

NERVOUS CONTROL OF RESPIRATION: OXYGEN-SENSITIVE ELEMENTS IN THE PROSOMA OF *LIMULUS POLYPHEMUS*

By CHARLES THOMPSON* AND CHARLES H. PAGE*

Department of Zoology, Ohio University, Athens, Ohio U.S.A.

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SUMMARY

1. Responses of oxygen-sensitive units in the prosomal haemal nerve of *Limulus polyphemus* were examined while varying the oxygen content of sea water bathing the intercoxal cuticle.

2. When exposed to high oxygen levels these units maintained a continuous background discharge of spikes. Unit activity was inhibited when oxygen content decreased. Upon reintroduction of oxygen tonic spike discharge resumed.

3. Mechanosensitive units with receptive fields on the prosomal shield or intercoxal cuticle were also present in the haemal nerve. Neither the mechanosensitivity nor the background discharge of these units was affected by changes in oxygen content.

4. It is proposed that the oxygen-sensitive respiratory reflexes of *Limulus* are an adaptation to existence in the intertidal zone. Published observations of the respiratory stress responses of many intertidal animals support this hypothesis.

INTRODUCTION

The horseshoe crab, *Limulus polyphemus*, has six pairs of opisthosomal appendages – the genital operculum and five pairs of gill appendages. Two book gills are situated on the posterior surface of each gill appendage. Rhythmic metachronal movements of the opisthosomal appendages circulate water over the book-gill lamellae. Both the frequency and amplitude of these ventilatory movements are proportional to the ambient oxygen tension (Waterman & Travis, 1953; Page, 1973). Under anoxic conditions ventilation ceases. When oxygen is introduced into oxygen-free sea water, respiratory ventilation resumes with a latency as brief as 5 sec, suggesting the presence of external oxygen receptors (Waterman & Travis, 1953). The observations of Hyde (1906) that rhythmic opisthosomal appendage movements were maintained during presumed anoxia resulting from removal of the heart and massive blood loss and of Page (1973) that oxygen responsiveness was not appreciably affected by cutting open all branchial blood sinuses also suggest that external oxygen receptors mediate this ventilatory reflex.

Page (1973) has shown that both the book-gill lamellae and the cuticle between the

* Present Address: Department of Physiology, Rutgers University, New Brunswick, N.J. 08903, U.S.A.

coxal segments of adjacent walking legs contain oxygen receptive structures. Sensory input from either set of oxygen receptors is sufficient to generate rhythmic movements of the opisthosomal appendages in response to the introduction of oxygen into oxygen-free sea water. Crabtree & Page (1974) have described oxygen-sensitive units in the nerve which innervates the book gills. Three classes are present: those which discharge in the presence of oxygen, those which are depressed by oxygen, and mechanosensitive units whose tactile sensitivity is oxygen-dependent.

In this report we describe a series of experiments which examined the oxygen-sensitive sensory system in the prosoma. We have detected oxygen-sensitive units in the nerve which innervates the intercoxal cuticle. Impulse discharge of these oxygen-sensitive units is inhibited by exposure of the intercoxal cuticle to anoxic sea water.

METHODS

Adult *Limulus polyphemus* (L.) (prosomal width of 15–20 cm) were purchased from the Marine Biological Laboratory, Woods Hole, Mass., and kept in circulating sea water (Dayno) at 18 °C. The animals were fed beef liver. Immediately before use each animal was bled through an incision made into the pericardial sinus at the prosomal–opisthosomal junction to reduce massive hemolymph coagulation. Next the prosoma was separated surgically from the opisthosoma and all walking legs were removed at the coxal–trochanter joint. After removing the coxal gnathobases the second and third walking legs were spread apart to expose the intercoxal cuticle and secured via hooks fastened to the carapace (Fig. 1). The fourth haemal nerve and the anterior ento-coxal branch of the fourth neural nerve were carefully exposed, severed proximally and subdivided into small bundles with glass needles. The animal was periodically rinsed with *Limulus* saline (Wyse, 1972) at 15 °C.

Afferent activity from the prosomal shield was eliminated by sectioning the fourth haemal nerve at the lateral margin of the intercoxal cuticle (see *E* in Fig. 1). The incision in the carapace was sealed with petroleum jelly.

The prosomal shield was secured so that the carapace formed a basin which was filled with saline to a depth covering the exposed nerves and intercoxal cuticle. The basin was separated into two sections with a dam of petroleum jelly (*A* in Fig. 1). The right-hand section was filled with sea water at air saturation. At the posterior end of the left portion of the carapace two inflow tubes were positioned to perfuse the basin with either air-saturated or nitrogen-saturated sea water. The slight tilt of the carapace permitted sea water to flow past the intercoxal cuticle and over the anterior edge of the carapace. Oxygen content could be maintained at levels greater than 5.0 ppm on the right side and near zero ppm on the left side.

Oxygen content corrected for salinity, temperature and altitude was measured with a Yellow Springs Instruments Model 51 A oxygen meter. The output from the oxygen meter was fed into a Bausch and Lomb VOM Chart Recorder. During trials the oxygen probe was placed next to the coxal segment of the second walking leg (see Fig. 1).

Responses recorded from fibre bundles in the anterior entocoxal nerve or the fourth haemal nerve with a suction electrode were fed into a Grass P-15 AC preamplifier, displayed on a Tektronix 502 A dual-beam oscilloscope and recorded on a Magnecord

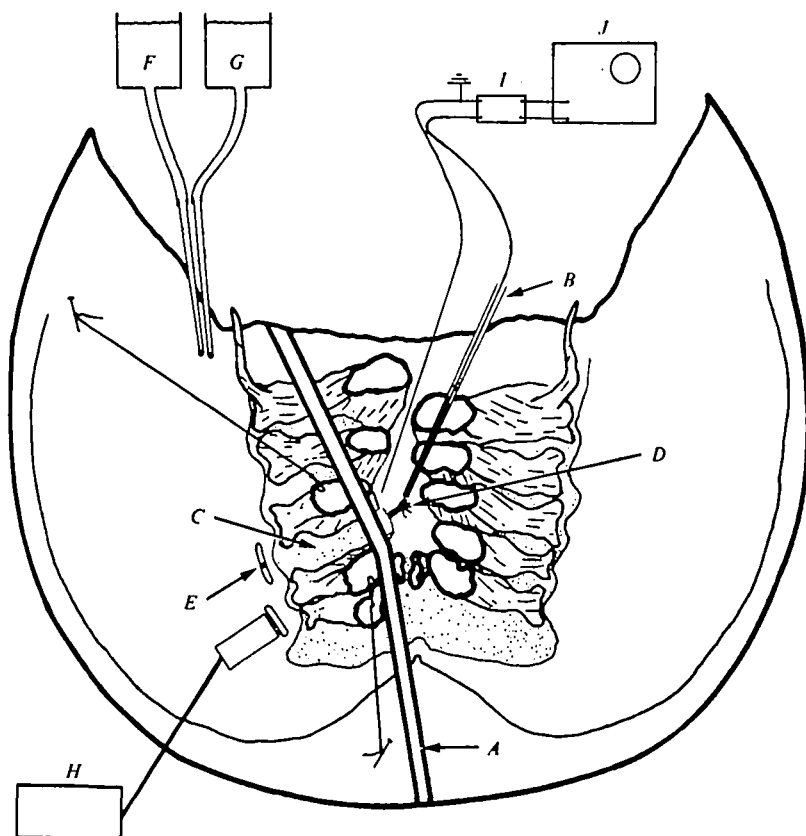


Fig. 1. The experimental preparation. *A*, Petroleum-jelly dam; *B*, suction electrode; *C*, intercoxal cuticular membrane between the coxal segments of the second and third walking legs; *D*, fourth haemal nerve; *E*, point at which the fourth haemal nerve was severed to eliminate the activity in axons innervating the carapace; *F*, nitrogen-saturated sea-water inlet; *G*, air-saturated sea-water inlet; *H*, oxygen electrode; *I*, preamplifier; *J*, oscilloscope.

1028 tape-recorder. A Grass C 4 oscilloscope-recording camera was used to obtain permanent records of tape-recorded data.

The temperature for all experiments ranged from 12 to 19 °C; it never varied by more than 3 °C in a single trial.

A blunt glass probe was used for tactile stimulation of the intercoxal cuticle and the prosomal carapace.

For morphological examination intercoxal cuticular membranes were excised and placed in 19% methylene blue in sea water for 5–10 h. Other intercoxal cuticular membranes were fixed in Zenker's Solution, embedded in paraffin, sectioned and stained with Mallory's Triple Stain.

RESULTS

A. Intercoxal cuticular receptor morphology

The intercoxal cuticle is covered with small spines between 100 and 500 μm in length (Fig. 6, Plate 1). The average density is 8 spines mm^{-2} . No other sensory structures were observed to be present in the cuticle stained with either methylene blue or Mallory's Triple Stain.

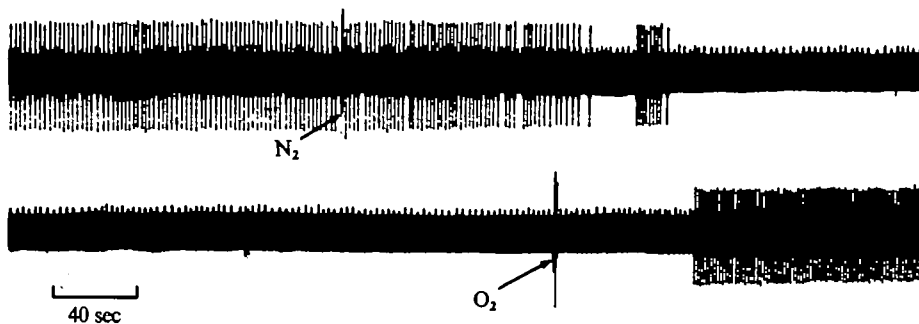


Fig. 2. A recording from an oxygen-sensitive unit in the intercoxal cuticle. During the initial portion of the record the intercoxal cuticle was perfused with sea water at air saturation. Arrows indicate times of introduction of oxygen-free sea water and its replacement with air-saturated sea water.

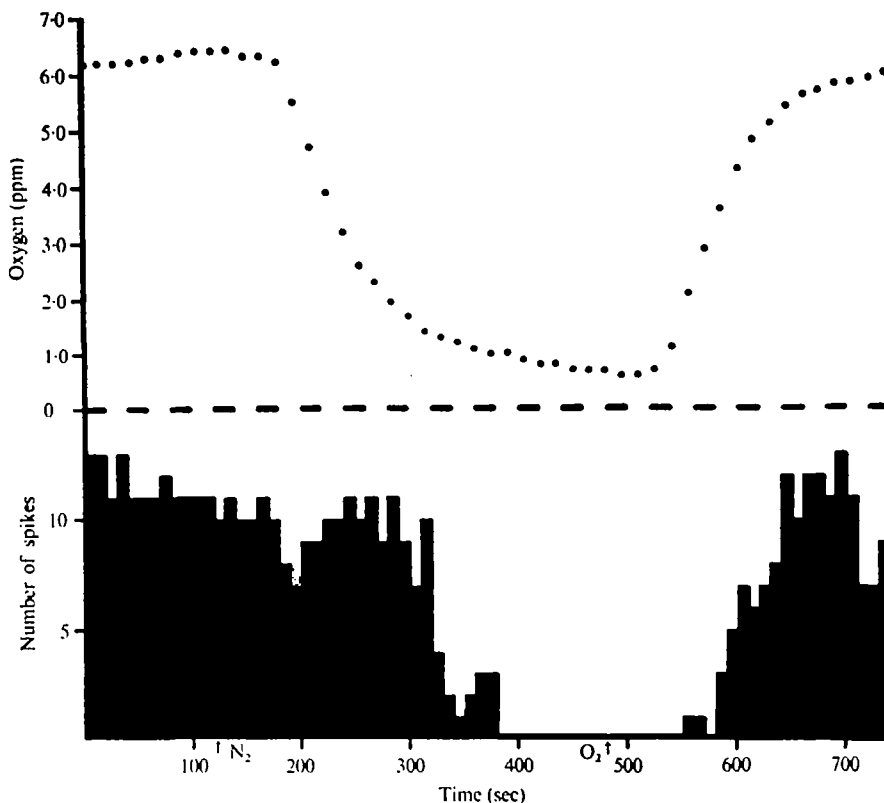


Fig. 3. This histogram shows spike activity of two units during a typical experimental trial. The oxygen electrode readings during this trial are plotted on the upper curve. From 0 to 120 sec the intercoxal cuticle was perfused with aerated sea water; from 120 to 480 sec with oxygen-free sea water; and from 480 sec to the end of the record with aerated sea water.

B. Sensory responses in intercoxal cuticle

Tactile units. Afferent axons from the intercoxal cuticle were always located within the posterior-ventral quarter of the haemal nerve. Tactile stimulation of the intercoxal cuticle evoked multi-unit bursts of action potentials 15–25 μ V in amplitude. Slight deflexions of the tips of the spines were sufficient to elicit a response.

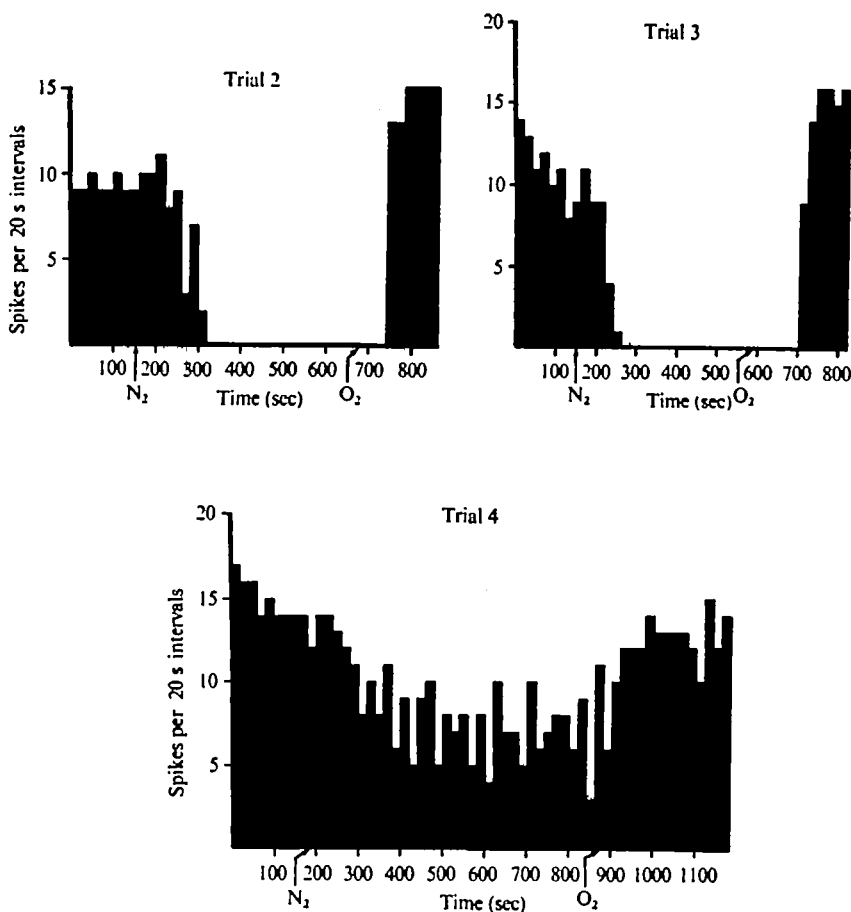


Fig. 4. Histogram showing adaptation of an oxygen-sensitive unit over three consecutive trials similar to those in Fig. 3. During the 15 min intertrial intervals the preparation was bathed in air-saturated sea water.

Oxygen-sensitive units. In the same nerve bundles which contained the intercoxal mechanosensitive spine units a second class of units was present which were oxygen-sensitive. These units were 60–90 μV in amplitude and displayed a steady rate of background activity of 0.2–1.0 impulses per sec. Unit discharge continued at a constant rate as oxygen content in the perfusing sea water was lowered from air saturation (6.0 ppm) to about 1.5 ppm. At this point the rate of firing began to decrease and the pattern of firing became irregular. The units ceased firing when oxygen levels were reduced below 1.0 ppm (Figs. 2, 3). When the intercoxal cuticle was perfused with aerated sea water, the units resumed firing.

Rapid adaptation to anoxia was characteristic of these intercoxal cuticular units. The influence of changes in oxygen content on the activity of these units diminished rapidly with repeated trials. In experiments where 5–10 min periods of exposure to oxygen-free sea water were separated by 20–30 min periods of high oxygen levels, complete inhibition of a unit was never observed to occur in more than three successive trials. After two or three trials a unit responded to the period of anoxic exposure by

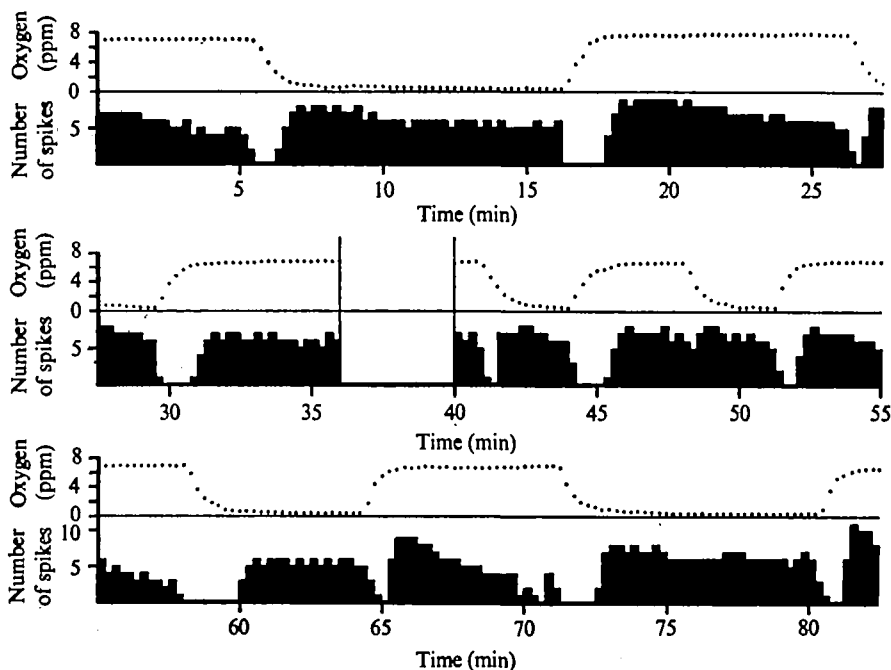


Fig. 5. Histogram of a unit whose activity was inhibited by changing oxygen content. The upper curve plots the oxygen levels during the experiment.

adopting an irregular bursting pattern which was generally associated with a decrease in firing rate (Fig. 4). After four or five successive trials it was found that exposure to oxygen-free sea water for periods of up to 20 min had no effect on the rate or pattern of activity.

These oxygen-sensitive units were insensitive to mechanical stimulation. Even vigorous tactile stimulation of the intercoxal cuticle did not alter the steady rate of background discharge.

In 14 preparations 20 units were observed which had an amplitude of 60–90 μV and ‘spontaneously’ discharged at a steady rate. These units were found in the same relative position within the fourth haemal nerve. Seventeen of the 20 units responded to varying oxygen content in the manner described above. Two of the units were unaffected by changing the oxygen content of the sea water and continued to fire steadily during periods of anoxic exposure. The remaining unit was inhibited by changing oxygen tension although it displayed a steady rate of activity at both high and low oxygen tensions (Fig. 5).

Effects of anoxia on mechanosensitive units. As a control, mechanosensitive units in the haemal nerve which innervated the intercoxal cuticle and the prosomal shield as well as tactile sensitive units in the ento-coxal branch of the fourth neural nerve which innervates the coxa were examined to determine if they exhibited any sensitivity to changes in oxygen tension.

The majority of neurones in the fourth haemal nerve innervate receptors in the prosomal carapace. Responses of these units could be elicited by tactile stimulation of the carapace. A background discharge rate of several spikes per sec was usually observed when recording from a bundle containing several such tactile-sensitive units.

When sustained pressure was applied to the carapace, these units responded with a phasic burst of 5–6 impulses. A similar response was elicited upon the release of pressure.

These prosomal tactile sensitive units were not affected by increasing (0.2–6 ppm) or by decreasing (6–0.2 ppm) the oxygen content of the sea water covering them. Neither their rate of background discharge nor their sensitivity to mechanical stimulation was altered by prolonged exposure to anoxic conditions.

Bursts of impulses were elicited in the anterior ento-coxal branch of the fourth neural nerve by tactile stimulation of the coxal segment of the third walking leg. Some of these units were 'spontaneously' active discharging impulses 2–3 times per sec. Application of stimulus pressure increased the rate of firing of some of the tonically active units.

No responses were observed to changing the oxygen content of the sea water covering the coxal segment. Exposure to oxygen-free sea water for prolonged periods did not affect the background discharge rates or the mechanosensitivity of these tactile-sensitive units.

DISCUSSION

The oxygen-sensitive units present in the haemal nerve described in this report are presumed to arise from sensory elements located in the prosomal intercoxal cuticle. This presumption is based upon three observations. First, responsiveness of oxygen-sensitive units recorded from the haemal nerve was maintained following surgical interruption of the nerve at a point immediately distal to the intercoxal cuticle (see Methods). Sectioning the nerve at this point eliminated the discharge of all units sensitive to tactile stimulation of the prosomal shield. However, units responding to tactile stimulation of the intercoxal cuticle as well as units sensitive to oxygen levels were not affected by this procedure. Secondly, the oxygen-sensitive units were always found in a fine bundle of the haemal nerve which ran in close proximity with the bundle containing those units sensitive to tactile stimulation of the intercoxal cuticle. Finally, Page (1973) has demonstrated that reflex oxygen sensitivity of the prosoma is dependent upon the integrity of the intercoxal cuticle. Inactivation of prosomal sensory structures other than the intercoxal cuticle did not affect the oxygen responsiveness of the prosomal appendages.

Histological examination of the intercoxal cuticle reveals only one class of cuticular sensory structures, the cuticular spines. Although these spines always responded to tactile stimulation, the oxygen-sensitive units were not affected by mechanical stimulation of the intercoxal cuticle. In addition, there appear to be very few oxygen-sensitive units (perhaps as few as three) in an intercoxal cuticle while there are between 1100 and 2000 spines. Therefore either a very small proportion of the total cuticular spine population is oxygen-sensitive, or the oxygen-sensitive elements are located in the subcuticle. The possibility that the oxygen receptors are free nerve endings in the cuticle has not been eliminated.

The observations that tactile-sensitive units in the prosomal shield, intercoxal cuticle and coxal segments of the walking legs were insensitive to anoxia as well as to changes in oxygen content provides a control for the possibility that the unit recordings of oxygen sensitivity were artifactual. These observations also provide additional

support for the results of Crabtree & Page (1974). They observed a small population of mechanosensitive units in the book gills whose tactile sensitivity was oxygen-dependent. That these book-gill units are unique is suggested by the absence of any sensory elements whose mechanosensitivity is oxygen dependent in the 300 prosomal units examined in the present study.

The prosomal oxygen-sensitive units described in this report resemble the Class I units described in the book gills of *Limulus* by Crabtree & Page (1974). Exposure to anoxic conditions inhibits spike discharge of both units. In response to the introduction of oxygen into oxygen-free sea water the book-gill units discharge a phasic burst of impulses whereas the intercoxal units respond to the consequent rise in oxygen tension by resuming their steady rate of spike discharge.

Crabtree & Page (1974) have proposed that the introduction of oxygen into oxygen-free sea water excites the Class I units, whose phasic discharge raises the level of excitation in the central ventilatory system, thereby initiating ventilatory beating of the gill appendages. Movement of the gill appendages evokes discharges of the oxygen-sensitive tactile units. The activity of these units maintains a level of excitation in the central ventilatory system which is sufficient to generate the rhythmic motor output required for ventilation. Presumably the haemal nerve units also provide a source of maintained excitatory input to the central ventilatory system during periods of high oxygen content.

The frequency of respiratory ventilation in *Limulus* is proportional to the logarithm of the ambient oxygen concentration from 0.1 to 2.0 ppm (Page, 1973). At oxygen levels above 2.0 ppm ventilatory frequencies remain constant at about 40 beats/min; at oxygen levels below 0.1 ppm, ventilation ceases. Oxygen-sensitive haemal nerve units are insensitive to variations in oxygen content in the range between air saturation and 1.5–2.0 ppm, for only after oxygen levels are reduced below 1.5–2.0 ppm does a noticeable decrease in their rate of firing occur. It is likely that the rate of firing of a unit, or of the entire population of units, is proportional to the ambient oxygen content over the same range that ventilatory frequency is proportional to the ambient oxygen levels. In this respect the oxygen-sensitive haemal nerve units are more closely correlated with the behaviour of the animal than Class I oxygen-sensitive gill nerve units.

As the frequency of ventilation of *Limulus* declines in response to decreasing environmental oxygen content, the animal becomes quiescent. Waterman & Travis (1953) proposed that this behaviour is an adaptation to existence in the intertidal zone where *Limulus* is frequently found. Anoxic conditions often develop in mud flats and tide pools where *Limulus* may be trapped at low tide. It is advantageous for *Limulus* to conserve energy and quietly await the return of favourable conditions with the next high tide. This hypothesis receives support from observations on the responses to respiratory stress of a wide range of intertidal organisms.

Reduced activity during anoxia has, for example, been observed in a number of organisms that inhabit brown seaweed which develops anaerobic conditions at low tide (Wieser & Kanwisher, 1959). Other examples of this behaviour can be found among teleost fish (Congleton, 1974), polychaetes (Dales, 1958; Mangum, 1970), shore crabs (Wallace, 1972), marsh crabs (Teal & Carey, 1967), mud shrimps (Thompson & Pritchard, 1969) and littoral beetles (Evans *et al.* 1971). There is a

marked similarity between the respiratory responses of *Limulus* to decreasing oxygen content in the ambient sea water and the ventilatory responses of littoral tube dwelling pericarideans (Gamble, 1970b), onuphid polychaetes (Dales, Mangum & Tichy, 1970) and bivalve molluscs (Collip, 1921; Salanki, 1966). Anoxic survival in a number of molluscs is correlated with their ability to reduce their metabolic demand by withdrawing into their shells (Theede *et al.* 1969).

It appears, therefore, that a large number of sedentary or slow-moving intertidal organisms tolerate anoxic conditions which are sufficient to kill sublittoral animals that are not exposed to the respiratory stresses characteristic of the intertidal zone. The investigators listed above have repeatedly observed that survival during anoxic exposure is associated with a decrease in activity (i.e. the animal becomes quiescent). In many intertidal animals reduced activity in response to anaerobic exposure is accompanied by a decrease in oxygen consumption. Several workers (Wieser & Kanwisher, 1959; Gamble, 1970a; Mangum, 1970) have reported that intertidal animals subjected to prolonged anoxic exposure become paralysed, presumably due to the accumulation of acidic end-products of anaerobic metabolism (Weiser & Kanwisher, 1959; Beadle, 1961). Tolerance of these end-products may be an adaptation which permits extended anaerobic survival.

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EXPLANATION OF PLATE

Fig. 6. Methylene blue stained intercoxal cuticle. Mark is 500 μ m.

