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Title: THE COMPARATIVE HISTOCHEMISTRY OF THE

DECAPOD ANTENNAL GLAND--ESTERASES,

PHOSPHATASES, AND GLYCOGEN

Abstract approved ███████████████████████

The distributions of alkaline phosphatase, acid phosphatase, non-specific esterases, and glycogen were described for the antennal glands of the freshwater crayfish, Pacifastacus, and the marine crabs Cancer and Pugettia.

Alkaline phosphatase is confined primarily to the luminal edge of the labyrinth in all of the above forms whereas acid phosphatase occurs in the coelomosac of Pacifastacus and Pugettia, and in the labyrinth and bladder of all three forms. In Pacifastacus the protein, horseradish peroxidase, injected into the hemocoel was subsequently localized in the phagocytic, acid phosphatase containing cells of the coelomosac.

Both acid phosphatase and alkaline phosphatase are localized at the luminal border of the labyrinth of the crayfish. This activity may represent a single enzyme which is still active at both an

alkaline and acid pH.

A sexual dimorphism was observed in the labyrinth of Paci-
fastacus. A very intense reaction for non-specific esterases sensi-
tive to 10^{-5}M E 600 and insensitive to 10^{-5}M eserine typified the
reaction in the male. The comparable reaction in the female was
much weaker and more diffuse.

The technique of gel electrophoresis was used to characterize
the dimorphism in the crayfish as well as those esterases in the
marine forms. The sexual dimorphism was observed in the antennal
glands of juvenile crayfish but was not present in the heart or hemo-
lymph.

Eserine and E 600 sensitive esterases were observed in all
remaining areas of the antennal glands of Pacifastacus, the coelomo-
sac, labyrinth, and bladder of Pugettia, and labyrinth and bladder of
Cancer.

Glycogen is localized primarily in the coelomosac and bladder
of Pacifastacus and in the bladders of the marine forms.

It was concluded that the labyrinth of the crayfish may be the
site of a sex related function, involving the metabolism and excretion
of a metabolite or hormone inherent in the physiology of the male.
It seems feasible that glycogen stored in the proximal portions of
the antennal gland constitutes an energy source for these and other
functions since glycogen can be released into the lumen of the gland

and reclaimed later in a more distal area.

No definite correlation could be established between the habitat and histochemistry of the antennal gland of the decapods from divergent environments.

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PHOSPHATASES, AND GLYCOGEN

by

Eugene George Fuller
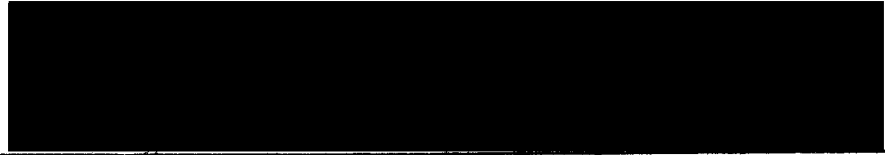
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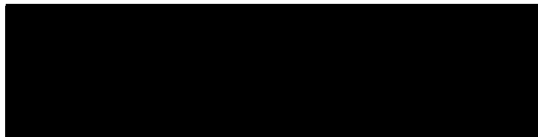the requirements for the
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THE COMPARATIVE HISTOCHEMISTRY OF THE DECAPOD ANTENNAL GLAND--ESTERASES, PHOSPHATASES, AND GLYCOGEN

I. INTRODUCTION

The morphological suggestion of secretory activity in the antennal gland (green gland) of decapods prompted many investigators to speculate on its physiological significance. Marchal (1892) assigned an exretory role to the antennal gland and speculated that the apical vesicles attached to cells and their counterparts lying free in the lumen of the labyrinth were vehicles for the excretion. The question of their significance was reopened by Riegel (1966b) who observed spheres, the formed-bodies, in the urine of the crayfish. He suggested that they represented a mechanism for the incorporation, excretion, and eventual digestion of large molecules in the antennal gland.

The comparative histology of the antennal gland was originally described by Marchal (1892) and some additional aspects were recorded by Balss (1944). Separate accounts of the excretory system exist for the crab, Cancer (Pearson, 1908), for the crayfish, Astacus (Peters, 1935; Malaczynska-Suchcitz and Ucinski, 1962), and for Cambarus (Maluf, 1939). Four major areas have been designated in the antennal gland. They are, in order of urine flow: the coelomosac, labyrinth, nephron tubule, and the bladder.

In a study of the fine structure of the antennal gland of Cambarus, Kümmel (1964a and 1964b) observed that the cells of the coelomosac possess similar morphology to that of the podocytes of the vertebrate glomerulus. This led him to propose the coelomosac as the site of filtration. The ultrastructure of the labyrinth revealed morphological features comparable to the vertebrate convoluted tubule such as numerous microvilli comprising a typical brush border and basal infoldings of the cell membranes. Mitochondria were not observed within the compartments of the basal infoldings (Anderson and Beams, 1956). In view of the ultrastructure of the crayfish antennal gland, the cuticle, which Pearson (1908) described in the labyrinth of Cancer, is probably also a brush border. Morse (unpublished) observed mitochondria compartmentalized in basal infoldings and confirmed a brush border in the labyrinth of Pacifastacus. A study by Beams, Anderson and Press (1956) of the nephron tubule revealed basal infoldings of the cell membrane with compartmentalized mitochondria and large apical vacuoles. No brush border was present. The apical and middle parts of the cells appeared devoid of any obvious material. This created the impression to some authors that the luminal border of the nephron tubule was incomplete (Malaczynska-Suchcitz and Ucinski, 1962).

Contrasting views have been set forth concerning the basic physiological mechanism for formation of the hypotonic urine in

the crayfish. The view held originally was that the primary urine was formed by a secretory process (Marchal, 1892; Maluf, 1939, 1941a, 1941b, and 1941c). However, recent data suggest a filtration reabsorption mechanism in crayfish comparable to that of the vertebrates (Peters, 1935 and Riegel and Kirschner, 1960). Micropuncture studies support a passive filtration in the coelomosac and active reabsorption in the distal part of the nephron tubule and bladder (Riegel, 1963 and 1965). Kirschner and Wagner (1965) also support filtration as the mechanism for urine formation and have defined the molecular limits of the filtration apparatus. Molecules smaller than 50,000 molecular weight are freely filtered but larger molecules are not. Flourescein labeled globulins found within the cells of the coelomosac led to the conclusion that although the coelomosac was the site of filtration the mechanism was different from the vertebrate nephron.

In light of these and other investigations which have established basic morphological and functional similarities between the antennal gland and the vertebrate kidney, the following equivalence may be said to exist: a. the coelomosac and glomerulus, b. the labyrinth and the proximal nephron tubule, c. the nephron tubule and distal convoluted nephron tubule, and d. the bladders.

Further homologies occur at the histochemical level. Alkaline phosphatase is associated with areas of reabsorption and is localized

primarily in the brush border of the convoluted tubule in the kidney of mammals (Gomori, 1941). Similarly, in the crayfish it has been noted in the brush border of the labyrinth (Moog and Wenger, 1952 and Malaczynska-Suchcitz and Ucinski, 1962) and in the lumen and epithelium of the labyrinth in Cancer (Beers, 1956). No qualitative differences were observed at different stages of molt in histochemically demonstrable phosphatases in the antennal glands of the crayfish (Kugler and Birkner, 1948).

In the mammalian kidney acid phosphatase stained most intensely in the proximal convoluted tubule. No glycogen was present except under pathological conditions (Wachstein, 1955). Malaczynska-Suchcitz and Ucinski (1962) noted acid phosphatase staining only in the coelomosac of the crayfish, and succinic dehydrogenase distributed through all areas of the antennal gland. Glycogen was reported in all areas but most concentrated in the labyrinth.

Although many morphological and physiological studies have been reported no comparative histochemical investigations have been performed. The present investigation was undertaken as a comparative study of decapods from several different environments to determine if the histochemistry was significantly different in the antennal glands of forms from widely divergent habitats.

The three decapods chosen for this investigation were the crayfish, Pacifastacus leniusculus Stimpson, the kelp crab, Pugettia

producta Randall, and the dungeness crab, Cancer magister Dana. The crayfish is freshwater, produces a dilute urine, and is capable of osmoregulation in spite of decreased or increased salt concentration of the environment. In contrast, the urine of marine decapods is generally isosmotic to the blood suggesting the antennal glands have a minor role in salt regulation. However, the elimination of excess magnesium ions is an important function of the antennal gland and may be accomplished in the bladder by a mechanism independent of the sodium pump (Gross and Capen, 1966). Some marine forms such as Cancer magister can regulate slightly in a dilute sea water while others, such as Pugettia, are always isosmotic to the environment (Lockwood and Peters, 1962).

II. MATERIALS AND METHODS

Crayfish were collected in the Alsea River and the Arboretum pond in MacDonald Forest near Corvallis, Oregon, and were maintained in circulating cold water tanks in the laboratory. The dungeness crabs were taken directly from the live traps of a commercial fisherman at Newport, Oregon and the kelp crabs were collected intertidally at Boiler Bay or Cape Arago, Oregon. The marine forms were brought into the laboratory and kept in a cold room at $12^{o}C$ in well aerated salt water for no longer than one week before sacrifice.

Some difficulty was encountered in obtaining marine animals in ample numbers, sizes, and representative sexes. Consequently most emphasis was placed on the crayfish which were readily obtained and kept in the laboratory with apparently no ill effects. A total of 169 animals were obtained and utilized as follows: 51 Pacifastacus, 10 Cancer, and 11 Pugettia for histochemistry; and 56 Pacifastacus, 15 Cancer, and 26 Pugettia were employed for gel electrophoresis.

Histochemistry

Bouin fixed tissue was infiltrated with paraffin, sectioned at 10 microns, and stained with hematoxylin and eosin for a general histological orientation.

The antennal glands for enzyme histochemistry were fixed in either 10% calcium formol or 30% sucrose in 10% formalin for 18 hours at 4°C. Tissues were rinsed with distilled water and infiltrated with 15% gelatin at 37°C for one and one-half to two hours. This was necessary due to the friable nature of the gland which was impossible to section otherwise. Sections were cut at 10 microns in a cryostat at -20°C, mounted on cold slides, and air dried for one to two hours prior to staining. Control slides in which the substrate was omitted from the incubation medium were made to evaluate any non-specific staining which may have occurred.

Alkaline phosphatase was generally demonstrated by the Gomori calcium-cobalt method, but some calcium formol fixed tissue of Pacifastacus was also stained by the azo dye method using fast violet B as coupler and alpha-naphthyl acid phosphate as substrate. Acid phosphatase was localized by the azo dye method using alpha-naphthyl acid phosphate as substrate and hexazonium pararosanilin as coupler (Barka and Anderson, 1963). In addition crayfish material was also stained for acid phosphatase using the Gomori method as outlined in Barka and Anderson (1963) and Lake's (1966) lead acetate modification.

The alpha-naphthyl acetate method using hexazonium pararosanilin as coupler was utilized to demonstrate the non-specific esterases (Barka and Anderson, 1963). An inhibitor, E 600

(diethyl-p-nitrophenyl phosphate) at 10^{-5}M in 0.1 M tris maleate

buffer pH 7.2 was used to distinguish arom-esterases from the

ali-esterases. Eserine sulfate at a concentration of 10^{-5}M both in

distilled water and in 0.1 M phosphate buffer pH 7.4 was used as an

inhibitor of cholinesterases (Pearse, 1961). In practice alternate

sections were placed in distilled water, eserine, and E 600 for one

hour prior to staining. Eserine was either incorporated into or

omitted from the incubation medium.

The procedure as outlined by Straus (1964a and 1964b) for the

simultaneous demonstration of acid phosphatase and the injected

plant enzyme, horseradish peroxidase, was employed with the ex-

ception that gelatin embedding was incorporated into the procedure.

Straus (1964c) proposed that the peroxidase taken up by the cells of

the mammalian convoluted tubule was initially incorporated into

phagosomes which migrated across the cell and fused with the lyso-

somes.

Seven male crayfish were injected with 4.5 mg. of horseradish

peroxidase in 0.5 ml. of physiological saline to determine if com-

parable relationships could be ascertained in the antennal glands.

Additional animals had been injected previously to determine the

appropriate dosage and time intervals between injection and sacrifice.

A pair of glands was taken at 1, 3, and 5 hours; 2 pairs were re-

moved at 10 and 24 hours. Sections were prepared as previously

described and subsequently stained for acid phosphatase and peroxidase.

Glycogen was localized using the periodic acid-Schiff (P. A. S.) procedure as outlined by Pearse (1961). Tissues from six crayfish representing different stages of molt were fixed in Gendre fluid and embedded in paraffin. Alternate sections were subjected to the P.A.S. test and to a one hour digestion in 1% diastase in distilled water to remove glycogen thereby distinguishing it from mucopolysaccharides which also react with P. A. S. reagents.

Gel Electrophoresis

Dissected antennal glands were weighed, homogenized in three parts of cold 0.7% saline and centrifuged at 12,000 x G for 20 minutes. In marine forms most of the bladder was lost during dissection. The supernatant was frozen and stored at -20°C until use. Generally the samples were held for no longer than two weeks before use.

Normally a 100 μl aliquot of supernatant was subjected to electrophoresis. Final results, with no apparent differences, were obtained when 50 μl aliquots from the supernatant of a gland homogenized in four parts of saline were used. In the case of extremely small antennal glands the entire supernatant constituted one sample.

The apparatus and gels were prepared according to the original recommendations of Davis (1964). A 7% acrylamide separation gel

at pH 8.7 and a 2.5% acrylamide stacking gel at pH 6.7 were used.

The sample gel was replaced by 0.1 ml. of 40% sucrose in tris glycine

buffer containing the supernatant. This mixture formed a layer im-

mediately above the stacking gel and was carefully overlayered with

the lighter tris glycine buffer.

The initial current, 0.5 milliamp per tube was increased to

1.5 milliamps after one hour at which time the sample had migrated

into the large pore stacking gel. Total time for electrophoresis was

about three hours at $6^{\circ}C$.

Characterization of the esterases was accomplished by a one

hour exposure to cold $10^{-5}M$ or $10^{-4}M$ eserine sulfate in 0.1 M

phosphate buffer at pH 7.4 or $10^{-5}M$ E 600 in 0.1 M tris buffer pH

7.2 prior to development of the gels. Control gels were soaked in

cold distilled water for the same length of time. Development of the

gels was accomplished in an incubation medium of the same composi-

tion used to demonstrate the esterases histochemically. Since the

staining time was 30 to 45 minutes at room temperature, eserine of

the proper concentration was always included in the incubation medium

of eserine inhibited samples to prevent reversal of the inhibitor dur-

ing the prolonged incubation. Following a distilled water rinse the

gels were photographed by trans-illumination to record the developed

electrophoretic patterns, some of which tended to fade in storage.

III. RESULTS

Histology

The histology of the decapod antennal gland with the exception

of Pugettia has been amply described in the papers already cited.

Examination of Pugettia revealed that the structure of the antennal

gland is similar to that of Cancer. Only a brief description will be

given here for orientation to the histochemical results reported. The

terminology as employed by Maluf (1939) will be used with some of

the possible synonyms included in parenthesis.

The excretory organ of the decapods lies in the anterior ventral

portion of the hemocoel. Its chief components are the coelomosac

(sacculus, end sac, first segment); labyrinth (cortex, second seg-

ment); a nephron tubule (labyrinth) composed of a thin, proximal

nephron tubule (third segment), and a thick, distal nephron tubule

(fourth segment); and a terminal bladder (fifth segment). The entire

tubule between the coelomosac and bladder was originally termed the

labyrinth. The coelomosac is of mesodermal origin and the remainder

of the gland is derived from ectoderm. In marine forms the nephron

tubule is absent and its presence in freshwater forms is associated

with the production of a dilute urine.

The antennal glands are devoid of muscle tissue and are com-

posed almost entirely of an epithelium. Some connective tissue cells

are present in the bladder wall, and in the coelomosac. Elements

of hemolymph are distributed to all parts of the gland through small

blood sinuses.

The coelomosac is characterized by a simple columnar epithelium with vacuolate cells. It is a well vascularized structure and in the crayfish it is supplied by a branch of the reno-antennal artery which nearly divides it into two chambers, each of which is partially subdivided by smaller septae. It is a very discrete structure and lies dorsal to the labyrinth and nephron tubule (Plate 1, Figure 1). However, in marine decapods it interdigitates ventrally with the roof of the labyrinth. Consequently, a typical section depicts numerous blood sinuses between the inner lightly-stained cells of the coelomosac and the outer darker cells of the labyrinth (Plate 1, Figure 4 and Plate 2, Figure 6).

The simple columnar epithelium of the labyrinth has a typical brush border, basal striations, and basally located nuclei. An apocrine type of secretory activity is manifest in the form of numerous apical vesicles which detach from the cells and lie free in the lumen of the gland (Plate 1, Figure 2). The labyrinth is supplied by branches of the reno-antennal and sternal arteries which lead to numerous small sinuses at the base of the epithelium.

The nephron tubule is divided into two parts on the basis of its histological appearance. The proximal nephron tubule has a

simple squamous epithelium in contrast to that of the distal nephron tubule which has a tall columnar epithelium. Brush borders were absent in all parts of the nephron tubule but there were basal striations. The cells of the distal nephron tubule possess extremely large and numerous apical vacuoles.

The bladder epithelium of the crayfish varies from a tall columnar to a squamous epithelium (Plate 1, Figure 3). It is columnar only at the junction with the nephron tubule and becomes squamous distally. Basal striations, brush borders, and apical vesicles are absent and the appearance of the cells is not that of a secretory epithelium. In marine forms the epithelium of the bladder is tall columnar with some apical vesicles (Plate 2, Figures 5 and 7).

Histochemistry

The stage of molt in Pacifastacus, determined by the size of the gastrolith, appeared to have no significant qualitative effect on the histochemistry of the antennal gland. Most of the marine forms exhibited no signs of an impending molt as indicated by a soft shell.

Fixation

The two fixatives used had widely divergent effects on the activity and distribution of enzymes. Decreased esterase and acid phosphatase activity resulted from fixation in 10% calcium formol as

compared to similar material fixed in sucrose formol. No acid

phosphatase staining granules were observed in tissues fixed with

calcium formol, and acid phosphatase staining was not detected in

Cancer antennal glands. Esterase staining was also significantly

decreased in the antennal glands of Pugettia, and in the bladder and

nephron tubule of Pacifastacus. Therefore, the results reported

below are primarily from tissues fixed in formol sucrose.

Acid Phosphatase

Abundant granules of acid phosphatase were observed in cells

lining the lumen of the coelomosac in the crayfish (Plate 2, Figure 8).

A positive reaction was noted in the coelomosac of the kelp crab

which appeared to mask entire cells (Plate 3, Figure 11). In

contrast, the coelomosac of the dungeness crab was negative to this

histochemical test (Plate 3, Figure 12).

In the labyrinth of Pacifastacus, acid phosphatase staining was

observed mainly in two areas of the cell. It was found at the luminal

edge and in granules at the basal portion of the cell around the nu-

cleus (Plate 3, Figures 9 and 10). The number and size of the gran-

ules varied to some extent among individuals. Pugettia exhibited

rather large granules in the labyrinth but no apical or luminal stain-

ing. A much less intense reaction appeared in the labyrinth of

Cancer and a diffuse staining reaction occurred within the lumen

of the labyrinth (Plate 3, Figure 12).

The nephron tubule and bladder of Pacifastacus were occasionally recorded as possessing a few smaller acid phosphatase staining granules. A markedly positive reaction was noted in the bladder of Pugettia in the form of numerous large granules located basally in the cell. In contrast, a diffuse staining reaction characterized the bladder of Cancer (Plate 4, Figure 13).

Peroxidase was incorporated within the acid phosphatase containing cells of the coelomosac (Plate 12, Figure 46). The intensity of staining, proportional to the amount of peroxidase phagocytized, increased as the interval between injection of peroxidase and sacrifice increased from 1 to 24 hours. Filtration of peroxidase was not rapid in the crayfish, presumably due to the low efficiency of an open circulatory system and the filtration mechanism. Consequently, peroxidase was still observed in the lumen of the gland 24 hours after its injection.

Peroxidase was not unequivocally observed in doubly-stained cells of the labyrinth although it was observed in its lumen (Plate 12, Figure 47). Possibly the peroxidase was taken up by the cells of the labyrinth but the size of the peroxidase containing vesicles was at the lower limit of resolution for the technique. Some rather large darker staining granules of acid phosphatase were observed in the labyrinth of crayfish injected with peroxidase.

Alkaline Phosphatase

Alkaline phosphatase was not detected in the coelomosac but was localized almost exclusively in the cells of the labyrinth of those decapods examined (Plate 4, Figures 14 and 15, and Plate 5, Figure 17). Sometimes the Gomori test was very intense and darkened the entire cell, but generally it was most intense at the apex of the cells. A positive reaction occurred in the lumen of the labyrinth in Cancer. Only in the kelp crab was a positive reaction noted in the bladder. This was detected in large granules at the base of the cells. Nuclei typically appeared slightly darkened by the Gomori reaction which is the characteristic false-positive reaction often noted in this reaction (Plate 4, Figure 16). When the azo dye method was used for alkaline phosphatase a positive reaction was noted only at the luminal border of the cells of the labyrinth. The nuclei were unstained.

Esterases

The esterases are a complex group of enzymes which exhibit overlapping substrate specificities. They are commonly separated into non-specific esterases, cholinesterases, and lipases on the basis of substrate specificity, and inhibitor and activator studies. Caution must be employed in applying the results of one study too liberally to the esterases of another organism. Accordingly, esterases sensitive to eserine at a concentration of 10^{-5} M are considered to be the

cholinesterases, while those esterases sensitive to 10^{-5}M E 600

are designated as ali-esterases (B-esterases) and the resistant ones

as the arom-esterases (A- or C-esterases). Inhibition is indicated

in the treated sample by a markedly decreased or abolished color

reaction after incubation in the substrate as compared to the color

developed in the untreated control.

Esterases sensitive to both eserine and E 600 were abundant

in the cells lining the lumen of the coelomosac in _Pacifastacus_ (Plate

5, Figures 20, 21, and 22). Only scattered individual cells were

positive for esterases in the coelomosac of _Pugettia_. They were

completely inhibited by eserine and E 600. A negative reaction was

observed in the coelomosac of _Cancer_ (Plate 7, Figure 28).

In _Pacifastacus_ a sexual dimorphism occurred with respect to

the non-specific esterases in the labyrinth. A very intense reaction

appeared in the male as compared to a much weaker diffuse reaction

in the female (Plate 5, Figures 18 and 19). The stain was generally

cytoplasmic, not particulate, and the apical vesicles stained intensely.

The intensity of the reaction was not diminished in either male or

female animals by treatment with 10^{-5}M eserine but it was abolished

completely by 10^{-5}M E 600 (Plate 5, Figure 20, and Plate 6, Figures

21 and 22).

No obvious differences were observed in the histochemistry of

the male or female of either _Pugettia_ or _Cancer_ under the conditions

of this investigation. Esterase staining was present in the labyrinth of _Pugettia_, and in the cells and lumen of _Cancer_. These reactions appeared partially sensitive to eserine and were nearly abolished by E 600. Some particulate E 600 resistant esterases were present in the bladder and labyrinth of _Pugettia_ (Plate 7, Figures 25, 26, 27, and 28, and Plate 8, Figures 29 and 30).

The bladder and nephron tubule of _Pacifastacus_ were observed to have a slightly positive reaction which was completely eserine sensitive (Plate 6, Figures 23 and 24). The bladders of both marine forms evidenced a positive reaction partially sensitive to eserine. E 600 treatment completely inhibited the esterases in _Cancer_ (Plate 8, Figures 31, 32, and 33). However, in _Pugettia_ some particulate, E 600 resistant staining remained.

Glycogen

Glycogen was present in the coelomosac, labyrinth, nephron tubule, and bladder of the crayfish. Special cells in the periphery of the coelomosac, and its septae and folds exhibited particularly intense staining for glycogen (Plate 9, Figures 34 and 35). These "glycogen cells" had no counterpart in the marine decapods. The bladder epithelium also evidenced a particularly intense staining reaction for glycogen in comparison to the labyrinth and nephron tubule which produced a rather weak reaction. Additional P. A. S.

positive diastase fast materials were present in the nephron tubule (Plate 9, Figures 36 and 37, and Plate 10, Figures 38 and 39).

In the marine forms glycogen was found in all major areas of the antennal gland but most intensely stained in the bladder (Plate 10, Figures 40 and 41, and Plate 11, Figures 42, 43, 44, and 45). Glycogen and other P.A.S. positive materials were also present in the lumen of the antennal glands of all forms. The mucopolysaccarides of the basement membranes, brush borders, and granules in the blood cells remained P.A.S. positive after diastase digestion.

Electrophoresis

This procedure was chosen to complement the enzyme histochemistry, particularly as relevant to the definition of the molecular species of esterases comprising the dimorphism in the antennal gland of Pacifastacus. The same reagents for staining and characterizing the esterases histochemically were employed to demonstrate those separated electrophoretically.

Separation was accomplished by the ability of the esterases to migrate through a polyacrylamide gel at a specific pH under the influence of an electric field. They were further characterized on the basis of their sensitivity to the specific inhibitors cited previously. One additional concentration of eserine, 10^{-4}M, was employed in this series of experiments.

Pacifastacus

A comparison of the patterns of esterases separated from homogenates of male and female green glands supported the histochemical observation of a sexual dimorphism (Plate 13, Figures 1, 4, 7, 9, 15, and 17). In the mature male, four bands of esterase (bands a, c, d, and e) were observed. Bands a and c, which occasionally appeared fused, were superimposed on a diffuse background reaction. This background reaction and a portion of band e were somewhat inhibited by exposure to eserine (Plate 13, Figures 1, 2, and 3). E 600 inhibited bands c and e, whereas a portion of a and all of d were uninhibited (Plate 13, Figures 7 and 8). In the female, a significant difference was observed in the bands comparable to a and c in the male. These bands, labeled a' and c' are not present in the same intensity and do not respond to the inhibitors in the same manner as those in the male. Band c' is sensitive to eserine while a' is insensitive to eserine and E 600 as is its E 600 resistant counterpart in the male (Plate 13, Figures 4, 5, 6, 9, and 10). The differences observed in bands a' and c' in female crayfish were probably due to individual variation. Bands d and e are similar in reaction to those in the male. It is to be noted that band d represents an E 600 resistant esterase (arom-esterase) which was not observed in sectioned material.

Crayfish of both sexes between 45mm and 49mm in total length were judged to be juveniles according to the size criteria of Mason (1963). They were examined to establish whether the dimorphism was present prior to their reproductive period. The electrophoretic patterns were very similar to those observed in adult animals (Plate 13, Figures 11, 12, 13, and 14).

The electrophoretic patterns of esterases in the heart were compared to those of the antennal glands from the same animal in both male and female crayfish to determine if the dimorphism was represented in organs other than the excretory organ. An examination of these esterases revealed a similarity in bands d and e but the dimorphism was not apparent in the heart (Plate 13, Figures 15, 16, 17, 18).

Pugettia

Four bands were generally present in the kelp crab (Plate 13, Figure 19). An additional band, s, occasionally appeared and possibly represented an esterase associated with the hypodermis which was inadvertantly included in the samples. Band m was sometimes present as just one band or as two and differed as to staining intensity among different individuals. All bands except s appear either completely or partially sensitive to eserine and E 600 (Plate 13, Figures 19, 20, 21, 22, 23, 24, 25, and 26). No obvious sex differences

were observed in the electrophoretic patterns but band r of the smaller animals was occasionally reduced or absent.

Cancer

Only adult male animals were observed. Three major bands were separated which appeared partially sensitive to eserine and to E 600 (Plate 13, Figures 27, 28, 29, and 30).

Variations in the electrophoretic patterns detected within a given species may be accounted for primarily on the basis of genetic heterogeneity within a group. However, the individual physiological state of the animal as well as experimental error may also have influenced the slight differences observed.

IV. DISCUSSION

A wealth of information has accumulated concerning the distribution of alkaline phosphatase in various animal tissues. Gomori (1941) originally described its distribution in a variety of mammalian tissues. In the kidney it is localized primarily in the brush border of the proximal convoluted tubule. Additional studies, extended to all classes of vertebrates, have confirmed this observation (Bishop, 1959; Browne, Pitts and Pitts, 1954; Longley and Fischer, 1954; and Moog and Wenger, 1952). Alkaline phosphatase has a similar distribution in those invertebrate kidneys which have a portion of the kidney comparable to the vertebrate proximal convoluted tubule. In the labyrinth of the antennal glands of crayfish, the nephridium of nemertean worms (Danielle and Pantin, 1950), flatworms (Dev, 1964; and Coil, 1958), and earthworms (Moog and Wenger, 1952), and the Malpighian tubules of insects (Bradfield, 1946), the convoluted portions of the tubule exhibited alkaline phosphatase activity.

It has been postulated that alkaline phosphatase takes part in the dephosphorylation of glucose phosphate in the reabsorption of glucose from the urine (Moog; 1946; Wilmer, 1944). However, its presence in the aglomerular kidney of the toadfish, which does not absorb glucose, led some authors to postulate that it has a more generalized function (Browne, Pitts and Pitts, 1950; and Longley, 1955).

Speculation that alkaline phosphatase plays a role in protein secretion in arthropods was advanced by Bradfield (1946).

Lysosomes are cellular organelles bound by a lipo-protein membrane and contain numerous acid hydrolytic enzymes. They have been found in every animal phylum and are involved in the autolytic or hydrolytic properties of the cell. Acid phosphatase has been used as a histochemical marker for lysosomes and has enhanced the study of these structures in cells at different physiological states. Some investigators urged that caution be used in the interpretation of such results and suggested additional biochemical evidence be employed before designating acid phosphatase containing granules as lysosomes (de Duve, 1963).

Some special considerations of fixation must be undertaken for the histochemical demonstration of lysosomes. It is necessary to preserve the lipoprotein membrane binding the lysosomes, and at the same time to expose the bound enzyme to substrate. Staining methods using a hypertonic calcium formol fixation and the Gomori's staining procedure for acid phosphatase have revealed droplets in rat liver cells and in the proximal convoluted tubule of the rat kidney (Holt, 1959).

Wachstein (1955), Wachstein, Meisel and Ortiz (1962), and Wachstein and Bradshaw (1965) studied the distribution of acid phosphatase in the kidney of several mammalian species. It is present

in one of two forms, either as a cytoplasmic, formalin sensitive

component, or as a formalin insensitive, granular component. The

most intense staining was observed in the proximal convoluted tubule.

Staining in the brush border was attributed to a formalin resistant,

non-specific alkaline phosphatase active at an acid pH. However, in

both adult and neo-natal guinea pigs acid phosphatase was observed

in the inner border of the collecting ducts. In addition, the presence

of a glycerophosphatase in granules suggested the necessity of further

study on the enzyme containing organelles.

Acid phosphatase had previously been reported only in the coelo-

mosac of the crayfish (Malaczynska-Suchcitz and Ucinski, 1962). This

was confirmed in the coelomosac but, in addition, acid phosphatase

staining granules were also observed in the labyrinth. The luminal

edge of the labyrinth was observed to stain for both alkaline phospha-

tase and acid phosphatase. Perhaps this was due to a phosphatase

which was active at both an alkaline and acid pH. McWhinnie and

Kirchenberg (1966) reported a phosphatase in the crayfish hepato-

pancreas which reflected two peaks of activity, one at pH 6.8 to 7.3,

and another at pH 8.0 to 8.5.

Granules staining for acid phosphatase, alkaline phosphatase,

and E 600 resistant esterases are often present in the bladder of the

kelp crab. It has been demonstrated histochemically that an E 600

resistant esterase, possibly a C-cathepsin, occurs in particulate

droplets (Shnitka and Seligman, 1961; Wachstein, Meisel and Falcon, 1961) and a C-cathepsin has also been identified biochemically in the particulate droplets of cellular homogenates (Straus, 1956).

Protein injected into the circulatory system of mammals is presumably filtered across the glomerulus and actively phagocytyzed by cells of the nephron and incorporated into the acid phosphatase containing lysosomes (Novikoff, 1961). The exact mechanism of incorporation of the protein into the lysosome is subject to debate. Straus (1964c)suggested that fusion of a protein ladened "phagosome" with lysosome occurred in the cells of the nephron of the rat previously injected with peroxidase. However, Miller and Palade (1964) found no ultrastructural evidence to support the fusion of separate particles.

Riegel (1966a) proposed a contrary view to the mechanism by which proteins and larger molecules were handled by the kidney. He proposed these large molecules were actively taken up from the blood and extruded into the lumen of the nephron in formed-bodies where they were further metabolized.

Analysis of the formed-bodies in the crayfish urine obtained by micropuncture reveals the presence of proteolytic enzymes, little or no acid or alkaline phosphatase, and short peptides. They are observed in the urine from all areas of the antennal gland except the bladder and probably originate primarily in the coelomosac

and labyrinth. Apical vesicles from the labyrinth presumably contained formed-bodies which are released when the apical vesicles rupture prior to entering the nephron tubule. Digestion presumably occurs within formed-bodies in the distal portions of the gland where they eventually burst and useful materials are reclaimed. Consequently, only smaller molecules are filtered by the coleomosac, the larger ones being taken up in pinocytotic vesicles and ultimately incorporated and digested in the formed-bodies (Riegel, 1966b).

Localization of peroxidase in the cells of the coelomosac confirmed the observations that the coelomosac exhibited phagocytic properties (Maluf, 1941c; and Malaczynska-Suchcitz and Ucinski, 1962). It also confirmed Kirschner's (1965) observation that protein was observed in cells of the coelomosac after injection. The molecular weight of peroxidase (44, 000) is within the molecular limits established by Kirschner (1965) for the filtration system of the coelomosac. It was observed within the lumen of the gland indicating it had been filtered or transported to the lumen in formed-bodies.

Although peroxidase was not unequivocally observed within the labyrinth some of the "lysosomes" appeared rather large and dense suggesting they had incorporated some protein. If the mechanism for excretion of large molecules proposed by Riegel (1966b) is true then it must be confined in large part to the coelomosac where most protein was localized intracellularly. The distribution of acid

phosphatase containing granules corresponds to those areas where

most of the phagocytosis presumably occurs.

In general the analysis of enzyme activities of representative

enzymes of the glycolysis and citric acid cycles within the different

segments of the antennal gland reveals that activities were highest in

the nephron tubule and lowest in the coelomosac which correlates with

the suspected functions of these areas, passive filtration in the coel-

omosac and reabsorption in the tubule (Keller, 1965). Glycogen dis-

tribution was inversely related to the activities of enzymes of the

glycolysis and citric acid cycle. It was least stained in the labyrinth

and nephron tubule and most abundant in the coelomosac and bladder

epithelium. In addition, glycogen was observed in the lumen of the

antennal gland. Perhaps it was metabolized very rapidly in the

labyrinth and nephron tubule as an energy source for the metabolic

functions which occurred there. Glycogen liberated into the lumen

of the antennal gland from the proximal storage areas may be

reclaimed and utilized by the distal portions of the antennal gland.

The blood vessels of the coelomosac probably constitute the most

immediate source of glucose for glycogen synthesis in the proximal

parts of the gland.

The complementation of the topographical distribution of the

esterases with an analysis of the molecular species of enzymes

present is desirable and informative. However, it must be noted

that the two procedures do not necessarily demonstrate the same entity. Histochemical techniques tend to localize the insoluble enzyme fractions whereas electrophoresis demonstrates the more soluble fractions (Markert and Hunter, 1959).

It is well established that the esterases represent a wide spectrum of isozymes with overlapping substrate and inhibitor specificities (Chessick, 1953 and 1954; Markert and Hunter, 1959). They are genetically controlled, somewhat variable in the same species, and exhibit sex differences (Ruddle, 1966; Ruddle and Roderick, 1965; Van Asperen and Mazijk, 1965; and Velthuis and Van Asperen, 1963).

In general, the functions of the esterases are still quite speculative. However, recent studies suggest that one of the esterases, cholinesterase, is functional in the active transport of sodium (Kirschner, 1953; Koblick, 1959; Koblick, Goldman, and Pace, 1962; and Fourman, 1966). It has also been implicated in the active transport of sodium in the crayfish because the injection of a cholinesterase inhibitor, eserine, reduced the uptake of sodium from the bladder (Kamemoto, Keister, and Spalding, 1962). Cholinesterase was determined to be most concentrated in the bladder and least concentrated in the coelomosac and labyrinth (Kamemoto and Keister, 1961). The non-specific esterases are usually isolated with the microsomes in cell fractionation studies and have been proposed to

function in protein synthesis (Markert and Hunter, 1959; Hunter, et. al., 1964).

The esterases have been investigated histochemically in the kidney of a number of mammalian species (Wachstein, 1955). Although some species differences were noted the enzymes were generally present in all areas of the nephron staining most intensely in the convoluted tubules.

Electrophoretic analysis revealed an ali-esterase in the kidney of the male mouse which was normally not present in the females or immature males. Injections of testosterone induced its appearance in these animals (Shaw and Koen, 1963). Similarly, a sexual dimorphism was also observed in the labyrinth of the crayfish which involved ali-esterases detectable by both histochemical and electrophoretic procedures. These esterases were present in the juvenile males suggesting their appearance was either not controlled by hormones inducing sexual maturity or if hormonally controlled they were induced early in development. The esterases were relatively specific to the antennal gland and were not found in heart tissue or its hemolymph. The function of the esterases responsible for the dimorphism in mice or crayfish is unknown. Shaw and Koen (1963), speculated they were associated with sex hormones. In the crayfish they may be associated with the metabolism of a more general substance inherent in the physiology of the male since they do not appear

to be influenced by the reproductive age of the animal.

Crayfish of both sexes exhibited E 600 resistant esterases in the electrophoretic patterns which were not represented in the marine forms. That these enzymes were not observed in sectioned material of the crayfish suggests that they represent formalin sensitive esterases. Such an E 600 resistant, formalin sensitive esterase has been reported in mammals (Holt, 1963).

A functional differentiation of the labyrinth is suggested by the available morphological, biochemical, and histochemical information. The apical vesicles of the crayfish exhibit very little phosphatase staining but pronounced esterase staining. In addition, the labyrinth of the male crayfish exhibits a distribution of esterases markedly different from the female suggesting the labyrinth was the site of a sex related function, possibly the excretion or digestion of a metabolite or hormone associated with the physiology of the male.

The antennal glands of those decapods studied exhibited many similarities of enzyme distribution suggesting basic similarities in function. A clear correlation could not be established between habitat and the histochemistry of the glands. Differences in the isozymes were present, particularly with regard to the E 600 resistant esterases of the crayfish, but it was not certain if this merely reflected a genetic difference or if it was related to the physiological adjustment to a different environment.

V. SUMMARY

1. The morphological features of the antennal gland were reviewed briefly and similarities were stressed between the vertebrate nephron and the antennal gland.

2. The distributions of the following histochemical components were described; acid phosphatase, alkaline phosphatase, esterases, and glycogen. The esterases were characterized on the basis of their sensitivity to specific concentrations of eserine sulfate and E 600.

3. The histochemistry of the esterases was complemented by the procedure of gel electrophoresis employing the same substrate, staining procedure, and inhibitors used in the histochemical tests.

4. The brush border of the labyrinth was the chief site of alkaline phosphatase activity. Acid phosphatase was found predominately in the coelomosac and labyrinth of the crayfish and in the labyrinth and bladder of marine forms. Granules staining for acid phosphatase may represent lysosomes.

5. Esterases were most intensely stained in the labyrinth of the crayfish and in the labyrinth and bladder of marine forms. The esterases in sectioned material were almost

completely inhibited by E 600 and showed selective sensitivity to eserine. A sexual dimorphism was observed in the esterases of the crayfish labyrinth. These esterases were not inhibited by eserine but were abolished by E 600. Two bands were observed in the polyacrylamide gels which corresponded to the esterases demonstrated histochemically in the labyrinth of the male crayfish. Additional E 600 resistant bands were observed in electrophoretic patterns of the crayfish antennal gland which were not present in sectioned material. The distribution of esterases separated electrophoretically was described in the marine forms.

6. Glycogen was most concentrated in the coelomosac and bladder of the crayfish and in the bladder of marine forms. It was also present in the lumen of the antennal gland.

7. Peroxidase was injected into the hemolymph of the crayfish and its localization was noted in the antennal gland. The coelomosac was the chief site of peroxidase uptake. It was observed in the lumen of the gland indicating it had passed through the filtration system.

8. No significant histochemical differences were observed in the antennal glands of forms from different environments.

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APPENDIX

APPENDIX

PLATE ABBREVIATIONS

B	bladder
C	coelomosac
GC	glycogen cells
NT	nephron tubule
DNT	distal nephron tubule
PNT	proximal nephron tubule
RA	renal branch of reno-antennal artery

<u>Plate 1</u>

Figure 1.   A hematoxylin eosin stained section of the
            antennal gland of <u>Pacifastacus</u>. The
            coelomosac (C) appears in the upper part
            of the photograph and is partially divided
            by a branch of the reno-antennal artery (RA).
            Two areas of the nephron tubule (DNT and
            PNT) may be observed between the coelo-
            mosac and labyrinth (L).   X 35.

Figure 2.   The labyrinth of <u>Pacifastacus</u> showing the
            apical vesicles both attached to cells and
            free in the lumen.   Stained with hematoxylin
            and eosin.   X 100.

Figure 3.   Section of the bladder of <u>Pacifastacus</u> at a
            point near its junction with the nephron
            tubule.   Stained with hematoxylin and
            eosin.   X 100.

Figure 4.   The relationships of the lightly stained
            cells of the coelomosac (C) to the darker
            cells of the labyrinth (L) may be observed
            in this section of the antennal gland of
            <u>Pugettia</u>.   Stained with hematoxylin and
            eosin.   X 100.

Plate 2

Figure 5. The columnar epithelium of the bladder (B) of Pugettia stained with hematoxylin and eosin. X 100.

Figure 6. Section of the antennal gland of Cancer demonstrating the coelomosac (C) and labyrinth (L) with small sinuses containing blood cells between them. Stained with hematoxylin and eosin. X 100.

Figure 7. Bladder epithelium of Cancer stained with hemotoxylin and eosin.

Figure 8. Azo dye stain for acid phosphatase in the coelomosac of Pacifastacus. Its lumen appears in the upper right corner of the photograph. The positive staining reaction appears as a dark granular material in the cells around the lumen. X 100.

Plate 3

Figures 9,10. Labyrinth of <u>Pacifastacus</u> stained for acid
            phosphatase with the azo dye method. Stained
            granules appear in the basal part of the cells
            around the nuclei. Note the intensely stained
            luminal border and limited staining in the
            vesicles in Figure 10. X 430.

Figure 11. Acid phosphatase staining in the antennal gland
            of <u>Pugettia</u> demonstrating the particulate nature
            of the reaction in the bladder (B) and labyrinth
            (L). Staining masks the cells in the coelomosac
            (C). X 100.

Figure 12. Section of <u>Cancer</u> antennal gland demonstrating
            acid phosphatase staining in labyrinth (L) and
            lumen but its apparent absence in coelomosac
            (C). X 100.

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Plate 4.

Figure 13. The bladder of Cancer showing a diffuse staining
            for acid phosphatase. X 100.

Figure 14. The Gomori reaction for alkaline phosphatase
            in the labyrinth of Pacifastacus. The luminal
            border of the labyrinth exhibits an intense
            black deposit of lead sulfide. X 100.

Figure 15. Two adjacent areas of the antennal gland of
            Pugettia showing contrasting alkaline phospha-
            tase reactions. The labyrinth (L) is positive
            and the coelomosac (C) negative. X 100.

Figure 16. A section of the bladder of Pugettia demon-
            strating alkaline phosphatase positive granules
            at the base of the cells. Nuclei show a false
            positive reaction to the Gomori reaction. X 430.

13

14

C

L

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16

Plate 5

Figure 17. The coelomosac (C) and labyrinth (L) of Cancer.
The former shows a negative reaction for alka-
line phosphatase whereas the latter is positive.
X 100.

Figure 18. A section of the antennal gland of a male
Pacifastacus showing an intense staining of
the labyrinth (L) for non-specific esterases.
X 35.

Figure 19. Comparable section from a female Pacifastacus
also stained for non-specific esterases demon-
strating the contrasting diffuse staining reaction
in the labyrinth. X 35.

Figure 20. Control section of a male crayfish stained for
esterases. Note the markedly positive reaction
in the labyrinth (L) which also appears in the
vesicles, and the positive staining in nephron
tubule (NT) and the coelomosac (C). X 100.

Plate 6

Figure 21. Comparable section treated for one hour in 10^{-5}M eserine prior to staining. Note positive reaction remaining only in the labyrinth. X 100.

Figure 22. Section treated with 10^{-5}M E 600. All staining is completely abolished by this treatment. X 100.

Figure 23. Section of the bladder of Pacifastacus stained for esterases. X 100.

Figure 24. Comparable section treated with 10^{-5} M eserine. X 100.

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Plate 7

Figure 25. Control section of <u>Pugettia</u> antennal gland
stained for esterases. Some cells in the
coelomosac (C) are stained as are the laby-
rinth (L) and the bladder (B). X 100.

Figure 26. Comparable section to the above treated in
$10^{-5}M$ eserine one hour prior to staining.
X 100.

Figure 27. A comparable section treated with E 600.
Note that some staining remains in particu-
lates in this section. X 100.

Figure 28. A control section of the antennal gland of
<u>Cancer</u> stained for non-specific esterases.
The coelomosac (C) is negative but the laby-
rinth (L) and material in the lumen exhibits
a positive reaction. X 100.

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Figure 29. Comparable section of <u>Cancer</u> antennal gland
treated with 10^{-5}M eserine. X 100.

Figure 30. A section as it appeared after 10^{-5}M E 600
treatment. X 100.

Figures 31, 32, 33. Sections of the bladder of <u>Cancer</u>
depicting the untreated control at left, eserine
treated section in the middle, and at the ex-
treme right the E 600 treated section. X 100.

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Plate 9

Figure 34. The P. A. S. positive reaction in the glycogen cells (GC) of the coelomosac (C) and in the lumen of the antennal gland in Pacifastacus. The basement membranes and nephron tubule (NT) also react positively.

Figure 35. A diastase digested section indicating the decrease in P. A. S. staining associated with the removal of glycogen. X 100.

Figure 36. A section of the labyrinth of Pacifastacus stained with P. A. S. to demonstrate the slight positive reaction in the labyrinth and nephron tubule. X 100.

Figure 37. Comparable section pretreated with diastase for glycogen removal. Note that a P. A. S. positive diastase fast reaction characterizes the brush border and basement membranes. X 100.

Plate 10

Figure 38. Bladder of Pacifastacus stained with the P. A. S.
            reaction. X 100.

Figure 39. Adjacent section treated with diastase before
            staining. X 100.

Figure 40. Section of Pugettia stained with P. A. S. X 100.

Figure 41. Diastase treated section comparable to the above.
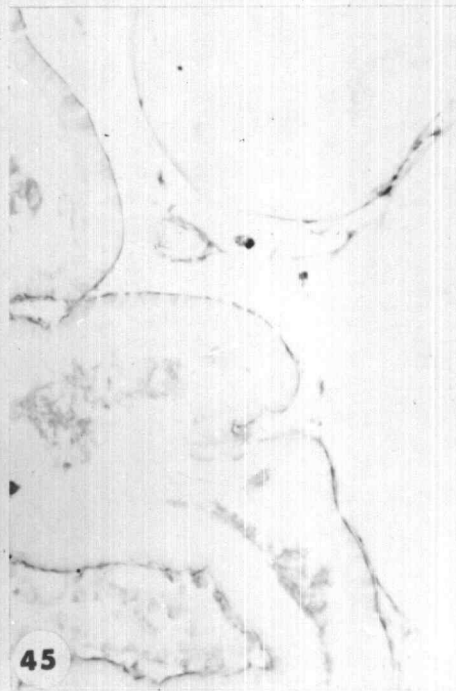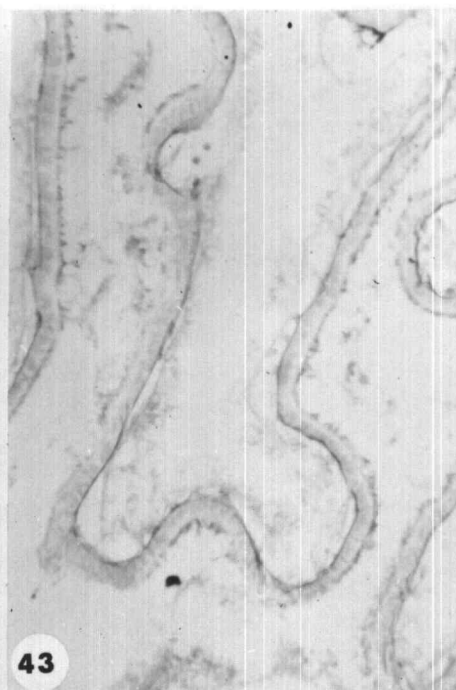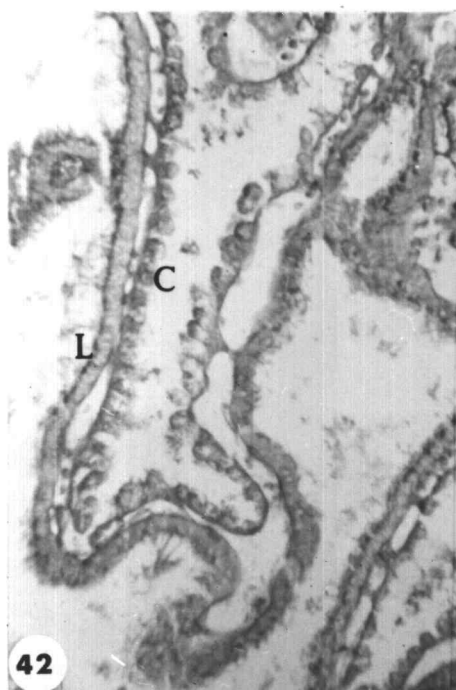   X 100.

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Plate 12

Double staining for acid phosphatase (red) and
peroxidase (dark blue) in portions of the an-
tennal gland of a male Pacifastacus.

Figure 46. The distribution of the two substances in the
coelomosac 10 hours after injection with
peroxidase. Heavy deposition of the peroxi-
dase at the luminal border. The artery of the
coelomosac may be seen in the lower right
hand corner of the photograph. X 100.

Figure 47. The labyrinth 24 hours after injection of
peroxidase. Note some peroxidase staining
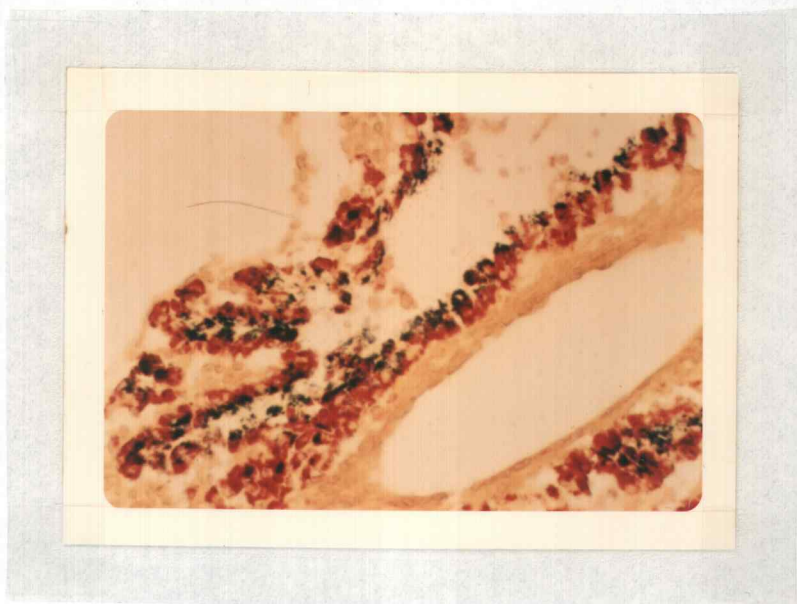in the lumen and the acid phosphatase staining
granules. X 430.

Plate 13

The electrophoretic patterns of the esterases separated in the polyacrylamide gels stained by the azo dye method using alpha naphthyl acetate as substrate and photographed by transillumination. All are from homogenates of the antennal gland except where otherwise stated. The adult crayfish were from 75 to 100 mm. in total length and the juveniles were 45 to 49 mm. in total length. The marine forms were measured across the widest part of the carapace. All Cancer crabs were six inches in width.

1. adult male Pacifastacus, control, 2. 10^{-5}M eserine, 3. 10^{-4}M eserine, 4. adult female Pacifastacus, control, 5. 10^{-5}M eserine, 6. 10^{-4}M eserine, 7. adult male Pacifastacus, control, 8. 10^{-5}M E 600, 9. adult female Pacifastacus, control, 10. 10^{-5}M E 600, 11. juvenile male Pacifastacus, 12. juvenile male Pacifastacus, 13. juvenile female Pacifastacus, 14. juvenile female Pacifastacus, 15. adult male Pacifastacus, control, 16. adult male heart, 17. adult female control, 18. adult female heart, 19. Pugettia female, 36 mm., control, 20. 10^{-5}M eserine, 21. Pugettia female, 35 mm., control, 22. 10^{-4}M eserine, 23. Pugettia male, 47 mm., control, 24. 10^{-5}M eserine, 25. 10^{-4}M eserine, 26. 10^{-5}M E 600, 27. Cancer male, control, 28. 10^{-5}M eserine, 29. Cancer male, control, 30. 10^{-5}M E 600.