

Unipolar Microtubule Array Is Directly Involved in Nurse Cell-Oocyte Transport

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The telotrophic ovariole of *Rhodnius prolixus* is richly endowed with microtubules (MTs). An extensive, stable array of MTs packs the trophic core and trophic cords which link the nurse cell compartments to the growing oocytes. This system is excellent to study MT-based transport as the MTs are believed to play a role in transport of nurse cell-produced mitochondria, ribosomes, and mRNAs to the oocytes. We investigated MT polarity and molecular MT motors in this unidirectional transport system. Hook decoration revealed that the MTs of the trophic core and cords have their plus (+) ends in the tropharium and minus (–) ends in the oocytes. Video differential interference optics (DIC) microscopy showed that vesicle transport was saltatory, ATP-dependent, and had an average velocity of 0.77 $\mu\text{m}/\text{sec}$ toward the oocyte. Transport was sensitive to 2 mM N-ethylmaleimide (NEM) and 50 μM vanadate and resistant to 1 mM 5'-adenylylimidodiphosphate (AMP-PNP) and 5 μM vanadate. We report that the unipolar, acetylated trophic cord MTs play a direct role in nurse cell-oocyte transport via a cytoplasmic dynein-like retrograde motor. *Cell Motil. Cytoskeleton* 36:355–362, 1997. © 1997 Wiley-Liss, Inc.

Key words: telotrophic ovariole; microtubule polarity; cytoplasmic dynein

INTRODUCTION

Polarized microtubule (MT) arrays are critical for outgrowth and function of specialized cellular processes such as neuronal axons and cilia and flagella. These unipolar MTs tend to be stable to maintain the cell asymmetry and in the case of axonal MTs, allow directional transport of organelles, MT polarity, along with motors such as kinesin and cytoplasmic dynein, provides the molecular basis underlying MT-based cellular transport. With the introduction of in vitro motility assays using differential interference optics (DIC) video microscopy with image processing one can assess which potential motors are responsible for particle transport based on their known sensitivity to selected pharmacological agents [Vale et al., 1985; Gilbert et al., 1985].

The meroistic ovary of the insect *Rhodnius prolixus* is ideal to study the development and function of polarized MT arrays. Each ovary contains 7 telotrophic ovarioles with an anterior trophic region of syncytial polyploid nurse cells (NC) in cytoplasmic continuity around a central MT-rich trophic core (C). The trophic core extends posteriorly by attenuated cytoplasmic bridges

called trophic cords (T) to a series of developing oocytes (O), with the most developed vitellogenic oocytes furthest away from the tropharium (Fig. 1) [for review see Huebner, 1984]. Trophic cords contain 30,000–50,000 MTs that are extremely stable [Huebner and Anderson, 1970; MacGregor and Stebbings, 1970; Hyams and Stebbings, 1977; Huebner, 1981]. Cords provide transport corridors to oocytes until mid-vitellogenesis when they close and MTs depolymerize as the cord retracts back to the tropharium [Bennett and Stebbings, 1979; Hyams and Stebbings, 1979]. Trophic cords supply the oocytes from their earliest growth stages so the cytoplasmic transport relative to oocyte differentiation is ongoing and highly regulated. Important is the MT polarity and the mechanisms which play a role in the mechanism of MT-based transport.

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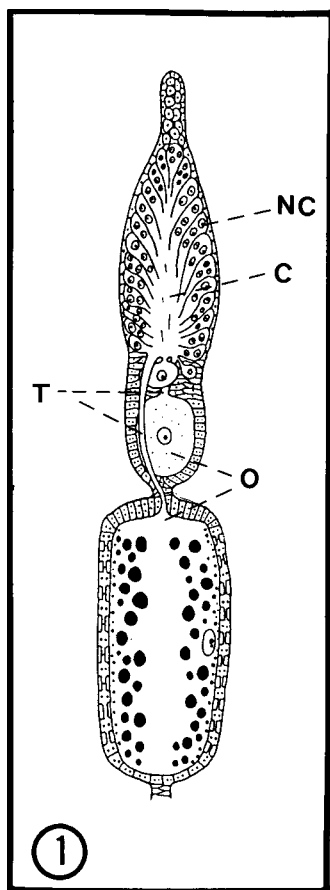


Fig. 1. Summary illustration of the adult *Rhodnius* telotrophic ovariole [based on Huebner, 1984]. See text for abbreviations.

We report here that the trophic cord MTs are oriented with the (–) ends of the MTs located in the oocytes and the (+) ends extending toward the tropharium. This polarity has structural implications on the assembly of the MTs as well as functional implications as to the directionality of transport. DIC video microscopy of these cord MTs clearly indicated that nurse cell–oocyte transport of mitochondria is MT-based and driven by a cytoplasmic dynein-like motor.

MATERIALS AND METHODS

Animal Rearing Techniques

A colony of *R. prolixus* was maintained at high humidity and 27°C in a controlled environment chamber [Huebner and Anderson, 1972]. The colony was fed using both a membrane feeding technique [Huebner et al., 1995] and on female New Zealand white rabbits. Ovaries spanning a range of developmental stages were dissected from mated adult females.

Hook Decoration of Trophic Cord MTs

Hook decoration was performed following the method of Heidemann and McIntosh [1980] and Steb-

bings and Hunt [1983]. *Rhodnius* ovarioles were microdissected to isolate trophic cords and placed in Hook buffer [500 mM PIPES (pH 6.94), 1 mM MgCl₂, 1 mM EDTA, 1.0 mM GTP, 0.5% Triton X-100 (or Brij-58), 0.5% NaDOC, 0.2% SDS, 2.5% DMSO, and 20 µg/ml RNase] containing 2 mg/ml brain tubulin. Tubulin was isolated from porcine brains following the twice-cycling protocol of Borisy et al. [1975]. Ovarioles were incubated in this final Hook buffer at 4°C for 20 min, room temperature for 5 min, and 37°C for 30 min. The ovarioles were fixed in 2% glutaraldehyde (GTA) in Pipes-EGTA-MgSO₄ (PEM) buffer for 45 min and processed for electron microscopy. Tissue was stained en bloc with 2% aqueous uranyl acetate. Embedded ovarioles were oriented such that trophic cord MTs would be cut in cross-section. Silver sections were stained with lead citrate and examined in a Hitachi H7000 scanning transmission electron microscope (STEM) in transmission electron microscopic (TEM) mode in 75 kV.

Motility Assays

Adult ovarioles were microdissected to isolate trophic cords attached to the tropharium. The tissue was transferred to motility buffer without ATP [35 mM PIPES (pH 7.4) and 5 mM MgSO₄] [modified from Dabora and Sheetz, 1988] and placed on slides. A coverslip, with corner drops of VALAP (Vaseline, lanolin, paraffin, 1:1:1) as spacers, was placed over the tissue. The VALAP droplets were slowly melted down by touching the corners of the coverslip glass using a heated wire just until the VALAP melted. This attached the coverslip to the slide preventing subsequent shifting or flattening of the coverslip and tissue. Ovarioles were exposed to various solutions by placing a drop of experimental solution at one edge of the coverslip and drawing away fluid at the opposite side using filter paper. Motility was observed in the presence or absence of 1 mM ATP, 1 mM 5'-adenylyimidodiphosphate (AMP-PNP), 2 mM N-ethylmaleimide (NEM), 5 µM sodium orthovanadate (NaVO₄), and 50 µM NaVO₄, all obtained from Sigma (St. Louis, MO). The inhibitors were diluted in motility buffer + ATP and added after motility was supported in buffer alone. Three trials for each inhibitor were run to assess sensitivity.

A Zeiss Photo I microscope especially equipped for high resolution DIC was used for observation and video recording. Microscopic illumination was with a 200 W mercury arc lamp and a heat filter and a narrow band pass mercury green line (546 nm) filter. The DIC optics consisted of a ×100 Plan objective (NA 1.25) equipped with an individual DIC Nicol slider and the matched DIC Nicol beam splitter in the condenser and the appropriate polarizers. Video images were obtained with a Hamamatsu CCD Model (C2400 with manual gain control and visualized using the Image 1 analysis system (Universal

Imaging Corp., West Chester, Pennsylvania). Videotape recordings were made on 0.5 in. S-VHS videotape using a Sony SV0-9500 MD videocassette recorder in real time for up to 30 min intervals. With Image 1, still frames at selected time intervals were sequentially captured and recorded to analyze direction and rates of vesicular movements. Distances were calibrated using a micrometer recorded at the same objective powers used in the motility assays. Normal motility was determined by tracking and capturing still frames of 20 different mitochondria at 1 sec intervals. The still frames of normal motility and inhibitor trials were photographed using a Polaroid Freeze Frame recorder and printed at the same magnification as micrometer images. Distances were measured and rates determined. Sensitivity to inhibitors was determined by observing significant increases or decreases in motility following treatment. From this information, the trophic cord MT-based motility could be characterized and compared to MT-based movement in other systems.

RESULTS

MT Polarity: Hook Decoration

The overall cellular organization of the telotrophic ovariole is illustrated in Figure 1. The MTs of the germ cell syncytium extend from the MT-laden trophic core (C) to each developing oocyte (O) via the trophic cords (T) into the individual oocytes. Determining the polarity of these cord MTs is essential to understanding transport in this highly regulated system where only a single oocyte is vitellogenic per ovariole at any one time. Heidemann and McIntosh [1980] showed that the curvature of protofilament hooks formed on endogenous MTs is a reliable method to determine the intrinsic polarity of the MTs. Many areas of trophic cords and trophic core were examined for MT polarity according to criteria used by Heidemann et al. [1981] and Redenbach and Vogl [1991]. Decorated MTs were classified as either "clockwise," "counterclockwise," or "ambiguous" (Table I). Clockwise-curving hooks indicate the observer is looking from the (+) toward the (-) end of the MT, while counterclockwise hooks indicate the observer is looking toward the (+) end of a MT [Heidemann and McIntosh, 1980]. Hooks which formed on hooks (rosettes), MTs containing hooks in both directions, and closed hooks were considered ambiguous. Percentages of hook configurations were determined by dividing the total hooks of either category by the total number of unambiguous decorated MTs (Table I).

We found that the majority of MTs, 93.5% (Table I), had counterclockwise hooks (Fig. 2). Tissue orientation was maintained throughout the procedure such that the observer was looking from the oocyte region toward the tropharium. These results indicate that trophic cord MTs

TABLE I. Hook Curvature for Oocyte-to-Nurse Cell Viewed MTs in Trophic Cords*

Section	MTs with counterclockwise hooks	MTs with clockwise hooks	MTs with ambiguous hooks
1	37	1	15
2	23	7	15
3	48	10	14
4	42	3	12
5	50	5	14
6	42	2	2
7	56	1	0
8	48	0	0
9	35	1	0
10	41	1	1
11	39	1	1
Total	461	32	74
Percent ^a	93.5	6.5	—

*The number of MTs with 3 categories of hooks detailed in text in 11 cross-sections viewed from the oocyte toward the tropharium region are shown.

^aThe total percentages of hooks were calculated for unambiguous decorated MTs.

are oriented with their (-) or slow-growing ends in the oocyte and their (+) or fast-growing ends toward the trophic core. Since most MTs were decorated with this hook configuration, the MT array was assumed to be unipolar.

MT-Based Transport: Motility Assays

Live tissue was used to determine MT and MT motor involvement in nurse cell-oocyte cytoplasmic transport as the size of the tissue limited biochemical analysis. Vesicles which moved were either spherical or elongated, rod-like organelles which were likely mitochondria (Fig. 3). Organelles varied in diameter from approximately 2 to 0.2 μm . Only some of the vesicles were moving at any one time along the MTs. Organelles within the cords moved in a MT-based fashion only toward the oocyte region (Fig. 3a-h). These vesicles moved smoothly for up to 20 μm with an average velocity of 0.77 ± 0.19 $\mu\text{m}/\text{sec}$ (mean \pm standard deviation, $n = 20$). Frequently the organelle movements were saltatory, exhibiting stops, starts, and minor oscillations. These observations were very similar to what has been reported in other systems [Vale et al., 1985]. Motility was not observed in either direction for the inhibitor 50 μM vanadate or 2 mM NEM characteristic of cytoplasmic dynein-based transport (Table II). With the other inhibitors (1 mM AMP-PNP and 5 μM vanadate) movement was not affected and organelles moved with the same frequency, rate, and direction as seen with motility buffer containing ATP alone (Table II). The directionality of transport was retrograde (toward the minus ends of MTs) similar to what one expects for cytoplasmic dynein-like motility (Table II). The rate of

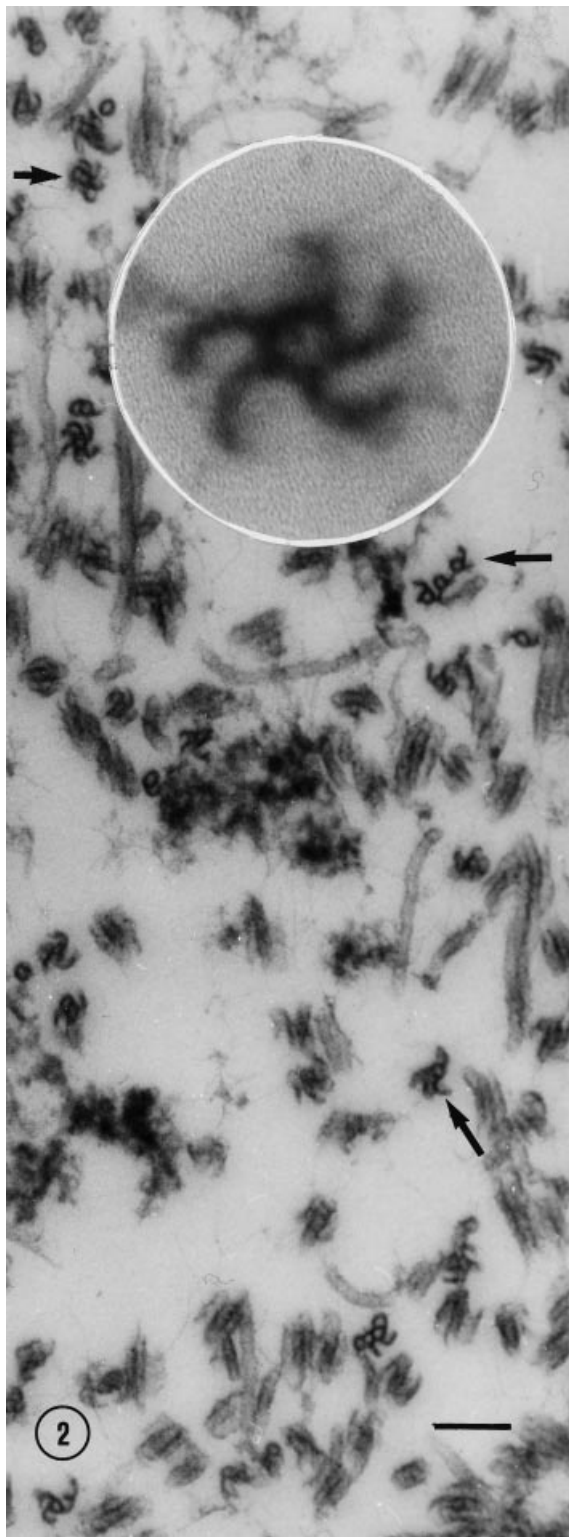


Fig. 2. Electron micrograph of a cross-section through a trophic cord after hook decoration. The majority of hooks are counterclockwise (arrows), indicating that the MTs have their (+) ends in the trophic core and their (-) ends in the oocyte. **Inset:** Higher magnification of a typical decorated MT. Bar = 100 nm.

movement was faster than that seen for kinesin (0.6 $\mu\text{m}/\text{sec}$) but slower than that reported in the literature for cytoplasmic dynein (1.25 $\mu\text{m}/\text{sec}$). Movement was ATP-dependent as known for both dynein and kinesin-based motility (Table II).

DISCUSSION

MT Polarity Relative to NC-Oocyte Dynamics

Nurse cells are the major supplier of many components for the oocytes so a unipolar transport mechanism is expected. The polarity of the trophic cord MTs was interpreted as having (-) ends in the oocytes and (+) ends in the tropharium based on hook decoration. The only other report on MT polarity in a telotrophic ovariole also found this polarity [Stebbins and Hunt, 1983]. Interestingly, in the polytrophic ovarioles of *Drosophila*, Therkauf et al. [1992, 1993] have shown that nurse cell-oocyte MTs also have (-) ends in the oocytes. This suggests that meroistic ovaries may have a universal mechanism of MT growth and polarity establishment for oogenesis. An important feature of the *Rhodnius* ovariole which contrasts with most other telotrophic systems studied thus far is its high degree of oogenesis regulation. Only the terminal follicle is in vitellogenesis with the penultimate ones arrested in previtellogenesis. The mechanisms that facilitate the enlargement and growth of the trophic cords during previtellogenesis and their dismantling when the terminal oocyte becomes isolated from the syncytium during vitellogenesis must be considered relative to the MT polarity.

Our findings regarding MT polarity and unpublished data showing that MT stability is linked to extensive acetylation raise questions about the origin, maintenance, and eventual disassembly of these dramatic MT arrays. The development of trophic cords begins at 6 days before the adult molt [Valdimarsson and Huebner, 1989]. The cord MTs grow from the oocyte compartments toward the tropharium. As the cords increase in width and length, the MTs increase in length and number with a slight decrease in packing density in growing oocytes compared to the small, quiescent oocytes [Valdimarsson and Huebner, 1989]. The total tubulin within the ovariole increases coincidentally with MT growth from 6 days before molt until 1 day before molt after which it remains constant [Valdimarsson and Huebner, 1989]. Whether the tubulin is nurse cell or oocyte produced is unknown. It is likely that the nurse cells produce this soluble component which then diffuses through the intracellular bridges to the oocyte where it is initially assembled (Huebner and Lutz, unpublished data). With continuous synthesis of tubulin while the MT arrays are being established, there likely is a relatively high pool of tubulin subunits compared to MT polymers. Conditions

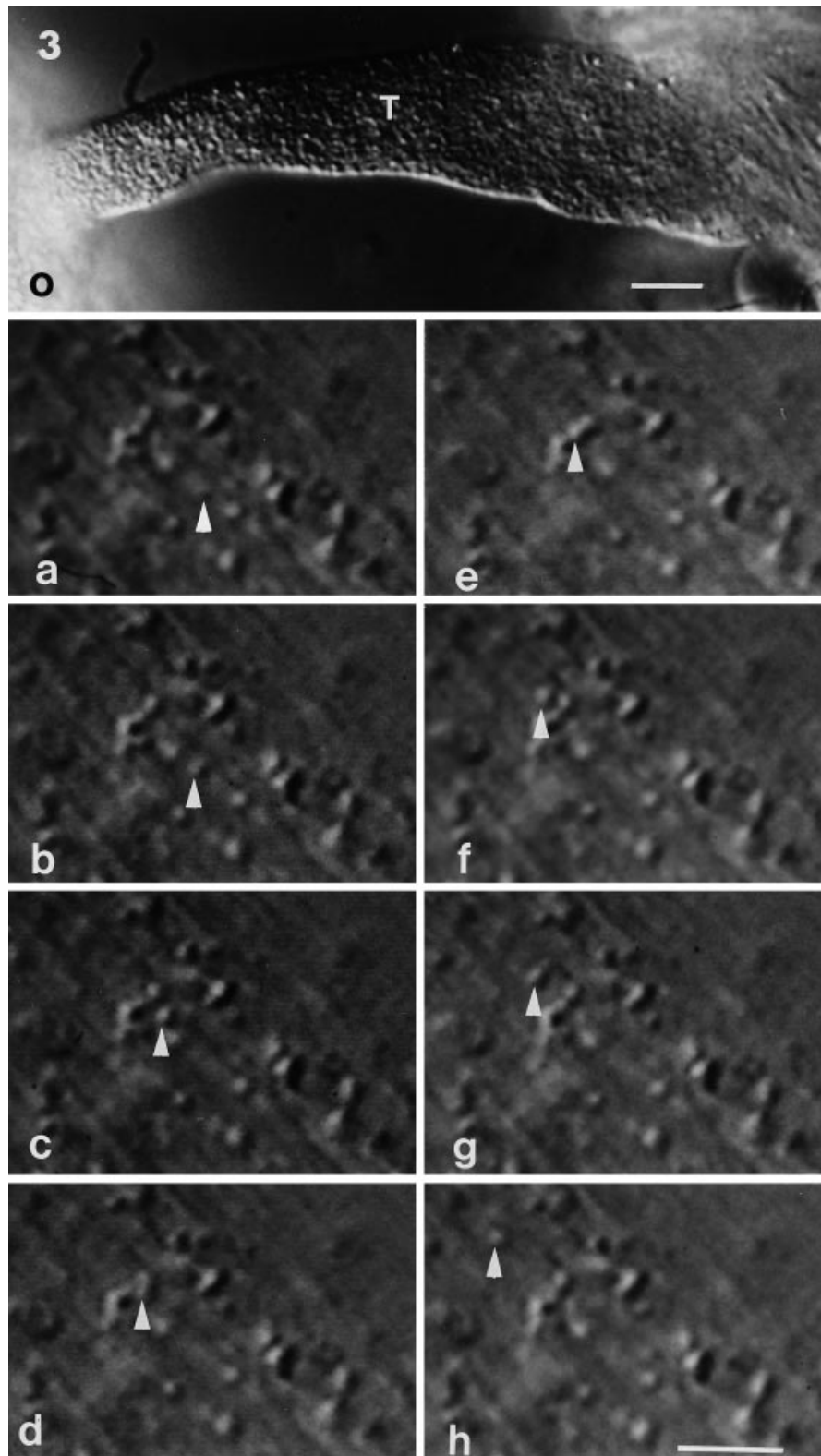


Fig. 3. Light micrograph of a microdissected trophic cord (T) showing insets (a-h) of Image 1 still frames from the video recorder at 1 sec intervals tracking the movement of mitochondria (arrowheads) along distinctly visible tracks through an isolated trophic cord. The oocyte (O) is in the upper left (top). Bar = 20 μm (top); 5 μm (a-h).

TABLE II. Comparison of Trophic Cord Motility Characteristics With Cytoplasmic Dynein and Kinesin-Driven Motility*

Characteristic	Trophic cord	Cytoplasmic dynein	Kinesin
Movement in retrograde direction	Yes	Yes	No
Rate of movement ($\mu\text{m}/\text{sec}$)	0.77	1.25	0.60
Movement ATP-dependent	Yes	Yes	Yes
Sensitive to 1 mM AMP-PNP	No	No	Yes
Sensitive to 5 μM vanadate	No	Yes	No
Sensitive to 50 μM vanadate	Yes	Yes	Yes
Sensitive to 2 mM NEM	Yes	Yes	No

*Data from a variety of sources summarized in Brady [1991].

which favor MT assembly would contribute to potential MAP binding and/or acetylation of the MTs to generate a stable trophic cord MT array. Since the trophic core contains the (+) ends of the cord MTs this implies a more dynamic MT domain. Like MTs in the polytrophic ovarioles of *Drosophila*, the dynamic instability of MTs in this region may allow populations to extend through ring canals in the 5th instar developing *Rhodnius* ovariole. These may perhaps become the initial (+) end stabilized MTs, prevented from disassembly, possibly via factors and MT-capping proteins in the nurse cell cytoplasm [Therkauf et al., 1992]. Ring canals associated with the development of the nurse cell syncytium have also been observed in 5th instar larval *Rhodnius* ovarioles [Yeow and Huebner, 1994].

Although the timing and oocyte origin of the cord MTs are known [Valdimarsson and Huebner, 1989], a nucleating center in the oocytes of telotrophic ovarioles has yet to be found. It is possible that newly formed MTs are free in the cytoplasm following nucleation like axonal MTs [Bray and Bunge, 1981]. Once MT assembly is initiated, elongation within the cords must also occur. Like axonal MTs the cord MTs are often longer than the diameter of the cell body/oocyte. Labeling newly assembled MTs showed that the MTs elongate from the (+) ends of preexisting MTs and no new polymers form de novo in the axon [Baas and Ahmad, 1993]. Acetylated MTs may act as the source of nucleation for nurse cell-produced tubulin via their (+) ends within the trophic cords, facilitating the elongation of the trophic cords during oogenesis.

This could explain the MT disassembly during trophic cord retraction. Redundancy of the trophic cords occurs at a time when the cortical MT network in *Rhodnius* oocytes undergoes dramatic alterations and reorganization at the onset of vitellogenesis [MacPherson and Huebner, 1993]. At the same time, Ca^{2+} ion channels appear to open at the apex of the oocyte, resulting in a Ca^{2+} influx [Diehl-Jones and Huebner, 1993]. This has

implications on the trophic cord MTs if indeed their (–) ends are free in the cytoplasm as they will begin to disassemble allowing cord closure and separating these MTs from the oocyte cortex MTs which remain. During vitellogenesis of the T (terminal) oocyte, the cord decreases in diameter and closes at the apex of the oocyte while the MTs pack into bundles [Huebner, 1981]. Soon after the closed cord very quickly retracts toward the tropharium [Huebner, 1981]. According to our polarity findings and observations on the stable redundant cord MTs, this would predict that the MTs disassemble at a faster rate in the trophic core where the (+) ends are located while the slow-growing (–) ends of the MTs move up toward the tropharium relatively intact. The MTs eventually all disassemble and tubulin subunits are presumably recycled into the growing ends of MTs in other cords. In most systems examined MT assembly and disassembly radiate away and toward the site of origin, respectively. The system in *Rhodnius* is unique because severance of the syncytial link precludes a disassembly back to the oocyte. The retraction of the elongate cords back toward the tropharium may also be aided by the cortical strands of F-actin found in the cortex of trophic cords [Huebner and Diehl-Jones, 1993].

Trophic Cord Cytoplasmic Transport: Cytoplasmic Dynein Involvement

The polarity of the trophic cord MTs suggests that retrograde nurse cell-oocyte transport is involved. Using video DIC microscopy and inhibitor studies, cytoplasmic dynein-like organelle translocation was confirmed. Study of nurse cell-oocyte transport in two other insect species revealed bidirectional movement of mitochondria in *Dysdercus intermedius* cords [Dittmann et al., 1987], but only retrograde movements in *Oncopeltus* [Stebbins and Hunt, 1987]. In *Rhodnius*, extensive electron microscopic studies have shown that the cords are filled predominately with ribosomes and mitochondria and MTs as the primary cytoskeletal component present in the interior of the cords [Huebner, 1981; Huebner and Gutzeit, 1986]. This suggests that the vesicles observed are likely mitochondria and the tracks which support their movement, the extensive MT array. This is the first report in *Rhodnius* that vesicle transport is motor-mediated along the trophic cord MTs. The polarity of the MTs thus provides the structural basis for the selective transport of nurse-cell components via a retrograde motor.

The rate of retrograde movement of 0.77 $\mu\text{m}/\text{sec}$ was faster than that if kinesin-based motility in squid axoplasm studies (0.6 $\mu\text{m}/\text{sec}$) [Paschal et al., 1987]. Although motility in trophic cords was slower than observed for cytoplasmic dynein (1.25 $\mu\text{m}/\text{sec}$), the rates observed for cytoplasmic dynein are from isolated, polymerized MTs in axoplasmic extracts, allowing maximum

activity [Schnapp and Reese, 1989]. Crowding or other factors within the isolated trophic cords may not allow for maximal motor rates. The rate and direction of transport were dynein-like. The inhibitor results also indicate a dynein-like motor. The results show that kinesin is likely not the motor responsible for MT-based transport due to its resistance to AMP-PNP and sensitivity to NEM. Vanadate inhibits cytoplasmic dynein low concentrations below 10 μ M but kinesin at least 5-fold higher concentrations in squid axoplasm extracts [Brady, 1991]. Motility in trophic cords was only affected by higher concentrations of vanadate in *Rhodnius*. Although low concentrations of vanadate did not affect transport this could be due to unique enzymatic characteristics of the presumptive motor or a failure of this inhibitor to affect motor functions due to penetration or dilution difficulties. Vanadate does not readily permeabilize membranes and has multiple cellular targets [Vallee and Shpetner, 1990]. With the exception of motility resistance to micromolar concentrations of vanadate, the results of the inhibitor studies further suggest that the trophic cord MTs are directly involved in cytoplasmic transport to the oocytes through the actions of an endogenous cytoplasmic dynein motor. This suggests an additional function of acetylation of stable MTs as these MTs may be selected for unidirectional transport to the oocytes via MT motors. Supporting evidence has been found in other systems. The distribution and development of acetylated MTs in vivo and in vitro have been shown to be consistent with a role in organelle transport as seen in the adult rat cerebellum axons [Cambray-Deakin and Burgoyne, 1987].

The generation of the acetylated, unipolar MT array and its motor interactions produce a physiologically significant spatial polarity in structure and metabolism essential for accumulation and compartmentalization of cytoplasmic components during oogenesis in this telotrophic ovariole.

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