the incubation medium after 2 hrs. A portion (20 μl) was treated with TCA and centrifuged. TCA precipitate was washed and dissolved in NaOH and the radioactivity estimated for the TCA supernatant and precipitate; and (c) in this experiment, 12 locusts, S. gregaria were used, 6 of which were fed 3 μCi of $^3$H cholesterol and midgut was excised 2 hr post feeding and incubated. The guts of remaining six insects were removed after 4 hrs post treatment and incubated. Incubations were carried out for 90 minutes in Heps medium with or without hemolymph.

Inhibitory effect of cycloheximide on incorporation of $^3$H amino acid in lipoproteins—The insects were subjected to the following treatments: (a) 3 locusts were injected 10 μl of (20 μg/μl) cycloheximide while the corresponding controls were injected 10 μl of distilled water. After 30 min thereafter, each locust was injected 3 μl (2 μCi) of $^3$H amino acid mixture and hemolymph collected after 1 hr. Hemolymph was diluted with buffer and an equal amount of 10% TCA was added. The solution was mixed thoroughly and centrifuged. Radioactivity was measured both in the TCA precipitate and supernatant. The precipitate was suspended in 1 ml of 5% TCA, sonicated and centrifuged. Radioactivity was estimated in both the precipitate and wash; (b) amino acid mixture (5 μCi) was fed to each of the 10 locusts used, 5 of which were fed a 100 μg cycloheximide 30 min before dissection. After 2 hr, hemolymph was drawn out from treated and untreated locusts. The alimentary canal was dissected and the midgut incubated in 2 ml Heps medium for 2 hr at 30°C in presence of 5 μg/ml of cycloheximide (=10 μg/locust) and the hemolymph and incubation media were processed for estimation of radioactivity as in (a). (c) 5 locusts were fed 5 μCi of $^3$H amino acid per locust and their alimentary canals removed. Only the midgut portion was placed in 2 ml Heps buffer for 90 min. The incubation medium was centrifuged and the radioactivity determined as above.

Inhibition of transport of cholesterol by cycloheximide—10 locusts were fed 5 μCi of radiolabelled cholesterol in the diet. After 90 minutes of feeding, 5 of these were fed leaves coated with 100 μg cycloheximide. Locusts were bled in 0.5 ml Heps buffer two hr later and 10 μl hemolymph collected. In another parallel experiment, the cholesterol fed locusts were also injected 100 μg cycloheximide in 5 μl water while corresponding controls were injected 5 μl distilled water. The midgut portion of the alimentary canal of the treated locusts was incubated for 3 hr in 2 ml Heps medium containing 10 μg cycloheximide at 30°C each and with out cycloheximide in case of controls.

Uptake and release of $^3$H cholesterol by midgut of L. migratoria—Locusts L. migratoria were starved...
for 24 hr and fed 5 µCi of \(^{1}H\) cholesterol with the diet. Six hr after feeding, maximum possible hemolymph was collected. Thereafter, the alimentary canal was removed and the midgut isolated for subsequent processing. Two sets of incubations were set-up. In one case, the weighed midgut which contained \(^{1}H\) cholesterol, was incubated in medium consisting of 50 µl unlabelled hemolymph from other locusts and 450 µl PBS buffer. In the second case, unlabelled weighed midgut isolated from other locusts was incubated in a medium consisting of 450 µl PBS buffer and 50 µl labelled hemolymph from locusts fed \(^{1}H\) cholesterol. Incubations were carried out at 30°C for 8 hr. Samples 5 µl were withdrawn from each incubation every 2 hr until 8 hr. At the end of incubation, the midgut was homogenized and radioactivity in all the samples of the media were removed.

Purification of cholesterol binding proteins from \textit{S. gregaria} midgut

\textbf{(i) Elution —} \(^{3}H\) cholesterol was subjected to gel filtration chromatography on Sepharose CL-6B-200 using PBS for elution. \(^{3}H\) cholesterol formed high molecular weight micelles, which were eluted after void volume. Radioactivity was estimated within micelles by passing 250 µl of the fraction through a 0.45µm Millipore filter.

\textbf{(ii) Solubilization —} Test tissue was excised and homogenized in following additives 6 hr after feeding two locusts on leaves coated with 5 µCi of \(^{3}H\) cholesterol; (i) Tris (200 µl of 0.01M) plus EDTA buffer (pH 8) and (ii) Triton X-100 (200 µl of 0.10%). Samples in both the additives were spun at 1,24,000 g for 45 min and the supernatant removed. The pellet was resolubilized in 100 µl of buffer and centrifuged for 15 min. The supernatant was collected in 5 µl aliquots which were used to determine radioactivity.

\textbf{(iii) Midgut binding —} The following experiments were conducted: (a) Cholesterol binding with midgut proteins: cytosolic preparation from midgut, ovary, testis and hemolymph were saturated in presence of an increasing concentration of labelled ligand \(^{1}H\) cholesterol). cytosolic homogenate (50 µl from midgut of \textit{Schistocerca gregaria} was incubated with \(^{1}H\) cholesterol in a total volume of 200 µl. Subsequent binding were allowed for 60 min, the bound cholesterol was separated from free cholesterol by centrifugation at 25,000 g at 4°C for 45 min. (b) the binding activity of midgut mucin was studied by isolating it from the midgut which was subsequently lyophilized and solubilized in different additives in Triton X-100. In order to further maximize the saturation, mucin was preincubated with an excess of cholesterol and bile salts. The binding characteristics of testis, ovary and hemolymph were also calculated. Unlabelled midgut proteins were isolated from midgut of both sexes. The proteins were eluted through gel filtration column and pooled fractions used for cholesterol binding assays. Varying amounts of \(^{1}H\) cholesterol ranging between 3.62\times10^{-9} - 3.62\times10^{-7} mol/l were incubated with 100 µl of pooled fractions.

The saturability of binding was obtained by adding increasing concentrations of \(^{1}H\) cholesterol to cytosolic preparations with an excess of unlabelled cholesterol during incubation. The specific binding was calculated by subtracting non-specific binding from total binding.

\textbf{(iv) Cholesterol binding assays —} Cholesterol was diluted with propylene glycol to prepare a solution of 36.2\times10^{-10} mol/l. The stock of unlabelled ligand was prepared by dissolving crystalline cholesterol in absolute alcohol to give a concentration of 1.81\times10^{-5} - 0.4 mol/l. The following two experiments were conducted: (a) binding sites were estimated by incubating a 100 µl of test homogenate with increasing concentrations (36.2\times10^{-10} mol/l - 3.62\times10^{-7} mol/l) of labelled ligand (cholesterol) which were incubated in 1.5 ml eppendorf tubes (Taron, India) at 37°C for 2 hours. The total assay volume in each tube was maintained at 200 µl with cold PBS (pH 7.4). After incubation, the tubes were centrifuged (Plastocraft Superspin, R, India) at 4°C for 30 min. The pellet was repeatedly washed at 4°C. Scintillation count of each vial was performed for 1 min on a Beckman Model (1989); (b) competitive binding assays were conducted using an excess of unlabelled cholesterol in order to saturate 50% of the binding sites present in the cell homogenate. Incubations were carried out for 30 min while maintaining other test conditions similar to the experiment mentioned earlier.

Cholesterol binding was calculated by Scatchard analysis (Scatchard, 1969). The parameters calculated were : total receptor concentration (Rt), dissociation constant (Kd), association constant (Ka), non-specific binding (Ns) and specific binding (Bs).

\textbf{(v) Gel filtration chromatography —} The fresh midgut membrane preparations were ultracentrifuged at 100,000 g (Sorvall-Ultra) for 45 min at 4°C. The supernatant was decanted in a separate tube. After determining the presence of proteins, a known quan-
buffer (pH 7.4) at 4°C. Fractions were collected at 15 min. intervals (4.6 ml) on a fraction collector (FRAC-100, Pharmacia).

Protein estimation of the eluted fractions were carried out as per Lowry et al. with absorbance measured at 280 nm. Radioactivity was determined for each fraction. For confirmation of the binding of 3H cholesterol with proteins, homogeneity of the experiment was maintained and specific activity of the tube confirmed in both the experiments. When radioactivity was observed in more than one fraction, they were pooled and assayed for cholesterol binding affinity to finally confirm the binding distribution of the radio-label. The supernatant proteins were subjected to gel electrophoresis (4.25% native) and molecular weights calculated.

(vi) Fractionation of cholesterol binding proteins—Starved locusts fed on 14C cholesterol were subsequently fed the plant diet. After 16 hr, the midguts were dissected, homogenized in cold PBS, sonicated and subsequently centrifuged. The supernatant was loaded on a gel filtration column and eluted with PBS buffer and absorbance at 280 nm noted and values compared with those obtained for proteins by Lowry’s method.22

(vii) Determination of molecular weights—Mixture of standard proteins were loaded on a gel filtration column. The proteins in the fractions eluted were estimated as in (vi) and elution volumes plotted against their log molecular weights.

Results

Uptake of 3H cholesterol by the locust, L. migratoria—Uptake of cholesterol by the hemolymph of adult L. migratoria was observed to be much higher (74.2%) in male than the female hemolymph when fed 3H cholesterol. However, the uptake by ovary on the basis of per mg weight was much higher (92%) than the testis. A very little difference in uptake was observed between the male and female fat bodies (Table 1).

Absorption of dietary cholesterol and its release in hemolymph—The density gradient ultracentrifugation of locust hemolymph fed S. gregaria 3H cholesterol showed two peaks of proteins, the first being broad and the second, a narrow peak (fractions 13-16) which coincided with a yellow protein band just below the surface. The radioactivity peak (3H cholesterol) coincided with the second narrow peak (Fig. 1) suggesting that cholesterol was bound to the first (3H cholesterol) to certain hemolymph protein. Also midguts of 3H cholesterol fed locusts S. gregaria incubated in Heps buffer reveal higher radioactivity in insects from which the guts were excised 2 hr post feeding (Table 2). The bulk of activity was observed in TCA precipitate dissolved in NaOH representing 4.64% of the 3H cholesterol given to each locust in the diet.

Inhibitory effect of cycloheximide on incorporation of 3H amino acids in proteins—When 3H amino acid mixture was injected in presence or absence of cycloheximide, the uptake was inhibited up to 50% in both sexes, indicating that the uptake of dietary cholesterol by the insects might be linked to de novo synthesis.

Table 1—Relative distribution of radio label in different tissues in vivo after feeding Jai leaves coated with cholesterol

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Tissues</th>
<th>Relative Dpm/mg</th>
<th>Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Hemolymph (Male)</td>
<td>3558 ± 1015</td>
<td>74.2</td>
</tr>
<tr>
<td>B.</td>
<td>Hemolymph (Female)</td>
<td>1238 ± 115.2</td>
<td>25.8</td>
</tr>
<tr>
<td>C.</td>
<td>Fat body (Male)</td>
<td>73.3 ± 7.78</td>
<td>54.3</td>
</tr>
<tr>
<td>D.</td>
<td>Fat body (Female)</td>
<td>61.6 ± 11.41</td>
<td>45.7</td>
</tr>
<tr>
<td>E.</td>
<td>Testis</td>
<td>49.2 ± 12.97</td>
<td>7.9</td>
</tr>
<tr>
<td>F.</td>
<td>Ovary</td>
<td>567.7 ± 7.84</td>
<td>92.0</td>
</tr>
</tbody>
</table>

Table 2—Absorption and release of 3H cholesterol from midgut of L. migratoria in Heps buffer

<table>
<thead>
<tr>
<th>Experiment (hr)</th>
<th>Incubation buffer</th>
<th>Radioactivity (dpm)</th>
<th>TCA precipitate dissolved in 0.1 N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 2</td>
<td>306360 ± 68027</td>
<td>1419 ± 140</td>
<td>21182 ± 1846</td>
</tr>
<tr>
<td>B 2</td>
<td>149883 ± 51313</td>
<td>585 ± 10</td>
<td>22030 ± 2822</td>
</tr>
<tr>
<td>C 4</td>
<td>71997 ± 26955</td>
<td>599 ± 30</td>
<td>10783 ± 1589</td>
</tr>
</tbody>
</table>

Locusts fed green grass coated with 3 μ mc 3H cholesterol. Alimentary canal removed 2 hr later and incubated in Heps buffer at 30°C.
heximide, a high amount of radioactivity was detected in the hemolymph of locusts (S. gregaria) although most of it was found in the TCA supernatant (Table 3). Only 3.3% of $^3$H amino acid was present in the TCA precipitate in controls while it was 1.08% in locusts treated with cycloheximide. In locusts fed $^3$H amino acid mixture, with or with out cycloheximide, a high level of radioactivity in the hemolymph was detected (Table 3). However, when it was treated with TCA, the label found in the TCA precipitate was only 1793 and 907 dpm in the control and treated groups respectively. Following incubation of the midgut portion, the incubation medium retained high levels of radioactivity. However, bulk of this $^3$H was present in the TCA supernatant and only 4.38% was found in the TCA precipitate in controls which decreased to 2.33% in cycloheximide treated insects (Table 4).

### Inhibitory effect of cycloheximide on release of cholesterol in vivo and in vitro

The hemolymph of the locusts (S. gregaria) fed $^3$H cholesterol and cycloheximide contained radioactivity which was almost 1/3rd of the amount found in the hemolymph of control insects (Table 5). Dialysis of the pooled media in which midgut proteins were incubated showed a 50% inhibition in the release of cholesterol (Table 5).

### Temporal transfer of $^3$H cholesterol by midgut

When midguts from locusts without any $^3$H cholesterol were incubated in media containing hemolymph from locusts fed $^3$H cholesterol, the data obtained showed that cholesterol from the incubation media was taken up by the midgut as the radioactivity in the media declined with time and the midgut contained 1581 dpm/mg after 8 hr incubation (Table 6). In case where midguts from locusts fed $^3$H cholesterol were incubated in PBS containing hemolymph from control locusts a high amount of radioactivity was released into the incubation media. At the end of 8 hr incubation the midgut contained 15293 dpm/mg of radioactivity thereby showing release of cholesterol into the incubation media.

### Cholesterol binding with tissue proteins of S. gregaria

Cytosolic preparations from midgut, ovary, testis and hemolymph of S. gregaria were examined for characterization of binding sites using increasing concentrations of the labelled ligand ($^3$H cholesterol) in presence of excess of unlabelled cholesterol. Transformation of these data to a Scatchard plot yielded a straight line demonstrating low binding and showed a dissociation constant of $1.64 \times 10^{-7}$ mol/l in case of midgut (Table 7). Delipidated mucin showed a higher binding i.e. $2.97 \times 10^{-8}$. Binding activities for mucin were $1.38 \times 10^{-8}$, and $1.24 \times 10^{-9}$ mol/l in the presence of unlabelled cholesterol and bile salts respectively. In testis, ovary and hemolymph, the values were $1.84 \times 10^{-8}$, $3.0 \times 10^{-8}$ and $1.23 \times 10^{-8}$ mol/l respectively.

### Table 3—Radioactivity released in the hemolymph of male locusts given $^3$H amino acid mixture and cycloheximide

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>Radioactivity (dpm) present in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TCA supernatant</td>
</tr>
<tr>
<td>A.</td>
<td>Control</td>
<td>221760 ± 15215</td>
</tr>
<tr>
<td>B.</td>
<td>Test*</td>
<td>225480 ± 21356</td>
</tr>
</tbody>
</table>

*Injected 10 μl (20μg/μl) cycloheximide/ water in controls

### Table 4—Incorporation of dietary $^3$H amino acid mixture in hemolymph proteins and release from midgut in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>hemolymph (dpm/10 μl)</th>
<th>TCA precipitate in 0.1 N NaOH</th>
<th>Medium (total)</th>
<th>TCA supernatant</th>
<th>TCA precipitate in 0.1 N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>48462 ± 4129</td>
<td>1793 ± 727</td>
<td>476928 ± 63044</td>
<td>126024 ± 15768</td>
<td>20912 ± 1270</td>
</tr>
<tr>
<td>Treated†</td>
<td>62939 ± 12442</td>
<td>907 ± 177</td>
<td>488344 ± 52212</td>
<td>125398 ± 12606</td>
<td>11376 ± 1840</td>
</tr>
</tbody>
</table>

†Injected 10 μl (20μg/μl) cycloheximide/ water in controls

### Table 5—Inhibitory effect of cycloheximide on release of dietary $^3$H cholesterol in hemolymph and by the midgut in incubation media

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19560 ± 8320</td>
</tr>
<tr>
<td>Treated†</td>
<td>7333 ± 1168</td>
</tr>
</tbody>
</table>
Purification of cholesterol binding proteins

(i) Gel filtration chromatography of \(^3\)H cholesterol: \(^3\)H cholesterol was subjected to gel filtration chromatography on Sepharose CL-6B-200 column using PBS buffer for elution. A single peak of radioactivity was obtained immediately after void volume between 133-165 ml of effluent as it formed high molecular weight micelles (Fig. 2).

(ii) Solubilization of cholesterol binding proteins in different additives: Tissues from locusts fed \(^3\)H cholesterol were homogenized in different additives. 0.1% Triton X-100 proved to be a good solubilizing agent of proteins from fat body, ovary and midgut tissues than EDTA + Tris buffer. A higher radioactivity was observed in the supernatant than in the resi-

Table 6 — Uptake and release of \(^3\)H cholesterol by the midgut of locusts\(^a\) from the incubation medium

<table>
<thead>
<tr>
<th>Duration of incubation (hr)</th>
<th>Uptake (dpm)</th>
<th>Release (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Midgut (dpm/mg)</td>
<td>Medium (dpm/mg)</td>
</tr>
<tr>
<td>2</td>
<td>3086 ± 26.51</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>2751 ± 177.4</td>
<td>5.5</td>
</tr>
<tr>
<td>6</td>
<td>2469 ± 178</td>
<td>5.0</td>
</tr>
<tr>
<td>8</td>
<td>1581 ± 62</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\(^a\)Locusts starved for 24 hrs and maintained on wet cotton swabs. Half of these were fed on 5 \(\mu\)Ci \(^3\)H cholesterol each and the rest were controls. Hemolymph was drawn out and midguts removed and weighed.

Table 7 — Binding characteristics of tissue proteins of *Schistocerca gregaria* and BmN cells with \(^3\)H cholesterol

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Total sites (Rt) Mol/L</th>
<th>Dissociation constant (Kd) mol/L</th>
<th>Association constant (Ka) mol/L</th>
<th>Non specific binding (Ns) mol/L</th>
<th>Specific binding (Sb) mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Midgut</td>
<td>4.77 x 10^8</td>
<td>6.4 x 10^7</td>
<td>2.03 x 10^9</td>
<td>2.52 x 10^9</td>
<td></td>
</tr>
<tr>
<td>B Pool ++</td>
<td>7.70 x 10^9</td>
<td>1.56 x 10^5</td>
<td>1.66 x 10^6</td>
<td>0.47 x 10^6</td>
<td></td>
</tr>
<tr>
<td>C Pool ++</td>
<td>12.8 x 10^8</td>
<td>4.48 x 10^8</td>
<td>6.22 x 10^7</td>
<td>8.54 x 10^7</td>
<td></td>
</tr>
<tr>
<td>D Mucin **</td>
<td>3.07 x 10^8</td>
<td>0.50 x 10^1</td>
<td>1.21 x 10^5</td>
<td>7.11 x 10^6</td>
<td></td>
</tr>
<tr>
<td>E Mucin ***</td>
<td>1.38 x 10^8</td>
<td>6.20 x 10^-4</td>
<td>1.38 x 10^6</td>
<td>2.79 x 10^7</td>
<td></td>
</tr>
<tr>
<td>F Mucin ***</td>
<td>1.24 x 10^9</td>
<td>4.40 x 10^-4</td>
<td>1.06 x 10^6</td>
<td>0.86 x 10^6</td>
<td></td>
</tr>
<tr>
<td>G Testis</td>
<td>1.84 x 10^8</td>
<td>1.73 x 10^-7</td>
<td>1.38 x 10^10</td>
<td>1.86 x 10^9</td>
<td></td>
</tr>
<tr>
<td>H Ovary</td>
<td>3.00 x 10^-8</td>
<td>6.29 x 10^-9</td>
<td>1.89 x 10^9</td>
<td>5.03 x 10^9</td>
<td></td>
</tr>
<tr>
<td>I Haemolymph</td>
<td>1.23 x 10^-6</td>
<td>0.83 x 10^-5</td>
<td>0.67 x 10^9</td>
<td>1.89 x 10^9</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Purified protein fractions (56-80) from the gel filtration chromatography from the previous experiment (Fig. 3)

\(^{++}\)Purified fractions (81-111) from the gel filtration chromatography from the same experiment.

**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 5% Triton X-100
due. Midgut proteins were less solubilized than those of fat body and ovary (Table 8).

(iii) Fractionation of cholesterol binding proteins from midgut: The midgut of locusts fed 3H-cholesterol were homogenized in PBS, sonicated centrifuged and the supernatant subjected to gel filtration on Sepharose CL-6B-200 column. The elution pattern at 280 nm showed two peaks one soon after void volume (fractions 39-51) and the second in fractions 91-113 (Fig. 3a). Two peaks observed coincided with the radioactivity whereby the first peak contained high level of 3H (Fig. 3b).

(iv) Binding of midgut proteins with 3H cholesterol: The midgut cytosolic preparations from control locusts were chromatographed as above on Sepharose CL-6B column which gave two protein peaks as

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Solubilization Buffer</th>
<th>Supernatant dpm</th>
<th>residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat body</td>
<td>EDTA + Tris</td>
<td>3343 (85%) ± 17.2</td>
<td>583 (14.8%)</td>
</tr>
<tr>
<td>Fat body</td>
<td>0.1% Triton X-100</td>
<td>13300 (63.5%) ± 397.2</td>
<td>7654 (36.5%)</td>
</tr>
<tr>
<td>Ovary</td>
<td>EDTA + Tris</td>
<td>5312 (82.19%) ± 37</td>
<td>4618 (34.6%)</td>
</tr>
<tr>
<td>Midgut</td>
<td>EDTA + Tris</td>
<td>163392 (36.18%) ± 743.8</td>
<td>284109 (63.8%)</td>
</tr>
<tr>
<td>Midgut</td>
<td>0.1% Triton X-100</td>
<td>166612 (37.6%) ± 335.1</td>
<td>275942 (62.3%)</td>
</tr>
</tbody>
</table>
above. Pooled fractions were used for cholesterol binding assays. Dissociation constants $1.56 \times 10^{-8}$ and $4.48 \times 10^{-8}$ M/L were calculated for the two peaks respectively (Fig. 4; Table 7).

(v) Determination of molecular weights: Molecular weights of CoBP (cholesterol binding protein) in peak one were estimated to be 17-32 kDa by plotting elution volume against log molecular weights (Fig. 5). The peak one proteins were subjected to SDS-gel electrophoresis which gave only one sharp band (Fig. 6).

Discussion

Absorption of dietary cholesterol in the alimentary canal of insects has been reported and the primary site of its absorption is the midgut25. The present study further confirms this absorption as dietary $^3$H cholesterol was detected in the locust hemolymph. The involvement of midgut in this absorption was shown by the release of dietary cholesterol by the midgut into the incubation media. It is also indicated that cholesterol binds with midgut tissue. Komnick12 reported binding of lipids with midgut epithelium in Aeshna cyanea. A higher uptake of $^3$H cholesterol by the ovary on per unit weight basis could probably be attributed to a higher sequestration of cholesterol for vitellogenesis in the ovary or transfer from the fat body apart from the additional requirement by the developing oocytes.

Dietary labelled amino acids were incorporated in hemolymph proteins and when the midgut portions containing amino acids were incubated in incubation media labelled proteins were detected in the media suggesting that proteins are synthesized in the midgut. The midgut proteins are known to be involved in the delivery of dietary cholesterol26,27 in the hemolymph after its assimilation inside the lumen10. Further, protein biosynthesis is inhibited by the protein synthesis inhibitor, cycloheximide23. This further suggests that inhibition of protein synthesis adversely affects cholesterol absorption and release. Fatty acid binding proteins have been isolated from the midgut of M. Sexta29.

The $^3$H cholesterol released in the hemolymph in vivo as well as the $^3$H cholesterol released from the midgut in vitro is bound to lipoprotein as shown by the radioactivity being present exclusively in the TCA precipitate. The dietary $^3$H cholesterol in hemolymph of the locusts was mostly present in the yellow band just below the surface on density gradient ultracentrifugation. While no such studies are available in insects, yet in higher animals involvement of lipoproteins in cholesterol influx and efflux has been reported30-33. Liver and intestine are known to synthesize and secrete lipoproteins34.

These cholesterol bound receptor/proteins which are solubilized in the additives were purified on a gel filtration column. Two main peaks were observed which are associated with a large proportion of the radioactivity. The comparison of the chromatogram of the proteins and of radioactivity gave two regions where the specific activity of the cholesterol was maximum after the void volume (fractions 32-38) containing high specific activity (32-106 dpm/µg), and specific activity (4-30 dpm/µg) in subsequent fractions (81-111).

The PBS extractable proteins of midgut tissue gave one major protein peak containing $^3$H cholesterol on gel filtration chromatography which had a molecular weight 17-32 kD. The insect FABP have molecular weight ~26 kD35. A lipophorin receptor of about 140 kD from the midgut of M. sexta has been partially purified and characterised36.

Binding studies suggest the presence of a large number of cholesterol binding sites that are available in the midgut lumen of S. gregaria which may be membrane bound proteins and play a key role in intracellular trafficking of cholesterol and its transport37. The dose dependent experiments show that the dissociation constant from the supernatant and fractionated proteins have nearly similar values in presence of radiolabelled ligand. The purified proteins showed a Kd value of $1.56 \times 10^{-8}$ mol/l. The competitive binding of $^3$H cholesterol studies in presence of 100 fold excess...
unlabelled ligand revealed a high non-specific binding. This amounts to about 20-35% of the total binding which consists of both true and non-specific binding and a low affinity specific binding.

Requirement of bile salts for absorption of cholesterol in mammals is well documented. However, cholesterol enters into the hemolymph in an esterified form inside the midgut and present study shows that the delipidated mucin exhibits a greater binding of the $^3$H cholesterol than the mucin isolated in presence of bile salts. The Kd values of delipidated mucin showed a higher saturation of binding ($3.28 \times 10^{-7}$ mol/l) in presence of bile salts than the cholesterol ($6.20 \times 10^{-8}$ mol/l).

The specificity of binding is indicated by the saturation of the analysed tissues to $^3$H cholesterol. The differences in binding of cholesterol by the midgut and mucin also indicates that surface absorption is higher in the midgut which clearly suggests that hydrophobicity is responsible for the binding of cholesterol with the protein either on the surface of membrane or in mucous proteins. This indicates the importance of 3-hydroxy group in the interactions which might have a marked specificity for binding.

Cholesterol binding specificity of rat recombinant liver fatty acid binding protein (L-FABP) and intestinal fatty acid binding protein (I-FABP) has been studied by Nemeez and Schroeder while the binding of palmitate, stearate, oleate and others to six FABPs from the intestine, heart, adipose and liver tissues from mammals were studied by Recheri et al. These studies reveal the dissociation constant (Kd) values to be between 2 to 1000 nM depending upon the tissue studied.

The results suggest that CBP is inhibited inside the midgut of S. gregaria after treatment with cycloheximide and there is a decrease in the uptake and release of cholesterol. Preliminary data suggest that there may be a number of cholesterol binding sites available in the ovary, testis, fat body cells and hemolymph. There is also a possibility that nuclear receptor proteins may help in transportation of cholesterol other than cytoplasmic receptor proteins. Yet we do not have an explanation for this type of characterization but it suggests that CoBPs play a significant role in cholesterol absorption. A proper identification and molecular characterization of these proteins may clear many unanswered questions in lipophorin biology.

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References


