

# HISTOCHEMICAL STUDIES ON TWO MILLIPED SPECIES<sup>1, 2</sup>

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## ABSTRACT

Histological and histochemical tests give similar results for the midguts of *Floridobolus penneri* (Causey, 1957) and *Narceus gordanus* (Chamberlin, 1943). The peritrophic membrane is composed of basic proteins and acid mucopolysaccharides. The epithelium contains basic proteins and large lipid concentrations, including glycolipids, phospholipids, and fatty acids. The luminal epithelial border gives reactions for protein-bound amino groups, tyrosine and phenolic compounds, and neutral fats. Protein-bound amino groups, glycoproteins, acid mucopolysaccharides, and bound lipids are found in the collagenous basement membrane. The circular and longitudinal muscle layers contain basic proteins, tyrosine and phenols, and bound fats. A large glycogen concentration occurs within the sheath membrane. This region, which is primarily basic protein, also gives positive reactions for protein-bound groups and both tyrosine and phenols.

Little work has been done on the internal anatomy of diplopods. Early anatomical studies included investigations by Verhoeff (1914), Ransom (1924), Attems (1926), Hefner (1929), and Miley (1930). Most biochemical analyses have included the whole animal and not particular organs or tissues. Siddiqui, et al. (1944) isolated dimethylglyoxime from a species of Indian diplopod. Bergmann (1949) reported that Ueno and Yamasaki had isolated a sterol from the saponifiable matter of millipedes. Blower (1951) found that the cuticle of diplopods was composed of two layers of chitinous materials. Recent studies on diplopods have included repugnatorial secretions. Some investigators of these substances were Schildknecht and Weis (1961), Monro, et al. (1962), and Wheeler, et al. (1964).

The purpose of this investigation was to compare the histology and histochemistry of the midgut of two milliped species, *Narceus gordanus* (Chamberlin, 1943) and *Floridobolus penneri* (Causey, 1957) and to determine the relative amount and distribution of carbohydrates, lipids, and proteins in each tissue layer. This work was considered important because, through it, a better understanding of the defense reactions of larval spiny-headed worms could be gained (Bowen, 1967).

## MATERIALS AND METHODS

Diplopods used in this study were obtained from the vicinity of the Archbold Biological Station, Highlands County, Florida, by Dr. Lawrence R. Penner. Fifteen *Narceus gordanus* and 25 *Floridobolus penneri* were decapitated and drained of coelomic fluid. The midguts were then removed and placed in a variety of fixatives.

Histological fixatives included Bouin's and Zenker's fluids and 10% formalin. Histochemical fixatives included: Carnoy's and Newcomer's solutions for carbohydrates; cobalt calcium formol and 10% formalin for lipids; and Bouin's, Carnoy's, and Zenker's fluids to demonstrate proteins. The tissues were dehydrated in alcohols, cleared in benzene, and infiltrated in paraffin at 60°C. for 12 hours. They were then embedded and sectioned between 8 to 10 microns on a rotary microtome. Frozen sections, prepared to demonstrate lipids, were sectioned to thicknesses of between 15 and 20 microns on a cryostat.

Mallory's aniline blue (McManus and Lowry, 1960) and Gomori's trichrome

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(Humason, 1962) stains were used to exhibit the fibrous components of the tissues. A modification of Van Gieson's picrofuchsin method (McManus and Mowry, 1960) determined the distribution of collagen. The Goldner-Foot modification of Masson's trichrome stain (Pearce, 1959) also demonstrated collagen. Sections utilizing these methods were dehydrated in alcohols, cleared in xylene, and mounted in Permount (Fisher Scientific Co., Fairlawn, N. J.).

Sections stained by the periodic acid Schiff (PAS) technique for carbohydrates were placed into four groups. The first group was subjected to the PAS reaction (McManus and Mowry, 1960). The second was exposed to Schiff's reagent without prior oxidation by periodic acid to determine the presence of free aldehydes. The third was acetylated from 24 to 48 hours with an acetic anhydride-pyridine mixture (McManus and Cason, 1950) to esterify reactive groups of the tissues. The final group consisted of acetylated sections that were saponified with an ammonical alcohol mixture (McManus and Mowry, 1960) for 24 hours. Best's carmine and Bauer's glycogen stains (McManus and Mowry, 1960), in addition to the PAS technique, determined the presence of glycogen. Control sections were incubated in a 0.5% solution of malt diastase (Gomori, 1952) for 20 minutes prior to staining. The Kelig extraction methods (Pearse, 1960), in conjunction with PAS staining, showed the amount of glycolipids. Sections incubated with 0.1% pepsin in 0.01N HCl (Gersh and Catchpole, 1949), followed by the PAS reaction, determined the presence of glycoproteins. The alcian-green staining procedure (Putt and Huskell, 1962) manifested the distribution of mucins and mucopolysaccharides. Control sections were methylated by the Fisher-Lillie techniques as outlined by Lillie (1965). Some methylated sections were subjected to the PAS reaction to distinguish mucins and mucopolysaccharides from other PAS-positive materials. Metachromasia of the tissues was determined by the standard toluidine-blue method (Pearse, 1960) at pH 5.5 and 7.0.

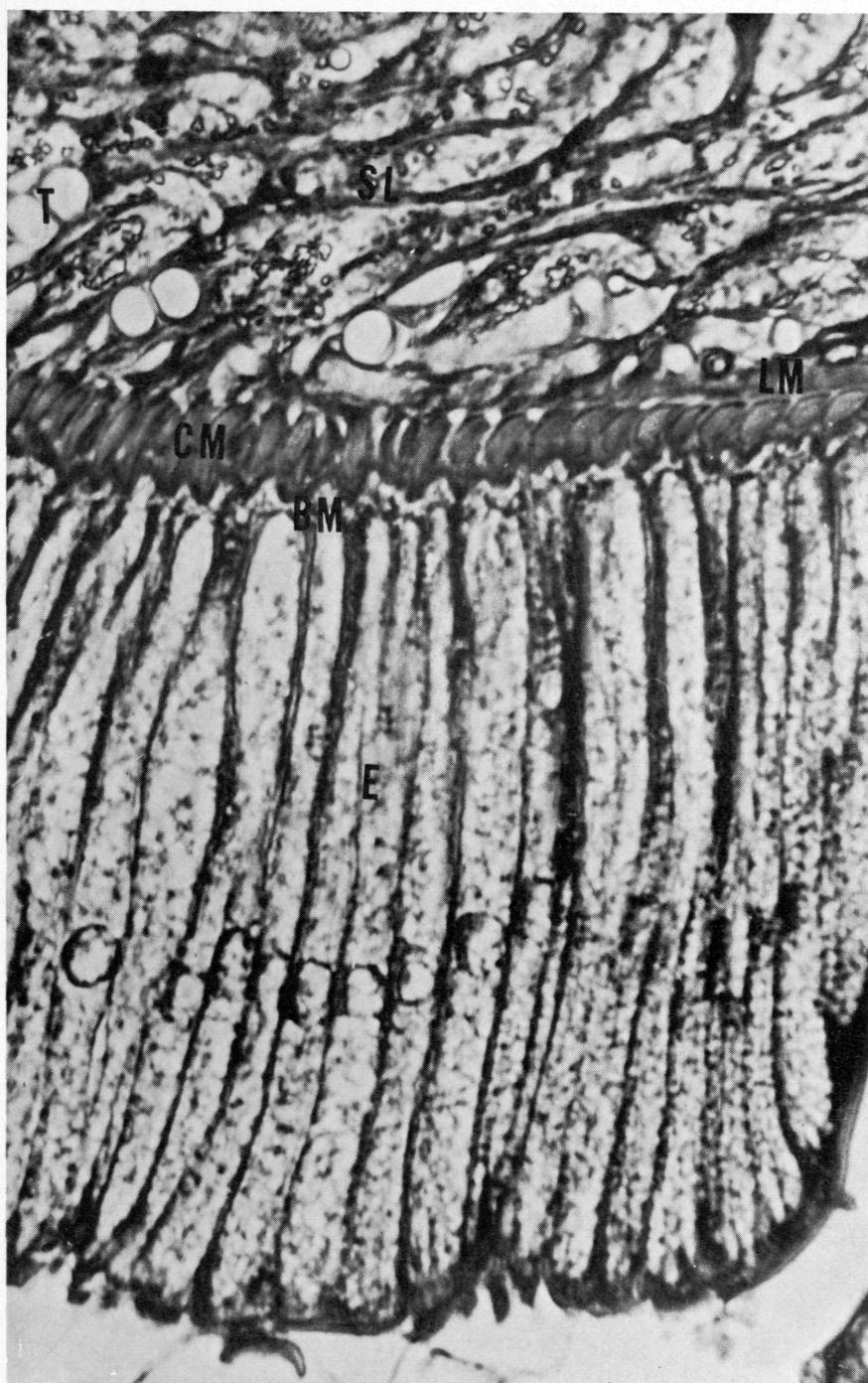
The propylene-glycol sudan method (Chiffelle and Putt, 1951) was used to determine the distribution of lipids. Sections incubated in a 50% methanol-chloroform solution at 60°C. for 24 hours served as controls for most lipid procedures. Neutral fats were distinguished from fatty acids by Mallory's nile blue A method (Humason, 1962). A general distribution of compound lipids was shown by the McManus sudan black B and the Berenbaum acetone-sudan-black methods (Pearse, 1960). Unsaturated fats were determined by the performic-acid Schiff reaction (McManus and Mowry, 1960). Control sections were brominated with a 2.5% solution of bromine in carbon tetrachloride (Lillie, 1954) for one hour prior to performic acid oxidation. The Menschik nile-blue method (Pearse, 1960) was used as the phospholipid procedure. The presence of melanin and lipofuchsin was resolved by the Lillie nile-blue method (Humason, 1962). Control sections were incubated in 10% H<sub>2</sub>O<sub>2</sub> for 24 hours at 25°C.

The mercuric bromphenol blue (Hg-BPB) method (Mazia, Brewer, and Alfert, 1953) detected the distribution of basic and total proteins in the tissues. These authors found that all proteins bound the dye in the presence of the mercuric salt, but only basic proteins bound the dye when the salt was omitted. Protein-bound amino groups were exhibited by the ninhydrin-Schiff reaction (Pearse, 1960). The distribution of phenols and tyrosine was determined by a modification of the Millon reaction (Gomori, 1952).

A qualitative scale was developed to demonstrate the relative number of reactive groups in tissues subjected to the Hg-BPB procedure and techniques utilizing Schiff's reagent. The scale ranged from a very strong reaction to a weak one. Mazia, Brewer, and Alfert (1953) and Hotchkiss (1948) found the intensity of these respective dyes to be directly proportional to the number of reactive sites in the tissues.

#### RESULTS

The histology of the midgut is similar in *Floridobolus penneri* and *Narceus gordanus*. The structure begins with a thickening of the epithelial and muscle

FIGURE 1. Midgut of *Floridobolus penneri*. x 1980.

BM—basement membrane

CM—circular muscle layer

E—epithelium

LM—longitudinal muscle layer

SL—sheath layer

T—tracheoles

layers as the esophagus valve. The midgut also expands in the posterior region adjacent to the pylorus.

#### *Peritrophic Membrane*

The peritrophic membrane lies laterad to the intestinal contents and appears to be delaminated from epithelial cells posterior to the esophageal valve. The structure measured 1.5 to 2.0 microns in thickness. The histological stains exhibited the mucoid nature of the structure.

The membrane gave a moderate reaction with the PAS technique. The reaction was not altered by diastase or methanol-chloroform, but a weak PAS reaction was observed following digestion with pepsin. The structure gave a positive reaction for mucins and mucopolysaccharides, but metachromasia was not manifested. Negative results were obtained for glycogen. Lipids were not demonstrated with any of the methods used. A weak positive reaction was given from the test for basic proteins, but no positive results were obtained for protein-bound amino groups, tyrosine, or phenols.

#### *Epithelium*

Pseudostratified columnar epithelium exists outside the peritrophic membrane. These cells measured 55 to 100 microns in length and 5.5 to 9.1 microns in width. *Floridobolus penneri* had smaller cells than *Narceus gordanus*. Epithelial cells of the esophageal valve and those anterior to the pylorus were generally larger than the cells throughout most of the midgut.

Histological stains exhibited polarity of the epithelial cell by showing three cytoplasmic regions of different staining intensities. The luminal end contained the most deeply stained region, occupying one-tenth to one-fifth the cell length. A lightly stained vacuolated area occupied the middle two-thirds of the cell. This region contained the nucleus, which was in a submedial position toward the basement membrane. The nucleus was round to oval and contained a large nucleolus. The epithelial region closest to the basement membrane was stained lighter than the luminal edge, but darker than the middle portion of the tissue.

The luminal epithelial border gave a moderate PAS reaction, but the remainder of the cell was essentially negative. Diastase and methanol-chloroform did not affect the reaction, but the PAS reaction was reduced from moderate to weak by pepsin. The epithelium did not exhibit glycogen or mucopolysaccharides. Metachromasia occurred at pH 7.0, but was absent at pH 5.5.

Most lipids were concentrated toward the luminal border of the cell and adjacent to the basement membrane. The border consisted primarily of neutral fats, but the remainder of the cell contained mostly fatty acids. Bound or compound lipids were found only at the luminal border and in the region adjacent to the basement membrane.

The luminal edge exhibited a very strong reaction with Hg-BPB, but no change in staining intensity was observed by omitting the mercuric salt, indicating the presence of basic protein. The remainder of the cell was stained moderately by this method. The border was the only epithelial region positive for protein-bound amino groups, tyrosine, and phenols.

#### *Basement Membrane*

The basement membrane, laterad to the epithelium, measured 2.0 to 3.0 microns in thickness. Histological stains indicated large amounts of collagen.

A strong PAS reaction was given by the membrane. The structure was not affected by diastase or methanol-chloroform. A weak PAS reaction resulted, following exposure to pepsin. The membrane gave a positive reaction for mucopolysaccharides and revealed beta metachromasia at pH 7.0. Glycogen stains had no effect on the tissue.

Staining reactions for neutral fats, fatty acids, and unsaturated fats were negative. Positive results were obtained for bound lipids. These fats were not in the form of phospholipids or glycolipids, but were composed of a protein-lipid complex.

The structure gave a strong reaction with Hg-BPB, which did not change in the absence the mercuric salt. A positive reaction occurred for protein-bound amino groups. Tyrosine and phenols were not demonstrated in the tissue.

#### *Circular and Longitudinal Musculature*

Each muscle layer, which consisted of two rows of fibers, measured 7.0 to 8.0 microns in diameter. The fibers were surrounded by thin layers of connective tissue. Histological stains showed the muscles to be striated.

The muscles were negative with all carbohydrate procedures used. The connective tissue gave a moderate PAS reaction, which was modified by pepsin, but not by diastase or methanol-chloroform. The connective tissue tested positive for mucopolysaccharides and gave gamma metachromasia at pH 5.5. Bound lipids were the only fats identified in the muscles.

The muscle layers reacted very strongly to Hg-BPB and were not affected by the omission of the mercuric salt. Protein-bound amino groups were not demonstrated. The muscles stained very deeply for tyrosine and phenols.

#### *Sheath Membrane Layer*

The sheath membrane covered the entire midgut. This layer was composed of large irregular cells with eccentric nuclei. The cytoplasm of some cells contained small to large vacuoles, but most contained yellow-brown pigmented granules that were not affected by histological stains. Many tracheoles and few connective-tissue fibers were interdispersed among the cells. Tracheoles continued into the haemocoele, but the connective tissue was limited to the sheath network. Bundles of longitudinal muscles, toward the periphery of the membrane, had diameters of 60 microns in *Floridobolus penneri* and 80 microns in *Narceus gordanus*. The muscles and their surrounding connective tissue gave staining reactions similar to those of other muscles and fibers in the midgut.

The sheath cells gave PAS reactions that varied from weak to very strong. The yellow-brown granules did not react to any of the histochemical stains used. Connective tissue fibers and tracheoles showed moderate staining with the PAS stain. Diastase removed most PAS-positive material from the cells, but the enzyme had no effect on the tracheoles and connective tissue. Methanol-chloroform did not affect the PAS reaction, but pepsin modified its intensity. All connective tissue contained mucopolysaccharides. Some tissue fibers exhibited gamma metachromasia at pH 7.0, and other fibers and the tracheoles gave beta metachromasia at pH 5.5. Diastase labile substances were stained by Best's carmine and Bauer's glycogen stains.

Most cells were fat free, although few exhibited large lipid concentrations. These fats were primarily neutral fats. Bound lipids occurred in the cells and fibers, but only the fibers were positive for phospholipids. The granules were negative for melanin and lipofuchsin.

The sheath network showed moderate staining by Hg-BPB and was unaffected when the mercuric salt was omitted. Protein-bound amino groups were exhibited in cell membranes. Sheath cells gave a positive Millon reaction, but connective tissue and tracheoles were negative.

#### OBSERVATIONS AND DISCUSSION

##### *Peritrophic Membrane*

The origin of the peritrophic membrane in *Narceus gordanus* and *Floridobolus penneri* is similar to that reported for *Parajulus impressus* by Hefner (1929).

Masson and Gilbert (1954) found that the membrane arose from the entire midgut surface in several species of British millipedes, and Snodgrass (1935) reported the same occurrence in most insects. A chemical similarity appears to exist between peritrophic membranes of spirobolidids and insects. The membrane of insects contains both mucoprotein and chito-protein (Wigglesworth, 1950), and it was found in this study to contain basic proteins, glycoproteins, and acid mucopolysaccharides in the millipedes. Pearse (1960) reported no histochemical difference between mucoproteins and glycoproteins.

### *Epithelium*

The epithelium revealed basic proteins and large lipid concentrations, including glycolipids, phospholipids, and fatty acids. The luminal border contained glycoproteins, neutral fats, and bound lipids. Glycogen in midgut cells of many insects might have contributed to peritrophic membrane formation (Wigglesworth, 1950), but glycogen was not found in the epithelium of the millipedes.

### *Basement Membrane*

The basement membrane demonstrated protein-bound amino groups, glycoproteins, and acid mucopolysaccharides.

### *Circular and Longitudinal Musculature*

Hefner (1929) reported that the ventricular musculature of *Parajulus impressus* was almost absent except in the pyloric valve region, and that it lacked striations. Miley (1930) observed no bands in the midgut musculature of *Euryrus erythropygus*. Randow (1924) reported that all muscles associated with the digestive tract of Julidae were striated. This present study revealed that circular muscles, longitudinal muscles, and peripheral muscle fascicles of the sheath network were striated. This study also indicated that muscles associated with the midgut of *Narceus gordanus* and *Floridobolus penneri* contain basic proteins, bound lipids, tyrosine, and phenolic compounds.

### *Sheath-Membrane Layer*

A single compact layer of sheath cells was observed in the genus *Julus* by Randow (1924) and in *Euryrus erythropygus* by Miley (1930). Both authors reported the absence of fat with osmic acid techniques, but in some cases Miley found lipids with Sudan III. Randow (1924) ascribed the function of glycogen storage to this layer.

The sheath membrane of *Narceus gordanus* and *Floridobolus penneri* was similar to that reported by Hefner (1929) for *Parajulus impressus*, with an open-network cellular arrangement. Neither Randow, Hefner, or Miley reported connective tissue associated with the membrane.

The cells of *Narceus gordanus* and *Floridobolus penneri* were rich in glycogen. Tests also indicated the presence of basic proteins, tyrosine, and phenols. Only a few cells demonstrated concentrations of neutral and bound lipids. The bound fats were not glycolipids or phospholipids; extraction techniques revealed the possibility of proteolipids. Connective tissue fibers were primarily composed of basic protein, glycoprotein, acid mucopolysaccharides, and phospholipids.

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