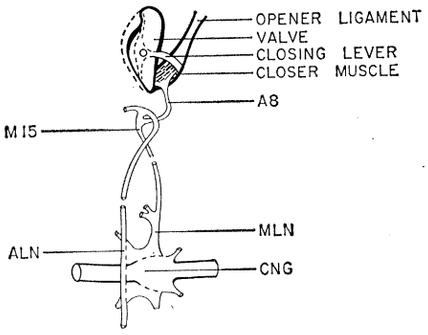

Fig. 4. Innervation of an abdominal spiracular closer muscle of a cecropia pupa [redrawn from Beckel (*6*)]. CNG, ganglion; ALN, anterior lateral nerve; MLN, mid-lateral nerve; A8, branch of ALN supplying the muscle; M15, branch of MLN supplying the muscle.

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 10mM 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.

Cilia were prepared from *Tetrahymena pyriformis* by a modification of the procedure of Watson and Hopkins (*4, 5*); the cilia were either converted to an acetone powder (*2*) or were used directly for nucleotide determination.

Outer fibers from isolated flagella were prepared according to Gibbons (*5*) by dialysis of the flagella for 36 to 48 hours against two changes of a buffer consisting of 1 m*M* tris-HCl, *p*H 8.0, 10^{-4}*M* EDTA, 3 m*M* KCl, and 0.01 percent 2-mercaptoethanol by volume. The outer fibers and membranes, having been freed of dynein and matrix protein through selective solubilization, were recovered by sedimentation at 35,000*g* for 30 minutes. To remove membrane fragments, we resuspended the pellet in 0.5 percent digitonin in 10 m*M* tris-HCl and extracted it twice for periods of 30 minutes. The material was then washed twice with 10 m*M* tris-HCl to remove residual digitonin. The outer fibers were recovered after each of these steps by centrifugation at 35,000*g* for 10 minutes. Protein determinations were carried out by the method of Lowry *et al.* (*6*). The isolated flagellum (and midpiece fragment) represented 27 to 29 percent of the total sperm protein, while the outer-fiber fraction constituted about 30 percent of the protein of the isolated flagellum.

For nucleotide determination, actively swimming sperm, isolated flagella, and outer fibers were each treated with 10 percent trichloroacetic acid (TCA) for 30 minutes at 0°C. The precipitated protein was removed by centrifugation, and the resulting supernatant was extracted six times with twice its volume of water-saturated diethyl ether to remove the TCA. We similarly extracted known quantities of nucleotides to determine loss; such loss consistently amounted to 45 to 55 percent. The residual ether was removed under reduced pressure, and the ultraviolet absorption spectra of the resulting solutions were determined. Both isolated flagella and outer fibers yielded a material whose spectrum was identifiable as that of a guanosine derivative (Fig. 1), but the nucleotide fraction from intact sperm appeared to be a mixture and required further separation.

The nucleotide samples were evaporated to dryness at room temperature with an air jet, dissolved in 50 μl of water, and chromatographed for 18 hours on Whatman 3MM paper, with an ascending solvent system consisting of isobutyric acid, ammonia, and water

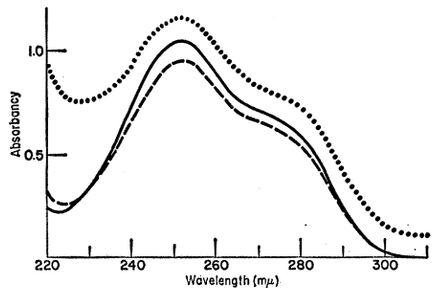

Fig. 1. Ultraviolet absorption spectrum of nucleotides from outer fibers of sea urchin flagella. The dotted line is the nucleotide soluble in TCA after ether extraction. The dashed line is a chromatographed sample of the TCA-soluble nucleotide and corresponds in R_F value to GTP. The solid line is the spectrum of a known sample of GTP. The *p*H in all cases is 7 to 8.

in the ratio of 57:4:39 by volume (*7*). Guanine, guanosine, guanine nucleotides, and adenine nucleotides were used as standards. Upon completion of the chromatography, the paper was air-dried, the spots were observed by their absorption at 257 mμ, and the positions of the samples were compared with those of the standards. For further identification, we exposed the chromatogram to HCl vapor in order to observe the characteristic fluorescence of guanine compounds under ultraviolet light at acid *p*H. For quantitative analysis, we removed the resolved components and standards by leaching cut segments of the paper for 30 minutes with 1 m*M* tris-HCl, *p*H 8.0, using adjacent segments of equal weight as blanks. Chromatographic separation resulted in the identification of guanine nucleotides in all preparations, while the intact sperm and the isolated flagella also contained adenine nucleotides. Spectra obtained after chromatography corresponded even better to those of guanosine derivatives than they did before the separation since extraneous material absorbing below 240 mμ was removed (Fig. 1).

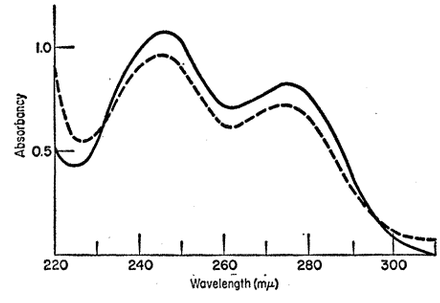

Fig. 2. Ultraviolet absorption spectrum of free base obtained by hydrolysis of chromatographed sample described in Fig. 1. The dashed line is the hydrolyzed and neutralized sample. The solid line is the spectrum of a known sample of guanine. The hydrolyzed sample yielded approximately 3 moles of inorganic phosphate per mole of guanine.

To further confirm that the base involved was guanine, we converted several of the chromatographed samples to the free base and inorganic phosphate by making the material 1*N* in HCl and hydrolyzing it for one hour at 100°C. The ultraviolet absorption spectra of the neutralized hydrolyzates were determined and were found to correspond to guanine (Fig. 2). In addition, phosphate determinations were carried out on the hydrolyzates by the method of Fiske and SubbaRow (*8*). The amount of base present was estimated from the absorbance at 250 mμ. The material identified chromatographically as guanosine triphosphate (GTP) contained 2.8 to 3.1 moles of phosphate per mole of base. That identified as guanosine diphosphate (GDP) contained 1.5 to 1.9 moles of phosphate, while the guanosine monophosphate (GMP) contained 1.2 to 1.5 moles of phosphate per mole of guanine. The deviations from the expected 2.0 to 1.0 moles in the latter two cases, respectively, probably reflect incomplete chromatographic separation.

The TCA-soluble nucleotides detected in living sperm were those of adenine and guanine, with the former accounting for about two-thirds of the total (Table 1). Adenosine triphosphate (ATP) made up about 50 percent of the adenine nucleotides; the remainder was adenosine diphosphate (ADP). Of the guanine nucleotides, GTP constituted only 12 to 18 percent, with the mono- and diphosphates occurring in roughly equal amounts. The total nucleotide content and the ratio among various nucleotides were estimated spectrophotometrically, employing the appropriate extinction coefficient (*7*).

Separation of the flagellum from the sperm resulted in the loss of nearly all of the adenine nucleotides and over half of the guanine derivatives. This may have been caused by the loss of the mitochondrial fraction and the loss of nucleotides through partially disrupted flagellar membranes. In this case, GTP was predominant and made up about two-thirds of the total nucleotide. The remainder was chiefly GDP, with only traces of GMP and ATP (Table 1).

In the preparations of outer fibers, only guanine nucleotides were found.

Table 1. Total and relative nucleotide content of whole sperm, flagella, and outer fibers. Total nucleotide is given in moles per 10^5 g of protein; nucleotides are given as mole fractions; guanine nucleotides are given in moles per 10^5 g of total sperm protein.

Compounds	Whole sperm	Flagella	Outer fibers
	Total nucleotide		
	4.0–4.3	1.3–1.4	1.6–2.0
	Nucleotides		
GTP	0.04/0.08	0.66/0.69	0.43/0.53
GDP	.10/0.19	.33/0.31	.20/0.29
GMP	.19/0.18	trace	.37/0.18
ATP	.35/0.28	trace	
ADP	.33/0.27		
AMP	trace		
	Guanine nucleotides		
	1.4–1.8	0.45–0.50	0.19–0.24

The ratios among the three nucleotides were quite variable, though all three were always evident (Table 1). Together they amounted to 1.6 to 2.0 moles/100,000 g of protein or 0.9 to 1.2 moles of nucleotide per mole of protein, a molecular weight of 60,000 being assumed for the outer-fiber subunit (*2, 3*). The nucleotide was tightly bound and did not exchange with radioactively labeled GTP in the medium (*9*). Dialysis of the outer fibers for 100 hours against 10 m*M* tris-HCl, *p*H 8.0, and $10^{-4}M$ EDTA, followed by several washes with the same solution, caused no significant loss of nucleotide. Prior depolymerization of the fibers with urea, followed by dialysis, resulted in the loss of nearly half of the nucleotide in a 40-hour period.

Somewhat similar results were obtained when the aforementioned analytical techniques were applied to the cilia of *Tetrahymena pyriformis*. Outer-fiber protein, extracted from an acetone powder of cilia (*2*), was separated from loosely bound nucleotide by gel-filtration on G-25 Sephadex. Both the protein fraction and the "unbound nucleotides" were then analyzed (Table 2). Purified outer-fiber protein contained between 0.5 and 0.8 mole of bound guanine derivative per mole of protein. The unbound fraction also consisted of guanine compounds, and together both fractions totaled nearly one mole of guanine derivative per mole of outer-fiber protein. In contrast to the flagellar protein, where only guanine nucleotides were found, the protein derived from cilia contained the nucleoside and free base, having only trace amounts of GMP and GTP.

Nucleotide analysis of whole cilia performed immediately after their isolation indicated the presence of a significantly larger amount of GTP and GMP than in the acetone powder protein, but guanine and guanosine were still predominant (Table 2). If native outer fibers from cilia bind nucleotides in the same manner as sperm flagella, the preponderance of free base and nucleoside over the nucleotide fraction in cilia is suggestive of hydrolysis during the isolation and subsequent extraction procedures. *Tetrahymena* produces a number of hydrolytic enzymes, and these may cause degradation during deciliation. In this same regard, it is perhaps significant that the outer fibers of the sperm flagella contain a much larger amount of GMP than do the isolated flagella from which they were derived (Table 1).

The ADP of the muscle protein actin is susceptible to phosphorylation or dephosphorylation only when the protein is in the depolymerized form. The outer-fiber protein apparently does not share this property. This difference suggests that the nucleotide in the outer fiber is bound to the protein by the guanine base, leaving the phosphoribosyl moiety accessible to degradation.

Since outer fibers constitute nearly 30 percent of the total flagellar protein and bind one mole of nucleotide per 60,000 g, it follows that the whole flagellum should contain 0.5 mole of bound nucleotide per 100,000 g of total protein; 1.3 to 1.4 moles were actually found. In cilia, about 0.6 mole of nucleotide per 100,000 g of total protein should be associated with the outer fibers; in this case, about 1 mole of guanine compounds were found in the whole cilium. Thus, guanine nucleotides or derivatives exist in an amount significantly in excess of that bound to the outer-fiber fraction. This "nucleotide pool" may represent a fraction loosely bound to the outer fibers and lost during the preparation; it may be associated with the fraction containing the central fibers and matrix removed during the preparation; or it may be located in the soluble phase within the isolated organelle. This last possibility appears least likely since nearly all of the adenine nucleotides are lost during the isolation procedure, while a sizable fraction of the guanine nucleotides are selectively retained.

The ratio of various nucleotides in sea urchin sperm does not differ significantly from that of bull sperm (*10*), nor even from such unrelated material as rat tissue (*11*); adenine and guanine nucleotides are the major components,

Table 2. Relative amount of guanine derivative in *Tetrahymena* outer-fiber protein and isolated cilia. Moles of guanine derivative per 10^5 g of protein.

Guanine compound	Outer-fiber protein		Whole cilia
	Bound	Unbound	
GTP	trace		0.10
GDP			.17
GMP	trace		trace
Guanosine	0.64	0.58	0.42
Guanine	.35	.22	.32
Total	.99	.88	1.01

but cytidine and hypoxanthine derivatives have also been detected in these sources. However, Hultin (*12*) has found ATP and uridine triphosphate as major components among sea urchin nucleotides, while Newton and Rothschild (*13*) reported adenine nucleotides and inosine in bull sperm. Plowman and Nelson (*14*) isolated an actin-like protein from starfish sperm flagella and found ATP in the ratio of 0.6 to 1.4 moles per 60,000 g of protein. Such findings may reflect differences in species, but more likely the apparent discrepancies are inherent in the methods of separation and identification of the component nucleotides. Proof of the universality of guanine nucleotides associated with the outer fibers awaits further comparative work, with techniques that unequivocally resolve the various nucleotides.

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