The distribution of the 50 kD centriolar antigen during centriolar assembly in chick ciliating epithelial cells and fragmentation of centrioles in rat pinealocytes.

by

José I. Sangerman

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Anatomy and Cell Biology in the University of Toronto

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Abstract

The distribution of the 50 kD centriolar antigen during centriolar assembly in chick ciliating epithelial cells and fragmentation of centrioles in rat pinealocytes

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José I. Sangerman
Doctor of Philosophy
Department of Anatomy and Cell Biology
University of Toronto
1997

Non-immune rabbit sera label centrioles and related basal bodies by immunofluorescence in a wide variety of cell types. One of these sera that recognize a 50 kD centriolar protein was used to localize it by immunofluorescence during assembly of centrioles in ciliating cells of chick trachea and during breakdown of centrioles in rat pinealocytes. The first sign that centriole assembly has begun was seen at embryonic day 16 (E16) when staining of both centrioles in apical part of some cells increased indicating that centriolar precursors and/or procentrioles had accumulated around both diplosomal centrioles. Also at E16 a second pattern was seen in some cells in which these diplosomal centrioles are associated with a larger stained region extending in a basal direction corresponding to the region occupied by the newly formed procentrioles. At E17 a new staining pattern consisting of small granules distributed throughout the apical end of ciliating cells was seen, indicating that procentrioles or recently matured centrioles at this stage are migrating to apical cell surface. At E18 first fully ciliated cells with stained basal bodies are seen. These results indicate that the 50 kD centriolar protein is present in centrioles at all stages of assembly. In contrast to newborn rat pinealocytes in which the two centrioles stain as single granules, in pinealocytes from 11-day-old and adult rats a larger region composed of structures of various sizes and shapes is labelled. These correspond to singlets, doublets, and triplets of MTs embedded in electron dense material visualized in these cells by EM. The finding that these structures contain the 50 kD centriolar protein indicates that they are derived from centrioles. Similar structures containing the 50 kD centriolar protein appear at a comparable time in cultured pinealocytes. Since both in situ and in vitro more material containing the 50 kD centriolar protein is present than can be accounted for by the fragmentation of the diplosomal centrioles the resulting fragments may be capable of generating more fragments. The above findings also suggest that the 50 kD centriolar protein is a component of the electron dense material in walls of centrioles between MT triplets since this material forms early and is present in procentrioles before MT assemble, is present in mature centrioles and basal bodies and around MTs in structures resembling fragments of centriolar wall.
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Dedication

To the members of my family in Oaxaca, Gloria (my mother) Ramon, July, Argelia, Doris (my brother and sisters) Karla, Paulina, and Gloria (mujе) (my nieces) and Ramoncito (my nephew). I thank all of them for their financial support and unconditional moral support.

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To the aboriginal people from Oaxaca and Canada, to the street kids from Toronto and Mexico City and to the gypsy kids from Madrid.
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<thead>
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<th>Abbreviations</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-(Diazabicyclo-2,2,2) octane</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>F(ab)</td>
<td>Antibody binding fragment</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filament</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney epithelium</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>MF</td>
<td>Microfilament</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing center</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCM</td>
<td>Pericentriolar material</td>
</tr>
<tr>
<td>pH</td>
<td>Negative logarithm of the hydrogen ion concentration in moles per liter.</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
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1

General Introduction.
Centrioles and basal bodies.

Studying cell division in the eggs of *Ascaris*, Boveri and Van Beneden working independently in 1887 first observed two dotlike structures which they called polar corpuscles and showed that they persist throughout the cell cycle. Their observations led them to view each corpuscle, now known to correspond to a centriole, as "an independent, permanent cell-organ, which, exactly like the chromatic elements, is transmitted by division to the daughter cell" (Wilson, 1925). Henneguy (1898) suggested that centrioles became basal bodies of flagella in spermatocytes and Lenhossek (1898) concluded that multiplication of the two mature preexisting centrioles had given rise to basal bodies of ciliated cells in the intestinal epithelium of lower vertebrates. Much later electron microscopy (EM) confirmed that the centrioles and basal bodies are related by showing that both are cylindrical structures about 0.2-0.3μm in diameter and 0.4-0.7μm in length with walls containing nine microtubule (MT) triplets. In some cell types such as *Chlamydomonas* centrioles become basal bodies by acquiring cilia at one stage of the cell cycle and the basal bodies become centrioles again at another by losing cilia and migrating toward the nucleus prior to the formation of the mitotic spindle (Johnson and Porter., 1968). In this thesis the term basal body will be restricted to structures with nine MT triplets located at the base of cilia. Similar structures lacking cilia will be referred to as centrioles.

Centrioles are the central components of the centrosome which is usually located in a perinuclear position close to the Golgi apparatus. In the centrosome the centrioles are surrounded by cytoplasmic foci referred to as MT-organizing centres (MTOC's) (Picket-Heaps, 1969) from
which the cytoplasmic and spindle MTs assemble. MTs are long, straight, relatively rigid structures that are one of the three major fiber systems which constitute the cytoskeleton of all eukaryotic cells (Avila, 1991). They are polar structures assembled from tubulin in which the tubulin subunits are arranged in a specific orientation growing faster at the plus end generally located closer to the cell surface than at the minus end which is anchored by the MTOC's in the centrosome. MTs determine the distribution and the organization of many cell organelles including the Golgi apparatus (Kreis, 1990; Rogalski and Singer., 1984), the endoplasmic reticulum (Terasaki, 1990), the mitochondria (Ball and Singer. 1982; Heggeness et al., 1978) and sometimes also that of the other two major components of the cytoskeleton the microfilaments (MF) (Bornens et al., 1989) and the intermediate filaments (IF) (Ishikawa et al., 1968; Klymkowsky et al., 1989).

**Centriolar functions.**

Very little is known about the functions of centrioles except that they can serve as basal bodies of cilia and flagella. Centrioles may participate in the concentration of certain centrosomal proteins such as gamma-tubulin that nucleates microtubule assembly to a particular region in the cytoplasm. Consistent with this view is the observation that "barrel-shaped" mitotic spindles in which gamma-tubulin is diffusely distributed at the ends of mitotic spindles are present in dividing acenriolar cells of a *Drosophila melanogaster* cell line. However a normal spindle with pointed ends at which gamma-tubulin is concentrated is present in the centriole-containing cells from which the acenriolar cell line was derived (Debec et al. 1995). These observations then, do
not support the idea that centrioles are simply "dispensable passengers" using the spindle only to separate equally between the two daughter cells at mitosis (Pickett-Heaps., 1971). The absence of centrioles in one of the Drosophila melanogaster cell lines (Debec et al., 1982), in mouse oocytes before the 8-16 cell stage (Szöllözi et al., 1972; Calarco-Gillam et al., 1983) and in most higher plant cells (Lambert., 1993) does, however, suggest that centrioles are not necessary for the division of certain cell types. In contrast Sluder (1989) and Rieder (1985) have obtained evidence that centrioles are necessary for the reproduction of sea urchin centrosomes by showing that centrosomes with centrioles replicate before each mitosis whereas, those without centrioles do not reproduce. Because of the cylindrical structure of centrioles, the arrangement of MT in their walls and their orthogonal orientation it has also been proposed that centrioles act as cellular sensors for locating signal sources in the environment (Albrecht-Buehler., 1990).

Centrioles, centrosomes and cell migration

Most animal cells including those which are capable of migrating contain a pair of centrioles which can serve as markers for the centrosomes. Several studies on the formation of multinucleated muscle cells are consistent with the idea that centrosomes and/or the centrioles play some role in cell migration. During myotube formation by fusion of myoblasts which are capable of migration and replication, the centrioles disappear and the nuclear envelope instead of the centrosome becomes the MT nucleating site (Connolly et al., 1985; Tassin et al., 1985). This suggest that centrioles and/or centrosomes may be necessary only for myoblast migration and/or proliferation but not for the functioning of the resulting stationary myotubes. Singer and
Kupfer (1986) and Hay (1989) have proposed a model in which both the centrosome and the Golgi apparatus play a role in determining the polarity of migrating cells. Since the centrosome is generally located close to the Golgi apparatus and since both are generally oriented toward the front end of migrating cells, according to this model the MTs radiating from the centrosome could transport vesicles from the Golgi apparatus toward the front end of migrating cells, for incorporation into the expanding cell membrane. Examples of migrating cells with centrioles and centrosomes preferentially oriented toward the front end of the cell are 3T3 cells (Albrecht-Buehler et al., 1979), neutrophils (Bessis et al., 1967; Malech et al., 1977), macrophages (Gudiina et al., 1988), newt eosinophils (Koonce et al., 1984) amoeboid cells of Dictyostelium discoideum (Swason et al., 1982), transformed avian otic epithelial cells (Carney et al., 1989), fibroblasts (Kupfer et al., 1983; Couchman et al., 1982), BHK cells in syncytia (Lewis et al., 1987) and endothelial cells migrating into a wound in vitro (Gotlieb et al., 1981; Mascardo et al., 1984), in organ culture (Rogers et al., 1986) and in situ (Rogers et al., 1992).

Centrioles are also preferentially oriented toward the leading edge of migrating neuronal precursor cells. Examination of such cells migrating along radial glia in the developing cerebral cortex of monkey by EM showed that the centrioles are positioned toward the front end of cells (Rakic., 1972). Centrioles are also located toward the front end in precursors of cerebellar granule cells migrating along radial glia in culture (Gregory et al., 1988).

Centrioles and centrosomes may therefore play a role in defining the direction of cell migration at least in some cell types. This is also supported by the finding that after irradiation
of their centrioles with an ultraviolet laser microbeam. the migration of newt eosinophils became slower and its direction changed more frequently (Koonce et al., 1984). It was suggested that the irradiation damaged the centrioles rather than the surrounding MTOCs, since MT assembly is restored soon after the irradiation whereas the migration rate never increased again after irradiation.

Centrioles, centrosomes and cell polarity

Van Beneden (1883) first suggested over a century ago that an imaginary line through the centrosomes and the center of the nucleus establishes an axis that gives the cell a polar organization (van Beneden, quoted by Wilson 1896). Subsequently a spatial correlation between the position of the centriole or the centrosome and a particular cytoplasmic or membrane domain of a polarized cell has been observed in many cell types. Conclusive evidence that the centrioles/centrosomes determine the polarity of highly polarized cells such as the neurons and epithelial cells however has not been obtained.

a) Neurons.

The polarity of neurons is specified by the position of their axons and dendrites. A spatial relationship between the position of the centrioles and the site of axon emergence has not been found. In one study more than 100 mouse retinal ganglion cells extending axons were reconstructed from electron micrographs of serial sections. In these cells, a particular relationship between the site of axon emergence and the position of their centrioles was not apparent (Hinds
and Hinds., 1974). Another study, in which 34 Ti1 pioneer neurons in the grasshopper were examined, did show that the axons grow from the side of the cell where the centrioles are located (Lefcort and Bentley., 1989). However, this relationship results from the fact that the spindle orientation in the mother cell is always perpendicular to the surface of the limb bud epithelium. Therefore the correlation between the centriole position and the site of axon emergence appears to be coincidental since the centrioles are automatically placed in that position during mitosis. A third study used cultured rat hippocampal neurons and tubulin antibodies to identify the position of the centrosome by noting the site from which MT repolymerize after their depolymerization by colcemid. This study showed that the centrosomes do not have a particular relationship to the site from which the axon emerges (Dotti and Banker., 1991). None of these studies therefore support the idea that a correlation or a causal relationship exists between the position of centrioles and the site of axonal emergence in developing neurons.

b) Epithelial cells.

The cell membrane of highly polarized epithelial cells is subdivided into an apical domain facing the external environment or the lumen of a cavity or duct and a basolateral domain in contact with neighbouring epithelial cells and the basal lamina. Tight junctions act as a "fence" separating the apical and basolateral domains which contain different membrane proteins and lipids. In addition to this asymmetric distribution of membrane constituents there is an apical-basal polarity in the distribution of intracellular organelles. In most epithelial cells the nucleus is typically located near the basal part of the cell whereas the Golgi apparatus, the centrosome and the centrioles are generally found on the apical side of the nucleus. A contractile belt composed
of bundles of MF encircles the apical end of the cell just below the tight junctions. The fact that epithelial cell polarity is not grossly distorted on MT disassembly suggests that other factors are more important than MTs in maintaining this polarity.

Experiments in Madin Darby canine kidney (MDCK) epithelial cells suggest that the establishment of polarity in these cells depends on specific external clues from the environment. MDCK cells (Rodriguez-Boulan et al., 1983) maintained in suspension are essentially nonpolar. However, when these cells settle on a substratum, the membrane in contact with the substratum becomes the basal plasma membrane by accumulating basement membrane receptors for extracellular matrix proteins such as collagen and laminin. The extracellular matrix protein laminin also appears to play an inductive role in the conversion of mesenchymal cells to epithelial cells in the developing kidney, since antibodies against laminin inhibit this transformation.

In addition to cell-substratum interactions the establishment of epithelial cell polarity also requires the formation of cell-cell contacts including tight junctions which as mentioned earlier are required for maintaining the polarized distribution of certain integral membrane proteins. Cell-cell adhesion molecules such as E-cadherin also play an important role in establishing epithelial cell polarity. For example disruption of cadherin function by low levels of Ca$^{2+}$ not only disrupts cell-cell contacts but also results in the redistribution of integral membrane proteins from particular membrane domains over the entire cell surface (Nelson and Veschnok., 1986).
With the establishment of cell-cell contacts between adjacent epithelial cells the distribution of integral membrane proteins and MT organization changes. Immunofluorescence studies using MDCK cells and tubulin antibodies showed that one day after plating, single cells have MTs radiating from a centrosome containing a pair of centrioles. By two days after plating when the cells had grown to confluence the two centrioles had separated and were no longer associated with the MTOC's. By the fifth day after plating, the centrioles had moved to the apical ends of the highly polarized MDCK cells (Bacallao., et al., 1989). In terminally differentiated columnar epithelial cells the MTOC's after detaching from the centrioles become scattered along the apical end of the cell (Bacallao et al., 1989). In these cells MTs are attached at their minus ends to the apically located MTOC's and run parallel to the apical-basal axis with their plus ends pointing toward the basal end of the cell (Bacallao et al., 1989). The apically located centrioles in terminally differentiated columnar epithelial cells are not associated with the MTOC's.

The precise role of MTOC's scattered along the apical ends of epithelial cells and MTs in the maintenance of epithelial cell polarity is not clear. The difference in composition between the basolateral and apical plasma membrane domains is maintained in large part by polarized intracellular sorting of proteins to these membrane domains. It appears that antimicrotubule agents interfere with the distribution and sorting of some integral membrane proteins (Eilers et al., 1989) but not others (Flemming et al., 1989).
Centrioles assemble in different ways and in association with different structures.

Since centrioles and basal bodies have a related morphology one would expect that they would be formed by a similar mechanism and from similar precursors in all cell types. Instead these organelles appear to form in different ways, in association with morphologically different structures. In interphase cells the centrioles are often found in an orthogonal orientation reflecting the fact that the new centrioles, termed procentrioles form by budding at right angles from the walls of pre-existing parent centrioles (Gall, 1961; Faure-Fremiet, et al. 1956). In ciliates the assembly of new centrioles destined to become basal bodies also occurs at right angles from the basal ends of pre-existing basal bodies (Dippel, 1968; Allen, 1969). During ciliogenesis in the trochophore of the annelid Nereis, dense plaques are present between the mature basal bodies and the procentrioles in a similar orientation i.e. at right angle to each other (Kalnins., 1967). Mizukami and Gall (1966) studying the multiflagellated sperm formation in the fern Marsilea showed that the immature centrioles could also form from the ends of tubules packed into a special organelle called the blepharoplast rather than preexisting centrioles.

In addition, in certain situations centrioles can also arise "de novo". Centrioles or basal bodies could not be found in the amoeboid stage of the amoeboflagellate Naegleria by electron microscopy but were present in the flagellated form (Schuster., 1963; Fulton and Dingle., 1971). Similarly centrioles were absent in the unfertilized sea urchin eggs (Verhey and Moyer, 1967; Longo and Anderson, 1968) but present in the asters of parthenogenetically activated eggs.
(Dirksen., 1961; Sacks and Anderson., 1970). As mentioned previously centrioles are also absent from the poles of the mitotic spindle and cytoplasm during the early divisions of mouse oocyte and appear only during the 8-16 cell stage (Szollosi et al., 1972; Maro et al., 1985; Schatten and Schatten., 1986).

**Single centrioles assemble in association with mature centrioles.**

In most eukaryotic cells a single centriole assembles near each of the mature centrioles prior to cell division as outlined below. In G1 phase of the cell cycle cells contain a mother and a daughter centriole in an orthogonal arrangement. This orthogonal arrangement is lost late in G1 phase and the two centrioles separate at the beginning of the S phase (Kuriyama and Borisy., 1981). During the S phase each of the centrioles acquires a short daughter procentriole which is assembled at right angles at a distance of 50-60 nm from the wall of the mother centriole near its proximal end (Robbins et al., 1968; Rattner and Phillips., 1973; Reviewed by Wheatley., 1982). The proximal ends of the procentrioles which assemble first, contain a "cartwheel" which consists of an axial tubular core with nine radially directed spokes to which the microtubules are attached (Vorobjev and Chentsov., 1982). Procentrioles with up to nine evenly spaced single microtubules in their walls which presumably guide the assembly of the other microtubules to form the triplets can be recognized at later stages (Vorobjev and Chentsov., 1982). After the triplets have formed, the diameter of the procentrioles increases with the unfolding of the 9 triplets for which a mechanism similar to the opening of an iris diaphragm has been proposed (Albrecht-Buehler., 1990). Elongation of the procentrioles occurs gradually and is completed at different times in
different cell types during mitosis (Vorobjev and Chensov., 1982). When the procentrioles reach their mature length the "cartwheel" inside their proximal ends disappears (Vorobjev and Chensov., 1982). Microinjection of biotinylated tubulin into synchronized porcine kidney epithelial cells early in G1 phase of the cell cycle and studies following its incorporation into the centrioles by immunofluorescence showed that after mitosis each daughter cell received one unlabelled (mother) and one labelled (daughter) centriole thus indicating that centrioles replicate semiconservatively (Kochansky and Borisy., 1990). The two mature centrioles present in interphase cells are termed diplosomal centrioles. In many cell types the oldest of these centrioles often becomes a basal body from which a non-motile primary cilium is assembled. In epithelial cells this occurs at the apical cell surface.

**Nucleic acids in centrioles**

The existence of replicating, DNA containing organelles such as mitochondria and chloroplasts suggested that centrioles may also have genetic information in the form of DNA and/or RNA which enable them to replicate. Investigations to determine whether or not centrioles and the related basal bodies contain DNA, however, have given conflicting results and evidence both in favour and against the idea that DNA is present in centrioles has been presented. In 1986 Ramanis and Luck showed that a mutation that affects basal body assembly in *Chlamydomonas* maps to a unique locus, the uni linkage group. *In situ* DNA hybridization experiments localized at least one copy of the DNA of this linkage group in each of the two basal bodies of *Chlamydomonas* (Hall et al., 1989). These observations however, have not been confirmed by
other investigators. Using immunogold labelling and antibodies to DNA, Johnson and Rosenbaum. (1990) found that in Chlamydomonas gold particles were distributed over the nucleus, chloroplasts and mitochondria but not over the basal bodies.

Other evidence suggesting the presence of nucleic acids in centrioles was obtained by Went (1977). He showed that treating sand dollar eggs with inhibitors of nucleic acid synthesis such as chloramphenicol, mercaptoethanol, actinomycin and 5-bromodeoxyuridine before the second cell division interferes with centriolar replication. Went (1977) proposed that DNA synthesis from an RNA template is required for centriolar replication. Biochemical evidence for the presence of functional RNA in the centrioles was presented by Heidemann and Kirschner (1975). In their experiments basal bodies isolated from Chlamydomonas and Tetrahymena injected into Xenopus eggs induced the formation of asters whereas, basal bodies treated with ribonucleases lost their aster-inducing activity. The loss of this activity was not accompanied by any observable changes in basal body structure. The authors proposed that RNA in the centriole could code for the mRNA of a specific protein that is required for aster formation.

Control mechanisms for centriolar assembly.

Centriolar replication normally occurs in synchrony with the cell cycle. Experiments using eggs from different organisms have shown that centriolar replication is controlled by the cytoplasm and does not require the nucleus. For example centrioles will continue to replicate in a normal fashion after the nucleus of a fertilized sea urchin or starfish embryos is removed.
(Sluder, et al., 1986; Picard et al., 1988) or when sea urchin (Sluder, et al., 1987) and *Drosophila* embryos (Raff and Glover., 1988) are injected with aphidicolin to block DNA synthesis and nuclear replication. Thus in zygotes neither DNA replication nor the nucleus is required for centriolar replication. In comparison to zygotes even less is known about the control of centriolar replication in somatic cells. Rose et al., (1993) have proposed that the somatic cell has an endogenous counting mechanism that monitors the absolute number of centrioles which not only ensures centriolar duplication but also limits it to a single round during each cell cycle.

Cell replication has been arrested in CHO cells (using hydroxyurea and aphidicolin) at the time in cell cycle when centriolar replication begins (G1/S boundary) and blocked for a period of time equivalent to four or five cell cycles. The arrested cells undergo multiple rounds of centriolar replication and in some of them which were arrested for 60 hrs up to 10 centrioles were found (Balczon et al., 1995). Analysis using northern blots and the cDNA encoding portion of the pericentriolar autoantigen (PCM-1) showed that centriole number increases when the production of PCM-1 transcripts is maximal and that centrioles do not replicate when the production of this mRNA is minimal. The authors concluded that centriolar replication in CHO cells is controlled by the availability of centrosomal subunits and that the somatic cell nucleus probably under the direction of cell cycle regulatory molecules directs cycles of centriolar replication by controlling the activation of centrosomal genes at the appropriate times during each cell cycle.
Sluder and Rieder (1996) made the observation that in an electron micrograph of CHO cells arrested at the G1/S boundary published by Balczon et al. (1995) the centrioles appeared to be single and were not associated with daughter centrioles. Since the average number of centrioles seen in these arrested cells increases on average by only one centriole per cell cycle, instead of geometrically as expected assuming that all the centrioles are able to replicate in each cycle. Sluder and Rieder (1996) believe that only the original mother centriole is mature and was able to replicate. They suggest that the newly formed daughter centrioles remain immature, are unable to replicate because the cell cycle was arrested and never acquire a functional replication template for centriole assembly. Sluder and Rieder (1996) believe that the components or conditions needed to construct a functional replication template for the daughter centriole(s) are not normally provided until some time after the G1/S boundary despite the continuous production of centriolar proteins. This mechanism would prevent daughter centrioles from rereplicating early in the event of a prolonged S phase induced by environmental factors.

The orthogonal orientation of centrioles also seems to be important for centriole replication. Centrioles isolated from calf thymocytes in which they are linearly instead of orthogonally oriented and closely associated with each other at their proximal ends (Tournier et al., 1991) are unable to replicate, when injected into Xenopus eggs. In contrast centrioles oriented at right angles to each other and further apart isolated from other cell types can replicate. From these observations Tournier et al., (1991) have proposed that the initiation of centriolar replication cannot occur as long as the two parent centrioles have a linear orientation and/or remain closely associated with each other.
Centriole assembly in ciliating epithelial cells.

Epithelial cells undergoing ciliogenesis are particularly well suited for studies of centriole replication because they produce a large number of centrioles which serve as basal bodies of cilia within a short period of time. Centriole replication during ciliogenesis has been examined at the ultrastructural level in rat (Dirksen and Crocker., 1966; Sorokin, 1968) and chicken trachea (Kalnins and Porter, 1969), in mouse (Dirksen, 1971), rabbit (McCarron and Anderson., 1973), monkey (Anderson and Brenner., 1971), human (Hagiwara., et al 1992) and cat oviduct (Verhage and Brenner., 1975), in epidermis and trachea of Xenopus laevis (Steinman., 1968), in the lamprey Petromyzon marinus kidney (Youson., 1982), in ductuli efferentes of chinese hamster (Chang et al 1979), in rat ependyma (Kalnins and Marshall unpublished observation) and in rabbit and rat olfactory neurons (Heist and Mulvaney., 1968; Popov and Tsyganova., 1996). These studies have indicated that centriolar assembly, is a highly complex process that generally takes place in the apical end of the ciliating cell (fig. 1). In the cell types studied, most of the immature centrioles or procentrioles are formed in clusters around electron dense structures and in association with fibrogranular material. In the chick (Kalnins and Porter., 1969) and duck (Marshall and Kalnins., unpublished observation) trachea, however, some of the procentriole clusters are also attached directly to the walls of diplosomal centrioles by electron dense structures (fig. 1) (Kalnins and Porter., 1969; Marshall and Kalnins., unpublished observation) similar to those forming the cores of procentriole clusters. This observation suggests that all of the procentriole clusters may form initially in close association with the diplosomal centrioles.
Fig. 1. Sequence of events in the assembly of centrioles suggested from studies by EM in the chick trachea. Condensation of ground substance accumulates along the two mature diplosomal centrioles located in the apical portion of the cell (a). These condensations organize into cylinders on the outside walls of the diplosomal centrioles. Procentrioles then assemble along the walls of the cylinders forming clusters (b). The clusters break away from the diplosomal centrioles as they are formed and become distributed within a few microns of the diplosomal centrioles (c). Throughout the formation of clusters, dense granules are present around the newly formed clusters of procentrioles (b, c, and d₁). As the procentrioles increase in length and acquire MT (d₂) the cores gradually disappear. The procentrioles meanwhile separate from the clusters (d₃) and become distributed throughout the apical portion of the cell where they complete their elongation. The mature centrioles line up with their longitudinal axes perpendicular to the cell surface and acquire ciliary vesicles, rootlets and cilia (e). The rootlets form first just below the recently matured centrioles in association with MT and only later become attached to the centriole. The ciliated border is finally formed when all of the cilia reach the mature length (f). 

After the procentrioles mature they separate from the clusters and migrate toward the apical cell membrane where they become aligned perpendicularly to the cell surface and become basal bodies by acquiring rootlets and cilia (Fig. 1). Although this general scheme is similar in all types of ciliating epithelial cells examined, there are considerable differences in opinion regarding the significance and function of the diplosomal centrioles and of the various structures associated with centriolar assembly during ciliogenesis.

**a) Core of procentriole clusters.**

Of particular interest for centriolar assembly is the core with which procentrioles are associated during early stages of centriolar assembly in ciliating epithelial cells. This structure has been called by different names in different tissues. These include "condensation forms" (Dirksen and Crocker., 1966; Dirksen, 1971; McCarron and Anderson., 1973); "deuterosome" (Anderson and Brenner., 1971; Hagiwara., et al 1992; Sorokin., 1968; Verhage and Brenner., 1975); "procentriole organizer" (Steinman., 1968); and "dense body" (Youson., 1982; Chang., et al 1979). An analogous structure found in a similar position relative to procentrioles during the formation of multiflagellated sperm in the fern *Marsilea* and in the cycad *Zamia* was called a "blepharoplast" by Mizukami and Gall (1966).

The core of clusters of procentrioles can vary considerably in morphology between species. In *Xenopus laevis* the core is a dense solid cylinder (Steinman., 1968) whereas in the chicken the cores are cylinders that have electron dense walls and a lighter staining central part in which an axial structure can often be detected (Kalnins and Porter., 1969). In contrast the cores
of procentriole clusters in ciliating cells of mammals are solid, electron-dense spheres varying in size from 90 to 550 nm in diameter. The largest so far observed are present in the mouse (Dirksen., 1971) medium sized ones are present in the rat (Dirksen and Crocker., 1966; Sorokin., 1968), cat (Verhage and Brenner., 1975), chinese hamster (Chang et al.. 1979), rabbit (McCarron and Anderson., 1973) and the smallest in the monkey (Anderson and Brener., 1971) and human (Hagiwara.. 1992) cells undergoing ciliogenesis. The morphology of the core however is similar in the different types of ciliating cells in each species. For example in both Xenopus trachea and epidermis (Steinman., 1968) a dense cylinder is observed whereas in both rat trachea (Dirksen and Crocker.. 1966; Sorokin., 1968) and in rat ependyma (Kalnins unpublished observation) a solid sphere is present at the center of procentriole clusters. Although the morphology of the core of procentriole clusters differs widely in different species, the procentrioles generated are basically similar in all of the ciliating cell types examined. The close spatial relationship seen between the procentriole and the core suggests that the morphologically different cores all contain common molecules that are responsible for initiating and sustaining centriole assembly. Since the walls of diplosomal centrioles are also capable of initiating procentriole assembly they are likely to contain similar components. The question of whether or not the structures present at the core of procentriole clusters contain precursors for the assembly of procentrioles remains unanswered.

b) Fibrogranular material.

One of the first signs that epithelial cells will undergo ciliogenesis is the appearance of fibrogranular material in the apical end of the cell composed of electron dense granules embedded in a lighter staining fibrous matrix. This fibrogranular material also referred to as the
"proliferative element" is believed by some to originate from pre-existing centrioles or basal bodies (Dirksen and Crocker., 1966) and by others from the nucleus (Anderson and Brenner., 1971). Because it appears before the procentriole clusters it has been suggested that the fibrogranular material is the precursor both for the cores of procentriole clusters (Dirksen and Crocker., 1966; Anderson and Brenner., 1971) and for the procentrioles (Anderson and Brenner., 1971; Youson., 1982). Although the fibrogranular material disappears as the procentrioles mature and separate from the clusters similar material reappears later when it was thought to contribute to the formation of MTs in the ciliary axoneme (Steinman., 1968) or to the formation of rootlets and/or basal feet (Kalnins and Porter., 1969). The exact role of these granules. if any. in the formation of the procentrioles. the cores of procentriole clusters, or the various other structures associated with the basal bodies remains to be determined.

c) Assembly of centrioles.

In the earliest stages of assembly that can be morphologically identified, procentrioles can be observed as short cylinders with fibrous walls lacking MTs (Kalnins and Marshall unpublished observation). Also before the MTs have formed in the walls of procentrioles a cart-wheel consisting of an axial tubular core with nine radially directed spokes is apparent in what will become the proximal end of the mature centriole (Kalnins and Porter., 1969; Anderson and Brenner., 1971; Kalnins and Marshall unpublished observation). The spokes of the cart-wheel may determine the positions where the first and innermost nine singlet MTs subsequently form. Later the second MTs are added to the singlets to form doublets and finally the third MTs are added to form the nine triplet MTs. As mentioned previously the cart-wheel has also been
observed at the proximal ends of daughter centrioles in dividing non-ciliated cells (Vorobjev and Chentsov., 1982). The fact that the cart-wheel disappears before the procentrioles reach their mature size (Sorokin., 1968; Kalnins and Porter., 1969; Perkins., 1970; Kalnins and Marshall unpublished observation), supports the hypothesis that its function may be to specify the position of the initial MTs in procentriole walls. After the centrioles increase in diameter and elongate they separate from the clusters and continue elongating as they migrate toward the apical cell surface. As in non-ciliated cells (Vorobjev and Chentsov., 1982), the diameter of centrioles destined to become basal bodies in ciliating epithelial cells also increases perhaps again by the unfolding of the 9 triplet MTs in a manner similar to the opening of an iris diaphragm as suggested by Albrecht-Buehler (1990).

**Loss and fragmentation of centrioles.**

In some terminally differentiated cell types which have lost the capacity to divide and migrate, centrioles are unnecessary and disappear or fragment at a particular stage of differentiation. Centrioles disappear in skeletal muscle cells (Connolly et al., 1985; Warren, 1974; Cartwright and Goldstein, 1982) and in *Drosophila* wing epidermal cells (Tucker et al., 1986) during differentiation. It has been suggested that centrioles in rat pinealocytes (Lin., 1970) become modified and structures interpreted as fragments of centrioles have also been observed in golden hamster (Lin, et al., 1987), guinea pig (Lin., 1972) and cat pinealocytes (Calvo et al., 1991), in human endothelial cells (Bytrevskaya., et al. 1992) and in ciliating and ciliated epithelial cells from hamster bronchioles (van der Steen et al., 1995), cat (Roperto, et al., 1994)
and pig oviduct (Roperto, et al. 1990), human trachea and bronchi (Moscoso et al., 1988; Lungarella., 1985), dog bronchi (Hoover, et al., 1989), and thymus of "nude" mice (Cordier., et al., 1974).

As mentioned previously ultrastructural and immunofluorescence studies using centriolar antibodies (Connolly et al., 1985) have shown that in the multinucleated myotubes that develop from the fusion of centriole containing myoblasts, the centrioles are absent and centriolar staining cannot be detected (Warren. 1974; Cartwright and Goldstein. 1982; Connolly et al.. 1985). In these myotubes the nuclear envelope instead of the centrosome acts as the MT nucleating site. Another example of a terminally differentiated cell type which loses centrioles at a particular stage in differentiation is the Drosophila wing epidermal cell. In these cells during the early stages of cell differentiation the centrosome which contains a pair of centrioles nucleates MT assembly. However in terminally differentiated cells apical electron dense plaques on the plasma membrane instead act as the MT nucleating sites (Tucker et al., 1986).

Ultrastructural studies have shown that pinealocytes of adult rats have structures resembling elongated centrioles and fragments of walls of centrioles (Lin., 1970). From these observations it has been proposed that centrioles of pinealocytes of rats first elongate and then fragment with age (Lin., 1970). Structures resembling fragments of centrioles have also been observed in adult pinealocytes of other species including the golden hamster (Lin, et al., 1987), guinea pig (Lin., 1972) and cat (Calvo et al., 1991).
Ultrastructural studies have shown that elongated centrioles and fragments of walls of centrioles are also present in endothelial cells from 2 to 40 year old human donors. In contrast to rat pinealocytes in which both mother and daughter centrioles appear to elongate and fragment, in human endothelial cells only the mother centrioles appear to do so (Bystrevskaya.. et al. 1992). Human endothelial cells from 30-40 year old donors, grown in cell and organ cultures contained what appear to be fragmented mother centrioles but intact daughter centrioles at right angles to each other at the spindle poles. This indicates that endothelial cells with abnormal centrioles were able to replicate (Bystrevskaya.. et al. 1992).

Ultrastructural studies of ciliating cells in bronchioles of new born hamsters have shown that some of the basal bodies of cilia are also incomplete (van der Steen et al., 1995). Cross sections of these basal bodies showed that they lack from 1 to 4 MT triplets in their walls. Serial cross sections of a basal body which lacked one MT triplet showed that the central part of this basal body contained an electron dense granule. One of the triplets of this centriole was attached to an electron-dense hollow cylinder (van der Steen et al., 1995). The authors of this study speculated that incomplete centrioles may be generated either by incomplete addition of MT triplets to preexisting ones during the formation of centrioles or by loss of MTs triplets from a fully mature centriole having 9 MT triplets in its wall (van der Steen et al., 1995).

Moscoso et al., (1988) examining centriolar replication in ciliating cells of human trachea and bronchi found that some of the centrioles, lacking a number of MT triplets, were oriented at right angles to other centrioles which also lacked triplets. Basal bodies lacking some of the nine
MT triplets have also been reported in ciliated epithelial cells of cat (Roperto, et al., 1994) and pig (Roperto., 1990) oviducts.

Basal bodies which lacked up to 4 MT triplets have also been found in ciliated cells of the nasal mucosa and/or bronchi of patients suffering from the immotile cilia syndrome which is a congenital disorder characterized by markedly impaired mucociliary transport (Lungarella et al., 1985). It was reported that up to 21% of the basal bodies from ciliated cells in these regions had an incomplete number (less than nine) of MT triplets in their walls (Lungarella et al., 1985). Longitudinal sections of some of these abnormal basal bodies showed a dense granule in their central part. Many striated rootlets were also seen in the cytoplasm of these ciliated cells (Lungarella et al., 1985). Fragments of basal bodies have also been found in ciliated epithelial cells of cats (Roperto., et al., 1994), dogs (Hoover, et al. 1989) and pigs (Roperto., et al., 1990) with the immotile cilia syndrome. They were also found in ciliated cells in cysts in the thymus of "nude" mice which carry the gene for hereditary dysgenesis of the thymus. In this condition by two to three months after birth the thymus is reduced to a group of cysts containing ciliated cells (Cordier., et al., 1974).
**General objectives.**

Although much information is available from ultrastructural studies on the assembly of centrioles during ciliogenesis in epithelial cells of many different species little is known about the spatial and temporal incorporation of centriolar proteins into the developing centrioles during their assembly. My primary objective therefore was to investigate when and where the 50 kD centriolar antigen recognized by non-immune rabbit sera first appears during the centriolar assembly in ciliating epithelial cells. I hoped that these studies would provide a start toward understanding centriolar assembly at the molecular level. Also since centriolar assembly during ciliogenesis in epithelial cells is likely to be similar to centriolar assembly in eukaryotic cells in general, I hoped that my findings from these studies may be applicable to centriole replication in all cell types.

My second objective was to investigate the distribution of the 50 kD centriolar antigen during centriole breakdown instead of centriole assembly. On the basis of EM studies several authors have suggested that pinealocytes in the adult rat do not contain normal centrioles. Instead cytoplasmic structures that were interpreted as fragments of centrioles were described. Using the antibody to the 50 kD centriolar antigen I investigated whether or not centrioles can be detected in adult pinealocytes and whether or not evidence could be obtained that the structures resembling fragments of centrioles present in the adult pinealocytes are indeed derived from centrioles. This was done by determining whether or not they contain the 50 kD antigen present in normal centrioles.
Having confirmed that the centrioles do fragment and that the fragments of centrioles are present and persist in adult pinealocytes I then attempted to determine whether the fragmentation is induced by neuronal or hormonal signals, or by factors intrinsic either to the pinealocytes and/or to the centrioles themselves. I therefore determined whether or not a similar loss and/or fragmentation of centrioles occurs or can be induced in pinealocytes _in vitro_. If it does or can be induced the system _in vitro_ could then be manipulated to study the mechanisms of centriolar loss and/or fragmentation and its control in greater depth.

**Specific objectives.**

In chapter two I examine centriole assembly in sections of 15 to 20-day-old chick embryo tracheas and in epithelial cells detached from such tracheas. Using the antibody specific for the 50 kD centriolar antigen and immunofluorescence, I determine changes in the distribution of this antigen during centriolar assembly. I investigate whether the clusters of procentrioles that can be visualized close to the diplosomal centrioles in electron micrographs of ciliating cells at early stages of centriolar assembly are likely to contain the 50 kD centriolar antigen. In an attempt to see more detail and to examine whole cells rather than sectioned ones, cells detached from ciliating chick epithelium were also examined using the MRC 600 Biorad scanning confocal microscope.

In chapter three I determined whether centrioles are present in the adult rat pinealocytes and whether the structures thought to be related to centrioles present in these cells contain the 50
kD centriolar antigen and therefore are likely to be derived from centrioles. To determine this I used sections of adult pineal glands and immunofluorescence staining with antibodies specific to this centriolar protein. EM was used to confirm that pinealocytes from the rats examined indeed have fragments similar to those previously described in the literature. If centrioles were absent and/or the fragments were related to centrioles a change could also occur in the distribution of MTOC's which nucleate MTs. I therefore determined whether a change in the distribution of MTOC's occurs in pinealocytes lacking normal centrioles in comparison to cells with normal centrioles using short-term cultures of pinealocytes from the adult rat pineal gland.

Having shown that pinealocytes of the adult rat contain fragments of centrioles. I then went on to determine whether a fragmentation of centrioles similar to that seen in vivo occurs or can be induced in cell cultures of pinealocytes. To do this I developed a long-term culture system capable of maintaining pinealocytes from 2-day-old rat pineal glands for periods of up to 70 days. Ultrastructural studies were undertaken to determine whether or not fragments of centrioles similar to those observed in vivo could be detected in pinealocytes in vitro. I also determined whether or not pinealocytes in vitro containing fragments of centrioles had the ability to nucleate MT assembly and where the MTOCs for such assembly were located in the cell.
Immunofluorescence study of centriolar assembly in chick ciliating epithelial cells using the antibody to the 50 kD centriolar antigen.
Introduction

From early studies of ciliating intestinal epithelial cells Lenhossek. (1898) had suggested that the multiplication of the two mature, preexisting diplosomal centrioles gives rise to basal bodies of cilia. Much later EM confirmed that the centrioles and basal bodies are related by showing that both are cylindrical structures about 0.2-0.3μm in diameter and 0.3-0.7μm in length with walls containing nine MT triplets. If the complexity of the centriole is similar to that of a cilium which contains at least 30 different proteins, only relatively few of its proteins have been identified. Those that have been identified include α- and β-tubulin from which the MT triplets are assembled (Cande, 1990), gamma-tubulin (Fuller, 1995; Dibbayawan, et al. 1995), cenexin which is present only in the older of the diplosomal centrioles (Lange and Gull, 1995) and a 50 kD protein identified by antibodies in sera from normal rabbits (Connolly et al., 1978; Turksen et al., 1982).

Gamma-tubulin is present both in the centrioles (Fuller, 1995; Dibbadayan et al., 1995) and in the surrounding MTOC's where it forms rings from which MTs assemble (Zheng et al., 1995; Moritz et al., 1995). When gamma-tubulin antibodies were used to localize this protein in ciliated cells of the trachea (Muresan et al., 1993) dots in the apical part of fully ciliated cells were observed and interpreted as stained basal bodies. However, no studies examining the distribution of α- β-tubulin, gamma-tubulin, cenexin (Lange and Gull., 1995) and the 50 kD protein (Connolly et al., 1978; Turksen et al., 1982) during assembly of centrioles in ciliating epithelial cells have been undertaken.
The 50 kD protein whose distribution is examined in this study is recognized by Western blotting in solubilized preparations of basal bodies from *Tetrahymena* and in extracts of chicken tracheal epithelial cells by some nonimmune rabbit sera (Turksen., 1982). These rabbit sera also stain centrioles and basal bodies by immunofluorescence in all species and cell types so far examined including endothelial cells (Rogers et al., 1985; Rogers et al., 1983), myoblasts (Connolly et al., 1985), neurons, fibroblasts, ciliated epithelial cells (Connolly and Kalnins., 1978) and in *Tetrahymena* (Turksen., 1982). The specificity of these rabbit sera for centrioles was established previously by immunofluorescence staining of cultured neurons. As expected two immunofluorescent stained dots which correspond to the pair of centrioles are seen in these cells (Connolly and Kalnins., 1978).

As described in the introduction, ultrastructural studies of ciliogenesis in many different species have indicated that centriolar replication is a highly complex process in which several hundred centrioles are assembled in a short period of time to provide basal bodies for cilia. In the ciliating cells studied most of the immature centrioles or procentrioles are formed in clusters around electron dense core structures in association with fibrogranular material. After the procentrioles mature they separate from the clusters and migrate toward the apical cell membrane where they become aligned perpendicularly to the cell surface and become basal bodies by acquiring rootlets and cilia (Anderson and Brenner., 1971; Chang et al., 1979; Dirksen, 1971; Dirksen and Crocker., 1966; Hagiwara et al., 1992; Kalnins and Porter., 1969; McCarron and Anderson., 1973; Steinman., 1968; Verhage and Brenner., 1975; Youson., 1982).
The investigators who have studied centriolar replication differ greatly in the extent to which they implicate the two pre-existing diplosomal centroles in procentriole assembly during ciliogenesis. From earlier studies of ciliating epithelial cells by light microscopy investigators concluded that basal bodies are derived specifically from the pair of mature diplosomal centroles that undergo repeated division after migrating to the apical part of the cell from a position near the nucleus (Lucas, 1932). In contrast in later studies of centriole assembly by EM no role in the assembly of the procentrioles was assigned to the two mature pre-existing diplosomal centroles (Stockinger and Cireli, 1965; Steinman, 1968). Sorokin (1968) concluded that the centroles destined to become basal bodies of cilia form by an "acentriolar pathway" and not from the pre-existing diplosomal centroles. He concluded that the cores, formed by the condensation of granules in the fibrogranular material, organized the growth of the procentrioles in the ciliating cells. He did not exclude the possibility, however, that the formation of the fibrogranular material may be under the remote influence of the diplosomal centroles. Dirksen and Crocker (1966) suggested that the fibrogranular material is produced directly by both the pre-existing diplosomal centroles and the recently matured basal bodies of fully ciliated cells and did not assign this function specifically to the two diplosomal centroles. Although Anderson and Brenner (1971) did not find the procentriole clusters in the vicinity of the diplosomal centroles they hypothesized that these centroles may be the initial site of synthesis of some type of "organizer substance" required for centriole assembly. The strongest morphological evidence that diplosomal centroles are involved in procentriole formation comes from studies of ciliating cells in the avian trachea showing that some of the procentriole clusters are directly attached to the wall of the diplosomal centroles and that all of them are found in close proximity to these centroles (Kalnins and Porter,
1969; Marshall and Kalnins, unpublished observations). These observations suggest that all of the procentriole clusters may be assembled at or very close to the surface of the diplosomal centrioles.

Although much information is available from ultrastructural studies on the assembly of centrioles during ciliogenesis in various types of epithelial cells from many different species, little is known about the spatial and temporal pattern of incorporation of centriolar proteins into developing centrioles during their assembly. In comparison to other known centriolar proteins such as cenexin and gamma-tubulin, the distribution of the 50 kD centriolar protein and its specificity for centrioles make it an excellent protein to use to study centriolar assembly. For example in contrast to cenexin which is present only in the older diplosomal centriole (Lange and Gull, 1995), the 50 kD centriolar protein is present in both diplosomal centrioles. Gamma-tubulin, on the other hand is present not only in the centrioles (Fuller, 1995; Dibbayawan, et al., 1995) but also in the surrounding MTOC's (Zheng et al, 1995; Moritz et al. 1995).

My first objective therefore was to investigate when and where the 50 kD centriolar protein recognized by non-immune rabbit sera first appears during centriolar replication in ciliating epithelial cells of the chick trachea. My second objective was to investigate where these sites are located in relation to the two preexisting diplosomal centrioles in the ciliating cells. My approach was to use immunofluorescence and a serum that specifically labels the 50 kD centriolar protein to stain sections of chick trachea and isolated cells detached from ciliating chick tracheal epithelium during the time in development, embryonic days 15 to 20 (E15-E20), when the
differentiation of ciliated cells is taking place. This approach also permitted me to examine a larger population of ciliating cells than could be examined by EM but at a lower resolution. I show that the 50 kD centriolar antigen appears very early during centriolar assembly in a region in the apical part of ciliating cells in close association with the two diplosomal centrioles. The relationship of the various staining patterns observed are compared with stages in centriolar assembly during ciliogenesis determined from previous studies by EM.

**Materials and methods.**

**Embryos.**

Fertilized eggs of white leghorn chickens were obtained from Glen Fenelon Farms (Toronto, Ontario) and incubated for 15 to 20 days in a humidified atmosphere at 37.8°C.

**Antibodies.**

Sera that label centrioles were obtained from New Zealand white rabbits. The sera were tested against proteins from epithelial cells of the chick trachea by Western blotting as previously described by Turksen et al (1984) except that molecular weights were determined using prestained low range standards (Bio-Rad, Richmond, CA), horseradish peroxidase HRP-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:800 in phosphate buffered saline (PBS) containing 0.05% Tween 20 was used as secondary antibody and specific proteins were detected using Amersham's (Oakville, Ontario) enhanced chemiluminescence (ECL) system according to manufacturer's instructions. Monoclonal antibodies to α-tubulin were purchased from Cedarlane
(Hornby, Ontario). Secondary antibodies including fluorescein-conjugated F(ab)$_2$ fragments of goat anti-rabbit and donkey anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (Mississauga, Ontario).

**Sections of trachea.**

After sacrifice by cervical dislocation, tracheas of 10 chick embryos at each of the following ages E15, E16, E17, E18, E19 and E20 were dissected, embedded in Tissue-Tek and frozen immediately in 2 methyl butane in liquid nitrogen. Cross sections approximately 7μm thick were cut with a cryostat, placed on coverslips and air dried.

**Preparation of intact tracheal epithelial cells.**

Tracheas of 20 chick embryos at each of the following ages E15, E16, E17, E18, E19 and E20 were removed and incubated in 50% glycerol in PBS, pH 7.0 for 24 hrs at 4°C. The tracheas were cut in half and their inside surfaces were scraped with a coverslip to remove the tracheal epithelium from which a suspension of epithelial cells was prepared in 50% glycerol in PBS.

**Immunofluorescence.**

Sections of E15-E20 chick tracheas and epithelial cells detached from such tracheas were used. Sections were first fixed in methanol and then in acetone both at -20°C for 5 min and then hydrated in PBS for 15 min. Detached cells were washed three times in PBS containing 10% glycerol. Both sections and detached cells were then treated with the rabbit serum which contained antibody to the 50 kD centriolar antigen diluted 1:30 in PBS for 30 min at room
temperature. In double immunofluorescence experiments the detached cells were treated in addition with monoclonal antibodies to α-tubulin diluted 1:500 in PBS. After washing the sections and the detached cells three times for 5 min each in PBS and in 10% glycerol in PBS respectively they were treated for 30 min at room temperature with fluorescein-conjugated F(ab)₂ fragments of goat anti-rabbit IgG diluted 1:40 in PBS. For double immunofluorescence labelling the detached cells were treated with a 1:1 mixture of fluorescein-conjugated F(ab)₂ fragments of goat anti-rabbit IgG diluted 1:40 in PBS and rhodamine-conjugated F(ab)₂ fragments of donkey anti-mouse IgG diluted 1:40 in PBS. Sections and detached cells were washed three times in PBS and 10% glycerol in PBS respectively. Sections of E15-E20 chick tracheas and epithelial cells detached from such tracheas from which treatment with the antibody to the 50 kD centriolar antigen was omitted treated with fluorescein-conjugated F(ab)₂ fragments of goat anti-rabbit IgG diluted 1:40 in PBS served as controls for single immunofluorescence experiments. In double immunofluorescence experiments the detached cells from which treatment with the antibodies to the 50 kD centriolar antigen and to α-tubulin were omitted treated with a 1:1 mixture of fluorescein-conjugated F(ab)₂ fragments of goat anti-rabbit IgG and rhodamine-conjugated F(ab)₂ fragments of donkey anti-mouse IgG both diluted 1:40 in PBS served as control. Sections and detached cells were mounted in Vinol containing 0.25% 1,4-(Diazabicyclo-2,2,2) octane (DABCO) to prevent bleaching and in 50% glycerol respectively and examined with a Zeiss Photomicroscope III (Zeiss, Germany) equipped with epifluorescence optics and FITC- TRITC-specific filters or with a MRC 600 Biorad scanning confocal microscope (Bio-Rad, UK). Photographs were taken with TMAX P3200 film, processed with TMAX developer and printed using Polycontrast III RC paper all from Kodak. The confocal images were recorded on optical
disks and then analyzed using the BioRad COMOS software package.

**Results.**

**Characterization of serum.**

Western blotting experiments showed that the serum used recognizes a 50 kD protein in extracts prepared from chick trachea epithelial cells which contained solubilized basal bodies of cilia (fig. 2). It has been previously shown that many sera from apparently normal rabbits that label centrioles also recognize a protein of the same molecular weight (50 kD) in similar extracts (Turksen et al., 1982).

**Immunofluorescence localization of the 50 kD antigen in ciliating epithelial cells of chick trachea.**

To confirm that the 50 kD centriolar antigen is present in the basal bodies of cilia, I double labelled ciliated cells removed from the E20 chick tracheal epithelium with antibodies to α-tubulin and to the 50 kD centriolar antigen. As expected the α-tubulin antibody stained cilia (fig 3b) and the antibody to the 50 kD centriolar antigen stained a band (fig. 3a) located at the base of cilia in the apical end of ciliated cells. This staining pattern confirmed that the 50 kD centriolar antigen is present in ciliated cells in the region where basal bodies of cilia are located, a region that is seen as a dense band when the same cell examined by phase contrast microscopy (fig 3c).
Having determined that the antibody to the 50 kD centriolar antigen stains basal bodies of cilia, I used this antibody to examine cells from the E15 to E20 chick tracheal epithelium. Examination of sections of the chick trachea and intact epithelial cells detached from the tracheas by immunofluorescence using the antibody to the 50 kD centriolar antigen showed different staining patterns. Some of these staining patterns were more common at earlier and others at the later ages examined. The different staining patterns observed are described below. In this description dots less that 1.2 \( \mu m \) in diameter visualized by immunofluorescence will be referred to as small dots whereas dots more than 1.2 \( \mu m \) in diameter will be referred to as large dots.

Examination of sections of the E15 chick trachea after staining with the antibody to the 50 kD centriolar antigen showed the presence of single small dots in most of the epithelial cells (fig 4a, 4b). Examination of similarly stained whole cells detached from the E15 chick tracheal epithelium also showed that a single small dot is visualized in each cell (fig. 7a). Phase contrast microscopy showed that the small dot is located at the apical end of the epithelial cells (fig 7b). No other staining patterns were observed in the cells from E15 chick tracheal epithelium.

In contrast similar immunofluorescent staining of sections of the E16 chick trachea showed three different patterns. In some epithelial cells single small dots (fig. 4c, 5) similar to those present earlier in the epithelial cells of the E15 chick trachea are seen. In addition to these small dots, however single larger dots are also seen in some of the cells in the E16 chick tracheal epithelium (fig. 5). Similar staining of a cell removed from the tracheal epithelium at E16 (fig. 7c) shows that a pair of larger dots are visualized in this cell. A phase contrast image (fig. 7d) of
this cell showed that the larger dots like the small ones are located at the apical end of the cell. Confocal microscopy of these cells (fig. 8) also showed the presence of two large dots located at the apical end of the cell. Staining was not detected in any other part of the cytoplasm in such cells.

In addition to the small dots and the large dots which, in intact cells, were always observed in pairs, examination of sections and whole cells removed from the E16 chick tracheal epithelium also showed a third pattern. In some of the cells, the larger dots were associated with a stained region which extended in a basal direction from these dots (fig. 4c, 5, 7e). Examination of these cells by phase contrast microscopy showed that in cells with this pattern the dot is again located at the apical end of the cell (fig. 7f). Confocal microscope examination of these cells showed the presence of two large apical dots. Also in the stained region extending basally from these dots brighter staining granules could sometimes be observed (fig. 9). Staining was not seen in any other part of the cell. Thus, in the E16 chick tracheal epithelium, cells showing small dots, cells possessing one or two large dots and cells in which the large dots are associated with a stained region are seen.

Examination of sections of the E17 tracheal epithelium after staining with the antibody to the 50 kD centriolar antigen showed that all three patterns described above could also be identified in some of the epithelial cells at E17. In addition in some cells a new pattern consisting of numerous small dots distributed throughout the apical ends of cells (fig. 4d) is observed. Similarly stained whole cells could be identified in preparations removed from the E17 chick
tracheal epithelium (fig. 7g) and comparison with images of the same cells obtained by phase contrast microscopy (fig. 7h) showed that these cells are non ciliated.

Examination of sections of E18, E19 and E20 chick tracheal epithelium after staining with the antibody to the 50 kD centriolar antigen showed that single small dots are present in some of the epithelial cells and numerous small dots distributed throughout the apical end are present in other cells. Cells containing the larger dots with or without the associated stained regions were not seen in cells of the E18, E19 and E20 chick tracheal epithelium. Instead a new staining pattern seen as a band was evident at the apical end of most of the epithelial cells (fig. 6a, 6b, 6c). Many of the whole cells detached from the E20 chick tracheal epithelium and examined by immunofluorescence and confocal microscopy showed a similar pattern. Moreover in many of the cells small dots (fig. 6c, insert, 7i, 10) are sometimes resolved in the stained band or row at the apical end of these cells. Examination of cells with this pattern by phase contrast microscopy showed that these cells are ciliated and that the staining is located at the bases of cilia (fig. 7j). No staining was seen in other parts of these epithelial cells.

Since the staining patterns observed in epithelial cells in frozen sections of E15 to E20 chick tracheas were similar to the patterns seen in isolated epithelial cells detached from such tracheas after incubating them in 50% glycerol (fig. 7), the glycerol treatment apparently did not change the distribution of the 50 kD centriolar antigen in these cells.
Discussion.

The studies described using the antibody to the 50 kD centriolar antigen to label epithelial cells in sections from E15 to E20 chick tracheas and epithelial cells detached from such tracheas showed that a number of different staining patterns can be visualized in these cells. Since a number of these staining patterns, each representing cells with centrioles in different stages of assembly, are seen in the same section, centriolar assembly in different epithelial cells is asynchronous.

In cells of the E15 chick tracheal epithelium a single small immunofluorescent dot is observed which corresponds to the pair of centrioles. This is in agreement with previous ultrastructural studies of centriolar assembly in ciliating cells which showed that at this stage of development (E15) only the diplosomal centrioles are present in epithelial cells of chick trachea (fig. 11, Kalnins and Porter, 1968). The diplosomal centriole pairs stained with the antibody to the 50 kD centriolar antigen appear to be approximately 1\(\mu\)m in diameter which is larger than would be expected from a pair of normal centrioles which were shown to be 0.3\(\mu\)m long and 0.2\(\mu\)m in diameter by electron microscopy (Kalnins and Porter, 1968). The centrioles appear larger in indirect immunofluorescence staining both because the structures containing the antigen are coated by two layers of antibody the outer one of which emits the fluorescence and because the structures labeled are emitting light.
The pair of larger dots present in some cells of the E16 chick tracheal epithelium also likely correspond to the diplosomal centrioles since examination of whole cells shows that two of them are present in each cell. The finding of one large dot or centriole in sectioned material could arise from removal of the other through sectioning. The fact that these dots are larger than the ones present at an earlier stage of differentiation of the epithelium (E15) suggests that material recognized by the antibodies to the 50 kD antigen has accumulated around the diplosomal centrioles at this stage in centriolar assembly. Electron micrographs from previous studies have shown that in some cells of the E16 chick tracheal epithelium fibrillar electron dense material accumulates around the walls of each diplosomal centriole (fig. 12). The two big dots stained with the antibody to the 50 kD centriolar antigen observed by immunofluorescence therefore likely correspond to the diplosomal centrioles and the electron dense material that surrounds them. The observation that the staining around both diplosomal centrioles increases suggests that both diplosomal centrioles participate in the formation of new centrioles.

The 50 kD centriolar antigen containing regions seen in some E16 cells (fig. 13) in close association with the larger dots probably correspond to the clusters of procentrioles seen extending from the diplosomal centrioles in a basal direction in ciliating cells by EM (Kalnins and Porter, 1968). Such procentriole clusters were located very close to the diplosomal centrioles and some of them were even attached to the walls of the diplosomal centrioles (fig. 14). This suggests that the 50 kD antigen is present in the clusters of procentrioles at an early stage of centriolar formation when the procentrioles are starting to assemble around the electron dense cylinders in the core of procentriole clusters and are still immature. From the
immunofluorescence studies it was not possible to determine which of the structures forming the clusters of procentrioles i.e. the cores and/or procentrioles are labelled with the antibody to the 50 kD centriolar antigen. To answer this question ultrastructural studies using immunogold labelled antibodies are needed.

The fact that mature diplosomal centrioles first accumulate the 50 kD centriolar antigen and the finding that the 50 kD centriolar antigen is later found in procentriole clusters assembled near the diplosomal centrioles suggests that diplosomal centrioles do play a key role in the assembly of new centrioles. The finding that the antigen is not detectable in parts of the cell other than the diplosomal region indicates that during ciliogenesis centriolar assembly only occurs in a very localized region of the cell around the two diplosomal centrioles. Studies by EM have shown previously that the new centrioles destined to become basal bodies of cilia in any one ciliating cell are all at the same stage of assembly indicating that centriole assembly in each cell is synchronous (Kalnins and Porter., 1969).

The non-ciliated epithelial cells with numerous small dots in the apical end first seen at E17 (fig. 15) probably correspond to the next stage in centriolar assembly when the newly assembled centrioles are migrating toward the apical surface of cells. Ultrastructural studies have shown that the more mature newly assembled centrioles that have acquired the nine microtubule triplets cells separate from the clusters and become distributed throughout the apical ends of the cells. These centrioles then elongate to their mature length as they migrate toward the apical cell membrane to become basal bodies of cilia.
The band of staining seen along the apical ends of fully ciliated cells from chick tracheal epithelium at E18, E19 and E20 (fig. 16) probably represents hundreds of stained basal bodies known to be present in this region. This is in agreement both with the observations by phase contrast microscopy and with previous ultrastructural studies which have shown that hundreds of basal bodies are located at the apical end of fully differentiated ciliated cells (Kalnins and Porter., 1969).

My studies of centriolar assembly in ciliating epithelial cells showed that the 50 kD antigen appears at a very early stage in the region where the new centrioles are assembled. Electron micrographs of early stages of centriolar assembly show that electron dense material is present in the walls of procentrioles before the MTs assemble. This suggests that the 50 kD centriolar antigen may be present in procentrioles before the MTs have formed. Immunogold staining with antibodies to the 50 kD centriolar antigen are needed to confirm that the 50 kD centriolar antigen appears first during centriole assembly i.e. is present in the walls of procentrioles before the appearance of MTs.

Once the newly assembled centrioles have become aligned perpendicularly to the cell surface and have acquired rootlets and cilia, assembly of new centrioles ceases. It could not be determined from this study if the diplosomal centrioles persist, fragment or disappear after the assembly of the new centrioles has been completed. Serial sections of epithelial cells at later stages in centriolar assembly at the ultrastructural level may be able to answer this question provided that the diplosomal centrioles can be distinguished from the newly assembled ones.
Only three studies have previously examined stages in centriolar assembly at the molecular level. Kochanski and Borisy.. (1990) studied centriolar assembly using biotinylated α- and β-tubulin in porcine kidney epithelial cells. They microinjected the biotinylated tubulin into synchronized cells in G1 phase of the cell cycle and followed its incorporation into centrioles by immunofluorescence and EM. New centrioles, observed by EM assembling at right angles to the mother centrioles, were shown to contain the biotinylated microinjected tubulin. The mother centrioles on the other hand had not incorporated the biotinylated tubulin.

The blepharoplast is a structure that is morphologically analogous to the core of a procentriole cluster, around which the assembly of centrioles during the multiflagellated sperm formation in the fern Marsilea and in the cycad Zamia takes place (Mizukami and Gall., 1966). Using immunogold labelling Pennel et al. (1988) found no evidence for tubulin in the blepharoplast. It was concluded that the blepharoplast rather than providing tubulin in the blepharoplast. It was concluded that the blepharoplast rather than providing tubulin may serve instead as a template for positioning of basal body triplets. Doonan, et al. (1986) studying centriole assembly by immunofluorescence in the fern Platyzoma microphyllum, however, found that tubulin is present at the periphery of blepharooplasts before procentrioles are formed suggesting that this tubulin may be used for the assembly of MT triplets during centriole assembly (Doonan, et al. 1986). The fact that most of the tubulin staining in Platyzoma microphyllum was detected at the periphery of the blepharooplast raises the question whether the staining could have come instead from MTs that run near the surface of the blepharooplast (Pennel et al. 1988). At the resolution of the light microscope it would be impossible to distinguish between these two alternatives. Hence the results from the immunogold labelling studies showing
that the blepharoplasts do not contain tubulin seem more plausible. To determine if tubulin and or the 50 kD centriolar antigen is present in the analogous cores of procentrioles in ciliating chick trachea further studies at the ultrastructural level would have to be undertaken.

Immunogold labelling showed that gamma-tubulin is present in the central parts of the centrioles as well as along the walls of centrioles of MDCK cells and in strands of fibrous material linking the daughter and parent centrioles after they had separated (Fuller, et al., 1995). Based on these observations Fuller, et al., (1995) suggested that a relocation of gamma-tubulin and other proteins required for centriole assembly first occurs from the center to the exterior wall of the parent centrioles where they form a template from which the MTs of daughter centrioles grow at right angles to the walls of the parent centrioles.

Using monoclonal and polyclonal antibodies that recognize a 96 kD centriolar protein, called cenexin, Lange and Gull., (1995) showed by immunofluorescence that in PtK2 cells the 96 kD protein is found only in one centriole of the centriolar pair present in G1 phase of the cell cycle. In S phase the cenexin positive and the cenexin negative centrioles separate and short daughter centrioles assemble at right angles to each parent centriole. Only one centriole of the resulting two pairs continues to be cenexin positive through the G2 phase. It is during the G2/prophase transition that the other or second parent centriole becomes cenexin positive. Thus, in prophase there are two cenexin positive centrioles, one in each pair of centrioles. The authors of this study believe that centrioles mature only after they become cenexin positive and that as a result each daughter cell receives both a mature and an immature centriole after mitosis. In
contrast to cenexin which is incorporated into the mother centriole only when it matures (Lange and Gull., 1995), the 50 kD centriolar antigen is present both on the two diplosomal centrioles and also on the immature centrioles destined to become basal bodies of cilia.
Conclusions

1. The first sign of centriole assembly is the increase in the amount of staining around the diplosomal centrioles indicating that centriolar precursors and/or procentrioles containing the 50 kD centriolar protein first accumulate around both diplosomal centrioles early in centriole assembly.

2. The other immunofluorescent staining patterns seen indicate that the procentrioles in clusters, centrioles migrating to the apical cell surface, and the recently matured basal bodies of cilia also contain the 50 kD centriolar protein.

3. Since the 50 kD centriolar protein is present very early during centriole assembly at sites where procentrioles are observed, in centrioles at different stages in assembly and also in mature centrioles and basal bodies, the 50 kD centriolar protein is likely a component of the electron dense material in centriolar walls.
Fig. 2. Immunoblot of SDS-PAGE of proteins from chicken tracheal epithelial cells after transfer to nitrocellulose and treatment with the serum used that labels centrioles and basal bodies of cilia by immunofluorescence, showing that the antibodies in the serum react with a 50 kD protein. Location of marker proteins ranging from 14 to 97 kD are indicated at right.
Fig. 3. Double immunofluorescent staining of a ciliated epithelial cell from an E20 chick trachea with the antibody to the 50 kD centriolar antigen (a) and antibody to α-tubulin (b) The same cell is seen by phase contrast microscopy in c. A band of staining (arrow) is seen at the apical end of the cell (a) below the cilia stained with antibodies to α-tubulin (b). By phase contrast microscopy (c) a dense band can be seen in this region (arrow). Bar=10μm.
Fig. 4. Immunofluorescence staining of cross sections of E15 (a, b), E16 (c) and E17 (d) chick trachea with the antibody to the 50 kD centriolar antigen. The centriolar antibody stains small dots (arrows) in (a, b, c and d) In (c) larger regions (open arrows) associated with larger dots (solid arrowhead) are stained in addition. In E17 chick trachea (d) numerous small dots are evident in the apical ends of some epithelial cells (large arrows). Lines in (a, b, c, and d, indicate the boundary between lumen (L) and epithelium (E) and epithelium (E) and connective tissue (CT). Bar=10\mu m.
Fig. 5. Immunofluorescent staining of a cross section of E16 chick trachea stained with the antibody to the 50 kD centriolar antigen. Different staining patterns are seen in this section. In some cells single small dots (arrows) are observed whereas, in others larger dots (large arrow) or regions (open arrows) associated with a dot (solid arrowhead) are stained. Lines indicate the boundary between lumen (L) and epithelium (E) and epithelium (E) and connective tissue (CT). Bar=10μm.
Fig. 6. Immunofluorescent staining of cross sections of E20 chick trachea (a,b,c) stained with the antibody to the 50 kD centriolar antigen. In (a) (b) and (c) irregular bands (open arrows) at the apical ends of fully ciliated cells are stained. A portion from a similar section at higher magnification showing a band at the apical end of fully ciliated cells cut obliquely in which smaller granules can be visualized is shown in the insert. Lines in (a, b. c and the insert) indicate the boundary between lumen (L) and epithelium (E) and epithelium (E) and connective tissue (CT). Bar=10μm.
Fig. 7. Immunofluorescent staining of intact epithelial cells from E15 (a, b), E16 (c, d, e, f), E17 (g, h) and E20 (i, j) chick trachea with the antibody to the 50 kD centriolar antigen (a, c, e, g, and i) with the corresponding phase contrast images (b, d, f, h, and j). These cells were selected and arranged to illustrate the most plausible sequence of changes in the distribution of the 50 kD centriolar antigen during ciliogenesis. Immunofluorescence staining with the antibody to the 50 kD centriolar antigen was present at the apical end of the cells which could be distinguished from the basal end because the basal end contained the nucleus. Dots are seen at the apical end of each of the two cells shown in (a) and (b). The larger pair of dots seen in (c) are also located at the apical end of the cell (d). One bright immunofluorescent stained dot (solid arrowhead) (e) associated with a strongly stained region (open arrow) is seen at the apical end of the cell (f). A cluster of bright dots (g) is seen at the apical end of a non ciliated cell observed by phase contrast microscopy in (h). A row of dots (i) is visualized along the apical end of the ciliated cell seen by phase contrast microscopy in (j). Cells showing similar staining patterns in sections of trachea are seen on the left. Bar=10μm.
Fig. 8. Confocal images of serial optical sections taken at 0.5μm intervals through a single epithelial cell from E16 chick trachea stained with the antibody to the 50 kD centriolar antigen. Two immunofluorescent dots at the apical end of this cell are visualized. Only one dot is seen in sections (b) and (c). The two dots are visualized in sections (d), (e) and (f). Only the second dot of the pair is seen in sections (g) and (h). Bar=10μm.
Fig. 9. Confocal images of serial optical sections taken at 0.5μm intervals through a ciliating epithelial cell from E16 chick trachea stained with the antibody to the 50 kD centriolar antigen. Regions (open arrowheads) showing immunofluorescence in sections (d), (e), (f), (g), (h), (i) and (k) are located close to large dots (solid arrowheads) seen in sections (g), (h), (i), (j) and (k). No other region of this cell is stained. Bar=10μm.
Fig. 10. Confocal images of serial optical sections taken at 1μm intervals through a single cell from E20 chick trachea stained with the antibody to the 50 kD centriolar antigen. A band of staining (arrow) is visualized by immunofluorescence at the apical end of this cell in sections b), c), d), e), f) and g). No staining is seen in other parts of this cell. Bar=10μm.
Fig. 11. Two cells detached from E15 chick tracheal epithelium and immunofluorescently stained with the antibody to the 50 kD centriolar antigen (a) and seen by phase contrast microscopy (b). The centrioles are seen as a dot at the apical part of the cell on the right (a). The centrioles in the cell on the left are out of the plane of focus. A section through the apical end of a similar cell is shown in the electron micrograph in (c) (Kalnins, unpublished) in which two diplosomal centrioles can be seen. Bar in a and b=10μm. Bar in c=250nm.
Fig. 12. A cell detached from E16 chick tracheal epithelium immunofluorescently stained with the antibody to the 50 kD centriolar antigen (a) and seen by phase contrast microscopy (b). The mature diplosomal centrioles are seen as a large dot at the apical end of the cell. A section through the apical end of another cell at a similar early stage in centriole assembly is seen in the electron micrograph in c (Kalnins, unpublished) which shows that dense material has accumulated around the diplosomal centrioles. Bar in a and b=10μm. Bar in c=250nm.
Fig. 13. Cell detached from E16 chick trachea immunofluorescently stained with the antibody to the 50 kD centriolar antigen (a) and seen in phase contrast micrograph (b). A large dot (solid arrowhead) associated with a larger stained region (open arrow) is seen in the apical end of the cell the end opposite to the basal end which contains the cell nucleus (a). A section through the apical end of another cell, at a similar stage in centriole assembly is seen in the electron micrograph (c) (Kalnins and Porter., 1969). It shows one of the diplosomal centrioles (C) associated with procentrioles (Pc) and cylinders (Cy). Bar in a and b=10μm. Bar in c=250nm
Fig. 14. A confocal image of one optical section of 0.5μm taken through a single cell detached from E16 chick trachea stained with the antibody to the 50 kD centriolar antigen. The diplosomal centrioles (C) are seen as two dots associated basally with a larger stained region (Pc). Sections through the apical ends of other cells that are at a similar stage in centriole assembly are seen in electron micrographs b and c (Kalnins and Porter., 1968). One of the diplosomal centrioles can be seen in an oblique section in b and another in cross section in c. Numerous clusters of procentrioles can be seen in the region extending basally from the diplosomal centriole in b forming a region that is similar to the one labelled (Pc) in a. Bar in a=10μm. Bar in b and c=500nm.
Fig. 15. Cell detached from the E17 chick trachea stained immunofluorescently with the antibody to the 50 kD centriolar antigen (a) and seen by phase contrast microscopy (b). Bright dots can be seen at the apical end of the cells. the end opposite to the basal end which contains the nucleus (a). A section through the apical end of another cell, at a similar stage in centriole assembly, is seen in the electron micrograph (c) (Kalnins and Porter. 1969) which shows that many centrioles detached from clusters at later stage of development than those in clusters are present in the apical end of the cell. Bar in a and b=10μm. Bar in c=500nm.
Fig. 16. Two ciliated cells detached from the E20 chick epithelium immunofluorescently stained with the antibody to the 50 kD centriolar antigen (a) and seen by phase contrast microscopy (b). Staining is seen as a bright row at the apical ends of the cells just below the cilia (a). The electron micrograph (c) (Kalnins and Porter, 1968) shows that basal bodies in fully ciliated cells are similarly distributed. Bar in a and b=10μm. Bar in c=3μm.
Distribution of the 50 kD centriolar antigen in rat pinealocytes \textit{in situ}.
Introduction.

Centrioles and the related basal bodies can be readily identified by EM as cylindrical structures with walls that contain nine blades of MT triplets (Reviewed by Albrecht-Buehler., 1992; Paintrand, et al., 1992). This structure of centrioles and basal bodies has been highly conserved during evolution and is found in different organisms and cell types. As mentioned in the introduction several proteins are known to be present in the centrioles, including tubulin (Cande., 1990), gamma-tubulin (Fuller., 1995; Dibbayawan. et al., 1995) and a 50 kD protein (Connolly, et al., 1978; Turksen et al., 1982). The 50 kD protein is recognized by Western blotting using non-immune rabbit sera which have been shown to stain centrioles and basal bodies by immunofluorescence labelling (Turksen et al., 1982). Using these rabbit sera the position of centrioles and basal bodies can be readily identified in a wide variety of cells from different species (Connolly and Kalnins., 1978; Turksen et al., 1982). In the previous chapter (Chapter 2) this antibody was used to follow the assembly of centrioles in cells undergoing ciliogenesis in the chick trachea.

Besides acting as basal bodies of cilia centrioles are also the central components of the centrosome and are normally surrounded by MTOC’s from which MTs radiate. In interphase cells, pairs of centrioles are often found in an orthogonal orientation reflecting the fact that the new centrioles or procentrioles arise by budding at right angles from the walls of the pre-existing parent centrioles (Gall., 1961; Fare-Fremiet., et al. 1956).

Very little is known about the function of centrioles except that they can serve as basal
bodies of cilia and flagella. Because of the cylindrical structures of centrioles, the arrangement of MT in their walls and their orthogonal orientation it has been proposed that they also act as cellular sensors to locate signal sources in the environment (Albrecht-Buehler, 1990). Centrioles may also participate indirectly in the concentration of certain centrosomal proteins such as the gamma-tubulin that nucleate MT assembly. Consistent with this view is the observation that gamma-tubulin staining is seen over a diffuse region or highly concentrated at the spindle poles of acentriolar cells and normal mitotic centriole-containing cells of a Drosophila melanogaster cell line respectively (Debec, et al., 1995). The existence of insect cell lines lacking centrioles and the fact that most plant cells can divide in absence of centrioles also indicate that centrioles are unnecessary for cell replication. In contrast Sluder (1989) and Rieder (1985) have obtained evidence that centrioles are, however, necessary for the reproduction of sea urchin centrosomes by showing that centrosomes with centrioles replicate before each mitosis whereas, those without centrioles did not reproduce.

In some terminally differentiated cells which have lost the capacity to divide centrioles are unnecessary and disappear at a particular stage of differentiation; these include skeletal muscle cells, Drosophila wing epidermal cells and pinealocytes. For example ultrastructural (Przybylski., 1971) and immunofluorescence (Connolly, et al., 1985) studies with antibodies to the 50 kD centriolar protein (Connolly, et al., 1985) have shown that in the multinucleated myotubes that develop from the fusion of centriole containing myoblasts, the centrioles are absent and centriolar staining with the 50 kD antibody cannot be detected (Warren, 1974; Cartwright and Goldstein, 1982; Connolly, et al., 1985). In these myotubes the nuclear envelope instead of the
centrosome acts as the MT nucleating site (Tassin, et al., 1985). In the terminally differentiated *Drosophila* wing epidermal cells that have lost centrioles apical electron dense plaques on the plasma membrane act as the MT nucleating sites (Tucker, et al., 1986).

Despite the fact that centrioles disappear in terminally differentiated skeletal muscle cells and *Drosophila* wing epidermal cells, intermediate stages in the breakdown of centrioles such as fragments of centriolar walls have not been found in these cells. Since structures resembling fragments of centriolar walls have been found in terminally differentiated rat pinealocytes by EM, (Lin, 1970), these cells were chosen to study the breakdown of centrioles. On the basis of ultrastructural studies it was suggested that the pinealocyte centrioles first become modified in the period between 3-6 weeks after birth by elongating to twice their normal length and then fragmenting. In the adult rat pinealocytes, structures interpreted as fragments of walls of the elongated centrioles referred to as sheaves in which a varying number of singlet, doublet and triplet MTs of different lengths are present can be detected. As many as ten of these sheaves were observed in one section of a pinealocyte, the longest being six times the length of a normal centriolar MT triplet. Similar structures have also been observed in pinealocytes of other species including the golden hamster (Lin, et al., 1987), guinea pig (Lin, 1972) and cat (Calvo, et al., 1991). Structures that resemble rootlets with striations having a periodicity of about 70 nm are also seen in close association with these fragments (Lin, 1972; Lin, 1970). In addition electron dense structures surrounded by the fragments of centriolar walls were found in the adult hamster pinealocytes (Lin et al., 1987). It has been suggested that modifications in or loss of the proteins that normally link the MT triplets may lead to the separation of the triplets in the centriolar wall.
and to the subsequent breakdown of centrioles (Calvo, 1991). In the pineal gland such a breakdown of centrioles occurs only in the pinealocytes since centrioles of pineal astrocytes remain normal (Lin, 1972; Calvo, 1991). Breakdown of centrioles occurs in rats exposed to light and also in the dark (Lin, 1970).

In this chapter, I investigated whether or not the structures resembling the fragments of centrioles present in the adult rat pinealocytes are derived from centrioles by determining whether or not they contain the 50 kD antigen present in normal centrioles. Parallel ultrastructural studies were undertaken to confirm that pinealocytes indeed have fragments, similar to those described in the literature, at the time they were examined by immunofluorescence. The results obtained show that in contrast to the new born rat pineal gland where immunofluorescence staining of pinealocytes with the antibody to the 50 kD centriolar antigen typically labels single dots, in the pinealocytes from the pineal gland of 11-day-old and adult rats a larger region composed of dots of various sizes is labelled. This staining pattern is consistent with the idea that the singlets, doublets and triplets of MTs visualized in adult pinealocytes by EM are derived from centrioles and indicates that the 50 kD centriolar antigen continues to be associated with the resulting fragments of centrioles.
Materials and methods

Animals.

10 Newborn, 10 11-day-old and 10 adult Sprague Dawley rats (Charles River, Canada) of both sexes and adult chickens were used. Husbandry was provided by the Division of Comparative Medicine at the University of Toronto according to the standards of the Canadian Council of Animal Care.

Antibodies.

Antibodies used in the experiments reported in this chapter were described under Materials and Methods in Chapter 2.

Frozen sections.

After experimental animals were sacrificed by asphyxiation in CO₂ in an euthanasia chamber, their pineal glands were removed, embedded in Tissue-Tek and frozen immediately in 2 methyl butane in liquid nitrogen. Sections approximately 7μm thick were cut using a cryostat, placed on coverslips and air dried.

Immunofluorescence.

Sections of pineal glands were first fixed in methanol and then in acetone both at -20°C for 5 min, hydrated in PBS for 15 min and then treated with non-immune rabbit serum diluted 1:30 in PBS for 30 min at room temperature. After washing three times for 5 min each in PBS the sections were treated for 30 min at room temperature with fluorescein-conjugated F(ab)₂
fragments of goat anti-rabbit IgG diluted 1:40 in PBS and then washed three times 5 min each in PBS. To stain nuclei, the PBS used for the last wash contained 1 μg of Hoechst 33258 per ml of buffer. Sections of pineal glands from which treatment with the antibody to the 50 kD centriolar antigen was omitted treated with fluorescein-conjugated F(ab')2 fragments of goat anti-rabbit IgG diluted 1:40 in PBS served as control. The sections were mounted in Vinol containing DABCO to prevent bleaching and examined with a Zeiss Photomicroscope II equipped with epifluorescence optics and FITC and Hoechst specific filters. Photographs were taken with a TMAX P3200 film, processed with TMAX developer and printed using Polycontrast III RC paper all from Kodak.

**Electron microscopy**

Rat pineal glands were fixed for 2 h at room temperature in 2.5% glutaraldehyde in 0.1M phosphate buffer, postfixed for 15 min in 0.5% osmium tetroxide (J.B. EM Services) in the same buffer at room temperature and dehydrated through a graded series of ethanol. The glands were then infiltrated in 1:1 Epon/propylene oxide before embedding in Epon. Ultrathin sections of the pineal gland were stained with lead citrate and uranyl acetate and viewed with a Hitachi 7000 transmission electron microscope at 75 kV.
Results

Characterization of serum.

The serum used for studies described in this chapter was characterized as described in the Results section of chapter 2

Distribution of the 50 kD centriolar antigen in pinealocytes in situ.

The rat pineal gland forms from an evagination of the roof of the diencephalon at E14 (Ariens Kappers, 1960) and in the newborn rat consists of differentiating pinealocytes surrounded by blood vessels and a thin connective tissue capsule. In the adult rat pineal gland approximately 82% of the cells are pinealocytes. 12% are glial cells and 5% are endothelial cells (Wallace, 1969). In addition, fibroblasts are present in the capsule and in the septa which project from the capsule into the pineal gland (Zimmerman and Tso, 1975).

A section of the new born rat pineal gland stained with the antibody to the 50 kD centriolar antigen shows that most of the centriole pairs are visualized as single immunofluorescently stained dots of similar size (fig. 17a). In agreement with previous studies (Lin, et al 1970) EM of the pineal gland at this stage of development showed that centrioles including those of pinealocytes have a normal morphology (fig. 18). EM also showed that centriole pairs in some pinealocytes were located in the perinuclear area whereas in others they were located further away from the nuclei in the apical end of the cells. In some of the pinealocytes one centriole of the pair at the apical end served as a basal body of a primary cilium (fig. 18).
In contrast, examination of sections of 11-day-old and the adult rat male and female pineal glands by immunofluorescence showed that in most of the pinealocytes the antibodies to the 50 kD centriolar antigen stain regions that vary considerably in size and shape in which smaller stained particles and some elongated fibers can be identified (fig. 17b, 17c). In comparison to the stained regions in pinealocytes from newborn rat pineal gland which are relatively small and homogeneous in size and shape, the stained regions in most of the 11-day-old and adult pinealocytes are larger and much more heterogeneous (compare fig. 17a with figs. 17b and 17c). In a minority of cells in the 11-day-old and adult pineal gland which probably were glial cells, endothelial cells or fibroblasts staining of dots similar in size to those seen in all cells in the newborn pineal gland were noted (fig. 17b, 17c).

Examination of the adult pinealocytes by EM revealed the presence of structures composed of variable numbers of MTs embedded in an electron dense matrix resembling material forming the walls of normal centrioles (fig. 19). The MTs in these structures previously referred to as bundles or sheaves (Lin., 1970) were several times the 0.4-0.7μm length of the triplets in a normal centriole (fig. 20). Cross sections of some of these structures showed that they resembled incomplete centrioles that lacked most of the MT triplets (fig. 19a, 19b, 19c, 19d). In addition to the triplet MTs, however, doublet and single MTs embedded in a similar matrix were also seen, some of them in an orthogonal arrangement (fig. 19b) that resemble in orientation normal replicating centrioles. Although generally these structures that resembled fragments of centriolar walls were visualized in clusters in a region close to the Golgi apparatus (fig. 19a) they were also sometimes seen at the apical ends of pinealocytes (fig. 21a). Structures that resemble
striated rootlets were also seen in both of these regions (fig. 21a, 21b, 21c). In some cells fragments of centrioles were observed in a perinuclear position, along processes and at the end of a process (not shown). Normal centrioles were never observed in adult pinealocytes. Although MTs that were not associated with the electron dense matrix, interpreted as cytoplasmic MTs, were also visualized in these regions, they did not obviously radiate from the MT containing structures resembling fragments of the centriolar wall (fig 22). No difference in the staining patterns could be observed between pinealocytes of male and female rats. In contrast to pinealocytes the other cell types such as endothelial cells and glial cells present in the adult rat pineal gland had normal centrioles with nine MT triplets in their walls (not shown).

In comparison immunofluorescence studies using frozen sections of the adult chicken pineal gland and the antibody to the 50 kD centriolar antigen showed that centriole pairs in chick pinealocytes are visualized as single immunofluorescent dots of similar size (not shown). Ultrastructural studies showed that centrioles of chick pinealocytes have normal morphology with nine triplets of MTs in their walls (not shown). Thus in contrast to rat fragmentation of centrioles does not seem to occur in the chick pinealocytes. Further work was therefore carried out on rat pinealocytes.

**Discussion.**

Since previous EM studies showed that fragmentation of centrioles occurs in adult rat pinealocytes (Lin, 1972), the distribution of the 50 kD centriolar antigen during such breakdown of centrioles was undertaken by immunofluorescence. My results suggest that the structures
containing MTs embedded in an electron dense matrix seen in adult rat pinealocytes by EM not only resemble segments of centriolar wall structurally but also contain the 50 kD centriolar antigen. These findings are consistent with the view that these structures composed of triplet, doublet and single MTs embedded in a dense matrix, are indeed centriole-related structures as suggested by others from ultrastructural studies (Lin, 1970). I have confirmed by EM that, like the centrioles, these fragments are usually located either in a perinuclear region near the Golgi apparatus or at the apical ends of pinealocytes (Lin, 1970). Since in the adult pinealocyte these structures occupy a larger region than the two centrioles in newborn pinealocytes this could account for some of the increase in size of the region stained by immunofluorescence with antibodies to the 50 kD centriolar antigen in adult pinealocytes as compared to the pinealocytes in the newborn rats. Moreover the fibers seen by immunofluorescence probably correspond to the sheaves or elongated bundles of MTs seen by EM. Taken together these findings are consistent with the view that the MT containing structures seen by EM in adult pinealocytes indeed result from the elongation and subsequent fragmentation of their centrioles. The electron dense structures surrounded by fragments of centriolar walls found in adult hamster pinealocytes (Lin et al, 1987) are not present in adult rat pinealocytes.

The regions stained with the antibody to the 50 kD centriolar antigen in sections of the adult rat pineal gland are of many different shapes and sizes and are composed of small granules and fibers. It appears also that much more material recognized by the antibody to the 50 kD centriolar antigen is present in the adult rat pinealocyte than can be accounted for simply by the elongation and subsequent fragmentation of the two centrioles. Since my ultrastructural studies
showed that some centriole fragments are oriented at right angles to each other in a configuration that resembles normal replicating centrioles. The possibility exists that these centriolar derivatives may also have the capacity to replicate. Observations which suggest that incomplete centrioles may also be able to replicate have been made by Moscoso et al. (1988). These investigators reported that in ciliating epithelial cells of human trachea and bronchi, centrioles lacking a number of MTs were oriented at right angles to other centrioles which also lacked some MT triplets.

It appears that after centrioles in adult rat pinealocytes elongate, they fragment and then replicate more fragments (i.e. triplets and doublets) either in a region close to the Golgi apparatus or near the apical end of the cell depending on the position of the diplosomal centrioles. At least in one cell, fragments were seen in a perinuclear position close to the Golgi apparatus, along cell processes and at the end of a process. This indicates that they may also migrate toward the apical ends of pinealocytes. The migration of centriolar fragments thus may be similar to the migration of centrioles reported in olfactory neurons (Heist and Mulvaney., 1968; Popov and Tsyganova., 1996) where centrioles replicate in a perinuclear position and then migrate toward the apical end of the cell where they become basal bodies of cilia.

The synthesis of hormones such as serotonin and melatonin is regulated by environmental lighting in both the newborn and the adult rat pineal gland (Zweig, et al., 1966) While in adults photic stimulation of the retina signals the pineal gland via a neuronal pathway, in the newborn rat, the pineal gland itself appears to be capable of detecting light. Experiments exposing blinded
newborn rats to light demonstrated that serotonin is produced in the pineal glands of these rats (Zweig, et al., 1966). Additional experiments in which blinded newborn rats with a hood covering their heads were exposed to light demonstrated that hooding prevented the production of serotonin by the pineal gland. These experiments suggest that the photic stimulation for the production of serotonin is detected by newborn rat pineal gland by a non-retinal route and that the pineal gland itself is capable of detecting light.

A sensory function such as reception of light has been proposed for primary cilia for which the older of the two diplosomal centrioles serves as a basal body. Primary cilia have 9+0 arrangement of MTs and are present in many cell types including cells of the mouse hypophysis (Barnes., 1961), and photoreceptor cells (Barnes., 1961). It has also been proposed that the pair of centrioles in an orthogonal orientation may function as cellular devices to locate infrared signal sources in the environment (Albrecht-Buehler and Bushnell, 1979; Albrecht-Buehler., 1981). Since newborn rat pinealocytes have a morphological similarity to retinal photoreceptors (Zimmerman and Tso., 1975) they may also have the capability to detect light. Rhodopsin, the molecule responsible for light absorption in retinal photoreceptors however has not been found in rat pinealocytes (Vigh-Teichman., 1986). It is therefore possible that the normal pair of centrioles in an orthogonal orientation in the newborn rat pinealocytes may, instead of rhodopsin, act as receptors of light for the regulation of serotonin and melatonin production in the new born rat pineal gland. Since centrioles are fragmented in the adult rat pinealocytes, these cells may have lost the capability to detect light which requires an intact pair of centrioles in an orthogonal orientation (Albrecht-Buehler and Bushnell., 1979; Albrecht-Buehler., 1981).
Previous studies in our laboratory showed that in skeletal muscle, myoblasts which contain centrioles and are capable of dividing and migrating lose centrioles soon after they fuse to form myotubes (Connolly, 1985). Fragments of centrioles, however, have not been observed in these terminally differentiated muscle cells, perhaps because they are rapidly destroyed. Cell replication in the rat pineal gland is very active just after birth and gradually decreases until it stops by postnatal day 13 (P13) (Wallace et al., 1969). After P12-17 the photoreceptor-like features of rat pinealocytes such as inner segments and primary cilia are lost (Zimmerman and Tso, 1975). My results from immunofluorescence studies suggest that centriolar fragmentation occurs in rat pinealocytes just before this happens at P11. All of these results suggest that centrioles in rat pinealocytes fragment after cell replication and cell migration has been completed and/or perhaps after the centrioles and/or cilia are no longer required for light detection.

EM of the adult rat pinealocytes showed that MTs do not radiate from the regions containing fragments of centriolar walls as they do from regions containing intact centrioles. This may indicate that the proteins that normally nucleate MT assembly, such as gamma-tubulin are not associated with the fragments of centrioles and have redistributed to other regions of the pinealocyte during or after the fragmentation of their centrioles.
Conclusions.

1. In rat pinealocytes after 11 days of age the 50 kD centriolar protein is present in granules and fibers instead of centrioles.

2. Structures resembling fragments of walls of centrioles but not centrioles are present in pinealocytes after 11 days of age. suggesting that the fragments contain the 50 kD centriolar protein and account for the change in the staining pattern observed.

3. An increase in the material stained with the antibody to the 50 kD centriolar protein may occur in rat pinealocytes after 11 days of age. The capacity of fragments of centriolar walls to replicate more fragments may account for this increase in the amount of material stained with the antibody to the 50 kD centriolar protein.

4. The 50 kD centriolar protein is a component of the electron dense material present around MTs in the structures resembling fragments of walls of centrioles.

5. In pinealocytes with centriolar fragments in situ MTs do not obviously radiate from the regions containing the fragments.
Fig. 17. Immunofluorescence staining of sections of the newborn (a) 11-day-old (b) and adult (c) rat pineal gland with the antibody to the 50 kD centriolar antigen. The stained dots (small arrows) in a are fairly uniform in size and correspond to a pair of normal centrioles. The regions stained with the same antibody in a section from the 11-day-old (b) and adult rat pineal gland (c) in contrast are larger and more variable in size and shape (large arrows) and many are composed of small granules and fibers (open arrows). Relatively few dots (small arrows) similar to those seen in the newborn pinealocytes (a) are seen in the 11-day-old (b) and adult (c) pineal gland. Bar=10μm.
Fig. 18. Electron micrograph of a section through a pinealocyte from the newborn rat pineal gland showing a pair of normal centrioles (arrow) in an orthogonal arrangement at the apical end of the cell. One of the centrioles serves as basal body of a primary cilium. An oblique section of another normal centriole from a pinealocyte is shown in the insert at a higher magnification. Bar=1.5μm.
Fig. 19. Electron micrographs of sections through pinealocytes from an adult pineal gland showing fragments resembling centriolar walls in which MT triplets in cross section embedded in electron dense material can be seen (large arrows) (a,b,c, and d). Parts of an adjacent Golgi apparatus can be seen in a. An elongated fragment oriented approximately at a right angle to another fragment cut in cross section is evident in b. Numerous structures resembling fragments of centriolar walls cut in oblique sections (small arrows) are seen in the regions shown in a and c. Bar=10μm.
Fig. 20. Electron micrographs of adjacent sections through a pinealocyte from an adult pineal gland showing two elongated bundles of MT embedded in an electron dense matrix (arrows). A bundle of MTs can also be seen in association with and oriented at right angles to one of them (open arrows). Bar=400nm.
Fig. 21. Electron micrographs of sections through the adult rat pineal gland. In a the apical ends pinealocytes are seen, one of them showing structures resembling fragments of centriolar walls (small arrows) and a striated rootlet (large arrows). Striated rootlets (large arrows) near centriolar fragments (small arrows) can also be seen in b and c. Bar=200nm.
Fig. 22. Electron micrograph of a section through a pinealocyte from adult pineal gland showing several fragments resembling parts of a centriolar wall (large arrows) in a region near the Golgi apparatus. Cytoplasmic MTs (small arrows), also present in this region, do not seem to be radiating from the fragments of centrioles. Bar=1.5μm.
Distribution of the 50 kD centriolar antigen in rat pinealocytes \textit{in vitro}.
Introduction.

Cell culture systems have been extensively used because they often provide more optimal conditions for studying cells. In culture, cells can be induced to differentiate and display many of the same morphological characteristics they express when they differentiate in vivo. The changes cells undergo when they differentiate in cultures can be readily examined under the microscope under optically ideal conditions. Moreover, the effect of adding and removing hormones or growth factors on cell differentiation can be studied.

Centriole loss has been shown to occur in some differentiating cell types both in vivo and in vitro. For example immunofluorescence studies using the antibody to the 50 kD centriolar antigen and skeletal muscle cells in culture showed that in vitro as in vivo myoblasts contain a normal pair of centrioles. However, when these cells fuse to form myotubes centrioles disappear both in vivo as well as in vitro (Connolly et al., 1985; Tassin et al., 1985; Przybylski, 1971). Bytrevskaya et al., (1992) showed that cultured human endothelial cells from 30-40 year old donors that contained fragmented mother and normal daughter centrioles were able to continue dividing in culture.

Results from studies described in the previous chapter showed that in contrast to the newborn rat pineal gland where immunofluorescence staining of pinealocytes with the antibody to the 50 kD centriolar antigen typically labels a single dot, in the pinealocytes from the pineal gland of 11-day-old and adult rats a larger region composed of centriolar fragments is labelled.
This latter staining pattern is consistent with the idea that the singlets, doublets and triplets of MTs visualized in adult pinealocytes by EM are derived from centrioles and indicates that the 50 kD centriolar antigen continues to be associated with these structures after the fragmentation of centrioles. It is not known if a similar fragmentation of centrioles occurs or can be induced in pinealocytes maintained in culture. If available, such a system would allow studies of centriole fragmentation in greater depth under the more controllable conditions in vitro.

I therefore determined whether or not a fragmentation of centrioles similar to that seen in situ occurs or can be induced in pinealocytes in vitro. In this chapter I show that fragmentation similar that in vivo also occurs in pinealocytes in vitro but not in fibroblasts present in the same cultures. I also show that in pinealocytes with fragmented centrioles, the regions containing the fragments are able to nucleate MT assembly.

Materials and methods.

Animals.

Two day old and adult Sprague Dawley rats (Charles River, Canada) were used to obtain pineal glands. Husbandry was provided by the Division of Comparative Medicine at the University of Toronto according to the standards of the Canadian Council of Animal Care.

Antibodies.

Antibodies used for these experiments were described in Chapter 2.
Cell cultures.

Cells from two-day-old and adult rat pineal glands were cultured on coverslips as previously described by Rowe., et al (1977) and modified by Steinberg., et al (1981). Briefly, after sacrifice 12 two-day-old or 12 adult rat pineal glands were dissected out and placed in PBS, pH 7.2, containing 0.25% crude trypsin. Glands were triturated and the fragments obtained were incubated for one hour with occasional agitation in DMEM (Dulbeco's modification of Eagle's medium-high glucose formulation) (Gibco, Burlington ON). Cells from the resulting cell suspension were grown at 36-37°C in 4% CO₂ in air on coverslips in DMEM containing 10% fetal bovine serum (Sigma, Mississauga ON) and 100 IU penicillin, 0.25 µg fungizone and 100 µg streptomycin (Gibco) per millilitre. The cells from the two-day-old rat pineal glands were cultured for periods of up to 74 days changing the culture medium once every three days and examined at different times after culturing. The cells from the adult rat pineal gland were maintained for 2 days in culture and then examined.

Immunofluorescence.

Cultured cells on coverslips from 2-day-old and adult pineal glands were fixed and stained with the antibody to the 50 kD centriolar antigen as described in chapter 2. For double staining the cultured cells were incubated in addition with monoclonal antibodies to α-tubulin diluted 1:500 in PBS. After washing 3 times in PBS 5 min each they were then treated with a 1:1 mixture of fluorescein-conjugated F(ab)₂ fragments of goat anti-rabbit IgG diluted 1:40 in PBS and rhodamine-conjugated F(ab)₂ donkey anti-mouse IgG diluted 1:40 in PBS. The cultured cells were washed 3 times in PBS and to stain nuclei, the PBS used for the last wash contained 1µg of
Hoechst 33258 per ml of buffer. Cultured cells on coverslips from which treatment with the antibody to the 50 kDa centriolar antigen was omitted, treated with fluorescein-conjugated F(ab)₂ fragments of goat anti-rabbit IgG diluted 1:40 in PBS served as controls for single immunofluorescence experiments. Cultured cells on coverslips from which treatment with the antibody to the 50 kDa centriolar antigen and to α-tubulin was omitted, treated with a 1:1 mixture of fluorescein-conjugated F(ab)₂ fragments of goat anti-rabbit diluted 1:40 in PBS and rhodamine-conjugated F(ab)₂ donkey anti-mouse IgG diluted 1:40 in PBS served as controls for the double immunofluorescence experiments. The cultured cells were mounted, examined and photographed as described in chapter 2.

**Electron microscopy.**

Cultured cells on coverslips were fixed for 10 min in 2% glutaraldehyde in 0.1M phosphate buffer at room temperature, postfixed for 15 min in 0.5% osmium tetroxide (J.B. EM Services) in the same buffer at room temperature and then dehydrated through a graded series of ethanol. The cultures were then infiltrated in 25, 50 and 75% Epon in ethanol before embedding in Epon. Ultrathin sections of cultured cells cut parallel to the coverslips, were stained with lead citrate and uranyl acetate and viewed with a Hitachi 7000 transmission electron microscope.

**MT depolymerization/repolymerization.**

MTs in cultured cells were depolymerized by keeping the cells at 0°C for 45 min and then allowed to polymerize by incubating the cells at 37°C for 30 sec, 3, 6, 9 and 12 min. MTs in cultured cells which were not depolymerized and kept at 37 °C served as control.
Results.

Characterization of serum.

Serum containing antibody to the 50 kD centriolar antigen used in the experiments described in this chapter was characterized as described in the results in chapter 2.

Distribution of the 50 kD centriolar antigen in pinealocytes in vitro.

To determine if a fragmentation of centrioles similar to that in vivo also occurs in pinealocytes in vitro, cells from 2 day old rat pineal glands with intact centrioles were cultured and examined at various time intervals up to 74 days. In such cultures two cell types, pinealocytes and fibroblasts were present. The majority of the cells present were the small round pinealocytes with nuclei that stained brightly with Hoechst (fig. 23b, d, f, and h). In contrast, the fibroblasts were elongated and relatively flat cells with larger oval-shaped nuclei that stained weakly with Hoechst (fig. 23f and h). The difference in staining and size of the nuclei allowed the two types of cells to be distinguished. Although some of the pinealocytes in these cultures grew attached to coverslips most of them grew in clusters on top of the fibroblasts (fig. 23d). Endothelial cells which can be distinguished from fibroblasts by their polygonal shape and elongated nuclei were not present in these cultures.

Examination of cells in 8-day-old cultures double stained with the antibody to the 50 kD centriolar antigen and Hoechst to visualize nuclei showed that the centrioles in the cultured pinealocytes (fig. 23a) and fibroblasts (not shown) could be visualized as single dots similar to centrioles in cells in newborn pineal gland (see fig. 17 previous chapter). However only one day
later, after 9 days in culture a dramatic change occurred in the staining pattern and instead of a single dot a cluster of dots of many different sizes is seen in the cytoplasm of the pinealocytes by immunofluorescence staining (fig 23c). The same pattern of staining could be seen in pinealocytes in 11(fig. 23e) and in 24 (fig 23g) day old cultures in which these cells visualized by phase contrast microscopy looked normal. In contrast the staining of centrioles in fibroblasts present in the same cultures remained unchanged (fig. 23e and g). These two different staining patterns with the antibody to the 50 kD centriolar antigen were observed in pinealocytes and fibroblasts up to 74 days of culturing, the longest period examined in this study.

Electron micrographs of pinealocytes from the 12-day-old culture showed that in some of the pinealocytes one of the pairs of centrioles appears normal with a complete set of MT triplets in its wall (figs. 24a and 25). However, the other centriole is clearly abnormal with some of the MTs in the centriolar wall changing their direction (fig 24b) or being shorter than normal (fig 25). In addition structures resembling fragments of walls of centrioles were also seen in the pinealocytes (figs. 26a, b, c and d). MTs radiated from both the region around the intact centrioles as well as from regions near the fragments of centrioles (figs 24a, 24b, 25, 26a,b,c, and d). All the centrioles of fibroblasts in contrast appeared normal with nine triplets of MTs in their walls (not shown).

To confirm that the pinealocytes with fragments of centrioles are indeed capable of nucleating MT assembly and to determine the site(s) from which such assembly occurs, MT assembly was induced at 37°C after depolymerization of the preexisting MTs at 0°C. The cells
were then fixed 30 sec, 3, 6, 9, and 12 minutes after incubation at 37°C and examined by immunofluorescence after double staining with antibody to the 50 kD centriolar antigen that labels centrioles and centriolar fragments and antibody to α-tubulin that stains the MTs. The results showed that MTs radiate from regions in the pinealocytes containing the centriolar fragments (fig 27a and b). MTs radiating from the regions staining with the antibody to the 50 kD centriolar antigen were also observed in pinealocytes in 2-day-old cultures of cells from the adult pineal gland after their MTs were similarly depolymerized and then allowed to reassemble (fig. 27c and d). No MTs were seen 3 seconds after incubation at 37°C.

Discussion.

Under the optically more ideal conditions of cell cultures, centriolar fragmentation in pinealocytes similar to the one that occurs in pinealocytes in situ was demonstrated. The centriolar fragmentation in pinealocytes from 2 day old rat pineal glands occurred suddenly after 8 days in culture. This corresponds to 10 days after birth which is approximately the time when centrioles in pinealocytes also fragment in vivo i.e. in 11 day old rats. The MT containing fragments of centriolar walls observed in longitudinal sections in electron micrographs of pinealocytes grown in culture were never as long as the ones observed in vivo. Also in vitro, sometimes one of the two centrioles appeared to have normal walls while the other was clearly abnormal or fragmented.

It is interesting that centriolar fragmentation occurs in pinealocytes in culture at about the same time as it occurs in vivo. This suggests that pinealocytes may be programmed for centriolar
fragmentation. The pinealocytes could for example activate cytoplasmic factors that lead to centriole fragmentation at a particular stage in differentiation. Since the centrioles of fibroblasts that are also present in these cultures and centrioles of cell types other than in pinealocytes in the intact pineal gland remain normal, they may simply lack the mechanisms that induces centriole fragmentation in pinealocytes. Alternatively it is possible that factors which normally prevent the fragmentation of the centrioles could be missing from the pinealocytes but present in the other cell types.

Since centriolar fragmentation in cultures of pinealocytes occurs in vitro, these cultures can now be manipulated to determine why centriolar fragmentation occurs, how it can be prevented and what effect its prevention would have on the structure and behaviour of pinealocytes.

EM of the adult rat pinealocytes in situ showed that MTs do not radiate from the fragments resembling centriolar walls. This may indicate that the proteins that nucleate MT assembly such as the gamma-tubulin may have become redistributed to other regions in the pinealocytes and is not associated with the centriolar fragments. EM of pinealocytes present in the 12-day-old cultures in contrast showed MTs radiating from the regions near the fragments of centrioles. The in vitro depolymerization/re polymerization experiments using 2-day-old cultures of cells from the adult rat pineal gland and 12-day-old cultures of cells from 2-day-old rat pineal gland confirmed and extended the observations by EM by showing that the MTs in vitro do assemble from regions that stain with the antibody to the 50 kD centriolar protein that contain the
fragments of centriolar walls.

Using antibodies to gamma-tubulin, Moudjou et al. (1966) have shown in human lymphoblasts, that this protein which nucleates MT assembly, is not only present in the centrosome but also in other parts of the cell cytoplasm. In fact 80% of the gamma-tubulin is found in the cytoplasm outside the centrosome. This gamma-tubulin has not been detected by immunofluorescence, probably because the protein is much less concentrated in the cytoplasm outside the centrosome than in the centrosome where it forms the MTOC's. In cell cultures, proteins that nucleate MT assembly such as gamma-tubulin which may be present in soluble form in the cell cytoplasm, may assemble to form a MTOC in a perinuclear region where fragments of the centriole are located. Alternatively it may be diffusely distributed in cytoplasm in vivo but aggregate in the perinuclear region when the pinealocytes are cultured. Manniotis and Schliwa (1991) demonstrated that in cultured African green monkey BSC1 cells from which the centrosomes have been microsurgically removed to produce centrosome-free karyoplasts a single new MTOC is reestablished after 30 hrs in culture. A single astral MT array radiated from this MTOC which was located in the perinuclear region where the centrioles and centrosomes would normally be expected. Gamma-tubulin in pinealocytes in culture may similarly redistribute from other regions of the cell to the perinuclear region to form a structure resembling a centrosome.

My EM studies of centrioles of rat pinealocytes in situ have shown that MTs in these cells do not radiate from the regions containing the fragments of walls of centrioles. This situation may be similar to the one found in fully polarized MDCK cells where MTs do not radiate from a
pericentriolar region but instead from MTOC's scattered along the apical end of the cell. In cultured rat pinealocytes where MTs radiate from the regions containing the fragments of centriole, on the other hand the situation may be similar to the one found in MDCK cells in culture before the establishment of cell junctions where centrioles have a perinuclear position from which the MTs radiate.
Conclusions.

1. A fragmentation of centrioles similar to that seen *in situ* occurs in pinealocytes *in vitro*.

2. This fragmentation *in vitro* occurs at the same time as *in situ* suggesting that the pinealocytes program the fragmentation of their centrioles at a particular stage of differentiation.

3. In pinealocytes with fragments of centrioles *in vitro*, the regions containing the fragments, unlike those *in situ*, nucleate MT assembly.
Fig. 23. Cells from 8 (a,b), 9 (c, d), 11 (e, f) and 24 (g, h) day-old-cultures immunofluorescence stained with antibodies to the 50 kD centriolar antigen (a, c, e, g) and with Hoechst to visualize the nuclei (b, d, f, h). In the 8-day-old culture (a,b) each pinealocyte has a pair of centrioles visualized as a single dot. The staining in pinealocytes, the cells with the smaller nuclei, in the 9 (c, d) 11 (e, f) and 24 (g, h) day old cultures instead is visualized as a cluster of smaller dots (open arrows) suggesting that their centrioles have fragmented. Centrioles of fibroblasts in contrast do not change and appear as single dots (arrows) (e, g). Bar=10μm.
Fig. 24. Electron micrographs of two adjacent sections from the centriolar region of a pinealocyte from a 12-day-old culture showing a normal centriole (arrow) and an abnormal centriole with MTs in its walls changing direction (open arrow). MTs (arrowheads) are seen radiating from the region around the centrioles. Bar=400nm.
Fig. 25. Electron micrograph of a section from a pinealocyte from a 12-day-old culture showing a normal centriole (arrow) and a fragment of a centriole (open arrow). MTs (arrowheads) are seen radiating from a region around these structures. Bar=400nm.
Fig. 26. Electron micrographs of sections through pinealocytes from a 12-day-old culture showing fragments resembling centriolar walls in various sections (open arrows in a,b,c, and d) containing MTs of different lengths. MTs (arrowheads) are observed radiating from regions around these fragments. Bar=400nm.
Fig. 27. Pinealocytes from a 12-day-old culture of cells from a 2 day-old pineal gland (a and b) and from a 2-day-old culture of cells from an adult rat pineal gland (c and d). The cultures were placed for 45 min on ice, and then incubated for 6 min at 37°C fixed and immunofluorescence stained with the antibody to the 50 kD centriolar antigen (a and c) and to α-tubulin (b and d). MTs (b and d) radiate from the regions labelled by the antibody to the 50 kD centriolar antigen (a and c). Bar=10μm.
General discussion and future studies.
In the studies described in the previous chapters I used immunofluorescence to localize the 50 kD centriolar protein during assembly of centrioles in ciliating cells of the chick trachea and during the breakdown of centrioles in rat pinealocytes.

I show that with the antibody to the 50 kD centriolar protein different staining patterns can be visualized in ciliating cells of the chick trachea. The following sequence of stages in centriolar assembly would be consistent with the staining patterns observed, with the temporal sequence in which they appear during ciliogenesis and with previous studies by electron microscopy (see fig. 1 chapter 1. and Kalnins and Porter., 1969). As expected the pair of centrioles i.e. the diplosomal centrioles contain the 50 kD centriolar antigen and are stained in epithelial cells of the E15 chick trachea before centriolar assembly begins. Because they are very close together they are visualized as a single granule. By E16 larger granules are visualized in the apical part of some of the cells while in other cells these larger granules are associated with a stained region extending in a basal direction from the granules. The first of these patterns showing an increase in the size of the diplosomal centrioles indicates that centriolar precursors and/or procentrioles have accumulated around both of the diplosomal centrioles in some of the cells. This is the first sign that centriole assembly has begun. The second pattern suggests a later stage in centriolar assembly. In ciliating cells with this pattern the newly formed procentriole clusters have migrated away from the diplosomal centrioles and now occupy a larger region near the diplosomal centrioles. By E17 a new staining pattern and therefore a later stage in centriolar assembly becomes apparent. In this pattern staining of small granules distributed throughout the apical end of the ciliating cells is seen. These granules probably correspond to procentrioles or
recently matured centrioles that have separated from the clusters and are now migrating to the apical cell surface. By E18 the first fully ciliated cells appear in which as expected a band of staining along the apical ends of cells in the region occupied by the basal bodies is apparent.

Previous ultrastructural studies of ciliating cells in the avian trachea suggested that the diplosomal centrioles may be involved in procentriole formation, since some of procentriole clusters were seen directly attached to the walls of the diplosomal centrioles and all of them were found in close proximity to these centrioles (Kalnins and Porter., 1969; Marshall and Kalnins, unpublished observations). My studies have shown that the 50 kD centriolar antigen is also present in the same region as the clusters of procentrioles i.e. in close association with the two diplosomal centrioles very early during centriolar assembly. The fact that no staining was observed outside this region strongly suggests that all of the procentrioles in a ciliating cell are assembled near the diplosomal centrioles.

In contrast to their distribution in chick trachea clusters of procentrioles have not been observed in close association with the two diplosomal centrioles in ciliating epithelial cells of the rat trachea by EM. The possibility exists, however, that centriolar assembly is similar in rat trachea but takes place more quickly thus decreasing the chances of seeing clusters of procentrioles in association with diplosomal centrioles especially by EM, which permits examination of a relatively small amount of tissue. Also in the chick trachea centriolar assembly takes place within a region around the diplosomal centrioles that is surrounded by a meshwork of IF (Kalnins and Porter., 1969). A similar IF meshwork is not observed around the diplosomal
centrioles and procentriole clusters in ciliating epithelial cells in rat trachea. This IF meshwork may prevent the procentriole clusters in the chick trachea from migrating away from the diplosomal centrioles whereas in the rat trachea the absence of this meshwork may permit clusters to disperse rapidly throughout the apical end of the ciliating cell. Using immunofluorescence rather than EM permits the examination of many more ciliating cells. If the procentrioles assemble in association with the diplosomal centrioles also in the rat trachea one might be able to detect the accumulation of the 50 kD centriolar protein around the diplosomal centrioles at an early stage in ciliogenesis by this approach.

The sequence in which the different proteins known to be present in the centrioles such as α-, β-, and gamma-tubulin, and cenexin, appear during centriolar assembly is not known. Now that the time of appearance of the 50 kD protein has been established, it would be interesting to know if the 50 kD centriolar protein appears before or after the assembly of α- and β- tubulins to form the MT of procentrioles. To establish this immunogold labelling studies using the antibody to the 50 kD centriolar protein at the ultrastructural level are needed. Similarly, double immunogold labelling using antibodies to the 50 kD centriolar protein and one of the other proteins present in the centrioles such as cenexin, and gamma-tubulin would provide information about the sequence of incorporation of these proteins in the procentrioles and lead to a better understanding of the assembly of these organelles.

Electron dense material of unknown function is observed by EM around some of the diplosomal centrioles before the appearance of clusters of procentrioles in ciliating cells of the
chick trachea (Kalnins and Porter., 1969). The larger granules stained at an early stage of
centiolar assembly suggesting that the 50 kD protein could be a component of this dense material
and that the new procentrioles could assemble from this material. Alternatively the procentrioles
themselves in clusters forming along the walls of the diplosomal centrioles could be labelled.
Only immunogold labelling could distinguish between these two possibilities. In any case the 50
kD centriolar protein is present in procentrioles at an early stage of assembly and also in mature
centrioles and basal bodies. This suggests that the 50 kD centriolar protein may be a component
of the electron dense material in the walls of centrioles in which the MT's are embedded. Since
this material is present in the procentrioles before the assembly of their MT's (Kalnins and Porter.,
1969) one would expect it to be present at an early stage in centriolar assembly. This
interpretation would also be consistent with the antibody to the 50 kD centriolar protein labelling
fragments of centriolar walls in the adult rat pinealocytes where similar electron dense material
is observed around the MT by EM.

It has been suggested that modifications in or loss of the proteins that normally link the
MT triplets may lead to the separation of the triplets in the centriolar wall and to the subsequent
breakdown of centrioles (Calvo., 1991). My results suggest that the fragments of centrioles in the
adult rat pinealocytes retain the 50 kD centriolar protein after the breakdown of the centrioles.
Thus, although present in the walls of the centrioles the 50 kD protein may not be the structural
protein that links the nine MT triplets together. Alternatively, the 50 kD protein could have
become modified thereby loosing its ability to link the MT triplets while retaining its antigenicity,
so that the fragments of centrioles continue to label with the antibody to the 50 kD centriolar
protein.

My immunofluorescence studies have shown that more material seems to be stained with the antibody to the 50 kD centriolar protein in adult than in the newborn rat pinealocytes. In addition my ultrastructural studies have shown that some fragments of centrioles are oriented at right angles to other centriole fragments. These results suggest that at least some fragments of centrioles in the rat pinealocytes may also be capable of inducing the assembly of more centriolar fragments. Ultrastructural studies of Fuller. et al., (1993) have shown that prior to centriolar assembly in cells, gamma-tubulin redistributes from the lumen of the centrioles to a region on the outside surface of their wall to nucleate the assembly of centriolar MT. The suggestion that in pinealocytes some of the centriolar fragments can induce the assembly of others would be strengthened if gamma-tubulin could be demonstrated on the fragments of centrioles in the region where they are associated at right angles with other MT containing fragments. Quantification of the immunofluorescent staining before and after centriolar fragmentation occurs could also provide information about the extent to which the amount of the 50 kD centriolar protein increases. Examination of serially sectioned pinealocytes on the other hand would enable one to establish the total number and length of centriolar fragments present after the fragmentation of centrioles, and help to determine whether they could have simply arisen from the elongation and fragmentation of preexisting centrioles or whether assembly of more fragments from preexisting fragments is required to account for those present.

Cell fusion experiments in cell cultures may also help to establish the mechanisms which
induce the fragmentation of centrioles in pinealocytes. By fusing pinealocytes before fragmentation of their centrioles with fibroblasts one could produce fused cells in which both the pinealocyte and fibroblast centrioles are fragmented. This result would indicate that factor(s) from the cytoplasm of pinealocytes induce the fragmentation of centrioles in fibroblasts. If in the fused cell both centrioles remain intact, this would indicate that centriolar fragmentation can be prevented in the pinealocytes by factors from the cytoplasm of the fibroblasts. Finally if the resulting cells retain a normal pair of centrioles derived from the fibroblasts and a pair of fragmented centrioles derived from the pinealocyte, this would suggest that centriolar fragmentation can not be induced or prevented by diffusible cytoplasmic factor(s). In this case the fragmentation may be due to a mechanism inherent in the pinealocyte centrioles themselves.
Summary of conclusions

1. An increase in the staining of diplosomal centrioles is the first sign of centriolar assembly in ciliating epithelial cells of the chick trachea. This indicates that centriole precursors and/or procentrioles containing the 50 kD centriolar protein first accumulate around both diplosomal centrioles.

2. The other staining patterns observed indicate that the procentrioles in clusters, centrioles migrating to the apical cell surface, and the recently matured basal bodies of cilia also contain the 50 kD centriolar protein.

3. The early appearance of the 50 kD centriolar protein during centriolar assembly and its subsequent distribution suggests that it may be associated with, or a component of, the centrioles.

4. Structures resembling fragments of centriolar walls are present in rat pinealocytes after 11 days of age but normal centrioles are absent. The structures stained with the antibody to the 50 kD centriolar protein have a similar appearance and spatial/temporal distribution as the fragments of centriolar walls suggesting that the centriolar fragments contain or are closely associated with the 50 kD protein.

5. Fragmentation of centrioles similar to that observed in situ occurs in rat pinealocytes in vitro at a similar time suggesting that pinealocytes program the fragmentation of their
centrioles at a particular stage of differentiation.

6. The regions containing the fragments of centrioles nucleate MT assembly in pinealocytes \textit{in vitro} but not \textit{in situ}.

7. The apparent increase in the material stained with the antibody to the 50 kD centriolar protein in rat pinealocytes after the fragmentation of the diplosomal centrioles both \textit{in situ} and \textit{in vitro} raises the possibility that the resulting fragments of centriole may be capable of generating more fragments.
References.


