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Histochemical and Biochemical Studies of the Hepatopancreas Peroxidase of the Freshwater Crayfish, *Cambarus robustus*

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ABSTRACT Crayfish are among the few invertebrate species reported to possess endogenous peroxidase activity. The enzyme is found within the hepatopancreas, the principal digestive and absorptive organ of the crustacean body. *Cambarus robustus*, a species found in abundance in the streams of western New York, was used in this study. Homogenates of 18 hepatopancreases were assayed for peroxidase activity using guaiacol as the substrate. Although present in all organs, peroxidase activity displayed a greater than 50-fold difference between the two extremes (0.05–2.72 units/mg protein). Histochemical examination using diaminobenzidine revealed peroxidase activity within a line of cells extending along the distal two-thirds of the lengths of all hepatopancreatic tubules. The cells function to synthesize the enzyme, sequester it within vacuoles of increasing size, and eventually secrete it into the tubule lumen. Since the tubule is constantly renewed by distal mitotic activity and concomitant proximal exfoliation, this histochemical technique permits not only the examination of the ontogeny of this peroxidase-positive cell line, but also offers additional insight into the mechanism of hepatopancreatic tubule renewal.

The crustacean hepatopancreas combines various functions of the vertebrate liver, pancreas, and small intestine. It stores a variety of metabolites and minerals, secretes all of the required digestive enzymes and bile acids, and is the primary site of nutrient absorption (Vonk, '60). It may also possess a population of phagocytic cells which removes foreign particles from the hemolymph (Reade, '68; Merrill et al., '79). The crayfish hepatopancreas consists of two lateral lobes each composed of a great number of hollow, blindly-ending tubules. The lumens of the tubules open into the collecting ducts of the corresponding lobe which, in turn, open into the midgut of the digestive tract. Each lobe is covered by a bordering membrane, a layer of dense connective tissue which is attached to the tips of the tubules and functions to confine hemolymph to the internal hemal sinus.

Each tubule is lined by a simple columnar epithelium. Pulse-labeling of hepatopancreatic nuclei with tritiated thymidine (Davis and Burnett, '64) confirmed the histological observations of Huxley (1880), who described the continuous process of tubule renewal as beginning at the "summit" of the tubule and proceeding through differentiation toward the collecting ducts. While unanimous agreement regarding the sequence of cellular ontogeny is lacking, most investigators use the nomenclature of Jacobs ('28) to describe the various cell types. E-cells (Embryonalzellen) are undifferentiated, mitotically-active cells located at the apex. It is widely held that these cells give rise to all remaining tubule cell types, either directly or indirectly. R-cells (Restzellen) are highly vacuolated acidophilic cells which contain lipid, glycogen, and copper inclusions (Dorman, '28; Ogura, '59; Miyawaki et al., '61). F-cells (Fibrillenzellen) possess extensive concentration of rough endoplasmic reticulum giving the cytoplasm a basophilic, fibrillar appearance (Davis and Burnett, '64). These cells may be involved in the elaboration of digestive enzymes (Loizzi, '71; Loizzi and Peterson, '71) and, perhaps, the absorption and storage of ionic iron (Ogura, '59; Miyawaki et al., '61;

Miyawaki and Tanoue, '62; Loizzi, '71). B-cells (Blazenzellen) are large secretory cells with a single supranuclear vacuole which displaces the nucleus and cytoplasm to the basal region of the cell as it develops. The abundance and activity of any given cell type are parameters which may vary from species to species, with the season of the year, or with the molt cycle or feeding behavior of a single individual.

While investigating the role of oxidative enzymes in the nitrogen metabolism of crustaceans, Hartenstein ('73) discovered peroxidase activity in the hepatopancreas of the freshwater crayfish, *Cambarus bartonii*. Peroxidases are a class of enzymes which utilize hydrogen peroxide to oxidize a wide variety of compounds (Saunders et al., '64). Dunfold ('82) has suggested that peroxidases first evolved in primordial organisms as a defensive measure against oxidation by toxic oxygen compounds. Later, once survival from oxidation was assured, the enzymes evolved specialized functions. At the present time the function of crayfish hepatopancreas peroxidase is unknown.

Preliminary studies (Merrill et al., '83) have shown that the hepatopancreas of *Cambarus robustus*, a local freshwater crayfish, possesses endogenous peroxidase activity. The objectives of our study were two-fold: 1) to sample individuals from a single population of this species and quantify, by biochemical assay, the activity of the enzymes; and 2) to utilize histochemical procedures to identify the cells responsible for the synthesis of the enzyme.

MATERIALS AND METHODS

Experimental animals

Mature, intermolt male crayfish, *C. robustus*, which were in good health and free of parasitic infestation, were used in the study. Animals used for the assay of peroxidase activity were collected from the same location (Oatka Creek, Scottsville, New York) on the same day. They were transferred to aquaria with continuously circulated and filtered water maintained at 15°C. They were sacrificed within one week of capture. Animals used in the histochemical studies were maintained under identical conditions but were fed a weekly diet of chopped fish.

Assay of peroxidase activity

Hepatopancreases from 18 cold-anesthetized animals were removed, weighed, and individually homogenized in a small volume of cold 6 mM phosphate buffer at pH 7.0. The homogenate was centrifuged at 40,000 × g for 60 min at 4°C. The supernatant was decanted and the pellet resuspended in cold buffer. Following a second centrifugation, the two supernatant fractions were combined and assayed spectrophotometrically for peroxidase activity by measuring the increase in absorbance at 470 nm upon oxidation of guaiacol. A total reaction volume of 3.0 ml contained the enzyme extract, 17 mM guaiacol, 2.4 mM hydrogen peroxide, and 6 mM phosphate buffer at pH 7.0. One unit of peroxidase activity was defined as that amount of homogenate which produced an increase in absorbance (A_{470}) of 1.0 optical density unit per minute as calculated from the initial rate of the reaction. Total protein was determined according to Lowry et al., ('51), using bovine serum albumin as the standard.

Light microscopy and histochemistry

Organs used for the histochemical demonstration of endogenous peroxidase activity were removed from cold anesthetized animals and rinsed in cold 0.1 M phosphate buffer at pH 6.5, containing 0.65% NaCl, 0.02% KCl, 0.012% $MgCl_2$ and 0.01% $NaHCO_3$. The salts used in this medium are present in the same proportions as those found in crayfish physiological saline (van Harreveld, '36), adjusted to be isosmotic with crayfish hemolymph (425 mOs/kg). A pH of 6.5 was selected because it represents the mid-point of the optimum pH range for peroxidase activity (Hartenstein, '73). The organs were then incubated for time periods of 15, 30, 45, and 60 minutes in buffer containing 0.1% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.015% hydrogen peroxide at 4°C. Controls were obtained by either incubating in medium devoid of hydrogen peroxide or by boiling the tissue for 30 minutes followed by incubation in the complete DAB medium. Following incubation, tissues were washed for 10 minutes in cold buffer and fixed in either neutral buffered formalin (NBF) or Bouin's fixative for a minimum of 72 hours. Individual tubules from NBF-fixed organs were isolated under a dissecting microscope and measured for length, diameter, and the distribution of DAB reaction product. Organs fixed in Bouin's fixative were dehydrated in ethanol, cleared in toluene, and embedded in paraffin. Serial sections of 7–10 μm were cut on a rotary

microtome and placed on albumin-coated microscope slides. Deparaffinized sections were either counterstained with 0.5% aqueous eosin, stained for ferric iron by the Perl method (McManus and Mowry, '60) or stained by the regressive hematoxylin and eosin method (Humason, '79).

RESULTS

Assay for hepatopancreas peroxidase activity

The hepatopancreases of 18 adult male crayfish of similar size and maturity were examined for wet weight, protein content, and endogenous peroxidase activity. Individual data, as well as means and standard deviations, are presented in Table 1. Hepatopancreas wet weights and total protein are very consistent among specimens. However peroxidase activity, although present in all animals tested, was highly variable with a greater than 50-fold difference in protein-specific activity between the two extremes. This variability could not be correlated with any readily-observable physical characteristics of the animal or the organ.

Histochemistry

Whole mounts

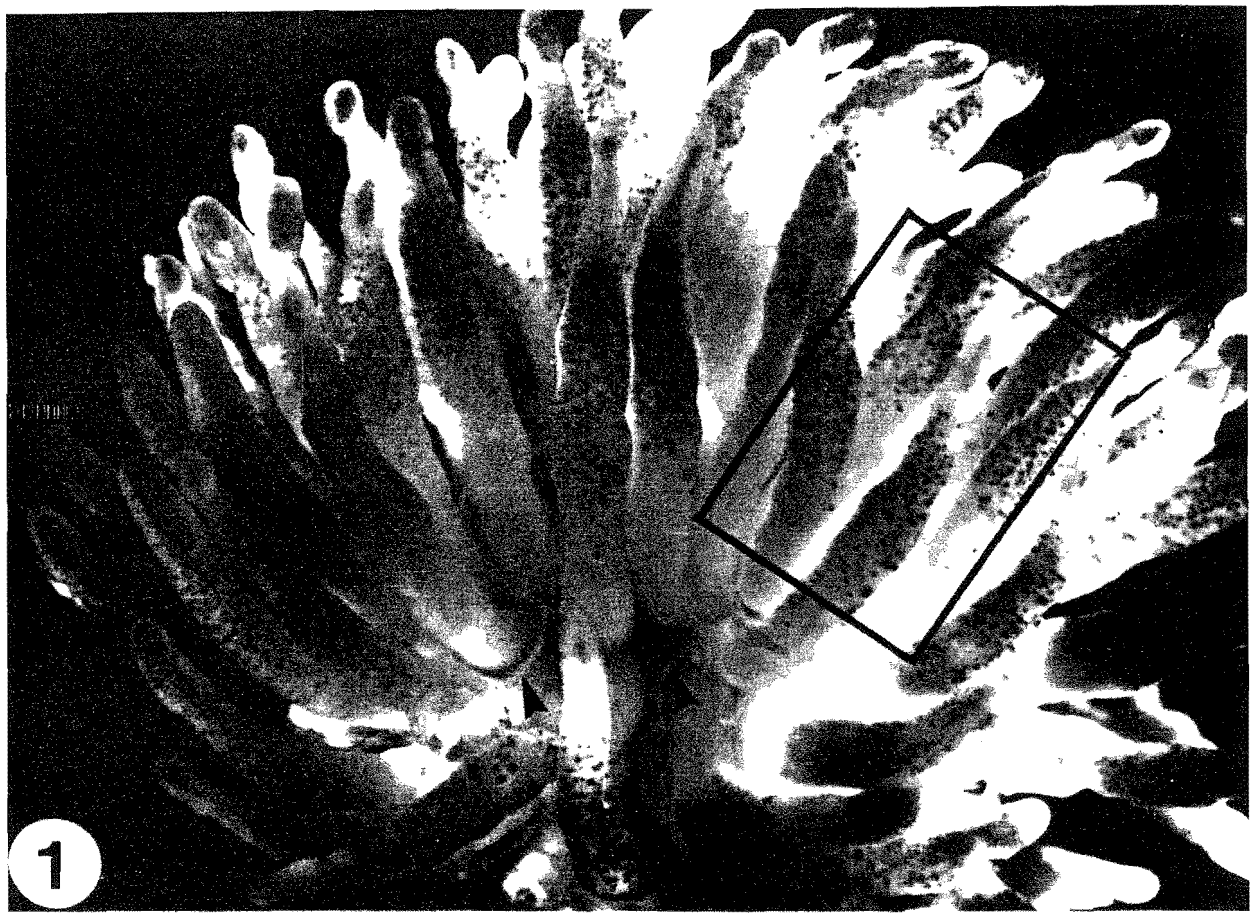
A widely used and highly sensitive histochemical assay for peroxidase activity involves the enzyme-catalyzed oxidation of DAB to a stable and highly dense precipitate (Graham and Karnovsky, '66). Figure 1 shows a portion of hepatopancreas incubated for 60 minutes in the complete DAB medium. The bordering membrane which forms an external covering over the organ has been dissected away to expose the individual tubules and interstitial vascular elements. The DAB reaction product, indicative of the site of peroxidase activity, is readily observed as spherical particles distributed randomly throughout the mid-section of most tubules. Figure 2 depicts a control portion of the same organ which was incubated in the absence of H_2O_2; the tubules are entirely devoid of peroxidase activity. Upon closer examination (Fig. 3),

Abbreviations

ac,	apical complex
BCZ,	B-cell zone
bm,	bordering membrane
dsnv,	developing supranuclear vacuole
DT,	distal tip
DZ,	distal zone
FeG,	ferric iron granule
grp,	granular reaction product
lrp,	luminal reaction product
PoRZ,	peroxidase reactive zone
PZ,	proximal zone
snv,	supranuclear vacuole
TZ	transition zone
vrp	vacuolar reaction product

TABLE 1. Peroxidase activity in the hepatopancreas of the freshwater crayfish, Cambarus robustus

		Hepatopancreas general data			Peroxidase activity	
Animal	Body weight (gm)	Wet weight (gm)	Total protein (mg)	Total protein/ wet weight (%)	Total units	Units/mg protein
1	41.6	2.1	54.8	2.6	99.3	1.81
2	39.9	3.9	99.9	2.7	272.0	2.72
3	37.0	2.7	76.2	2.8	63.3	0.83
4	27.1	2.2	72.5	3.3	19.7	0.27
5	29.6	2.5	77.0	3.9	154.0	2.00
6	20.6	2.2	79.2	3.6	61.7	0.78
7	26.1	2.9	87.4	3.0	31.0	0.35
8	23.2	2.3	77.2	3.4	10.9	0.14
9	35.8	2.6	83.7	3.2	54.2	0.65
10	24.6	2.2	86.7	3.3	69.6	0.84
11	25.2	2.3	93.9	4.1	26.0	0.28
12	26.2	1.8	70.4	3.8	3.4	0.05
13	36.0	3.3	106.2	3.2	41.2	0.39
14	32.6	2.9	117.4	4.0	66.8	0.57
15	20.9	2.3	87.3	3.8	69.6	0.80
16	21.0	1.9	80.3	4.2	6.9	0.09
17	20.4	2.1	74.2	3.6	22.0	0.30
18	26.6	2.0	79.6	4.0	76.0	0.95
Mean	28.6	2.5	83.3	3.5	63.8	0.77
S.D.	6.9	0.5	14.2	0.5	63.9	0.72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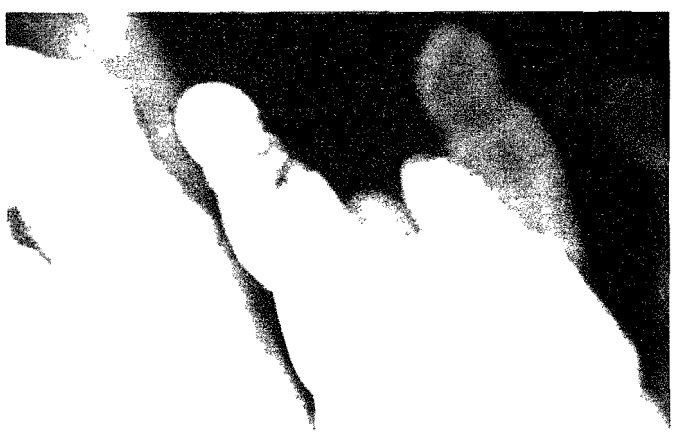
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the peroxidase-reactive particles exhibit variations in staining intensity and size. They range from 10 to 60 μm in diameter with a tendency for the smaller particles to be distributed toward the apical region of the tubule.

Fifty tubules from a hepatopancreas incubated in the complete DAB medium were selected at random and separated from the main body of the organ. A series of measurements were made which led to the construction of the generalized tubule shown in Figure 4. The mean dimensions and standard deviations of all 50 observations are presented in Table 2. With only slight variability, tubules appear as thin projections with a gradually-tapering diameter and a blindly-ending apex. On the basis of the distribution of peroxidase activity, it was possible to define three distinct and relatively consistent zones within each tubule. The proximal zone (PZ), which appears devoid of peroxidase activity, extends from the attachment of the tubule on the body of the organ to the point where the reaction product is first apparent. This zone accounts for 37% of the tubule length. The middle region, which we termed the peroxidase-reactive zone (PoRZ), occupies 46% of the total length. The distal zone (DZ), which extends to the apex and occupies the remaining 17% of the tubule length, appears devoid of particulate peroxidase activity. Many tubules display peroxidase reaction product as a faint, thin line through the center of the distal zone.

Histology

A longitudinal section through one of the hepatopancreatic lobes of *C. robustus* reveals several complete tubules (Fig. 5). The border-

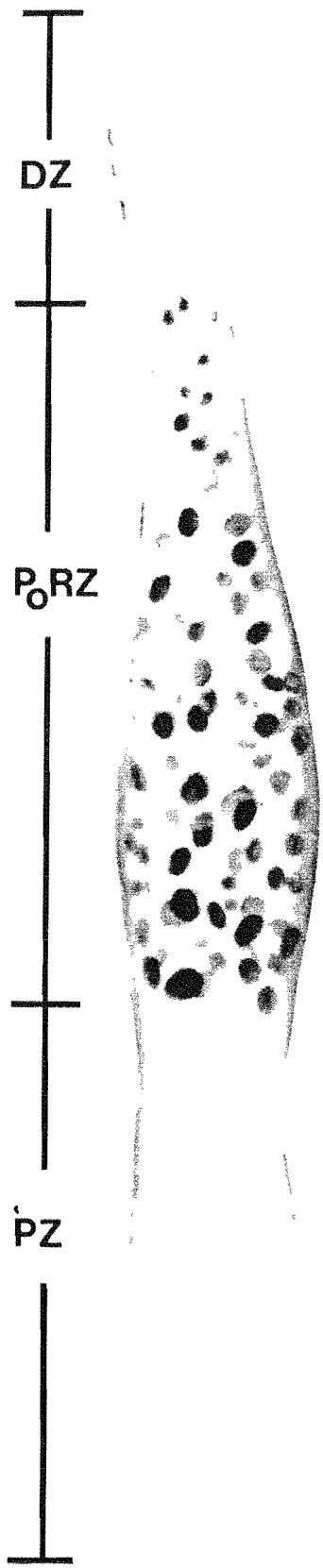


Fig. 4. This sketch depicts the distribution and physical characteristics of peroxidase reaction product within a typical hepatopancreas tubule. Measurements and zonation reflect the mean values of fifty observations (see Table 2). PZ, proximal zone; PoRZ, peroxidase reactive zone; DZ, distal zone. ×50.

Fig. 1. This portion of crayfish hepatopancreas was incubated in the complete DAB medium. Peroxidase reaction product appears as dark spots (brown), giving the tubules a speckled appearance. The bordering membrane which normally encircles the organ has been removed to expose the tubules and internal vasculature (arrows). ×14.

Fig. 2. Hepatopancreas tubules incubated in a DAB medium lacking hydrogen peroxide. ×48.

Fig. 3. Higher magnification of tubules shown in Figure 1 (box) demonstrating variations in the staining intensity and size of peroxidase-positive granules. ×40.

ing membrane which surrounds the organ is intact, accounting for the compact state of the organ and making visualization of individual tubules difficult. The different cell types comprising the tubule epithelium are responsible for the variegated histological profile. Each tubule is composed of four zones or regions distinguished on the basis of cell composition (Loizzi, '71). Beginning apically they are: distal tip, transition zone, B-cell zone, and proximal zone. The parallel organization of tubules in the section of hepatopancreas (Fig. 5) permits the identification of the two largest zones, the proximal zone (PZ) and B-cell zone (BCZ) of several neighboring tubules. Their relatively small sizes require that the distal tips (DT) and transition zones (TZ) of the tubules be viewed at a higher magnification (Fig. 6).

TABLE 2. Dimensions of 50 hepatopancreatic tubules

	Mean (mm)	Standard deviation (mm)
Length	3.48	0.44
Proximal diameter	0.25	0.02
Distal diameter	0.12	0.02
Proximal zone	1.28	0.31
Peroxidase reactive zone	1.62	0.24
Distal zone	0.58	0.09

The distal tip is populated with E-cells, undifferentiated columnar cells that may be stratified in regions of mitotic activity. The common appearance of mitotic figures is evidence that the distal tip serves as the growth center of the tubule. Although not apparent in tubule whole mounts, certain E-cells appear to possess peroxidase activity, manifested as finely-granular reaction product (grp) scattered throughout the apical cytoplasm (Fig. 7).

The transition zone (Figs. 6, 8–10), is the region where cells newly derived from the nearby embryonic distal tip gradually differentiate and mature. Its considerable histological variability prompted us to subdivide

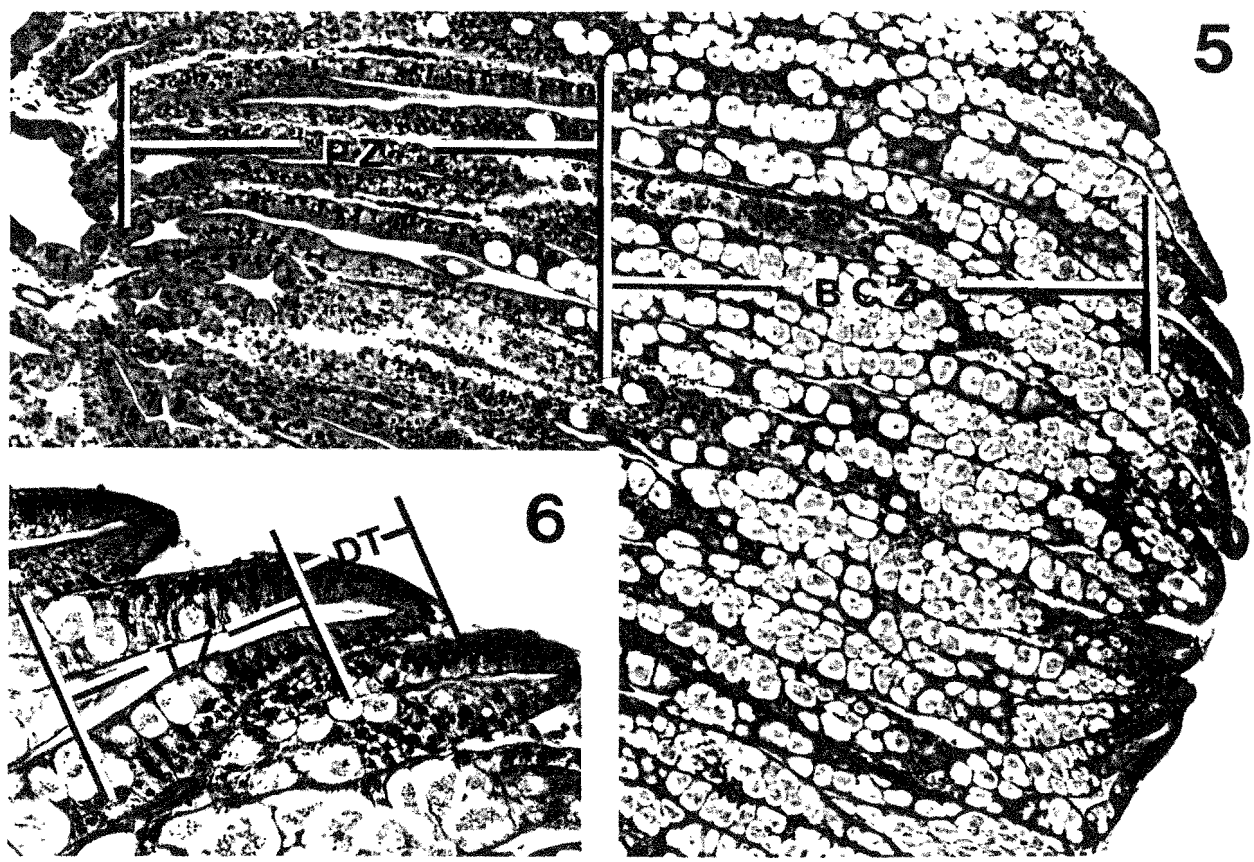


Fig. 5. Longitudinal section of a portion of a hepatopancreatic lobe depicting the histological zonation of the tubules. The bordering membrane is intact, accounting for the parallel alignment and compressed nature of the tubules. H & E stain. PZ, proximal zone; BCZ, B-cell zone. ×36.

Fig. 6. Higher magnification of the distal regions of three tubules shown in Figure 5 depicting the transition zone (TZ) and distal tip (DT). ×120.

this zone into distal, middle, and proximal regions. Distally, reaction product appears as discrete, compact granules typically ranging from 1 to 2 μm in diameter. Although always found in the supranuclear cytoplasm, they may appear both isolated or packed into tight clusters (Fig. 8). Generally, the number and size of these granules within a given cell increase correspondingly with the distance of the cell from the distal tip of the tubule. Within the middle segment of the transition zone the trend toward larger (upwards of 4 μm in diameter) and more numerous granules is apparent (Fig. 9). In addition, developing supranuclear vacuoles (dsnv) appear within some of the peroxidase-reactive cells. Within these vacuoles, peroxidase reaction product displays both a granular and a dispersed nature, suggesting that the development of the supranuclear vacuole may involve the fusion of several peroxidase-positive granules. Figure 10 shows three cells, located within the proximal region of the transition zone, which display reaction product. One of these cells contains a large supranuclear vacuole filled with coarse, somewhat flocculent reaction product. To distinguish it from the granular form we termed it vacuolar reaction product (vrp). These cells also show a thick band of activity located along their luminal margins. This region corresponds to the apical complex, described by Loizzi ('71) as containing a microvillar border, dense cytoplasm and pinocytic channels and vesicles of various sizes.

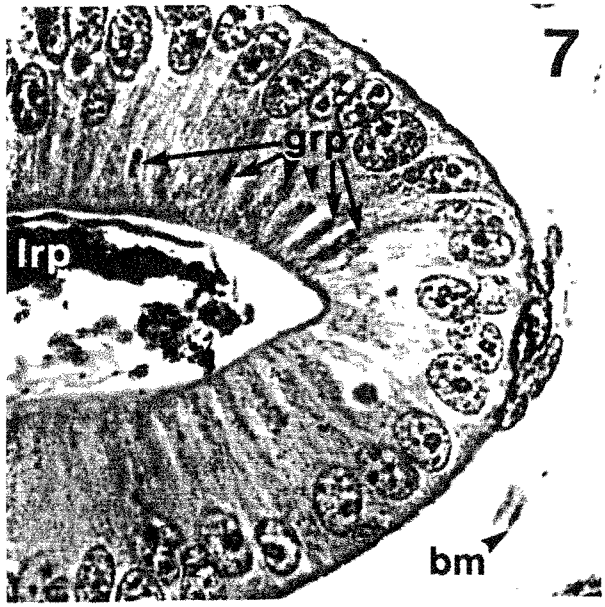
The B-cell zone is clearly the most distinctive region of the tubule (Fig. 5). Although a minority of R-cells and F-cells are found scattered throughout the zone, they are obscured by the B-cells with their enormous (0.05 × 0.09 mm) supranuclear vacuoles and crescent-shaped basal nuclei. Peroxidase activity is found within the vacuoles of most of the B-cells exposed to the complete DAB medium. The reaction product is flocculent (Fig. 11). In addition, the apical complex of each cell is strongly peroxidase-positive.

The B-cell zone ends abruptly, giving rise to a proximal zone containing cells which, on the basis of staining preferences (Fig. 5), appear to be F-cells and R-cells in various stages of activity or degeneration. We observed a significant number of cells with intense cytoplasmic basophilia that possessed a single vacuole which stained for ferric iron (Fig. 12). These cells appear to correspond to the Fe-cells reported first by Ogura ('59). Owing to the presence of a few iron particles, these cells are first visible near the end of the B-cell zone. It is apparent that as these cells migrate through the proximal zone they accumulate iron quickly. Cells forming the lining of collecting ducts possess ovoid vacuoles of approximately 15 μm in diameter showing a core of intense iron reactivity (Fig. 12). All cells of the proximal zone are totally devoid of peroxidase activity, although the presence of peroxidase-positive luminal contents (lrp) throughout all exposed tubules proves that the required reactants penetrated the tubule cells at this level.

DISCUSSION

The hepatopancreas of the freshwater crayfish, *C. robustus*, was examined both biochemically and histochemically for the presence and cellular location of endogenous peroxidase activity. Although the enzyme was found in all animals assayed, there was considerable individual variability, with a greater than 50-fold difference in protein-specific peroxidase activity between the two extremes (Table 1). While our attempts to standardize the experimental protocol were very rigorous, certain factors such as state of health, pre-capture diet or feeding frequency may serve either to induce or suppress enzyme synthesis. In addition, while all of the animals used in the study were adult and intermolt, the exact age and stage of the molt cycle of each animal was unknown. There is evidence from studies of the lobster (Travis, '55, '57) and blue crab (Johnson, '80) that the histology of the hepatopancreas varies with the molt cycle. While concomitant changes in biochemical activity have not been reported, it is conceivable that the variability we observed may represent normal fluctuations, perhaps cyclical, in the physiological state of each individual. Finally, the levels of enzyme activity may simply reflect a biochemical state which is unique to each organism.

In our histochemical study of the hepatopancreas of *C. robustus*, the distribution of peroxidase activity, manifested as the dark brown reaction product of diaminobenzidine oxidation, allowed us to establish firmly the existence of a previously unidentified population of cells within the hepatopancreatic tubule which, throughout various stages of differentiation, functions to synthesize, elaborate, store, and then secrete peroxidase. Since the tubule is constantly renewed by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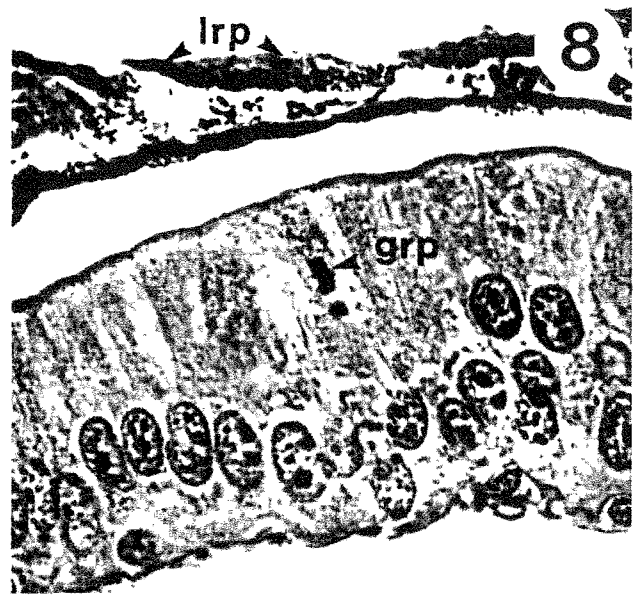
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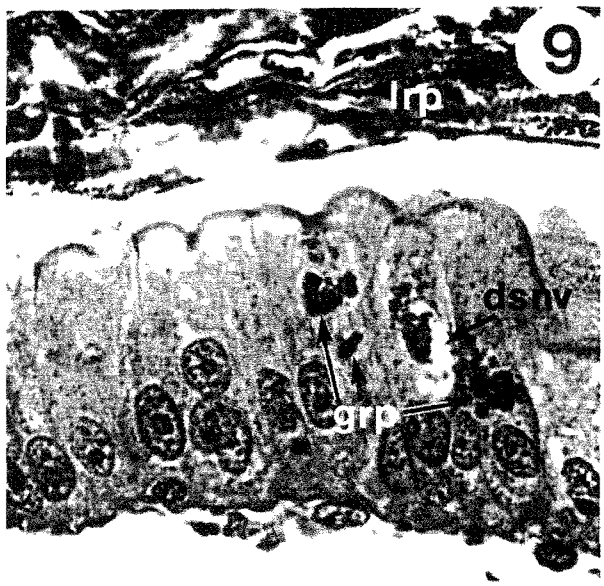
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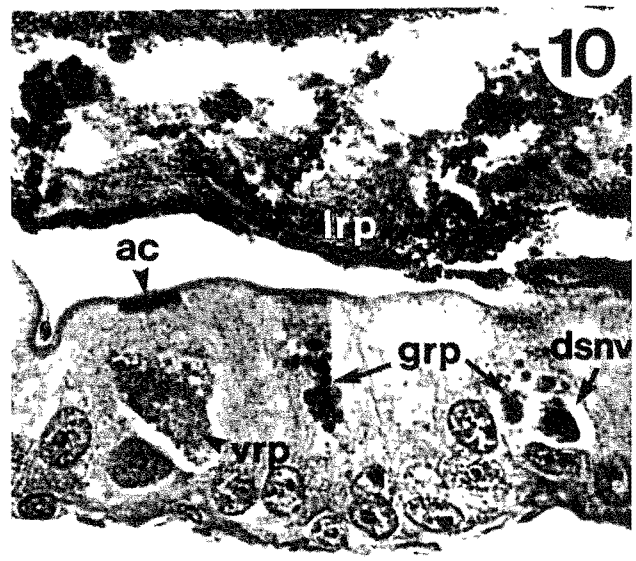
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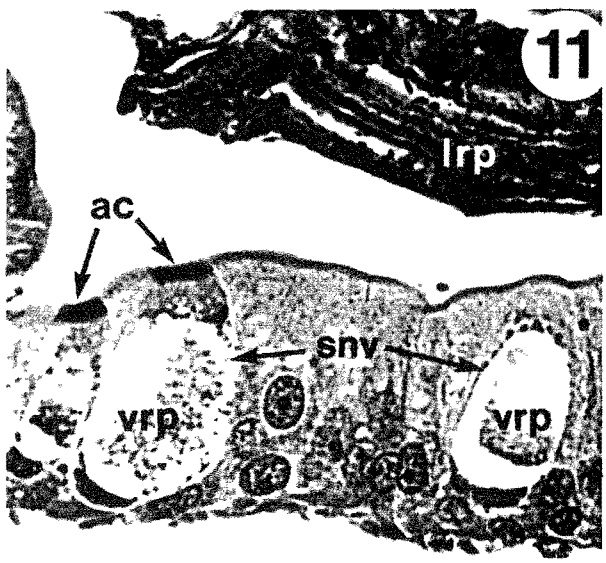
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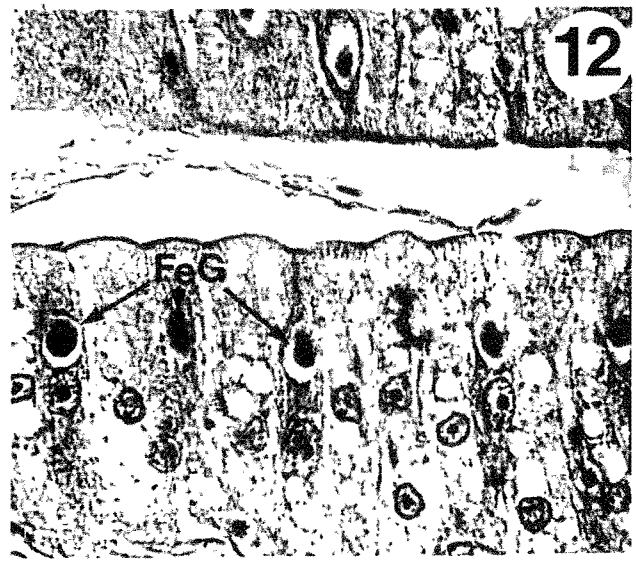
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FeG

mitotic activity in the distal tip and concomitant cell death along the proximal segment, the determination of the location and distribution of peroxidase activity provides not only a means of tracking the migration of this specific cell line, but offers additional insight into the mechanism of tubule renewal. Because the lobster and other closely related crustacean species lack hepatopancreas peroxidase activity (Wurzinger and Hartenstein, '74), we have chosen to examine our findings in the light of other studies dealing with just the crayfish hepatopancreas, and to pay particular attention to reports on the structural and functional interrelationships among the various cell types comprising the hepatopancreatic tubule.

Since the publication of Huxley's classic monograph on the crayfish (1880), descriptive studies of the hepatopancreas have been numerous and often contradictory. From detailed histological work on the hepatopancreas of *Astacus leptodactylus*, Hirsch and Jacobs ('28, '30) proposed that E-cells give rise to two distinct cell lines, R-cells and F-cells, with the latter differentiating into B-cells, secretory cells which use a holocrine mode of secretion to deliver digestive enzymes to the tubule lumen. Following his discovery of copper and iron sulfide in the ash of the hepatopancreas of *Procambarus clarkii*, Ogura ('59) used histochemical procedures to distinguish three mature cell types which he named Cu-cells, Fe-cells and B-cells. Cu-cells with prominent copper granules are clearly R-cells. Miyawaki et al. ('61) demonstrated that the copper content of these cells was directly related to the concentration of copper in the water. Likewise, Ogura's ('59) B-cells were identical to the B-cells of Jacobs ('28) and Hirsch and Jacobs ('28, '30) except that he proposed their progenitors to be "certain inconspicuous cells" in the distal tip and not F-cells. Possessing a prominent ferric iron granule and pronounced cytoplasmic basophilia, the Fe-cell was shown to be abundant along the collecting ducts and proximal regions of the tubules but with a reduction in number and a decrease in the size of its iron granule toward the apex of the tubule. He concluded that Fe-cells evolve from apical basophilic cells which gradually accumulate iron as they migrate proximally. Those basophilic cells match the description of Jacobs' ('28) F-cells. Davis and Burnett ('64) tracked the course of cell migration in the hepatopancreas of *Procambarus blandingii acutus* at several time periods after the introduction of tritiated thymidine. Unlike the histological findings of the authors cited previously, they found only a single cell type, which they called an absorptive cell, in the transition zone between the embryonic cells and the secretory and fibrillar cells of the B-cell zone. They concluded that the various tubule cells represent sequential stages in the ontogeny of only a single cell line that develops over a period of 5 days and involves the direct conversions of E-cells to absorptive (R) cells to secretory (B) cells to fibrillar (F) cells to prox-

Fig. 7. Longitudinal section through the distal tip of a tubule. Peroxidase reaction product is visible within the lumen of the tubule (lrp) and as discrete granules within the apical cytoplasm of E-cells (grp). bm, bordering membrane. Eosin counterstain. ×600.

Fig. 8. Longitudinal section through the transition zone just proximal to the distal tip of a tubule. A single cell has accumulated peroxidase reaction product within a collection of dense granules (grp). lrp, luminal reaction product. Eosin counterstain. ×612.

Fig. 9. Longitudinal section through the middle region of the transition zone of a tubule shows three cells with peroxidase activity. In two of these cells reaction product appears as discrete granules of various sizes. The third cell shows a developing supranuclear vacuole (dsnv) displaying a dispersed reaction product. Eosin counterstain. lrp, luminal reaction product, grp, granular reaction product. ×612.

Fig. 10. Longitudinal section through the transition zone immediately distal to the B-cell zone of a tubule. An incipient B-cell displays a highly reactive apical complex (ac). The vacuolar reaction product (vrp) within its enlarged supranuclear vacuole is finely dispersed and flocculent. Two additional peroxidase-positive cells show development similar to cells in Figure 9. lrp, luminal reaction product; grp, granular reaction product; dsnv, developing supranuclear vacuole. Eosin counterstain. ×612.

Fig. 11. Longitudinal section through the B-cell zone of a tubule shows three B-cells recognized by their large supranuclear vacuoles (snv) and crescent-shaped basal nuclei. Each displays typical flocculent vacuolar reaction product (vrp). Two show stained apical complexes (ac). Eosin counterstain. lrp, luminal reaction product. ×600.

Fig. 12. Longitudinal section through the proximal zone of a tubule shows several fibrillar cells with prominent ferric iron granules (FeG). Vacuolated R-cells are interspersed in this region. Eosin counterstain. ×612.

imal necrotic cells. Like Ogura ('59), Davis and Burnett ('64) found ferric iron granules within the fibrillar cells of the proximal zone; however, they also reported the presence of tiny iron deposits within the secretory vacuoles of B-cells.

Loizzi ('71) and Loizzi and Peterson ('71) conducted histochemical, cytochemical, and ultrastructural investigations of the hepatopancreases of two crayfish species, *Procambarus clarkii* and *Orconectes virilis*. Taken collectively, their data provide substantial support of the two-cell-line differentiation scheme proposed by Hirsch and Jacobs ('28, '30). They found two morphologically distinct cell types within the transition zone and concluded that both differentiate independently from distal E-cells and must give rise to all remaining tubule cells, including B-cells. They did not examine the fine structure of E-cells to discover if distinctive features might allow the identification of precursors of both cell lines. They found R-cells, the more numerous of the two, to possess a fine structure which suggests an integration of the various functions of intestinal absorptive cells and hepatocytes of vertebrates. Glycogen and lipid inclusions were abundant, whereas rough endoplasmic reticulum and Golgi bodies were confined to a few cisternae scattered near the basal region of the cell. The less abundant F-cells had a strikingly different morphology. Shown earlier by pulse-labeling with tritiated uridine to be active in protein synthesis (Davis and Burnett, '64), young F-cells were shown to possess an extensive rough endoplasmic reticulum with dense accumulations of ribosomes, dilated cisternae, and large numbers of Golgi bodies. Loizzi ('71) confirmed the presence of Fe-cells with the electron microscope but identified them as young F-cells. He proposed that as the F-cell matures the cisternae of its endoplasmic reticulum and Golgi bodies dilate and small vesicles associated with these organelles expand and coalesce to form a supranuclear vacuole containing primary digestive enzymes. Evidence of pinocytic activity at the microvillus border suggests that F-cells accumulate luminal materials by bulk transfer. He proposed that this mechanism could account for the uptake of ferric iron found in the vacuoles of some of these cells. With their expanding supranuclear vacuoles, F-cells within the proximal portion of the transition zone clearly represent intermediate forms in the differentiation of B-cells from F-cells. According to Loizzi ('71), B-cells deliver the contents of the supranuclear vacuole to the tubule lumen by an apocrine mode of secretion. The immediate fate of the resulting spent cell, which migrates with R-cells into the proximal zone and subsequently degenerates, was not closely examined and is therefore not fully understood. He did not report on the presence of proximal zone Fe-cells.

We believe that hepatopancreatic renewal in *C. robustus* involves the development, from embryonic E-cells, of at least three functional cell lines: the R-cell (absorptive) line, the secretory cell line, and the Fe-cell line. Our observations of the R-cell line appear to support those of Hirsch and Jacobs ('28, '30), Ogura ('59) and Loizzi ('71). These acidophilic cells accumulate lipid, glycogen and, other metabolites and migrate the full length of the tubule. The remaining two lines develop and maintain prominent cytoplasmic basophilia. The secretory cell line is the larger of the two. Using peroxidase activity as a marker for this line, early secretory cells can be observed within the embryonic tip (Fig. 7). To the best of our knowledge, this is the first report of a technique that appears sensitive enough to permit the identification of a specific cell line within this population of cells thought previously to be undifferentiated and, therefore, indistinguishable. As the peroxidase-positive cells are displaced proximally into the transition zone, the enzyme continues to be synthesized and is packaged into spherical granules or vesicles which increase in size and number within the cytoplasm. Clearly basophilic at this stage, these cells match earlier descriptions of F-cells. A supranuclear vacuole gradually develops, possibly through the fusion of membrane bound vesicles. As the vacuole expands the peroxidase reaction product loses its dense, compact appearance and becomes flocculent. In addition, an intensely-staining apical complex develops. If the apical complex does consist of pinocytic vesicles as reported by Loizzi ('71), its peroxidase reactivity may be due to the uptake of luminal reaction product rather than the accumulation of enzyme synthesized within the cell. The intensely staining particles of the peroxidase reactive zone of tubule whole mounts (Figs. 1,3) are unquestionably the supranuclear vacuoles of B-cells and their maturing F-cell precursors in the proximal transition zone. The abundance of reaction product within tubule lumens, the

abrupt termination of the peroxidase reactive zone (PoRZ), and the absence of even a trace of peroxidase activity within the proximal zone supports previous conclusions that B-cells are the principal secretory cell of the hepatopancreas and suggests that the act of secretion is a dramatic and highly efficient process. Following secretion, which we assume is not a holocrine process since we never observed whole B-cells within the collecting ducts or tubule lumens, the spent cell migrates into the proximal zone where it eventually degenerates. It is clear from this study that the developmental sequence of this cell line is identical to the E-cell to F-cell to B-cell differentiation sequence described by both Hirsch and Jacobs ('28, '30) and Loizzi ('71).

The identity of Fe-cells and their ontogenic relationship to the other tubule cells are not entirely clear. Because peroxidase enzymes possess a ferriporphyrin prosthetic group, we initially suspected that the activity of this enzyme would correspond to the location of the ferric iron granules of reported Fe-cells. Accordingly, we stained sections of DAB-reacted hepatopancreas for ferric iron in order first to locate the Fe-cells and then to determine if their iron granules displayed peroxidase activity. We found a population of iron-containing basophilic cells which was abundant within the proximal zones and which gradually disappeared near the proximal end of the B-cell zone (Fig. 12). These cells were totally devoid of peroxidase activity. In fact, at no time were we able to superimpose the blue stain for ferric iron over the peroxidase reaction product of any cell. Loizzi's ('71) micrographs showing iron granules within developing F-cells support the conclusion of Ogura ('59) that Fe-cells develop from fibrillar basophilic cells near the distal tip of the tubule. However, neither investigator reported, as did Davis and Burnett ('64), the presence of ferric iron in B-cell vacuoles. This would clearly be the case if all F-cells, including those with iron granules, differentiated into B-cells. Therefore, we are suggesting that the Fe-cell line and the secretory cell line represent distinct populations which develop from transition zone basophilic (F) cells. Prior to the use of peroxidase activity as a marker for the secretory cell line, they could not be readily distinguished, a fact that may have contributed to the conflicting reports cited earlier. Perhaps fewer in number initially, precursor Fe-cells begin only slowly to accumulate iron in small vacuoles which may be evident with the electron microscope (Loizzi, '71) but which are below the level of resolution by light microscopy. Outnumbered and obscured by the expanding supranuclear vacuoles of the secretory basophilic cells, Fe-cells become barely visible only as they leave the B-cell zone. There, near to the collecting ducts where iron from ingested food is readily available, they quickly establish a prominent iron granule. Their relative abundance in the proximal zone can be explained by longevity coupled with the rapid termination of the secretory cell line. It is possible that either environmental or physiological factors influence the relative numbers of transition zone basophilic cells that become either Fe-cells or secretory F-cells. Further support for this proposal must await the completion of electron microscopic studies currently underway in our laboratory.

This brings us to a consideration of the function of crayfish hepatopancreas peroxidase. In a survey of 53 invertebrate species, Wurzinger and Hartenstein ('74) reported a positive correlation between the presence of peroxidase activity and organisms that 1) were terrestrial and dwelled in burrows or detritus, and/or 2) fed largely on materials rich in lignin or tannins. We have shown that the crayfish hepatopancreas peroxidase is a secretory enzyme. Peroxidases from other secretory glands, namely the vertebrate salivary glands and mammary gland, are microbicidal when reacted with hydrogen peroxide and a halide under acidic conditions (Klebanoff and Luebke, '65; Klebanoff, '68; Slowey et al., '68; Belding et al., '70). Presumably, these enzymes function to reduce the growth of microorganisms in saliva and milk. Although homogenates of the crayfish peroxidase are not microbicidal under identical conditions (Merrill and Glenister, '80), the enzyme may still be a component of an in vivo defense mechanism that requires a unique set of conditions to be effective. Interestingly, the wood-inhabiting isopod, *Oniscus asellus*, reported by Wurzinger and Hartenstein ('74) to possess a very active hepatopancreas peroxidase, has a digestive tract that not only processes ligneous compounds, but is also totally devoid of microorganisms (Boyle and Mitchell, '78). It is possible that hepatopancreas peroxidase is required for the effective digestion of materials present in the crayfish diet and that any microbicidal action may represent a secondary adaptation.

At the present time the elucidation of the function of this enzyme requires further study.

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LITERATURE CITED

Belding, M., S.J. Klebanoff, and C.G. Ray (1970) Peroxidase-mediated virucidal systems. Science *167*:195–196.

Boyle, P.J., and R. Mitchell (1978) Absence of microorganisms in crustacean digestive tracts. Science *200*:1157–1159.

Davis, L.E., and A.L. Burnett (1964) A study of growth and cell differentiation in the hepatopancreas of the crayfish. Dev. Biol. *10*:122–153.

Dorman, H.P. (1928) The morphology and physiology of the hepatopancreas of *Cambarus virilis*. J. Morphol. *45*:505–535.

Dunford, H.B. (1982) Peroxidases. Adv. Inorg. Biochem. *4*:41–68.

Graham, R.C., and M.J. Karnovsky (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. *14*:291–302.

Hartenstein, R. (1973) Characteristics of a peroxidase from the freshwater crayfish, *Cambarus bartoni*. Comp. Biochem. Physiol. *45B*:749–762.

Hirsch, G.C., and W. Jacobs (1928) Der Arbeitsrhythmus der Mitteldarmdrüse von *Astacus leptodactylus*. I. Methodik und Technik: Der Beweis der Periodizität. Z. Wiss. Biol. *8*:102–144.

Hirsch, G.C., and W. Jacobs (1930) Der Arbeitsrhythmus der Mitteldarmdrüse von *Astacus leptodactylus*. II. Wachstum als primärer Faktor des Rhythmus eines polyphasischen organischen Sekretionssystems. Z. Wiss. Biol. *12*:524–558.

Humason G.L. (1979) Animal Tissue Techniques. San Francisco: W.H. Freeman.

Huxley, T.H. (1880) The Crayfish. An Introduction to the Study of Zoology. Cambridge, Massachusetts: The MIT Press.

Jacobs, W. (1928) Untersuchungen über die Cytologie der Sekretbildung in der Mitteldarmdrüse von *Astacus leptodactylus*. Z. Zellforsch. *8*:1–62.

Johnson, P.T. (1980) Histology of the Blue Crab, *Callinectes sapidus*. New York: Praeger.

Klebanoff, S.J. (1968) Myeloperoxidase-halide-hydrogen peroxidase antimicrobial system. J. Bacteriol. *95*:2131–2138.

Klebanoff, S.J., and R.G. Luebke (1965) The antilactobacillus sytem of saliva. Role of salivary peroxidase. Proc. Soc. Exp. Biol. Med. *118*:483–486.

Loizzi, R.F. (1971) Interpretation of crayfish hepatopancreatic function based on fine structural analysis of epithelial cell lines and muscle networks. Z. Zellforsch. *113*:420–440.

Loizzi, R.F., and D. Peterson (1971) Lipolytic sites in crayfish hepatopancreas and correlation with fine structure. Comp. Biochem. Physiol. *39B*:227–236.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*:265–275.

McManus, J.F.A., and R.W. Mowry (1960) Staining Methods. Histologic and Histochemical. New York: Harper and Row.

Merrill, D.P., and R.F. Glenister (1980) Crayfish hepatopancreas peroxidase: Absence of a microbicidal function. J. Invertebr. Pathol. *35*:214–216.

Merrill, D.P., S.A. Mongeon, and S. Fisher (1979) Distribution of fluorescent latex particles following clearance from the hemolymph of the freshwater crayfish, *Orconectes virilis* (Hagen). J. Comp. Physiol. *132*:363–368.

Merrill, D.P., T.R. Tiersch, R.S. Austin, and R.A. Reife (1983) A biochemical and histochemical study of hepatopancreas peroxidase of the crayfish, *Cambarus robustus*. Am. Zool. *24*:975 (Abstract).

Miyawaki, M., and S. Tanoue (1962) Electron microscopy of the hepatopancreas of the crayfish, *Procambarus clarkii*. Kumamoto J. Sci. Series *B6*:1–12.

Miyawaki, M., M. Matsuzaki, and N. Sasaki (1961) Histochemical studies on the hepatopancreas of the crayfish, *Procambarus clarkii*. Kumamoto J. Sci. Series *B5*:161–169.

Ogura, K. (1959) Midgut gland cell accumulating iron or copper in the crayfish *Procambarus clarkii*. Annot. Zool. Jpn. *32*:133–142.

Reade, P.C. (1968) Phagocytosis in invertebrates. Aust. J. Exp. Biol. Med. Sci. *46*:219–229.

Saunders, B.C., A.G. Holmes-Siedle, and B.P. Stark (1964) Peroxidase. Washington, D.C.: Butterworths.

Slowey, R.R., S. Eidelman, S.J. Klebanoff (1968) Antimicrobial activity of the purified peroxidase from human parotid saliva. J. Bacteriol. *96*:575–579.

Travis, D.F. (1955) The molting cycle of the spiny lobster, *Panulirus argus* Latreille. II. Pre-ecdysial histological and histochemical changes in the hepatopancreas and integumental tissues. Biol. Bull. *108*:88–112.

Travis, D.F. (1957) The molting cycle of the spiny lobster, *Panulirus argus* Latreille. IV. Post-ecdysial histological and histochemical changes in the hepatopancreas and integumental tissues. Biol. Bull. *113*:451–479.

van Harreveld, A. (1936) A physiological solution for freshwater crustaceans. Proc. Soc. Exp. Biol. Med. *34*:428–432.

Vonk, H.J. (1960) Digestion and metabolism. In T.H. Waterman (ed): The Physiology of Crustacea. New York: Academic Press, pp. 291–316.

Wurzinger, K-H., and R. Hartenstein (1974) Phylogeny and correlations of aldehyde oxidase, xanthine oxidase, xanthine dehydrogenase and peroxidase in animal tissues. Comp. Biochem. Physiol. *49B*:171–185.