The Cytochemistry of the Amoebocytes and Intestinal Epithelium of *Venus mercenaria* (Lamellibranchiata), with Remarks on a Pigment resembling Ceroid

By SUMNER I. ZACKS

Jeffries Wyman Scholar, Harvard University, 1952–3

(From the Department of Anatomy, Harvard Medical School, Boston 15, Mass., U.S.A.)

With two plates (figs. 1 and 2)

**SUMMARY**

The properties of the amoebocytes and intestinal epithelium of *Venus mercenaria* were studied by a variety of cytochemical procedures designed to demonstrate proteins, enzymes, carbohydrates, and lipids.

The cytoplasm of the amoebocytes contains specific granules which are constantly present and which are interpreted as being atypical mitochondria. Identification of their mitochondrial nature rests on their staining with Janus green B, their positive reaction for phospholipid by Baker’s test, and the presence of dehydrogenase activity. Unlike typical mitochondria, the specific granules are eosinophil. Protein-bound carbonyl groups and disulphide and sulphydryl groups are present in both the specific granules and the cytoplasm. The sulphhydryl groups may in part be associated with the presence of dehydrogenase, lipase, and serum cholinesterase. Amoebocytes also contain glycogen and a material that is resistant to diastase and positive to the periodic acid/Schiff test; this material may be a neutral polysaccharide, unsaturated lipid, or mucoprotein.

Cytoplasmic structures which are inconstantly present in amoebocytes include sudanophil droplets, neutral red vacuoles, metachromatic granules, and granules of an excretory pigment resembling ceroid. The sudanophil droplets may be stored neutral fat or lipid associated with the Golgi apparatus. The neutral red vacuoles are not preformed inclusions, but form as the dye accumulates within the cells. Metachromatic granules, which are confined solely to the intestinal amoebocytes, consist of phagocytosed intestinal mucus liberated from goblet cells.

The histochemical reactions of the columnar intestinal epithelium suggest that these cells may be active in the digestion and absorption of nutrients, since eosinophil granules, lipid droplets, alkaline phosphatase, lipase, and serum cholinesterase are present in them.

Masses of a ceroid-like excretory pigment and goblet cells containing mucus are present between the columnar intestinal epithelial cells. The pigment contains phospholipid and apparently arises as an oxidized end-product of lipid metabolism.

**AMOEBCYTES** are granulocytes which are found in large numbers in the blood, connective tissues, gills, cardiac muscle, and intestinal mucosa of invertebrates. They have been the subject of numerous morphological and chemical studies and many speculations have been advanced concerning their nature and functions (Metschnikoff, 1884; Haughton, 1934; Takatsuki, 1934; Ohuye, 1938; Yonge, 1923, 1926a, 1926b, 1946).

The present study concerns the cytology and cytochemistry of the amoebocytes and columnar intestinal epithelium of the quahog, *Venus mercenaria*. A ceroid-like excretory pigment found in these cells as well as in other tissues of *Venus*, was also investigated. Interest in these topics arose from a previous investigation of esterolytic enzymes in *V. mercenaria* (Zacks and Welsh, 1953).

The individual amoebocyte is a nucleated cell 9–12 μ in diameter, which, in recently drawn blood, rounds up and appears as a sphere with small projecting spikes 1–2 μ long. After remaining in contact with the surface of a glass slide for several minutes, or in tissue-spreads, the amoebocytes extend pseudopods; by means of these they are capable of slow motion. Within their cytoplasm, small hyaline granules as well as yellow, refractile granules of excretory pigment are seen. The numerous, even-sized, hyaline granules are constantly present in *Venus* amoebocytes and therefore will be termed specific granules to distinguish them from granules of excretory pigment and other cytoplasmic inclusions which are inconstant constituents.

The chemical nature of the cytoplasmic granules of these amoebocytes has interested a few investigators, but no one appears before to have studied the granules by cytochemical methods. By these procedures it was hoped that information might be obtained concerning the chemical nature of the granules and their relations to the enzymatic activities of the cells. The chemical cyto-logy of the columnar intestinal epithelium was investigated with particular reference to its role in digestion and absorption of nutrients. The nature of the excretory pigment which is present in the amoebocytes, intestinal epithelium, and other tissues of *Venus* was similarly investigated by histochemical means. This pigment, which occurs in the form of granules in the amoebocytes and amorphous clumps in the intestine, has been described as a degradation product of chlorophyll (MacMunn, 1900) and as echinochrome (Durham, 1891). Since the chemical nature of this pigment is poorly understood, it was hoped that histochemical tests might contribute to its characterization.

**Materials and Methods**

Fresh specimens of *V. mercenaria* were purchased from a fish dealer. Whole hearts were excised and placed either in sea-water for supravital staining or in various fixatives. Hearts prepared in this way were convenient objects for the study of both amoebocytes and intestinal epithelium, since the heart is wrapped around a segment of intestine.

*Supravital methods.* For supravital staining, whole hearts were placed in sea-water containing *Janus green B* at 1:10,000 or *neutral red* at 1:10,000 and stained for 2 hours at 29° C. A fragment of atrial wall was then spread under a coverslip on a glass slide for examination. This preparation was chosen because the thin network of atrial muscle fibres enmeshed and supported the amoebocytes, thus allowing a favourable opportunity for observing pseudopod formation and amoeboid motion.
Methods for the staining of proteins. Basophil structures were studied in Zenker-fixed material. Deparaffinized sections were stained in methylene blue solutions buffered at pH 2.3, 4.3, 6.4, and 7.4 according to the method of Singer and Morrison (1948). Similar sections were also stained by Mallory’s eosin and methylene blue. The Feulgen procedure, followed by counterstaining with light green, was applied to deparaffinized sections to detect desoxypentose-nucleic acid (DNA). Protein-bound sulphydryl and disulphide groups were sought in deparaffinized sections of hearts fixed in 80 per cent alcohol containing 1 per cent. trichloracetic acid (Barrett and Seligman, 1952). Sections were stained with and without prior reduction in (NH₄)₂S, to allow identification of both sulphhydril and disulphide groups.

Methods for the detection of enzymes. Gomori’s acid and alkaline phosphatase methods (1939, 1941), as modified by Dempsey and Deane (1946), were applied to alcohol- and acetone-fixed deparaffinized sections as well as to fresh frozen sections. Sections were incubated for 30 minutes, 1, 3, 6, and 24 hours in solutions of glycerophosphate buffered to pH 4.5 and 9.5.

Dehydrogenase activity was detected in fresh hearts after incubation for 20 minutes in blue tetrazolium chloride (pH 7.4), a method modified from that of Rutenburg, Gofstein, and Seligman (1950).

Substances capable of reducing ferricyanide to ferrocyanide were studied by applying the ferric-ferricyanide reaction of Lillie and Burtner (1953) to fresh and formalin-fixed sections.

Methods for the detection of carbohydrates and mucopolysaccharides. Glycogen and polysaccharides were stained by the periodic acid / Schiff (PAS) method of McManus (1946) and Hotchkiss (1948), after fixation of hearts in Rossman’s fluid. Control sections were treated with diastase before application of this procedure. Metachromasia was investigated by staining with 0.5 per cent. toluidine blue after 4 per cent. lead acetate fixation (Holmgren, 1940; Jorpes, Holmgren, and Wilander, 1937).

Methods for the detection of lipids. The distribution of lipids was studied after staining frozen sections of fresh and formalin-fixed hearts with Sudan black B. Phospholipids were identified in frozen sections of hearts fixed in formaldehyde-calcium and in weak Bouin’s fluid (Baker, 1946). Carbonyl groups were sought in frozen sections of material fixed in buffered 10 per cent. formalin and stained by the method of Ashbel and Seligman (1949). Control sections were extracted with acetone to remove lipid-containing carbonyl groups. Cholesterol and cholesteryl esters were investigated in frozen sections by the Schultz procedure and acid-fast substances were identified in material fixed in formalin, alcohol, and Zenker’s fluid, by the carbol-fuchsin procedure of Lillie (1948).

Methods for the detection of birefringence and fluorescence. Birefringence and fluorescence were studied in fresh-frozen and formalin-fixed sections.

Method for the detection of iron salts. To detect the presence of iron salts material fixed in 80 per cent. alcohol and in Zenker’s fluid was embedded in paraffin, sectioned, and treated with potassium ferrocyanide (Lillie, 1948).
Supravital staining. Spreads of atrial muscle stained in 1:10,000 Janus green B exhibited amoebocytes which were uniformly filled with deep blue-green granules 1–2μ in diameter. The harmlessness of this dye was shown by the slow extension of pseudopods from the cells. Withdrawal of pseudopods was never observed in supravitaly-stained or fresh, unstained preparations. After 3–5 minutes, the specific granules appeared red-violet in colour, and eventually assumed the red colour of the reduction-product of Janus green B, diethyl safranin. Recolouring of the granules did not occur when the atrial fragments were re-exposed to atmospheric oxygen.

The cut ends of the intestine appeared deep blue-green after staining in Janus green B and this staining was almost entirely confined to the intestinal epithelium. A faint blue-green colour was observed in the ventricular muscle, but the intestinal muscle was nearly devoid of staining.

When neutral red (1:10,000) was applied supravitaly for 2 hours, the amoebocytes were filled with deep brick-red inclusions of various sizes (fig. 1, A). The majority of the neutral red inclusions were larger and less uniform in size than the granules stained by Janus green B. The brick-red colour of the neutral red inclusions suggested that their pH was close to neutrality.

Excretory pigment was unstained by Janus green B and neutral red.

Basiphilia. In sections stained in methylene blue at pH 7.4 and 6.4, cardiac and intestinal muscle fibres, goblet cell mucus, and excretory pigment were stained deep blue, whereas the cytoplasm of the amoebocytes was moderately stained blue. After staining with methylene blue at pH 4.3, the intestinal muscle fibres were coloured grey-blue, amoebocyte cytoplasm was stained light blue, and masses of excretory pigment appeared blue-green. Goblet cell mucus was metachromatically stained deep violet at pH 3, but excretory pigment and cardiac and intestinal muscle fibres and amoebocyte granules were unstained. However, the cell plasma of the amoebocytes was faintly stained blue. At pH 2, nothing was stained.

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**Fig. 1 (plate).** A, atrial muscle spread, illustrating the appearance of amoebocytes supravitaly stained by neutral red. Large and small irregular vacuoles are present in each cell.

B, transverse section through the intestine of *Venus mercenaria*, stained by the Feulgen reaction and counterstained by light green. The typical ciliated columnar epithelium, as well as numerous amoebocytes and accumulations of excretory substance, are illustrated. Numerous amoebocytes are scattered between the intestinal muscle fibres.

C, an amoebocyte stained by the Feulgen procedure and counterstained with light green. The cytoplasm is packed with green-stained specific granules.

D, a goblet cell after application of the Feulgen procedure. The goblet cell contains red-stained mucus and the nuclear chromatin is coloured red-violet.

E, higher magnification of the field shown in B. Masses of excretory pigment composed of globules of yellow or brown material embedded in a green-stained matrix are illustrated.

F, transverse, alcohol-fixed section of *Venus* intestine after 3 hours' incubation in glycero-phosphate at pH 9.5. Alkaline phosphatase activity is located in the distal portion of the epithelium. Excretory pigment is unreactive yet some clumps of this material appear dark in the photograph due to the intrinsic colour of the pigment.
Fig. 1
S. Zacks
Fig. 2
S. Zacks
Eosin and methylene blue. After staining with eosin and methylene blue (pH 5.3), the specific granules of the amoebocytes were stained red. Occasional amoebocytes contained a few blue granules of various size. The oval, peripherally placed nucleus contained strongly basophil clumps of chromatin.

The intestinal epithelium was composed of tall, ciliated, pseudostratified columnar cells which rested on a thick basement membrane. The proximal and middle portions of each epithelial cell were basophil and numerous dust-like eosinophil granules were present in the distal zone. The intestinal surface of the epithelial cells was equipped with cilia which were 7-10µ in length. The nucleus was located in the middle or proximal part of the cells. Amoebocytes containing specific granules were gathered in great numbers on the basement membrane in spaces between the epithelial cells. Numerous amoebocytes were also present between the smooth muscle fibres of the intestinal musculature. Occasional goblet cells, containing deeply basophil mucus, were present between the columnar epithelial cells.

Masses of various sizes (7–30µ) of amorphous, strongly basophil excretory pigment were also present in the spaces between the epithelial cells. Small granules of the same material were scattered between the cardiac muscle fibres.

Desoxyribonucleoprotein. The Feulgen procedure stained the nuclei of the muscle fibres, epithelial cells (fig. 1, b) and amoebocytes, but the specific granules of the amoebocytes were not coloured (fig. 1, c). The cytoplasm of the goblet cells was stained deep red in contrast with the violet colour of the nuclei (fig. 1, d). The cytoplasm of the amoebocytes and portions of the excretory pigment masses were stained by the acid dye, light green, used as counterstain. The excretory pigment masses in the intestinal epithelium were composed of unstained yellow or orange globules embedded in a green-stained matrix (fig. 1, e).

Protein-bound sulphydryl and disulphide groups. After (NH₄)₂S reduction, the intestinal epithelium, intestinal muscle cells, and cardiac muscle reacted positively. The amoebocytes also contained sulphydryl groups, as indicated by a positive reaction of their cytoplasm. It could not be ascertained whether the reactive material was localized in both the specific granules and cell plasma of the amoebocytes, or only in the cell plasma.

Fig. 2 (plate). A, a goblet cell after 3 hours' incubation in glycerophosphate at pH 9.5. The goblet cell mucus shows alkaline phosphatase activity.
B, transverse section of the intestinal epithelium illustrating the appearance of an amoebocyte and two masses of excretory pigment stained by the PAS-procedure.
C, amoebocytes stained by Sudan black B, showing lipid droplets.
D, amoebocytes stained by the Baker procedure showing numerous phospholipid granules in the cytoplasm.
E, transverse section of Venus intestine after staining for phospholipid. The masses of excretory pigment contain phospholipid granules embedded in a matrix of unstained material.
F, ultra-violet light photograph illustrating the intense fluorescence of the excretory pigment and the less intense fluorescence of the intestinal epithelial cells.
positively when reduction with (NH₄)₂S had been omitted, thus indicating the presence of free-SH groups as well as S–S groups in these cells. By both procedures, the excretory pigment was unreactive.

**Alkaline and acid phosphatases.** Alkaline phosphatase activity was more intense in fresh-frozen sections than in alcohol-fixed sections. After 30 minutes’ incubation, the distal ends of the columnar epithelial cells were stained brown (fig. 1, F) but the amoebocytes and excretory pigment were unreactive. After 6 hours of incubation the intestinal epithelium was intensely stained, but only an occasional amoebocyte could be found which contained one or two brown granules. In alcohol-fixed sections, the goblet cells were stained brown after 3 hours of incubation (fig. 2, A).

Acid phosphatase activity was not demonstrable in any of the tissues investigated.

**Dehydrogenase.** After 20 minutes’ incubation in blue tetrazolium chloride, granules of blue formazan pigment were present in the amoebocytes and the cardiac and intestinal muscle fibres.

**Ferric-ferricyanide reaction.** After 10 minutes in this reagent, the cardiac muscle and intestinal epithelium were tinged light green, the amoebocytes were colourless, and the masses of excretory pigment were coloured deep blue-green.

**Periodic acid / Schiff reaction.** The PAS procedure produced intense red staining of the cardiac and intestinal muscle fibres, goblet cells, and excretory pigment. The amoebocytes were filled with many red granules of various sizes. Fig. 2, B illustrates the appearance of an amoebocyte and masses of excretory pigment stained by this procedure. After the exposure to the action of diastase to remove glycogen, staining of both cardiac and intestinal muscle fibres and amoebocytes was considerably reduced. However, goblet cells and excretory pigment were deeply stained despite exposure to diastase.

**Staining with toluidine blue (metachromasia).** Muscle fibres, the specific granules of the amoebocytes, and the excretory pigment were stained faintly blue.

Metachromatic granules and amorphous clumps were abundantly present in the intestinal lumen and epithelium as well as in amoebocytes located between the epithelium. However, large numbers of amoebocytes located in the intestinal and cardiac musculature were devoid of metachromatic granules.

**Lipids.** After the staining of atrial spreads in Sudan black B, each amoebocyte contained up to a dozen black droplets of variable size. The larger droplets were occasionally U shaped and associated with cytoplasmic vacuoles. A tendency toward perinuclear localization occurred (fig. 2, c). After 15 minutes’ extraction in acetone or hot alcohol (60° C.), the sudanophil droplets could no longer be demonstrated.

The amorphous masses of excretory pigment were stained green-black or grey-green by Sudan black B and small black sudanophil droplets were present in the apices of the intestinal epithelial cells.

**Phospholipids.** Each amoebocyte contained 2–50 black granules of uniform
size (fig. 2, D) after staining by Baker’s method for phospholipids. In many amoebocytes, these granules were not as numerous as the specific granules which were stained by supravital Janus B or eosin. After pyridine extraction, the nuclei of the amoebocytes and muscle cells were stained, but the granules were unstained. These results indicate that the amoebocyte granules contain phospholipid. It should be emphasized, however, that in many amoebocytes, the phospholipid granules represented but a small fraction of the full complement of granules stained by Janus green B or eosin.

Black-stained granules and clumps were embedded in the yellow masses of excretory pigment present in the intestinal columnar epithelium which was otherwise unstained (fig. 2, E).

**Carbonyl groups.** The distal halves of the columnar intestinal epithelial cells reacted strongly for carbonyl groups, but the basal halves stained only faintly. The musculatures of both heart and intestine were unreactive. The specific granules as well as the cell plasma of the amoebocytes were moderately stained. In control preparations extracted with acetone, the outer portions of the epithelial cells remained reactive, but the masses of excretory pigment were stained red-orange, a result attributable to non-specific solution of the blue azo dye in the lipid component of the excretory pigment (Nachlas and Seligman, 1949). Carbonyl-staining was reduced in amoebocytes after acetone extraction.

The Schultz test for cholesterol and cholesterol esters was negative in all of the cell elements studied.

**Acid-fast substances.** After several hours’ extraction in dilute hydrochloric acid, red masses of excretory substance and occasional small granules in amoebocytes were present.

**Birefringence and fluorescence.** Neither epithelium, excretory pigment, nor amoebocytes were birefringent when examined under the polarization microscope.

In ultra-violet light, the middle and basal portions of the columnar intestinal epithelial cells showed light blue fluorescence, whereas the apical, granular portion of the cells exhibited red-violet fluorescence. The large, amorphous masses of excretory pigment situated on the basement membrane and between the epithelial cells (fig. 2, F), as well as occasional small granules in the amoebocytes, showed intense yellow fluorescence. Similar fluorescent granules were scattered between the intestinal muscle fibres. Much of this material seemed to be free, but a smaller fraction was located within amoebocytes.

**Ferrocyanide reaction (iron salts).** Sections stained by this means were completely negative, no blue or green deposits of Prussian blue being observed.

**Discussion**

The nature and properties of the various structures in the cytoplasm of amoebocytes

A variety of cytoplasmic inclusions which exhibit distinctive histochemical properties and enzymatic reactions were present in the amoebocytes of
V. mercenaria. These included specific granules (constantly present), neutral red vacuoles, sudanophil droplets, pigment granules, and metachromatic and PAS-positive materials, all of which were inconstantly present. Besides the reactions shown by these various inclusions the cell plasma itself manifested certain staining properties.

**Cytochemical reactions of specific granules in amoebocytes**

Supravital staining with Janus green B showed great numbers of even-sized specific granules which reduced the dye to diethyl safranin, thus indicating the presence of hydrogen-donor enzymes. That this reaction was not reversible was seen in the failure of the specific granules to become coloured again when exposed to atmospheric oxygen. The fact that these granules stained with Janus green B and were capable of reducing this dye when oxygen was excluded, indicated that they are of mitochondrial nature. Furthermore, dehydrogenase activity associated with the specific granules was indicated by the oxidation of blue tetrazolium chloride. The mitochondrial nature of the specific granules was also suggested by the fact that many of the granules reacted positively with Baker's test for phospholipid. However, the specific granules were eosinophil, a staining reaction which does not occur in typical mitochondria. The foregoing observations suggested that amoebocyte specific granules represent an atypical variety of mitochondria.

Diastase-labile as well as diastase-resistant PAS-positive granules were present in the amoebocytes. The substances responsible for this reaction are thought to be compounds containing 1, 2 glycol linkages which are oxidized by periodic acid to form aldehyde groups which then react with the Schiff reagent (Hotchkiss, 1948). According to Leblond (1950), only substances insoluble in water and fat substances can be considered to persist after exposure of the tissues to the reagents employed in fixation and paraffin embedding. These substances include glycogen, which can be removed by pretreatment of the sections with diastase, and mucopolysaccharides and mucoproteins which are not removed by diastase. Lillie (1950), Wolman (1950), and Pearse (1953) have shown that certain lipids also yield a positive PAS-reaction which is retained after exposure to the action of diastase. Unsaturated lipids or other substances containing hydroxyl and amino groups on two adjacent carbon atoms might be expected to yield a positive PAS-test.

Since much of the material which was stained by the PAS procedure was removed by the action of diastase, it appears that these cells contain glycogen. However, amoebocytes also contained granules of diastase-resistant material.

The nature of the diastase-resistant material in amoebocytes is not clear. This material may be neutral polysaccharide, since evidence of strongly acidic groups of acid mucopolysaccharides is lacking, or mucoprotein, phospholipid, or other unsaturated lipid. The presence of neutral fat and phospholipid as well as PAS-positive excretory pigment has been demonstrated in Venus amoebocytes. Furthermore, since serum cholinesterase is present in amoebocytes (Zacks and Welsh, 1953), it is of interest that cholinesterase is
thought to be a mucoprotein and that practically all sites possessing cholinesterase activity are PAS-positive (Gomori, 1951). Thus the diastase-resistant material may be unsaturated lipid or mucoprotein.

The presence of ketonic carbonyl groups of proteins or proteo-lipids was indicated rather than ketonic groups associated with lipids (Seligman and Ashbel, 1951) since the amoebocytes were still reactive after acetone extraction.

**Sulphydryl and disulphide** groups were present in both the specific granules and cell plasma of amoebocytes. These groups are important in binding proteins and prosthetic groups (Barron, 1951), cell division and growth (Brachet, 1950), cell permeability (Lefevre, 1948), and in enzymatic activity (Barron, 1951). Several sulphydryl enzymes have been demonstrated in amoebocytes. The presence of lipase in the amoebocytes of several molluscs has been shown by Yonge (1926a) and Takatsuki (1934), and recently by histochemical procedures by Zacks and Welsh (1953). Serum cholinesterase is also present in the amoebocytes of *Venus* as indicated by carbonaphthoxycholine iodide hydrolysis (Zacks and Welsh, 1953). In addition, Takatsuki (1934) has demonstrated enzymes capable of attacking starch, glycogen, maltose, lactose, sucrose, salicine, and gelatine in extracts of *Ostrea* amoebocytes.

Among the numerous enzymes requiring SH-groups for their activity are cholinesterase, lipase, esterase, β-amylase, and carboxypeptidase (Barron, 1951). Of this group, cholinesterase, lipase, and an unidentified dehydrogenase have been detected in *Venus* amoebocytes, and amylase, protease, and lipase have been demonstrated in the amoebocytes of other species (Yonge, 1926a; Takatsuki, 1934). Since several sulphydryl enzymes are present in amoebocytes, it seems that the sulphydryl groups demonstrable in these cells may be partially attributed to these enzymes.

**Metachromasia in amoebocytes.** The absence of metachromatic granules in amoebocytes outside the intestine suggests that intestinal amoebocytes which contain metachromatic granules had phagocytosed some of the goblet cell mucus. Metachromatic staining is regarded as evidence for the presence of acid mucopolysaccharides (Holmgren and Wilander, 1937; Wislocki, Bunting, and Dempsey, 1947), an important constituent of mucus from several sources. Mucus is used by lamellibranchs to trap food particles to aid their ingestion by amoebocytes and digestive diverticula (Yonge, 1926a).

**Sudanophil droplets in amoebocytes.** Sudanophil droplets of various size were present in nearly all the amoebocytes. In every case these droplets could be distinguished from the specific granules by their relatively small number and by their size and position within the cell. Since the sudanophil droplets were extractable by cold acetone and hot alcohol, they appeared to consist of neutral fat.

The presence of lipids in lamellibranch amoebocytes has been investigated by Yonge (1926, a and b), Takatsuki (1934), and others. Yonge believes that amoebocytes and digestive diverticula play an exclusive role in fat ingestion and digestion and that no fat is digested extracellularly in the stomach. Thus,
lipid droplets present in amoebocytes may represent ingested fat globules. Another interpretation is that of Gatenby and Hill (1934), who regard similar sudanophil droplets in *Helix* amoebocytes as elements of the Golgi apparatus. The perinuclear position frequently assumed by the sudanophil droplets in *Venus* amoebocytes might suggest a similar interpretation.

Neutral red vacuoles in amoebocytes. In addition to specific granules and lipid droplets, amoebocytes contained large numbers of various sizes, cytoplasmic inclusions which were stained by neutral red. That these inclusions were not the same as the specific granules stained by Janus green B was indicated by their lack of uniformity of size and shape. Also the large numbers of granules staining with Janus green B precluded the possibility that both these granules and neutral red bodies could both be present in the cell as preformed inclusions. Furthermore, it is generally agreed that supravital Janus green B rarely, if ever, stains structures which are stained by neutral red. Bensley (1911) observed that Janus green B stained mitochondria in acinar cells of the guinea-pig pancreas, whereas neutral red stained granules of prozymogen and zymogen. Gatenby (1931) stated that many cells collect and aggregate neutral red into vacuoles while it is passing through the cytoplasm, and Gatenby and Hill (1934) concluded that the neutral red inclusions of *Helix* amoebocytes were not pre-existent structures. In the case of *Venus* amoebocytes, the objects which stained with neutral red appear to be vacuoles filled with dye, rather than preformed cytoplasmic inclusions.

Pigment granules in amoebocytes. Amoebocytes frequently contained highly refractile yellow granules which appear to be identical with the excretory pigment described by Yonge (1923, 1926, *a* and *b*) and others in various lamellibranchs. These pigment granules were easily distinguished from the specific granules of these cells. Larger granules and clumps of this material were also found scattered throughout the intestinal and cardiac musculature and in especially large masses in the intestinal epithelium. The histochemistry of the excretory pigment will be considered more fully in the discussion of the intestinal epithelium.

The nature and properties of Venus intestinal epithelium. The central importance of intracellular digestion in the amoebocytes in lamellibranch nutrition has been questioned by Nelson (1933), Mansour (1946), and Mansour-Bek (1946). These workers believe that extracellular digestion occurs in the alimentary tract of these animals. Yonge (1926*a*) states that 'no evidence of any absorption in the epithelium of the gut or any free surface in the mantle cavity, other than by the agency of phagocytes was found'. Observations made on the cytochemistry of *Venus* intestinal epithelium may contribute to this question.

Red-violet granules resembling secretory granules were observed in the distal zone of the columnar epithelial cells in sections stained by eosin and methylene blue, and this region was marked by red-violet fluorescence in ultra-violet light. The eosinophil, fluorescent granules of each cell corresponded in position with the major site of alkaline phosphatase activity. This
enzyme functions in the dephosphorylation of several organic phosphates including hexose diphosphate, nucleic acid, lecithin, and glycerophosphate. Deane and Dempsey (1945) described the localization of alkaline phosphatase in the apical zone of duodenal epithelium in several vertebrate species after incubation of sections in glycerophosphate. Alkaline phosphatase activity was also seen in the supranuclear or Golgi region of these cells. These authors concluded that their studies supported the concept that intestinal epithelium contains enzymes capable of dephosphorylating intermediate substances in normal metabolism. Kosman, Kaulbersz, and Freeman (1943) reported that alkaline phosphatase was secreted by the dog duodenum and jejunum and that it probably functioned in the digestion of monophosphoric esters of food. Lecithin phosphatase is also present in duodenal epithelium and is thought to be of importance in digestion of phospholipids (Dempsey and Deane, 1946). Furthermore, glycerophosphatase is thought to be involved in fat absorption, since glycerophosphatase is believed to be an intermediate state in the breakdown and resynthesis of neutral fat (Bloor, 1943). Thus, it appears that the alkaline phosphatase activity detectable in the intestinal epithelium of Venus may be associated with possible digestive and absorptive functions of these cells. Further evidence for the absorptive role of the epithelium was seen in the sudanophil droplets which were present in the distal portion of the epithelium and ketone-containing lipid which was demonstrated in the middle portion of the epithelium. Gutheil (1912) observed fat globules in the intestinal epithelium of Anodonta and concluded that the epithelium functions in absorption, and Yonge (1926a) observed fat globules in the stomach and midgut epithelium of Ostrea after feeding on diatoms, but concluded that the amoeocytes transmitted the fat to the epithelial cells for storage. Although Yonge (1926, a and b) has denied the existence of digestive and absorptive activity in the epithelium, the presence of eosinophil granules, sudanophil droplets, alkaline phosphatase, lipase, and serum cholinesterase suggests that these functions may be present.

The nature of the goblet cell mucus. Since the goblet cells of the intestinal epithelium were strongly basophil and retained their stainability by methylene blue in solutions buffered below pH 4 and were metachromatic as well as PAS-positive after diastase digestion, it appears that they contain typical mucus. The goblet cells were stained red by the Feulgen procedure, a colour unlike the violet stain produced in the nuclei. The basis of this atypical Feulgen reaction is obscure, since this reaction is quite specific for DNA.

The nature and properties of excretory substance. Numerous investigators have noted the presence of yellow, brown, or green pigment granules in amoeocytes and other tissues (Metschnikoff, 1884; Grobben, 1887; MacMunn, 1900; Yonge, 1926, a and b). MacMunn (1900) referred to this material as enterochlorophyll, which he believed to be a derivative of ingested chlorophyll, and Durham (1891) suggested that this pigment was an excretory product composed of degraded echinochrome. Since the appearance of the pigment varied in different cells, Durham concluded that transformation of the
pigment occurred in the amoebocytes. Similar pigments were observed by J. H. List (1890) and T. List (1902). J. H. List (1890) reported that the pigment masses were not constant and that their nature depended on the food ingested by the animal since starved animals had few pigment inclusions. Yonge (1926b) observed that globules of green and brown material appeared in amoebocytes after diatoms were ingested but disappeared during starvation. The brown colour of the pigment was attributed to ingested chlorophyll and products of chlorophyll degradation. In the amoebocytes and free in the intestinal and cardiac musculature of Venus, excretory pigment appeared as small yellow granules, but in the intestinal epithelium this material occurred in large yellow or yellow-brown heterogenous aggregates which were composed of pigment granules embedded in a matrix.

**Table 1. Comparisons of the histochemical reactions of ceroid and excretory pigment**

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<th>Excretory pigment</th>
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<td>Colour</td>
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<td>+</td>
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<tr>
<td>Iron (Prussian blue)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ultra-violet fluorescence</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAS after exposure to diastase</td>
<td>+</td>
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</table>

*Ceroid-like nature of the excretory substance.* The description of ceroid by Endicott and Lillie (1944) is strongly reminiscent of the excretory pigment found in amoebocytes and intestinal epithelium of Venus. These authors identify ceroid by the following characteristics:

1. Golden yellow colour in unstained sections.
2. Resistant to fat solvents.
3. Stained by basic dyes.
4. Not stained by acid dyes.
5. Sudanophil in paraffin sections.
6. Acid fast.
7. Reduces alkaline silver nitrate, but not the ferric-ferricyanide reagent.

In addition Lee (1950) observed that ceroid in rat and mouse livers is variably PAS-positive, and Popper, György, and Goldblatt (1944) reported that ceroid possesses bright yellow or golden brown fluorescence when examined in ultra-violet light. Table 1 compares the histochemical properties of ceroid and excretory pigment.

The excretory material appears to be very similar to ceroid as described by Endicott and Lillie (1944) and others with one exception, namely, that unlike
excretory pigment, ceroid fails to stain when exposed to the ferric-ferricyanide reagent (Endicott and Lillie, 1944). However, Pearse (1953) states that this property may be acquired during the oxidation of lipids.

The origin and nature of ceroid. Ceroid was first discovered in cirrhotic livers of nutritionally deficient rats by Lillie and his associates (1941, 1942), who believed that it was a product of abnormal metabolism. It was found that ceroid accumulation was inhibited by feeding on casein, choline, and methionine. Subsequently, other workers concluded that ceroid was a lipoprotein derived from the necrotic remnants of liver parenchymal cells (György and Goldblatt, 1942; Lee, 1950), and that its presence was due to vitamin E deficiency (Victor and Pappenheimer, 1945). This pigment is found in many vertebrate tissues other than cirrhotic liver (Firminger, 1952; Deane and Fawcett, 1952; Lillie, 1941, 1942; Wolf and Pappenheimer, 1945).

Ceroid is believed to be a mixture of several substances (Lee, 1950; Pearse, 1953). Pearse (1953) regards ceroid as a member of a general group of pigments termed lipofuscins which are derived by oxidation from lipids or lipoproteins. Different staining reactions are given as the lipoidal material becomes progressively oxidized. The PAS-reaction is positive in the intermediate stages of the oxidation of lipids and Schiff-reacting aldehydes are produced from unsaturated phosphatides by periodic acid.

Casselman (1951) observed the formation of ceroid-like substances in vitro from unsaturated fat and from fatty acids and their esters, but never from saturated fats or hydrocarbons. The production of the pigment was prevented by the presence of antioxidants such as α-tocopherol and hydroquinone. Casselman concluded that 'whenever conditions are such that unsaturated fats accumulate in tissues to such an extent that a relative lack of biological antioxidant results, autoxidation of the fats and their conversion to ceroid pigment are favored, and that ceroid and the lipofuscin pigment of vitamin E deficiency may be fundamentally similar'.

The occurrence of a ceroid-like pigment in the amoebocytes and intestine of Venus was unexpected. The excretory pigment appears to be a lipofuscin closely related to ceroid observed in cirrhotic and vitamin E deficient animals. This material is formed either as an oxidized by-product of lipid metabolism in the intestine or as a by-product of intracellular digestion in the amoebocytes. The relation of excretory pigment to digestion is indicated by the disappearance of this pigment in starved animals. In the case of Venus, the excretory pigment may arise in part from the oxidation of phospholipids which can be demonstrated within the masses of excretory pigment.

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