Control of Spiracles in Silk Moths by Oxygen and Carbon Dioxide

Abstract. *Spiracles of insects open in high carbon dioxide tensions and close in high oxygen tensions. However, the targets of these gases in insects have never been identified. In diapausing pupae of the cecropia silk moth carbon dioxide acts primarily and directly on the spiracular apparatus itself (muscle or neuromuscular junctions), whereas oxygen has as its primary target the central nervous system. The spiracle behaves as an independent effector in response to carbon dioxide; this is quite different from the situation in vertebrates, where carbon dioxide acts primarily on the central nervous system. The roles of various nerves in controlling spiracular activity are discussed.*

Gas exchange in insects occurs principally through the spiracles. When the spiracles open oxygen enters, and carbon dioxide leaves the tracheal system. Some insects, such as locusts and honeybees, ventilate the tracheal system by gross bodily movements. Others, such as caterpillars and silk moth pupae, have no obvious respiratory movements other than movements of the spiracular valves, and they breathe by a combination of diffusion and mass flow of gases past the spiracular valves. Since insects, like all small terrestrial organisms, are subject to desiccation, natural selection has favored mechanisms that enable insects to keep their spiracles closed as much as possible to prevent water loss. The spiracles open only enough for adequate gas exchange.

What controls spiracular movements? In mammals respiratory movements are controlled primarily by carbon dioxide acting on the respiratory center in the medulla and secondarily by low oxygen tensions (and high carbon dioxide tensions) acting on chemoreceptors of the carotid sinus and the aorta (*1*). In insects, carbon dioxide and oxygen also control respiratory movements, and the spiracles of most insects open in high carbon dioxide tensions, and close in high oxygen tensions. However, the targets of these gases are uncertain (*2*). To identify these sites of action we have used pupae of giant saturniid moths. These insects offer three advantages: (i) their large size permits convenient surgery; (ii) each spiracle is operated by a single closer muscle, which is opposed by an elastic ligament; and (iii) there is discontinuous respiration in which spiracles exhibit several distinct kinds of behavior in "slow motion."

In diapausing pupae of the cecropia silk moth (*Hyalophora cecropia*) respiration takes the form of a continuous uptake of oxygen and a periodical release of metabolic carbon dioxide in "bursts" (Fig. 1) (*3*). During a burst the spiracular valves open widely, permitting carbon dioxide to leave and oxygen to enter the tracheal system. The burst is followed by a brief period of valve constriction, as a consequence of which intratracheal oxygen tension falls and intratracheal dioxide tension rises. Constriction is followed by a prolonged period of valve opening and closing—"flutter"—during which the intratracheal oxygen tension remains constant while the carbon dioxide tension continues to increase. When the intratracheal carbon dioxide tension reaches a triggering threshold the valves open, and another burst begins (Fig. 1).

Table 1. Responses of spiracular values to simultaneous systemic and central gas flows. In these experiments only the detracheated fourth abdominal ganglion received the central flow. The fourth right abdominal spiracle, the third and fifth right abdominal spiracles, and the third and fifth abdominal ganglia received the systemic flow. RAS, right abdominal spiracle.

Composition of gas mixture (% of atm)				Spiracular valve response		
Central		Systemic		4RAS	3RAS	5RAS
pO_2	pCO_2	pO_2	pCO_2			
5	7	5	3	Flutter	Flutter	Flutter
5	3	5	7	Open	Open	Open
21	7	21	3	Closed	Closed	Closed
21	3	21	7	Open	Open	Open
5	15	5	3	Flutter	Flutter	Flutter
5	20	5	3	Flutter	Flutter	Flutter
5	20	5	3	Flutter	Flutter	Flutter
5	3	21	3	Flutter	Closed	Closed
21	3	5	3	Closed	Flutter	Flutter
3	3	50	3	Open	Closed	Closed
50	3	3	3	Closed	Open	Open

When the intratracheal partial pressure of O_2 (pO_2) falls below about 5 percent, the spiracles flutter. Artificially increasing intratracheal pO_2 above about 20 percent suppresses fluttering, but discontinuous respiration persists, apparently controlled now by carbon dioxide alone. These and other facts led to three hypotheses: (i) spiracular fluttering is controlled by the intratracheal oxygen tension, whereas wide spiracular opening is controlled by the intratracheal carbon dioxide tension; (ii) these gases affect spiracular behavior by separate mechanisms; and (iii) the primary target of oxygen is the central nervous system, whereas the primary target of carbon dioxide is the spiracular apparatus (*4*). To test these hypotheses the following procedures were developed.

A pupa was immobilized with strips of melted paraffin applied along the abdominal segments except over the spiracular openings. An 8-cm length of polyethylene tubing was inserted past the spiracular valve of the second right abdominal spiracle (the "inlet" spiracle), and a similar cannula was placed in the sixth left abdominal spiracle (the "outlet" spiracle). Specific gas mixtures were introduced into the tracheal system through the inlet spiracle; the cannula in the outlet spiracle prevented the buildup of pressure within the animal and provided a path for free flow of gas. The flow rate was adjusted to compensate for the oxygen uptake of the pupa and to ensure a uniform and constant intratracheal oxygen tension. By this means the entire tracheal system could be perfused at a constant rate with a gas of known oxygen and carbon dioxide content (systemic flow).

Following this procedure, the fourth abdominal ganglion was prepared to receive a separate gas flow. The ganglion was exposed surgically and supported (Fig. 2); all tracheal connections between the ganglion and the rest of the insect were severed; that is, the ganglion was "detracheated." Experimental gas mixtures, flowing from a delivery bell lowered over the ganglion preparation, perfused the ganglion

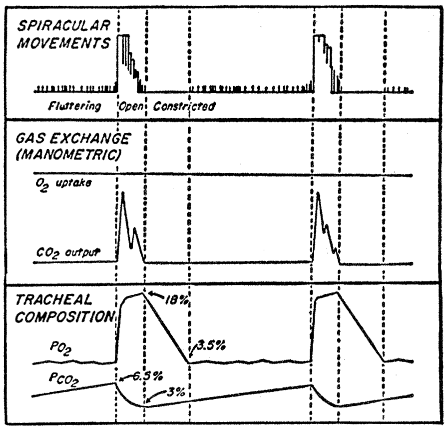

Fig. 1. The relationships between spiracular movements, gas exchange, and intratracheal gas composition during a respiratory cycle. A "burst" of CO_2 output occurs when the spiracular valves are *open*; intratracheal pO_2 rises and pCO_2 falls. Following the burst, valves *constrict*; pO_2 falls, and pCO_2 begins to increase. During the period of valve *flutter* pO_2 remains constant, while pCO_2 continues to increase. [Levy and Schneiderman (3)]

surface and the tracheal stumps that remained attached to the ganglion. Thus, the ganglion received its own gas supply by way of its tracheal stumps (central flow), while the rest of the insect was perfused simultaneously with a separate gas mixture (systemic flow). Spiracular response to gas flows was determined by following the leading edge of a spiracular valve with the hairline of an ocular micrometer and automatically recording changes in the position of the hairline (*4, 5*). These experimental techniques were used to examine the effects of oxygen and carbon dioxide on spiracular behavior, to locate the sites of action of each gas, and to examine the nervous control of spiracular behavior. Six hundred perfusions of between 20 and 240 minutes duration were conducted on 59 pupae. About 700 hours of visual observations and records of valve movements were completed.

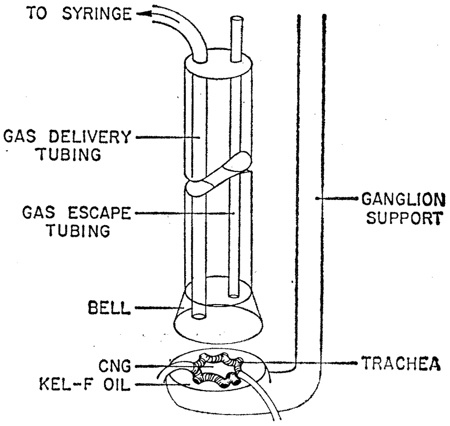

Fig. 2. Diagram of assembly for supporting the fourth abdominal ganglion and perfusing it with an experimental gas mixture. Gas was perfused at a constant rate from a motor-driven syringe through the gas delivery tubing; when the bell was lowered to meet the bowl of the ganglion support, the ganglion was perfused with the gas mixture. The gas escape tubing permitted excess gas to escape. Kel-F oil was used to prevent desiccation and sticking of the ganglion to its support.

What are the effects of various mixtures of oxygen and carbon dioxide on spiracular behavior? To answer this question, we perfused the same gas mixture systemically and centrally. The results of 12 perfusions of 20 minutes each are shown in Fig. 3. At carbon dioxide tensions above 14 percent, the valves remained open regardless of the oxygen tension. At oxygen tensions below about 3 percent, the valves remained open as a result of anoxia. At oxygen tensions above about 35 percent the valves remained constricted unless the carbon dioxide tension was above 14 percent, whereupon the valves opened. Between oxygen tensions of 3 and 35 percent the spiracles exhibited three sorts of behavior—constriction, flutter, or opening—depending on the gas mixture. An examination of the curves in Fig. 3 reveals an interaction between carbon dioxide and oxygen. Thus, in a gas mixture containing 10 percent oxygen and 12 percent carbon dioxide, the valves opened; when the carbon dioxide tension was 8 to 11 percent the valves fluttered. And when the carbon dioxide tension was less than 8 percent the valves constricted. These curves define the gas mixtures in which particular modes of spiracular activity occur and in which discontinuous respiration is possible. Not only does each gas affect spiracular behavior, but the effect of each may be modified by its interaction with the other. Thus, oxygen tension affects the pCO_2 necessary to open a valve widely, and carbon dioxide tension affects the pO_2 that is required for initiating fluttering.

These results led us to ask the following question: As the carbon dioxide tension increases in a pupa, what part of the insect—the nervous system or the spiracular apparatus—responds first to carbon dioxide to evoke a change in spiracular behavior? Various gas mixtures were perfused over the fourth abdominal ganglion, and a different gas mixture was perfused systemically. Thus, in the fourth abdominal segment the spiracular muscle and myoneural junctions received the systemic flow, whereas the ganglion received its own central flow. In the case of the other spiracles both the ganglion and spiracles received the same gas flow. A systemic flow of 5 percent oxygen and 7 percent carbon dioxide caused all valves to open (Table 1). But a central flow of 5 percent oxygen and 7 percent carbon dioxide did not cause the fourth right abdominal spiracle (4RAS) to open. Nor did central flows of up to 30 percent carbon dioxide induce the 4RAS to open. It is clear that the ganglion is quite insensitive to carbon dioxide, whereas the spiracle itself is very sensitive. In the normal life of the insect, spiracular constriction causes the intratracheal carbon dioxide tension to rise. The spiracle will open whenever the intratracheal carbon dioxide ten-

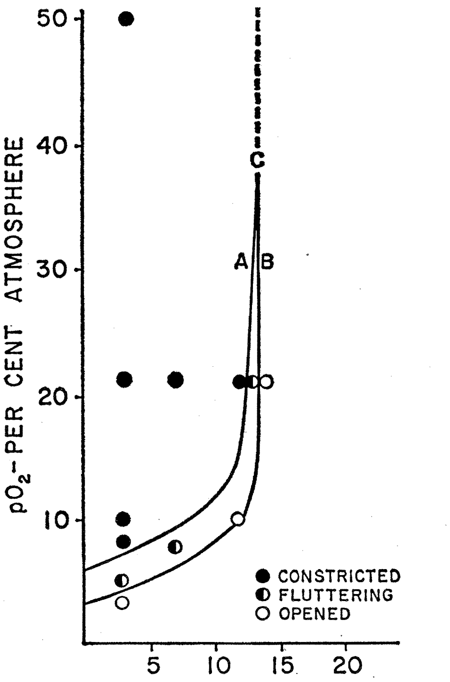

Fig. 3. Oxygen-carbon dioxide interaction curves for a diapausing cecropia pupa. The range of pO_2 and pCO_2's over which valves were constricted, fluttering, or open is delimited by the two curves. To the left of curve A lie those pO_2 and pCO_2's at which valves were constricted (solid circles). The half-shaded circles that lie in the area between curves A and B denote those pO_2 and pCO_2's at which valves fluttered. Points to the right of curve B (open circles) indicate the pO_2 and pCO_2's to which valves responded by opening. Discontinuous respiration—constriction or flutter—occurred at points to the left of curve B.

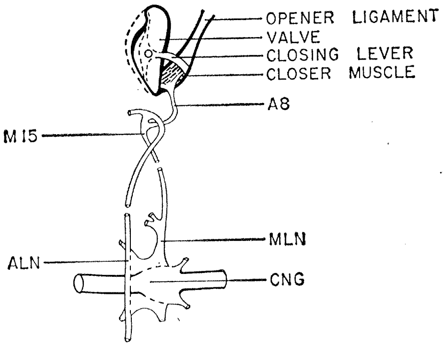

Fig. 4. Innervation of an abdominal spiracular closer muscle of a cecropia pupa [redrawn from Beckel (*6*)]. CNG, ganglion; ALN, anterior lateral nerve; MLN, mid-lateral nerve; A8, branch of ALN supplying the muscle; M15, branch of MLN supplying the muscle.

sion gets high enough to affect the spiracle itself.

The target of carbon dioxide is the spiracular apparatus, but what is the primary target of oxygen? During most of the life of the pupa the spiracles are constricted, and intratracheal oxygen tension falls. Does the central nervous system or the spiracular apparatus itself respond first to falling oxygen tension to cause a change in spiracular behavior? The behavior of 4RAS may be dictated by perfusing the fourth abdominal ganglion with a gas mixture of appropriate oxygen concentration (Table 1); the pO_2 within the spiracular tracheae serving the 4RAS is ignored by the insect. The adjacent control spiracles of the 4RAS, however, respond to the oxygen tension of the systemic gas flow. The ganglion is far more sensitive to changing oxygen tensions than is the spiracle.

The spiracular muscle of each functional abdominal spiracle receives axons from its segmental ganglion by way of the mid-lateral nerve (MLN) and from the anterior ganglion via the anterior lateral nerve (ALN) (Fig. 4) (*6, 7*). Since the ganglion is sensitive to changing oxygen tensions, what control does it exert over spiracular activity via these nerves? Previous studies have shown that when one or several abdominal ganglia are extirpated, the spiracles in the denervated segments remain closed in air for at least 42 days after the denervation. They open in low oxygen tensions and in high carbon dioxide tensions, but they never flutter (*5*). Thus, in the absence of innervation by both MLN and ALN the spiracular muscle contracts. To determine what happens if only MLN is cut we transected the mid-lateral nerve from the ganglion to the spiracular muscle where the nerve leaves the ganglion, and the spiracular responses of 4RAS and its contralateral and adjacent controls to gas perfusions were observed at intervals for 30 days. The partially denervated 4RAS opened in low oxygen tensions and in high carbon dioxide tensions, but remained closed at oxygen tensions at which its controls fluttered. Apparently the partially denervated spiracle, like the fully denervated spiracle, can open and constrict (*5, 8*), but flutter is suppressed by denervation.

Where does central nervous control of spiracular valve activity reside? It has been suggested that in a cecropia pupa the spiracular valves of each segment are controlled principally by the ganglion in the next anterior segment and that "the spiracular muscle must be almost continuously driven by the nerve impulses coming through ALN" (*7*, p. 332). However the following experiments suggest another possibility. Cutting the nerve cord either anterior to, posterior to, or both anterior and posterior to the fourth abdominal ganglion had no effect on the response of the third, fourth, or fifth right abdominal spiracle to various gas mixtures. The 4RAS, even when it was isolated from nervous connections with any ganglion except its own, continued to respond to the oxygen tension of the central flow mixture, while its controls responded to the oxygen tension of the simultaneous systemic flow. Therefore, the principal nervous control of a spiracular closer muscle resides in the ganglion located in the same pupal segment.

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Guanine Nucleotide Associated with the Protein of the Outer Fibers of Flagella and Cilia

Abstract. *Flagella from sperm of the sea urchin* Strongylocentrotus droebachiensis *and cilia from* Tetrahymena pyriformis *contain guanine nucleotides bound to the outer-fiber fraction in the ratio of one mole of nucleotide per mole of protein subunit.*

The outer fibers of the classical "9 + 2" arrangement in cilia and flagella (*1*) are composed of subunits which resemble the muscle protein actin in molecular weight and amino acid composition (*2*). Outer fibers of sperm flagella are made up of subunits having similar properties (*3*). We now report that the protein of the outer fiber contains a bound nucleotide in the amount of 1 mole/60,000 g of protein. Unlike actin, in which the bound nucleotide is adenosine diphosphate, this protein is associated with a guanine nucleotide.

Flagella were prepared from the sperm of the sea urchin *Strongylocentrotus droebachiensis* by homogenization of the cells in either seawater containing $10^{-4}M$ ethylenediaminetetraacetate (EDTA) or in $1.0M$ sucrose containing $0.1M$ NaCl and $10 mM$ tris-(hydroxymethyl)aminomethane hydrochloride (tris-HCl) buffer, *p*H 8.0. The sperm heads were separated from the tails by differential centrifugation. In the case of the seawater preparation, the homogenate was sedimented at 1000*g* for 5 minutes. The resulting pellet, consisting chiefly of heads but containing an appreciable number of flagella, was resuspended, and the centrifugation was repeated. The combined supernatant fluids from these centrifugations were sedimented at 10,000*g* for 5 minutes to recover the flagella. The flagella were resuspended, and the entire procedure was repeated. After these steps were taken, the final preparation containing flagella was free of contaminating heads when observed in the phase-contrast microscope. The procedure in which we used sucrose involved essentially the same steps except that the period of centrifugation was increased to 10 to 20 minutes and the final pellet was resuspended in 2.5 mM $MgCl_2$ and 30 mM tris-HCl, *p*H 8.0, and spun for 5 minutes at 1000*g* to remove residual heads. Flagella prepared in seawater were used for the preparation of outer fibers, while those isolated in the salt-sucrose solutions were analyzed directly for nucleotide.