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# Serum Changes in the Blue Crab, *Callinectes sapidus*, Associated With *Paramoeba perniciosa*, the Causative Agent of Gray Crab Disease

GILBERT B. PAULEY, MARTIN W. NEWMAN, and EDITH GOULD

*ABSTRACT—Hemolymph from healthy blue crabs* (Callinectes sapidus) *was compared with that of animals infected with* Paramoeba perniciosa, *the causative agent of gray crab disease. There was a significant difference (P = 0.01) between normal and gray crab sera in both the protein and the glucose values. Analysis of infected blue crab serum by both immunoelectrophoresis and acrylamide gel electrophoresis showed altered patterns of total protein and hemocyanin that could be accurately correlated with the severity of the disease.*

A previously unknown disease of the commercially important blue crab (*Callinectes sapidus*) which killed 20-30 percent of the animals held in commercial shedding tanks was described by Sprague and Beckett (1966). When viewed ventrally, moribund and dead specimens had a translucent gray appearance, hence the name "gray crab disease." Sprague and Beckett (1968) ascertained that the etiological agent of the disease was an amoeba, subsequently named *Paramoeba perniciosa*.

The amoebae cause lysis of muscle and blood cells and may completely replace the normal blood cells in the hemolymph prior to death of the crabs (Sprague et al., 1969; Sawyer et al., 1970). Epizootiological studies have shown the disease is ephemeral, occurring each summer in the latter part of June, and has a restricted range from Maryland to North Carolina (Sawyer, 1969; Newman and Ward, 1973).

In another commercially important crustacean, *Homarus americanus*, it has been found that bacteria, *Gaffkya homari*, cause serious alterations in hemolymph chemistry (Stewart et al., 1969; Stewart and Cornick, 1972). Therefore, a study was undertaken to determine whether the hemolymph from blue crabs infected with *P. perniciosa* differed from that of normal crabs. This paper reports our findings on the changes observed in the serum glucose, serum protein, and serum electrophoretic patterns in infected *C. sapidus*.

## MATERIALS AND METHODS

The crabs, *C. sapidus*, used in this study were obtained from Chincoteague Bay, near Greenbackville, Va. Crab sampling began on 26 May 1972, with six subsequent samplings during June 1972. The sample size varied from 4 to 26 animals, with a total of 84 animals being examined. Control sera for immunoelectrophoresis were obtained from uninfected Chesapeake Bay crabs, taken near Oxford, Md.

The posterior portion of the carapace was swabbed with 70 percent ethanol before withdrawing hemolymph from the pericardial cavity with a sterile 5.0 or 10.0 ml syringe and 20-gauge 1½-inch needle. A drop of hemolymph from each animal was placed on a microscope slide and spread evenly in a thin film. This was allowed to air dry for 1-5 min and then placed in 10 percent neutral-buffered formalin seawater. Smears prepared from gray-appearing crabs were fixed in separate containers to avoid the possibility of amoebae floating off the slide and contaminating adjacent slides. Hemolymph smears were stained for 10 min with Giemsa[1] stain diluted 1:5 with phosphate buffer, pH 6.8, and examined for the presence of *P. perniciosa*. The slides were then scored as normal, light, moderate, or heavy infection, based on the relative number of amoebae present as described by Newman and Ward (1973). Hemolymph from healthy crabs, placed in a test tube, was allowed to clot around an applicator stick after which the clot was easily removed. Because the hemolymph of most crabs infected with *P. perniciosa* does not clot, it was centrifuged at 5,000 rpm for 10 min at 4°C, to remove cellular debris. The resultant sera were passed through Swinnex-25 millipore filters (0.45 $\mu$pore size) into sterile tubes, divided into 1.0 ml lots, dated, and frozen at −25°C. Sera from individual crabs were always kept separate.

Two rabbits were used for each antiserum produced. Freund's complete adjuvant (Difco) was mixed with an equal amount of crab serum and administered weekly for 5 weeks as 1.0 ml subcutaneous and 2.0 ml intramuscular injections in the rabbits. Two weeks after the final injection, antiserum was collected by cardiac puncture, filter-sterilized, and frozen at −25°C until used. Antisera were made against the sera from 13 infected crabs from Chincoteague Bay and from two normal male and two normal female crabs from Chesapeake Bay. Immunoelectrophoresis and staining were carried out as described by Pauley (1974).

The total protein concentration in the serum of 66 crabs was analyzed by the Folin-Ciocalteau method (Lowry et al., 1951). Serum glucose was determined for 60 crabs by the method of Hultman (1959). Because of the small sample size of some groups and because they were not homogeneous by analysis of the variance using the *F* distribution (Snedecor, 1956), no statistical tests for significance were attempted for the various groups other than between pooled

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

*Gilbert B. Pauley was with the Pathobiology Investigations section of the Middle Atlantic Coastal Fisheries Center, National Marine Fisheries Service, NOAA, Oxford, MD 21654. He is now with the Washington Cooperative Fishery Unit, College of Fisheries, University of Washington, Seattle, WA 98195. Martin W. Newman is with the Pathobiology Investigations section of the Middle Atlantic Coastal Fisheries Center, National Marine Fisheries Service, NOAA, Oxford, MD 21654. Edith Gould is with the Experimental Biology Investigations section of the Middle Atlantic Coastal Fisheries Center, National Marine Fisheries Service, NOAA, Milford, CT 06460.*

normal and pooled heavily-infected gray crab samples. The *t*-test for comparing the means of two randomized groups of unequal size was used for statistical analysis of this data according to the procedure outlined by Snedecor (1956).

Aliquots (ca. 3 $\mu$l) of serum from individual blue crabs were subjected to electrophoresis at 4°C on 7 percent acrylamide gel columns, pH 9.1, with sample and stacker gels of 3 percent acrylamide, pH 5.2. Electrode buffer was 0.005M Tris (hydroxymethyl aminomethane) - 0.038 M Glycine, pH 8.3. Running time was 60 min at 1 milliampere/column followed by 70-75 min at 3 milliamperes/column with constant current. Both gel formulas and electrophoretic procedure are based on the work of Davis (1964) and have been fully described by Gould and Medler (1970).

Subsequent to electrophoresis, gels were stained for total protein or for copper. The stain used for total protein was Amido Schwartz 10 B (Buffalo Black), 1 percent in 7.5 percent acetic acid. Gels were destained by passive diffusion in several changes of methanol—glacial acetic acid—distilled water (5:1:5) for a total of about 20 h. Hemocyanin sites on the gels were marked by a stain for copper using an aqueous tetrazolium-cyanide solution (Gould and Karolus, In press), that proved to be faster and more consistently reliable than the classic rubeanic acid stain (Horn and Kerr, 1969).

## BIOCHEMICAL ANALYSIS

Concentrations of total protein and glucose in the serum of heavily infected and uninfected crabs used in this study are listed in Table 1. The range, standard deviation, standard error, and mean of these samples are graphically presented in Figures 1 and 2. The distribution of the values was skewed in some groups toward the lower values, such as the glucose of normal females, where the value of the mean minus one standard deviation exceeded the lower limit of the range. As indicated in the Materials and Methods section, statistical tests of significance were performed only between pooled normal serum values and pooled heavily infected gray crab values. There was a significant difference ($P = 0.01$) in both protein and glucose values between the normal and the gray crabs. Serum concentrations of protein and glucose could often be correlated with the intensity of infection (Table 2).

**Table 1.—Concentrations of total protein and glucose in normal and heavily parasitized blue crab serum.**

| | Total protein (mg/ml) | | | | | Glucose (mg/100 ml) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. | Range | Mean | Std. dev. | Std. error | No. | Range | Mean | Std. dev. | Std. error |
| Normal | | | | | | | | | | |
| Males | 11 | 25.0-67.0 | 45.4 | 15.1 | 4.5 | 11 | 10.5-66.4 | 34.7 | 18.4 | 5.5 |
| Females | 11 | 5.3-22.4 | 14.6 | 5.6 | 1.7 | 15 | 7.9-74.4 | 24.4 | 18.6 | 4.8 |
| Sponge | 10 | 3.9-55.4 | 31.0 | 15.7 | 5.0 | 6 | 31.8-130.3 | 74.4 | 37.2 | 15.2 |
| All | 32 | 3.9-67.0 | 30.3 | 18.0 | 3.2 | 32 | 7.9-130.3 | 37.3 | 28.9 | 5.1 |
| Gray | | | | | | | | | | |
| Males | 23 | 2.8-18.5 | 9.4 | 4.6 | 1.0 | 18 | 0-61.1 | 14.1 | 15.8 | 3.7 |
| Females | 11 | 2.8-13.7 | 7.4 | 3.9 | 1.2 | 9 | 0-29.2 | 9.4 | 10.4 | 3.5 |
| All | 34 | 2.8-18.5 | 8.8 | 4.4 | 0.8 | 28 | 0-61.1 | 11.1 | 14.1 | 2.7 |

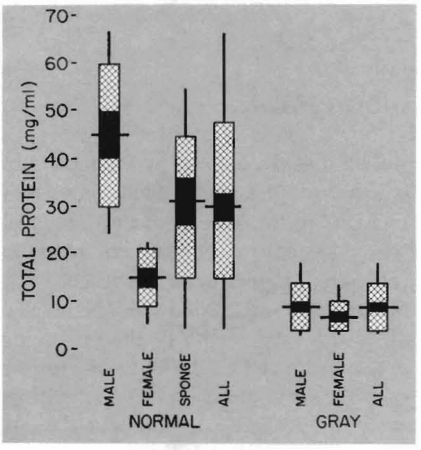


**Figure 1.—Graphic presentation of the range, standard deviation, standard error, and mean of total serum protein concentration in normal and heavily parasitized blue crabs.**

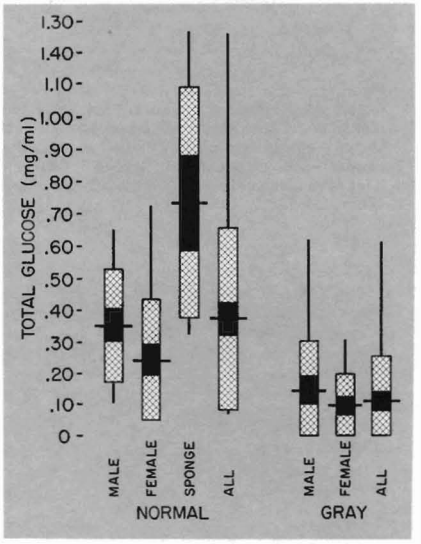


**Figure 2.—Graphic presentation of the range, standard deviation, standard error, and mean of total serum glucose concentration in normal and heavily parasitized blue crabs.**

## IMMUNOELECTROPHORESIS

The normal immunoelectrophoretic patterns of male and female crabs were different (Figs. 3, 4). Infection of *C. sapidus* by *P. perniciosa* caused a progressive loss of serum protein as observed by immunoelectrophoresis, which was apparently related to the severity of the infection (Figs. 3, 4). There was some overlap in the appearance of the immunoelectrophoretic patterns of normal and lightly infected animals which were often not distinguishable (Fig. 5). Although no heavily infected female crabs were analyzed by this method, because of a shortage of hemolymph, there was never any doubt about a correct diagnosis with this method when hemolymph was tested from moderately or heavily infected male crabs.

## ACRYLAMIDE GEL ELECTROPHORESIS

Typical electropherograms for male and female blue crabs with varying degrees of *P. perniciosa* infection show

**Table 2.—Blood glucose, blood protein, and degree of infection by *Paramoeba perniciosa* in the exemplar blue crab hemolymph used for acrylamide gel pherograms.**

| Pherogram no. in figures 6 and 7 | Date of capture | Sex | Blood glucose (mg/100 ml) | Blood protein (mg/ml) | State of infection observed in Giemsa stained blood smears |
|---|---|---|---|---|---|
| 1 | 6/30/72 | ♀ | — | 2.8 | Heavy infection |
| 2 | 6/28/72 | ♀ | 2.5 | 6.5 | Moderate infection |
| 3 | 6/23/72 | ♀ | 3.9 | 17.5 | Light infection |
| 4 | 5/26/72 | ♀ | 37.1 | 43.9 | Normal |
| 5 | 6/28/72 | ♂ | 0.0 | 2.8 | Heavy infection |
| 6 | 6/30/72 | ♂ | 5.2 | 7.4 | Moderate infection |
| 7 | 6/16/72 | ♂ | 11.9 | 15.6 | Light infection |
| 8 | 6/9/72 | ♂ | 21.2 | 33.6 | Normal |

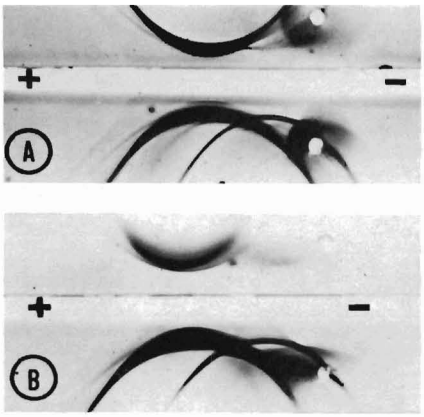


Figure 3.—Immunoelectrophoretic patterns of female blue crabs. A shows a lightly infected female at top and a normal female on the bottom. B shows a moderately infected female at top and a normal female on the bottom. Central trough contains rabbit antiserum against normal female crab serum.

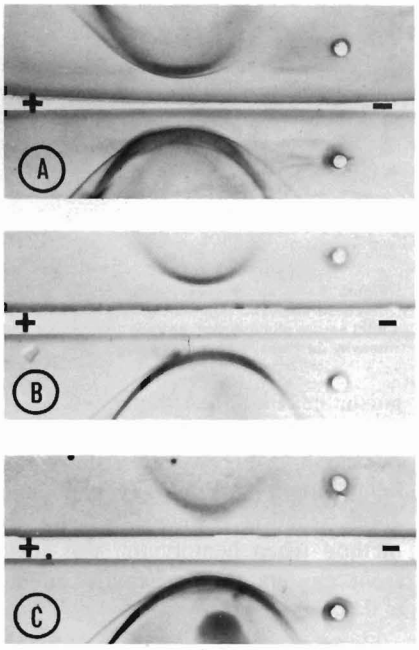


Figure 4.—Immunoelectrophoretic patterns of male blue crabs. A shows a lightly infected male at the top. B shows a moderately infected male at the top. C shows a heavily infected male at the top. Normal male patterns are on the bottom in all cases. Central trough contains rabbit antiserum against normal male crab serum.

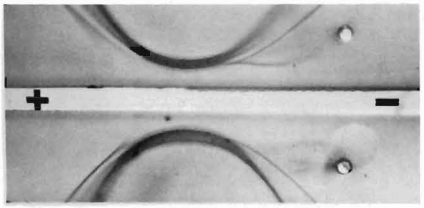


Figure 5.—Lightly infected male at top shows an immunoelectrophoretic pattern that is the same as the normal male at bottom. Rabbit antiserum against normal male crab serum is in central trough.

that both protein and serum copper decline (Figs. 6-9) in relation to the severity of the infection. The disappearance of the hemocyanin (HCy) copper (Figs. 6, 7) from the hemolymph of infected crabs is more rapid than the disappearance of the protein moiety (Figs. 8-10), a circumstance probably reflecting the relative proportions of copper and protein in the HCy molecule. The two fast HCy's ( $\alpha -1$ and $\alpha -2$) in the normal examples, Figures 6 and 7, are the last proteins to disappear from the blood of infected crabs.

## DISCUSSION

The disease caused by *P. perniciosa* is systemic (Sawyer, 1969) and effects changes in the blue crab that resemble the alterations seen in lobsters (*Homarus americanus*) infected by the bacterium *Gaffkya homari*. Sawyer et al. (1970) observed a decline of hemocytes and a reduced ability of crab serum to coagulate when infected with *P. perniciosa*. These changes have also been observed in infected lobsters (Stewart et al., 1969; Stewart and Rabin, 1970). Diet alone is capable of influencing crustacean hemocyte numbers (Stewart et al., 1967), and the presence of actively phagocytizing cells in invertebrates does not insure pathogen destruction (Cornick and Stewart, 1968; Pauley et al., 1971). However, in the case of *P. perniciosa* in blue crabs, these changes probably demonstrate the pathogen's ability to overcome two of the most important defense mechanisms of crustaceans: phagocytosis and coagulation (Bang, 1970; Sindermann, 1971).

The normal glucose values obtained in this study for *C. sapidus* (7.9-130.3 mg/100 ml, $\bar{x}$ = 37.0 mg/100 ml) are much lower and show a greater range than those listed by Florkin (1960) for this species. However, Lynch and Webb (1973b) have found a considerable variation in the serum glucose level of blue crabs throughout the year. It is known that lack of food will cause significant hypoglycemia in *C. sapidus*, but this stress factor alone is not capable of reducing the glucose level to zero (Florkin, 1960), a value observed in several diseased blue crabs in this study. Stewart and Cornick (1972) found a virtual disappearance of glucose from the hemolymph and a reduction in total carbohydrates in *H. americanus* infected with *G. homari*. The reduction of glucose which we observed in *C. sapidus* may be attributed, at least in part, to uptake and utilization by *P. perniciosa*, resulting from the hosts' inability to compete successfully for their own nutrients, as in the case in lobsters infected with *G. homari*.

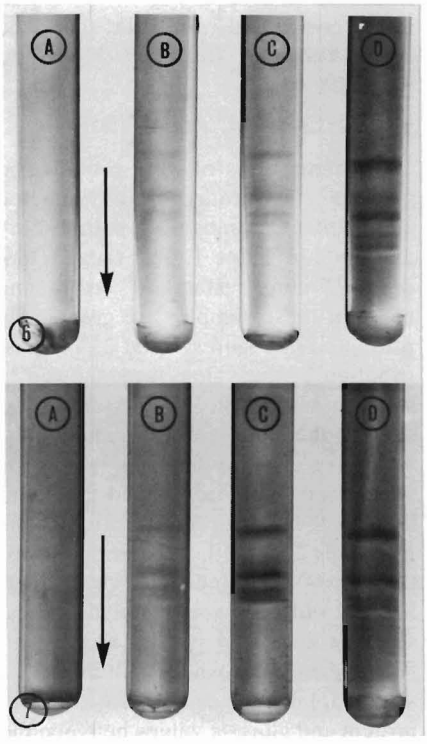


Figure 6.—Pherogram of female crab hemolymph stained for copper. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

Figure 7.—Pherogram of male crab hemolymph stained for copper. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

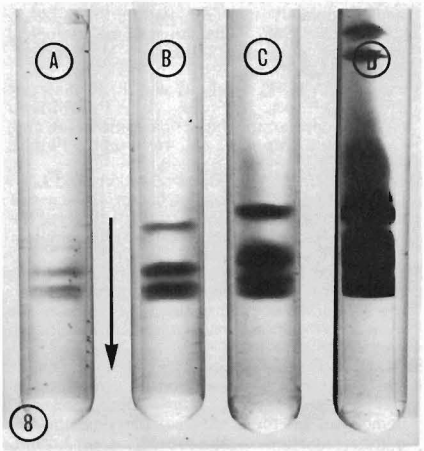


Figure 8.—Pherogram of female crab hemolymph stained for total protein. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

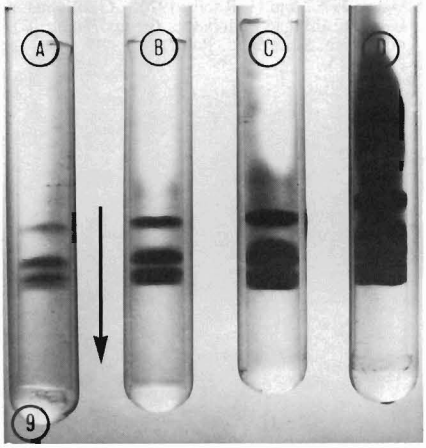


Figure 9.—Pherogram of male crab hemolymph stained for total protein. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

Normal total protein values obtained in this study for *C. sapidus* serum (3.9-67.0 mg/ml, $x$ = 30.3 mg/ml) are in close agreement with the normal values observed in lobsters (Stewart et al., 1967) but are somewhat lower than those values observed in the blue crab by other investigators (Lynch and Webb, 1973a). In lobsters infected with *G. homari*, there is a significant, but not drastic, decline in serum protein, which is not regarded as a serious factor in the deterioration of the animals' physiological condition (Stewart et al., 1969). In blue crabs, the drop from 30.3 mg/ml to 8.8 mg/ml in infected animals is significant and probably accounts, in part, for the extremely weakened condition and rapid deterioration of crabs infected with *P. perniciosa*. Stewart et al. (1967) have shown that protracted diet changes can cause severe serum protein losses in the American lobster. However, in the infected blue crabs, diet change alone probably would not account for the large drop in serum protein, because the diseased crabs are captured in baited crab pots within 24 h of examination, indicating that they feed actively into the final stages of the disease. The amoebae are probably either pinocytosing serum proteins of the crab or secreting proteolytic enzymes which hydrolyze them into small peptides and amino acids, which may then be consumed by the parasite. The loss of fibrinogen as part of the total serum protein by either of these methods would account for the failure of the infected crab hemolymph to clot.

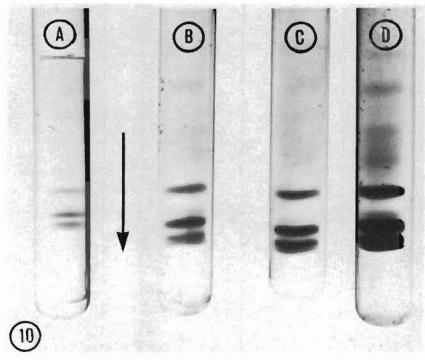


Figure 10.—Pherogram of male crab hemolymph stained for total protein. Hemolymph samples are from the same three infected animals whose immunoelectrophoretic patterns are shown in Figure 4. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

The secretion of powerful enzymes by the parasite would also account for the extensive cellular lysis observed in diseased animals. The rapid destruction of the slow HCy ($\beta$) during infection by *P. perniciosa* does not occur with lyotrophic agents, such as urea, in which case it is the fast HCy ($\alpha$) that are destroyed initially (Gould and Karolus, In press). This is further evidence that the amoebae, directly or indirectly, are altering covalent bonds of molecules within the host. Since hemocyanin is the oxygen binding and transporting molecule of crustaceans, the loss of hemocyanin indicates there probably is insufficient oxygen binding and transport. Death, therefore, may be due to a combination of insufficient oxygen and nutrients.

Minor electrophoretic changes were observed in infected lobsters (Stewart et al., 1969), but these irregularities were not greatly pronounced at the time of death. Analysis of infected blue crab serum by both immunoelectrophoresis and acrylamide gel electrophoresis showed significantly altered patterns of total protein and hemocyanin that could be correlated with the severity of the disease in all but 4 of the 79 cases analyzed. Acrylamide gel protein patterns corroborate the results obtained with immunoelectrophoresis (Fig. 10). Copper electropherograms may also be sensitive indicators of infection because of the rapid disappearance of hemocyanin during the disease. Although electrophoretic analysis is probably not specific for this infection, we recommend acrylamide gel electrophoresis of the serum to determine the degree of stress because of its relative ease of performance and clarity of results.

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