

## Branchial water- and blood-flow patterns and the structure of the gill of the crayfish *Procambarus clarkii*

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Water flow within the branchial chamber, blood flow within the gill filaments, and the fine structure of the gill epithelium have been determined in the crayfish *Procambarus clarkii*. The epithelium of the podobranch filaments, which is 3.15-8.70  $\mu$  thick, represents the blood-to-water diffusion distance. Blood flow within the filament is arranged in a bidirectional system that is first cocurrent and then countercurrent to the flow of water irrigating the gills. Morphological evidence suggests that gas exchange might occur in both directions. Shunting of blood occurs within the gill filament and its significance in countercurrent blood flow and gas exchange is discussed.

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On a déterminé l'écoulement d'eau dans la chambre branchiale, l'écoulement de sang dans les filaments branchiaux et la structure fine de l'épithélium branchial, chez l'écrevisse *Procambarus clarkii*. L'épithélium des filaments podobranchiaux, de 3.15 à 8.70  $\mu$  d'épaisseur, représente la distance de diffusion du sang à l'eau. L'écoulement de sang dans le filament se fait par un système bi-directionnel qui est d'abord dans le sens du courant d'eau qui irrigue les branchies, puis dans le sens inverse. D'après la morphologie, il semble que les échanges gazeux puissent se faire dans les deux directions. Le sang emprunte des dérivations à l'intérieur du filament branchial et on discute de l'importance de ce phénomène en relation avec l'écoulement du sang à contre-courant et l'échange gazeux. [Traduit par le journal]

### Introduction

Despite the recent reawakening of interest in the respiratory physiology of the decapod Crustacea, only a fragmentary picture yet exists of the factors influencing gaseous exchange across crustacean gills. Particularly poorly known is the structure and function of the gills themselves and how this relates to the existing experimental data on gill irrigation and oxygen extraction from branchial water.

Oxygen extraction is low in the brachyuran species that have been investigated. Hughes *et al.* (1969) measured oxygen extractions of the 10-30% from *Carcinus maenas*, and Johansen *et al.* (1970) reported extractions of 10-30% for *Cancer magister*. Both studies suggest the existence of countercurrent gas exchange between water and blood, and the former authors attribute the low percentage extractions to low membrane permeability, uneven ventilation and perfusion of the gills, and considerable anatomical dead space.

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Few investigations of the function of the gills of macrurans, which differ considerably in structure from the "lamellar" brachyuran gills, have been undertaken, although existing experimental evidence indicates that branchial gas exchange in macrurans can approach the exchange capabilities of fish that possess a countercurrent respiratory system. Indeed, Lindroth (1938), Larimer (1961), Larimer and Gold (1961), and McMahon *et al.* (1974) have reported oxygen extractions up to 59-79% for the crayfish. Although little is known of the respiratory mechanisms embodied in the gills of these decapod Crustacea, our analysis of the largely anatomical data from studies on crayfish gills by Huxley (1896) on water flow over the gill, by Bock (1925) on gill morphology and blood flow, and by Fisher (1972) on the gill fine structure indicates that a countercurrent mechanism for gas exchange could operate within the crayfish gill. The objective of the present investigation was to examine the pattern of branchial blood and water flow with relation to gill structure in the crayfish *Procambarus clarkii*.

## Materials and Methods

The crayfish, *Procambarus clarkii*, were obtained from commercial sources and reared at 20°C in tap water. Healthy intermolt adults 10–12 cm in length were used for this study.

### Water Flow Within the Branchial Chamber

The gills were exposed by removing a portion of the branchiostegite overlaying the branchial chamber with a dental burr and drill. A 2-mm-wide strip of the ventral-most portion of the branchiostegite was left intact so that the inhalant apertures of the branchial chamber formed by the branchiostegite margin and the pereopod bases would not be altered. A 0.4-mm thick piece of transparent plastic sheet was heat-molded to a shape and size conforming to the excised portion of the branchiostegite and firmly fixed to the carapace over the hole with dental wax (Hughes *et al.* 1969). Extreme care was taken to ensure that the plastic replacement branchiostegite did not alter the volume or shape of the branchial chamber. Through this window in the branchiostegite, all six podobranchs, the epibranchial space, and the scaphognathite were visible. The removal of a portion of the branchiostegite and its replacement with a plastic duplicate appeared to have no adverse effects on the crayfish, and some windows remained in place for up to 1 month.

A crayfish was then placed in a transparent Plexiglas box 3.5 cm wide, 4.0 cm deep, and 14.0 cm long and was secured by a thread tied through a small hole drilled in the rostrum. The shape of the box was such that the chelae and pereopods were limited to only small movements. Cannulae were introduced into the Plexiglas box and advanced under the crayfish until their tips were positioned within 2 mm of the branchial inhalant apertures at the pereopod bases. India ink particles in water (a 2% dilution of commercially available India ink) were then selectively injected with a syringe into the different cannulae at a rate just sufficient to tint the inhalant water in the apertures. Water currents in the branchial chamber were recorded using a 16 mm camera and color film.

### Blood Flow Within the Podobranch Filaments

A crayfish with a window over the branchial chamber was placed in the Plexiglas box as described above. The intact podobranch filaments of the crayfish were examined through the transparent "branchiostegite" with a Wild M5 microscope using magnification of 125–300 times. The filaments themselves are diaphanous and hemocytes 5–8 microns ( $\mu$ ) in diameter circulating in the almost transparent blood provided convenient "markers" which illustrated the blood flow direction and velocity.

### Light Microscopy

Podobranchs from the first to third pereopods were excised from living, healthy crayfish and immediately examined with a Wild M5 microscope fitted with a 35-mm camera. Single podobranch filaments were fixed in 10% formal saline and then embedded in paraffin wax. Iron haematoxylin and eosin were used to stain the 5- $\mu$  sections cut from the blocks, which were subsequently examined in a Wild M20 microscope equipped with a Mka4 camera and microphotomat.

### Electron Microscopy

Podobranch gill filaments were fixed with 1% osmium tetroxide in Millonig's phosphate buffer at pH 7.3 for 1 h. In some preparations this fixative was modified by the addition of 5% sucrose (Fisher 1972). Fixed material was embedded in Araldite, from which thin sections were cut. The sections were stained in ethanolic uranyl acetate for 30 min and in lead citrate for 15 min (Reynolds 1963). The sections were examined using an AFI 801b transmission electron microscope.

## Results

The positioning, points of articulation, and gross anatomy of the pleurobranch, arthrobranch, and podobranch units which constitute the gills of *Procambarus clarkii* are similar to those described for other species of crayfish (Huxley 1896; Bock 1925) and will not be elaborated further in the present study. The podobranch, which in *Procambarus clarkii* is generally much larger than either the pleurobranch or arthrobranch, was chosen for detailed investigation, since its size and surface area relative to other gill components suggest an importance in gas exchange.

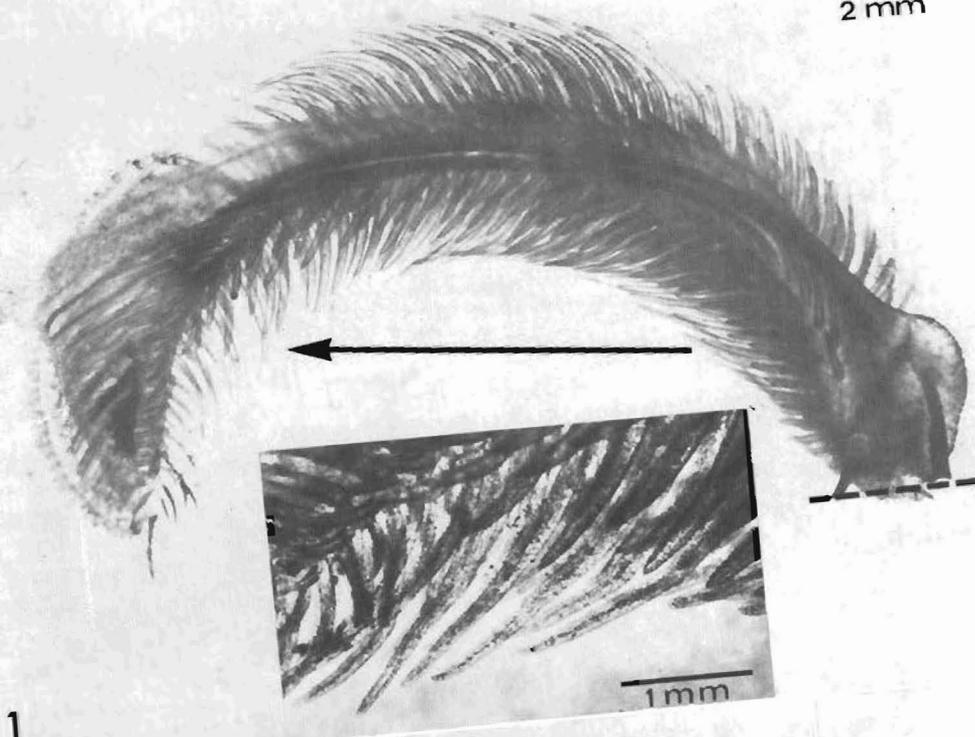
With the exception of the first maxilliped and the fourth pereopod, all thoracic appendages give rise to a podobranch at their points of articulation to the thoracic body wall. The podobranchs, which range from 1.0 to 2.5 cm in length, are positioned vertically in the branchial chamber with the larger podobranchs associated with the more posterior appendages.

One hundred to 200 filaments arise from the lateral surface of the podobranch in alternate rows covering all but the distal tip (Fig. 1).

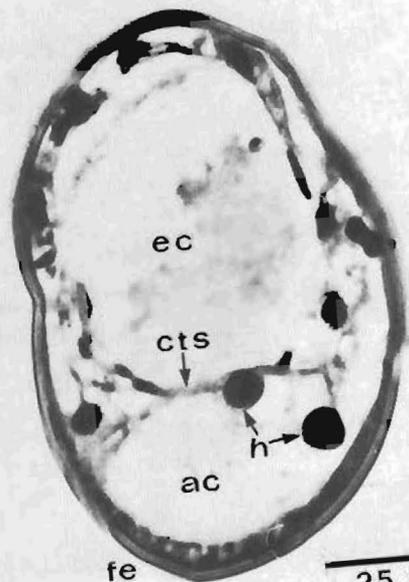
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FIG. 1. The podobranch of *Procambarus clarkii*. The point of excision is represented by the broken line. The direction of water flow over the podobranch is indicated by the arrow. 6 $\times$ . Insert; Podobranch filaments near central region of podobranch shaft. 18 $\times$ . FIG. 2. Transverse section of a podobranch filament. The filament is divided by a connective tissue septum into a narrower afferent channel carrying blood to the distal end of the filament and an efferent channel returning blood to the peripheral canal of the podobranch. The septum is narrowest in the filament lumen (4–14  $\mu$ ) but widens to 20–30  $\mu$  as it approaches the filament wall. *ac*, afferent channel; *cs*, connective tissue septum; *ec*, efferent channel; *fe*, filament epithelium; *h*, hemocyte. 745 $\times$ .

2 mm



1



2

25 μ

These filaments are relatively uniform in size and shape, being about 2.0–3.0 mm long and 75–100  $\mu$  in diameter. The medial surface of the podobranch, which is loosely covered for its entire length by a prominent membranous fold of the podobranch epithelium, is devoid of filaments and lies against the thoracic body wall. The filaments do not extend perpendicularly from the surface of the podobranch shaft, but over most of their length are deflected dorsally in a plane nearly parallel to the axis of the shaft.

#### *Water Flow Within the Branchial Chamber*

Negative water pressures in the branchial chamber generated by the scaphognathite cause water to be drawn into the branchial chamber through the inhalant apertures lying between the limb bases at the ventral margin of the branchiostegite (Fig. 3). A considerable portion of the inhalant water is shunted between each podobranch dorsally into the epibranchial space. However, the tendency of the podobranchs to overlap and the positioning of the most ventral filaments diverts water into the filament mat. The

distal (dorsal) quarter of the podobranch, which is free of filaments, curves anteriorly and it has a ridged surface formed by several longitudinal folds of the epithelium (Figs. 1 and 3). As water emerges from the most dorsal row of filaments, the epithelial folds, which are parallel to the axis of the podobranch, channel the water along the podobranch and into the epibranchial space.

The observed velocity of the water entering the branchial chamber and in the currents on either side of the podobranchs was 4.5–5.0 cm/s. The velocity of water diverted into the filament mat decreased to 1.0–2.5 cm/s. In the epibranchial space, water from the currents in between the podobranchs mixes with water which has flushed the filament mat. Water velocity increased up to 5.0–6.0 cm/s as the epibranchial space progressively narrows into the excurrent canal. (These observations were made on stressed animals whose high rate of scaphognathite beat resulted in a greatly elevated irrigation volume and water velocity within the branchial chamber.) Reversals in water flow through the branchial chamber de-

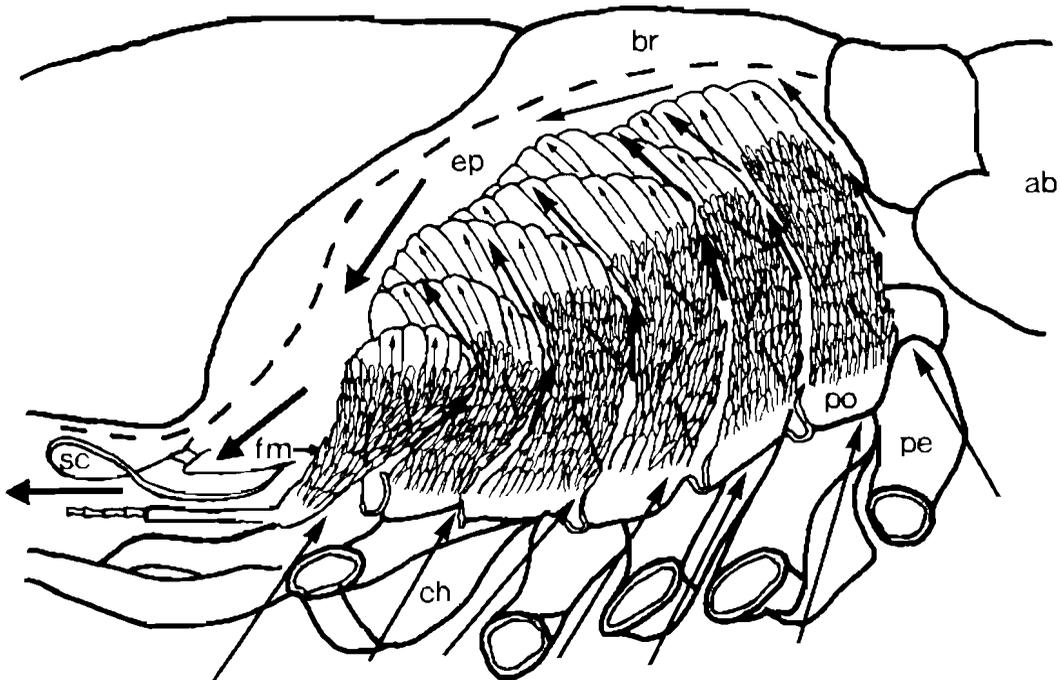


FIG. 3. Water flow within the branchial chamber of the crayfish. The broken line indicates where the branchiostegite has been removed. The arrows indicate the direction of water flow, with the larger arrows representing greater flow. *ab*, abdomen; *br*, branchiostegite; *ch*, cheleped; *ep*, epibranchial space; *fm*, filament mat; *po*, podobranch; *pe*, perciopod; *sc*, scaphognathite.

scribed for other Crustacea (Arudpragasam and Naylor 1966; Hughes *et al.* 1969; Wilkens and McMahon 1972) were never observed in *Procambarus clarkii*.

#### Blood Flow Within the Podobranch Filaments

Internally, each filament is divided longitudinally into two separate channels by a septum of connective tissue (Fig. 2). This septum is 5–14  $\mu$  thick towards the middle of the filament lumen

but widens to 20–30  $\mu$  as it approaches its point of attachment to the filament epithelium. Within the filament, blood flow is bidirectional. The septum of connective tissue forms the medial wall of an afferent channel carrying deoxygenated blood towards the distal end of the filament and an efferent channel returning the blood from the filament to the peripheral canal of the main podobranch body (Fig. 4). The septum is not continuous throughout the length of the filament. At distinct, evenly spaced points along its attachment to the filament epithelium are small orifices about 10–17  $\mu$  in diameter. Deoxygenated blood crosses laterally from the afferent channel through the orifices in the relatively thick portion of the septum into the efferent channel (Fig. 4). These "scalloped" edges of the septum form the lacunae described by Fisher (1972). No attempt was made to quantify flow through the filaments, although visual estimates based on observation of hemocytes in the blood indicate that a significant fraction of the blood from the afferent channel passes to the extreme end of the filament, where it crosses to the efferent channel in a larger terminal lacuna. However, the apparent magnitude of the shunt through the lacunae along the length of the connective tissue septum varied considerably from crayfish to crayfish. The blood velocity was highly variable within the podobranch filament. Blood velocity in the narrower afferent channel was about 0.30–0.75 cm/s but it was reduced to 0.15–0.40 cm/s in the wider efferent channel which returns blood to the peripheral canal of the podobranch shaft. Lacunae were often momentarily occluded by hemocytes which had become wedged into them. Because of the possibility of the hemocytes' passage being retarded, an estimate of blood velocity in the lacunae was not made.

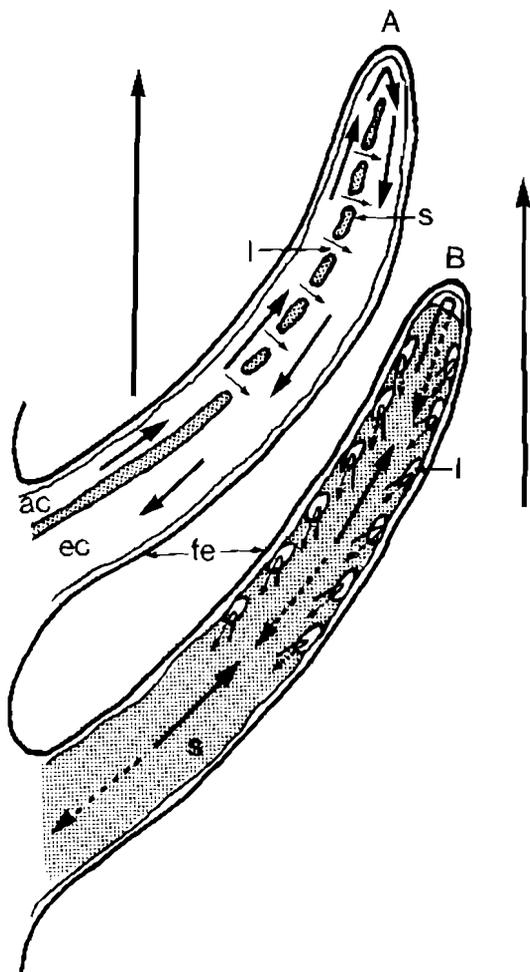


FIG. 4. A diagrammatic illustration of blood flow within the podobranch filaments. Two filaments are shown. The septum (shaded) in filament A is rotated 90° from filament B. Blood flow in the afferent channel of filament B is shown by solid arrows and in the efferent channel by broken arrows. The direction of water flow over the filaments is indicated by the large vertical arrows. See text for detailed explanation of blood flow in the filaments. *ac*, afferent channel; *ec*, efferent channel; *fe*, filament epithelium; *l*, lacunae; *s*, septum.

#### Blood Flow/Water Flow Relationships

The pattern of blood flow through the filaments of *Procambarus clarkii* is largely the same as that determined for *Potamobius astacus* by Bock (1925) and *Astacus pallipes* by Fisher (1972). However, in these previous studies on crayfish the orientation of the water which irrigates the filaments to the bidirectional blood flow within them has not been examined. Each filament lying on the podobranch shaft is constantly flushed with water drawn into the

branchial chamber through the inhalant apertures. The direction of water flow over the filaments is nearly parallel to their longitudinal axis (Fig. 4). Hence, blood in the afferent channel moving towards the distal tip of the filament flows in nearly the same direction as the water moving over the filament. However, after passing laterally through the lacunae of the septum into the efferent channel, the blood returning along the length of the filament to the peripheral canal of the podobranch shaft runs counter to the direction of water flow. Thus, in *Procambarus clarkii*, the pattern of blood movement involves a combination of cocurrent and countercurrent flow within a single podobranch filament.

#### *Fine Structure of the Podobranch Epithelium*

A fibrous basement membrane forms the basal boundary of the filament epithelium (Figs. 5 and 6). The basement membrane, which varies considerably in thickness from 0.05 to 0.20  $\mu$ , is relatively uniform in arrangement and rarely protrudes into the tissue underlying it.

The cellular constituent of the filament epithelium enveloped by the basement membrane is highly variable. In certain areas of the filament epithelium the space between the granular basement membrane and the chitinous endocuticle is occupied by a single layer of cells from 1.5 to 6.0  $\mu$  thick. In other areas both the basal and apical plasma membranes of these cells are highly folded and interdigitated (Figs. 5 and 6). These interdigitations form extracellular channels 500–1000 Å wide on the basal membrane surface, which may extend for 5–10  $\mu$  and penetrate to within 1–2  $\mu$  of the cuticle. The apical plasma membrane is highly folded in areas where basal folding is prevalent, and outward extension of the apical plasma membrane may approach to within 0.1  $\mu$  of the endocuticle (Fig. 5). The cytoplasm of the cells of the gill filament epithelium contains mitochondria and ribosomes, which are sometimes displaced by extensive diffuse electron-transparent deposits (Fig. 5), and a very great number of vesicles which may be as large as 2.3  $\mu$  in diameter. The presence of large vesicles near either surface of the epithelial cells occasionally straightens out the folded apical or basal membranes (Fig. 5).

The cuticle itself is composed of the innermost

endocuticle, the exocuticle, and the external epicuticle. Together they form a cuticular structure 1.5–2.5  $\mu$  thick. However, pore canals extend into the cuticle to within 0.5  $\mu$  of the external environment (Fig. 6).

The basement membrane (0.05–0.20  $\mu$  thick), a cellular fraction of highly folded or largely vacuolar cells (1.5–5.0  $\mu$  thick), a layer of granular electron-dense material (0.1–1.0  $\mu$  thick), and a chitinous cuticle (1.5–2.5  $\mu$  thick) form a water-to-basement-membrane barrier 3.15–8.70  $\mu$  in thickness in the podobranch filaments of *Procambarus clarkii*.

#### Discussion

The gas permeability of the podobranch filament is dependent on both its cellular organization and the diffusion distance between water and blood. Crayfish possess a chitinous cuticle whose relative impermeability to water and ions no doubt contributes to the preservation of the ionic and osmotic balance of the animal but also may restrict the passage of oxygen and carbon dioxide. This restriction is minimized by the effect of hydration, which strikingly increases the rate of diffusion of oxygen through chitin (Krogh 1919), and by a substantial reduction in the thickness of the filament cuticle. Travis (1963), Green and Neff (1972), and others have described extensive penetration of the crustacean cuticle by pore canals, whose density may approach  $4 \times 10^6/\text{mm}^2$  of epidermal cell surface and may penetrate up to 75% of the cuticular thickness. Thus, pore canal penetration, which has been observed in *Procambarus clarkii* (Fig. 6), may further enhance the diffusion of gases through the chitinous cuticle.

Assuming that the basement membrane defines the margin of the hemocoel, the overall blood-to-water diffusion distance in the podobranch filaments of *Procambarus clarkii* is 3.2–8.7  $\mu$  (Figs. 5 and 6). This is comparable to the 1.4–5.7  $\mu$  attributed to *Astacus pallipes* by Fisher (1972). However, the actual blood-to-water barrier may be smaller. Copeland (1967) has described gaps in the basement membrane of the metepipodite of *Artemia salina* which allow the blood to pass into a complex system of sinuses linked by extracellular channels formed by apical and basal plasma membrane folding. As a consequence of this outward encroachment of

the hemocoel, the blood-to-water diffusion distance in *Artemia salina* is only 1–2  $\mu$ , although the actual water-to-basement-membrane distance is about 4  $\mu$ . Similar plasma membrane folding has been observed in the filament epithelium of *Procambarus clarkii* forming extracellular channels up to 0.2  $\mu$  wide (Fig. 6). No discrete system of extracellular sinuses has yet been observed in the podobranch epithelium of the crayfish but large extracellular spaces do occur in some regions (Figs. 5 and 6). Gaps in the basement membrane are not consistently observed but in certain areas of the filament epithelium the basement membrane is only 0.05  $\mu$  thick (Fig. 5). This observation concurs with that of Fisher (1972) for the crayfish *Astacus pallipes*. It is not known to what extent the basement membrane restricts the movement of hemolymph proteins into the filament epithelium, but clearly an encroachment of the hemocoel into the epithelium would reduce the blood-to-water diffusion distance.

A significant proportion of the water drawn into the branchial chambers flows at a relatively high velocity between the podobranchs through to the epibranchial space without flushing the filament mat. These water shunts may be considered as anatomical dead space similar to that in the posterior part of the gill chambers of *Carcinus maenas* (Hughes *et al.* 1969) or water flowing between the tips of the gill filaments of fishes (Hughes 1966a). McMahon *et al.* (1974) have shown that the percentage of oxygen extracted from the branchial water flow is low (20–28%) in stressed crayfish exhibiting high pumping rates similar to those rates observed in the present investigation. In contrast, percentage oxygen extraction from the branchial water is higher (40–61%) in relatively unstressed animals with reduced pumping rates and it is tempting to speculate that under such conditions the pro-

portion of inhalant branchial water diverted into the filament mat is substantially increased.

Some of the water actually diverted into the filament mat may not make functional contact with the filament surface and so may represent Hughes's (1966a) diffusion dead space. However, water diverted from the main branchial shunts into the filament mat at the podobranch base usually flushes the entire filament mat before emptying into the epibranchial space. Hence, the water may contact several filaments sequentially, and water which occupied diffusion dead space as it passes over one filament may not necessarily be within the diffusion dead space as it passes over the next. Also, additional water is continually diverted from the shunts into the filament mat along its length and this may maintain the oxygen content of water flushing the more distal filaments.

The velocity of water passing through the filament mat was 1.0–2.5 cm/s. Thus, water entering the filament mat at the base of the podobranch is in contact with filament epithelium for 0.8–2.0 s. This period compares favorably with estimates of the length of time that water is in contact with the secondary lamellae of the teleost gill filament (Randall 1970).

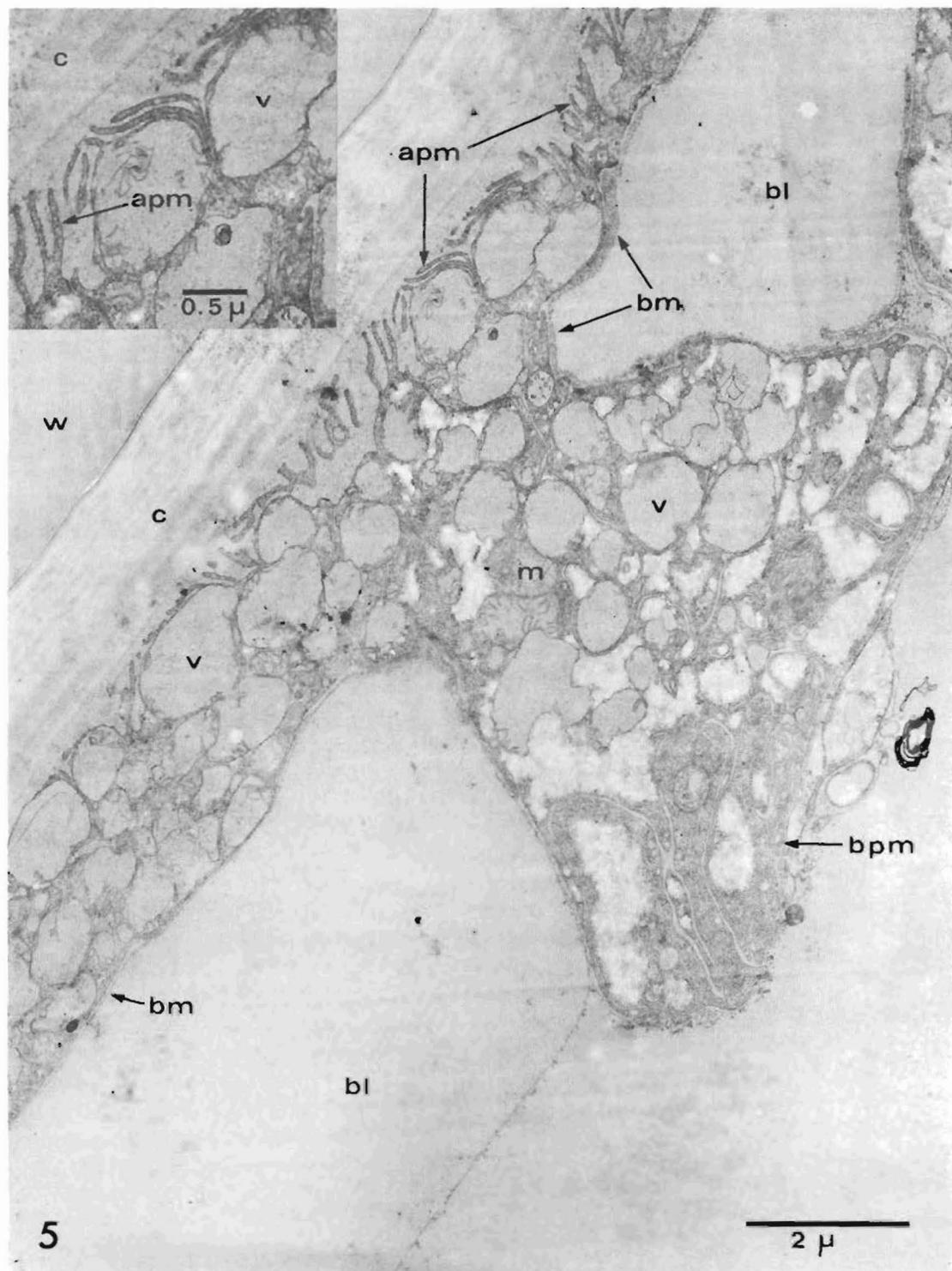
Countercurrent blood flow systems have been reported for many diverse respiratory organs such as the gills of fishes (Randall 1970 for review), the mammalian placenta (Metcalfe *et al.* 1967), and the crab gill (Hughes *et al.* 1969). In these systems, blood flows countercurrent to the flow of the respiratory medium along the entire length of the respiratory surface. The arrangement of blood and water flow initially proposed by Bock (1925) and corroborated in the present investigation for the crayfish gill is markedly different. Blood flow is initially cocurrent to branchial water flow in the afferent channel but blood returns in a nearly counter-

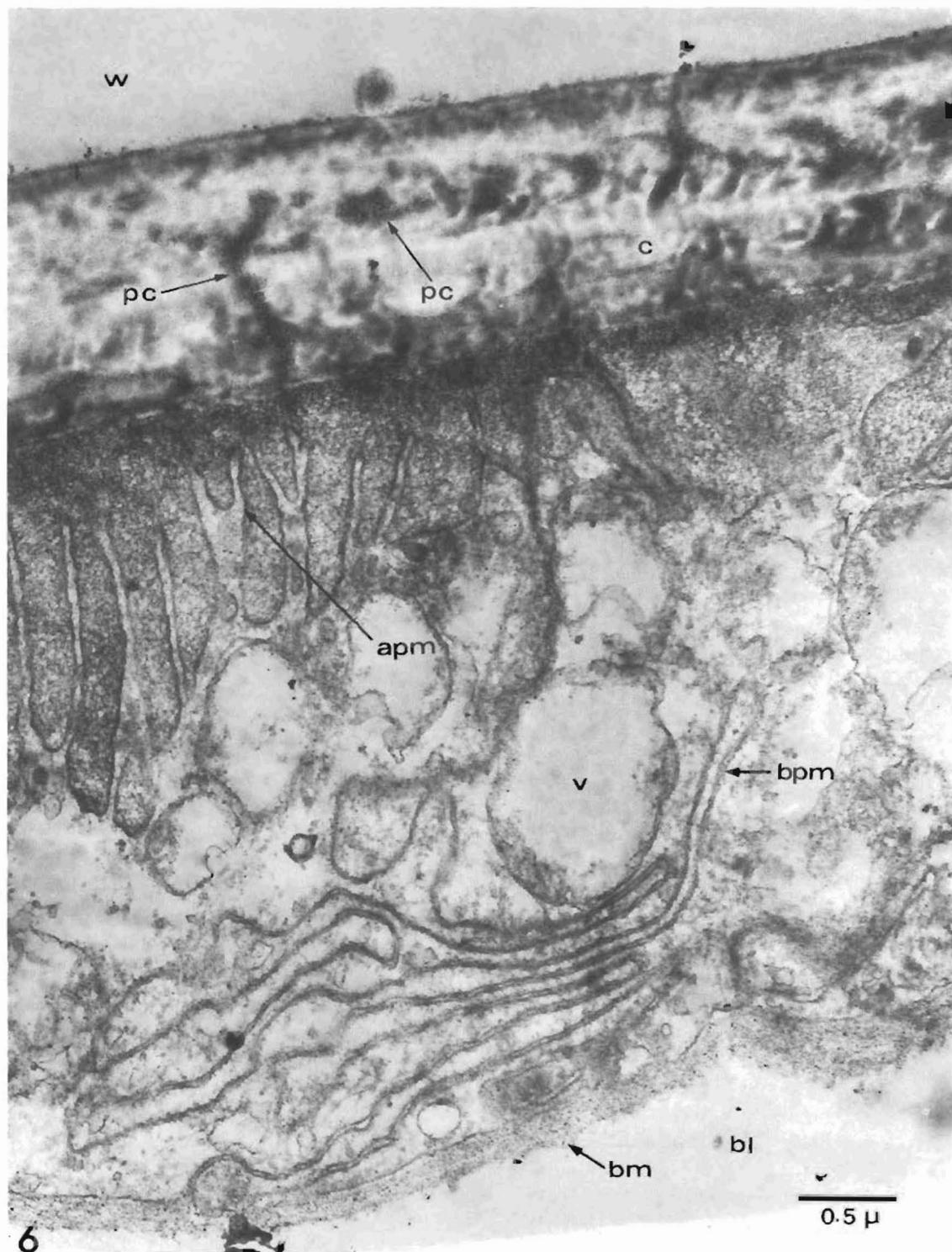
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FIG. 5. Transverse section of filament epithelium in the vicinity of a lacuna. The epithelium is composed of a basement membrane, an underlying layer of cells which may be relatively simple with large vacuoles, or complex with highly folded basal and apical plasma membranes, and a chitinous cuticle. *apm*, apical plasma membrane; *bl*, blood; *bm*, basement membrane; *bpm*, basal plasma membrane; *c*, cuticle; *m*, mitochondria; *v*, vesicle; *w*, water, i.e. external environment. 11 600  $\times$ .

Insert: Area of the filament epithelium with extensive apical plasma membrane folding; *apm*, apical plasma membrane; *c*, cuticle; *v*, vesicle. 19 200  $\times$ .

FIG. 6. Transverse section of filament epithelium where extensive folding of both basal and apical plasma membranes occurs. Pore canals containing the fibrous material can be seen to penetrate the cuticle. *apm*, apical plasma membrane; *bl*, blood; *bm*, basement membrane; *bpm*, basal plasma membrane; *c*, cuticle; *pc*, pore canal; *v*, vesicle; *w*, water. 30 000  $\times$ .





current direction to branchial water flow in the efferent channel. Both vessels appear to have similar characteristics for gaseous exchange, although the consistently larger efferent channel has slightly greater surface area exposed to branchial water (Fig. 2) (Bock 1925; Fisher 1972). The system is further complicated by the presence of lacunae in the connective tissue septum (Fig. 4) (Fisher 1972), through which we have observed the admixture of blood in the efferent channel. Fisher (1972) states that the filament lacunae are the sites of blood oxygenation. However, the thinness of the epithelium of the afferent and efferent channels of *Procambarus clarkii* indicates that gas exchange could occur along the length of both channels. It is also apparent that the afferent and efferent vessels within the filament are separated by a relatively thin membrane (Fig. 2) and if diffusion occurred freely between channels they could themselves constitute an internal countercurrent loop.

The respiratory system thus revealed in *Procambarus clarkii* is complex, and in the absence of measurements of blood flow either in the main filament channels or through the lacunae it is impossible to attempt a complete functional interpretation. However, the data presented do allow some speculation on the potential of the system for gaseous exchange.

Clearly, the presence of countercurrent flow between branchial water and blood in the efferent filament channel shows a potential for highly effective gas exchange at this site. However, this potential could be limited by cross diffusion of gases over the connective tissue septum intervening between efferent and afferent channels or by admixture of efferent and afferent blood shunted across the lacunae. Diffusion of oxygen between afferent and efferent channels is probably of less importance. At most, the area of contact between the two channels is equivalent to about 30% of the total epithelial area available for gas exchange. Also, the septum between the channels is biconcave (Fig. 2) (Bock 1925; Fisher 1972) and is probably thin enough for substantial gas exchange only along the central axis. On morphological grounds, it appears that cross-diffusion of gases across the central septum may be insignificant when compared with total exchange in the filament.

Shunting of blood via the lateral lacunae is potentially of much greater importance. Sub-

stantial shunting would limit the exchange capacity of the podobranch filament through a reduction of its functional length, since a smaller portion of blood would travel to the terminal lacuna at the distal tip of the filament before returning countercurrently in the efferent channel (Fig. 4). However, under certain circumstances such a reduction in functional length of the filament could prove beneficial to the crayfish. The gills of freshwater Crustacea in particular are major sites of ionic regulation. Clearly, any reduction in functional filament length would reduce ion loss because blood would be exposed to the highly hypotonic ambient medium over a reduced surface area. If the proportion of total filament blood flow shunted through the lacunae could be controlled, this would provide a system which could be balanced to suit the crayfish's immediate respiratory and ionic requirements by an adjustment of functional podobranch filament length. The observed proportion of blood shunted through the lacunae of the filament varied considerably between animals in the present investigation, but no mechanism for controlled shunting of blood can be suggested as yet.

#### Acknowledgments

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