

MICROTUBULE PROTEIN DURING CILIOGENESIS IN THE MOUSE OVIDUCT

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ABSTRACT

A colchicine-binding assay and quantitative sodium dodecyl sulfate gel electrophoresis have been used to determine the changes which occur in microtubule protein (tubulin) concentrations in the particulate and soluble fractions of mouse oviduct homogenates during that period of development when centriole formation and cilium formation are at a maximum. When mouse oviducts, at various ages after birth, are homogenized in Tris-sucrose buffer, tubulin concentration is partitioned between the soluble (70%) and particulate (30%) fractions. During the period of most active organelle formation (3–12 days), there is a marked increase in colchicine-binding specific activity, in both the soluble and particulate fractions. Microtubule protein concentration increases from 16 to 24% in the soluble fraction, declining to 14% in the adult. In the particulate fractions, microtubule protein concentration increases from 16 to 27%, leveling off at 16% in the adult. We have concluded from these observations and from electron microscopy that colchicine-binding activity in the particulate fractions is related to the presence of centriole precursors in the pellets of homogenized oviducts from newborn mice. These data further suggest that centriole precursor structures are conveniently packaged aggregates of microtubule protein actively synthesized between 3 and 5 days, and maintained at a maximum during the most active period of organelle assembly.

INTRODUCTION

The complex morphogenetic events which occur in ciliated Protozoa and differentiating vertebrate ciliated epithelia during ciliogenesis have been well established for a variety of cell types (1, 2, 10, 11, 13, 14, 19, 20, 22, 23, 25, 31, 33, 34). Centriole formation, for all practical purposes, is the earliest recognizable event in ciliogenesis. In vertebrates, each ciliated cell, for example, requires about 300 centrioles to provide for the basal bodies which in turn function as assembly sites for the ciliary axonemes. During centriole formation several types of morphological precursors, having little resemblance to the mature organelle, have been

identified (12, 13). It was suggested that microtubule protein (tubulin), the fundamental biochemical component of centrioles, basal bodies, and cilia, could be synthesized and packaged into precursor structures in order to expedite, in some manner not understood, the simultaneous assembly of a large number of centrioles (13, 14, 25).

Although these ultrastructural studies contributed considerable information to the events preceding and during centriole formation as well as to ciliary growth, they could not account for the manner in which microtubule protein is polymerized into microtubules, and how these in turn are

assembled into the mature organelles. Polymerization *in vitro* of microtubules from microtubule protein has been shown for the model system, brain tubulin (7, 38). Since it has been demonstrated that pools of microtubule protein exist in sea urchin eggs (26, 35), *Chlamydomonas* (29), and *Tetrahymena* (28, 36), this suggests that aggregation from these tubulin pools could occur *in vivo* through similar mechanisms by which they occur *in vitro* (8, 30). It has also been postulated that the formation of organelles, whose structural unit is the microtubule (i.e., mitotic apparatus, centrioles, cilia), could occur through a self-assembly process (21, 35), ostensibly from the existing tubulin pools in the cell.

Colchicine, a plant alkaloid, binds specifically to the tubulin dimer, and for this reason has been used to measure the tubulin concentration of various tissues (9, 40, 42, 43). Accurate measurements of colchicine-binding material have been obtained not only in soluble, but in particulate fractions of embryonic chick neural tissue (4, 5), mouse brain (17), and aggregated tubulin structures from sea urchin eggs (39). It is possible to determine accurately the amount of tubulin in a tissue extract in the presence of other proteins, although these latter may account for a large proportion of the total proteins in the homogenate.

In the mouse oviduct, cilium formation begins immediately after parturition. As early as 1 day after birth there are already present a few randomly scattered ciliated cells on the surface of the oviduct (16). The number of ciliated cells steadily increases, accelerating at 5–8 days after birth, and achieving in the 12–14-day old suckling the number of ciliated cells found in the adult (15). The largest number of centriole precursors, however, are present between 5 and 8 days after birth (13).

The existence of microtubule protein pools and their relation to centriole precursors and mature organelles continues to provoke difficult questions. In order to clarify some of these questions we have modified a colchicine-binding assay and, in conjunction with quantitative polyacrylamide gel electrophoresis, we have determined the concentrations of tubulin in the soluble and particulate fractions of homogenates from mouse oviducts obtained at various ages after birth. We have correlated the biochemical studies with electron microscope observations in order to explore the possibility that centriole precursors are aggregates of microtubule protein. With the data obtained we have also hoped to assess the changes in mi-

cro-tubule protein levels and relate these to the known morphological events which occur during ciliogenesis in the developing mouse oviduct.

MATERIALS AND METHODS

Homogenization and Fractionation

Oviducts were dissected from 3-, 5-, 8-, and 12-day old newborn and adult Swiss-Webster mice. They were washed with 2-ml homogenization buffer and stored at -70°C until needed. 200 oviducts each for the newborns and 20 for the adult were homogenized in 2–3 ml of buffer with a hand-operated glass homogenizer. In each set of experiments the same buffer was used throughout the whole procedure, either phosphate (0.02 M potassium phosphate, pH 6.8, 1.0 mM GTP) or Tris-sucrose (0.02 M Tris-HCl, pH 6.8, 1.0 M sucrose, 1.0 mM GTP, 0.01 M MgCl_2).

Soluble (S) and particulate fractions (p_1 , p_2) were obtained by differential centrifugation, Fig. 1 summarizing this procedure. The temperature was maintained at 4°C throughout. The low- and high-speed pellets were washed once with the corresponding buffer, and tubulin was extracted from them by homogenization in 1.7 ml of buffer containing 0.1 M KCl (39). The samples were further allowed to extract for 15 min, after which they were clarified by centrifugation at 13,000 *g* for 10 min. The supernates (p_1 , p_2) were removed and assayed for colchicine-binding activity.

Porcine brain tubulin was prepared and purified according to the methods described by Shelanski et al. (30). These preparations were used, for purposes of comparison, in conjunction with the oviduct soluble and particulate fractions.

Colchicine-Binding Assay

To determine colchicine-binding activity in soluble and particulate fractions, 0.01 ml of 5×10^{-4} M [^3H]colchicine (approximately 60,000 cpm) was added to 0.5-ml aliquots of oviduct protein and incubated at 37°C for 90 min. Protein concentrations in these fractions measured 0.2–0.6 mg/ml as determined by the method of Lowry et al. (24), with bovine serum albumin as a standard. Samples from the soluble fractions were adjusted to contain 0.1 M KCl in order that they would have the same final salt concentration as extracts from the particulate fractions.

The amount of bound colchicine was determined by a modification of the diethylaminoethyl (DEAE) Sephadex A-50 method of Weisenberg (39) and Frigon and Lee (18). After a 90-min incubation the assay mixture was diluted to 1 ml with buffer and cooled at 4°C to stop the reaction. It was passed through a 0.5×2.0 cm column (Pasteur pipet) of Sephadex which had been previously equilibrated with the same buffer used in the assay. The [^3H]colchicine-protein complex was adsorbed to the column and the unbound colchicine removed with 8-ml

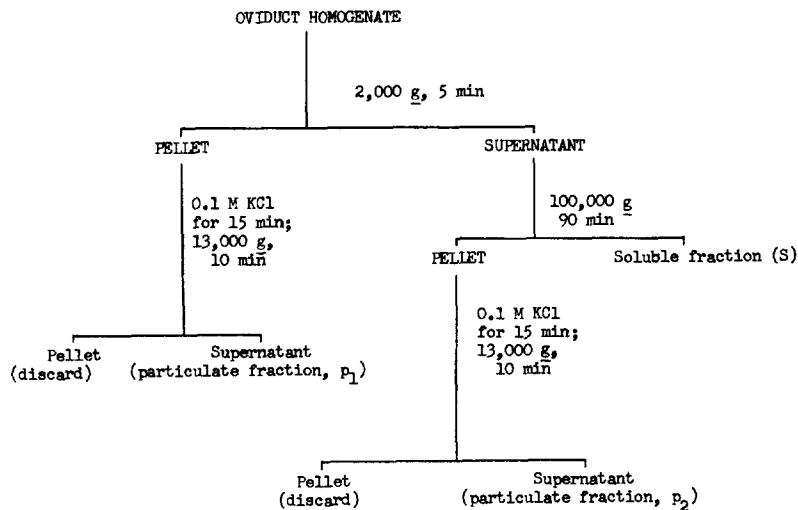


FIGURE 1 Homogenization scheme for the preparation of the soluble (S) and particulate fractions from the low-speed (p_1) and high-speed (p_2) pellets obtained from mouse oviduct homogenates.

buffer. The column was eluted with 10-ml Scintisol, a liquid scintillation counting solution, and the radioactivity determined with a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). For the time-decay colchicine-binding assay, the method as described by Bamberg et al. (4) was used.

Acrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and the molecular weight determinations were carried out as described by Weber and Osborn (37). Soluble fractions from newborn and adult oviducts were examined on 7.5% gels (5×120 mm) containing 0.1% SDS. Samples (0.3 mg/ml protein) were preincubated with 1% SDS, 5 M urea, 0.1 dithiothreitol (DTT), and 0.1 M sodium phosphate buffer, pH 7.0, at 100°C for 5 min. Aliquots (30 μg) of this mixture were applied to each tube. For purified brain tubulin, 7–30- μg aliquots were used. The running buffer was 0.1 M sodium phosphate, pH 7.0, containing 0.1% SDS. Tap water (about 15°C) was circulated around the gel tubes for cooling purposes. After electrophoresis, gels were removed from the tubes, stained overnight with Coomassie brilliant blue (0.1% in 7.5% acetic acid and 40% methanol) and destained electrophoretically in 7.5% acetic acid and 5% methanol solution. Gels were scanned with a Zeiss spectrophotometer (Carl Zeiss, Inc., New York) equipped with a linear transport system. The wavelength used for the dye-protein complex was for its maximum absorbance, or 550 nm. The areas under the protein bands were estimated with a DuPont curve analyzer (E. I. DuPont de Nemours & Co., Wilmington, Del.).

Estimation of Tubulin Concentrations

For the soluble fractions, the percent tubulin of the total soluble cell protein can be determined by using the

information obtained from the gel scans. When the total area for the whole protein sample is set as 100% in the curve analyzer, the percent area for the tubulin can be obtained. It is then possible, utilizing the data for colchicine binding of the particulate fractions, to estimate their percent of tubulin by the following relationship (5):

$$\begin{aligned} & \text{concn in particulate fraction (\%)} \\ &= \frac{\text{sp act of particulate fraction}}{\text{sp act of soluble fraction}} \\ & \quad \times \text{concn in soluble fraction (\%)} \end{aligned}$$

This makes the assumption that the colchicine-binding activity of tubulin in the particulate fractions is similar to that in the soluble fractions.

Electron Microscopy of Tissues and Pellets

Oviducts from mice at various ages after birth were fixed *in situ* with 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) to which 0.05% CaCl_2 was added just before use. Subsequently the fimbriae were dissected from the rest of the oviduct tubules and postfixated in 2% OsO_4 in acetate-Veronal buffer (pH 7.4) for 1.5 h and stained in block with 1.5% uranyl acetate in 0.2 M sodium maleate buffer (pH 5) for 1.5 h. The tissues were dehydrated in a graded series of acetones and embedded in Spurr's low-viscosity plastic medium (32). Pellets obtained from the low- and high-speed centrifugations were fixed in 2–3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.05% CaCl_2 and prepared by the same methods used for the whole tissues. Thin sections were stained with uranyl acetate and lead citrate by standard procedures.

RESULTS

Appearance of Centriole Precursors

A previous report on centriole formation in developing ciliated epithelium of the mouse oviduct described several types of precursors which begin appearing about 3 days after birth, increase in frequency between 5 and 8 days, and almost disappear 12 days after birth (13). As part of the morphogenetic scheme, particularly in ciliated epithelia, these precursors assume various aspects, consisting of fibrogranular masses of various sizes (Figs. 2, 3, and 5), electron-dense condensation forms (Figs. 4 and 5), and condensation forms associated with immature centrioles (Fig. 6). Because these interesting morphogenetic events take place in oviducts of mice 3–12 days of age, we have chosen for our studies this rapid period of organelle proliferation.

Colchicine-Binding Assay

Colchicine-binding activity has been widely used as an assay for microtubule protein (6, 9, 18, 40). Recently a time-decay colchicine-binding assay has been developed which accounts for the variation in binding activity decay rates and reflects the true concentration of microtubule protein present in cell extracts (4, 5). This has been described as the *initial binding capacity*, and was determined by extrapolating to zero time of incubation. When a time-decay curve was done for colchicine binding of oviduct soluble fractions, colchicine-binding activity did not decay with an increase in preincubation time under the conditions and protein concentrations used. It was therefore not necessary to determine *initial colchicine binding* activity by the time-decay assay procedure for every fraction from oviduct homogenates. Under the circumstances accuracy could be achieved by the single-point colchicine-binding assay.

As used in our experiments, the DEAE-Sephadex method was reproducible within 7% in duplicate or triplicate samples. Colchicine binding of soluble fractions from oviducts homogenized in Tris or phosphate buffer was linear for the range of protein concentrations used.

Distribution of Tubulin in Soluble and Particulate Fractions

When colchicine-binding activity was measured in the soluble and particulate fractions of oviducts homogenized in phosphate buffer, only a small portion of the total activity was recovered in the particulate fractions from either the low- (p_1) or high- (p_2) speed pellets (Fig. 7). Compared to the

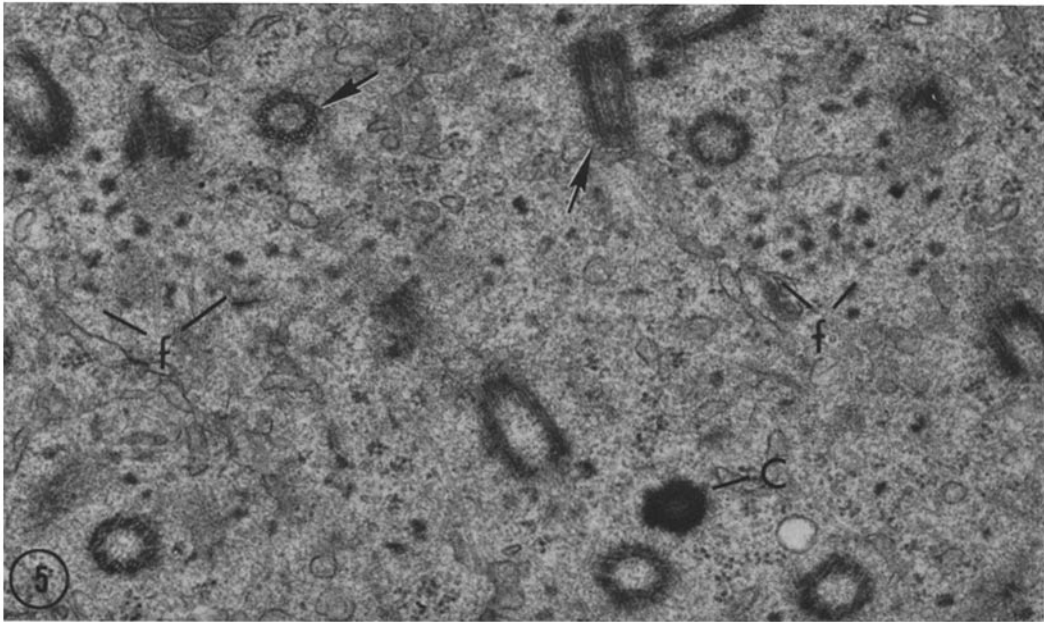
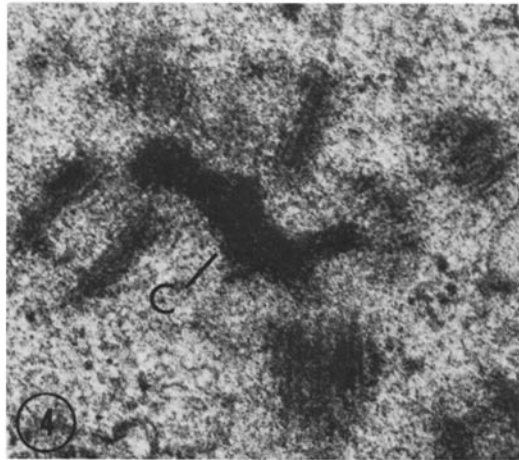
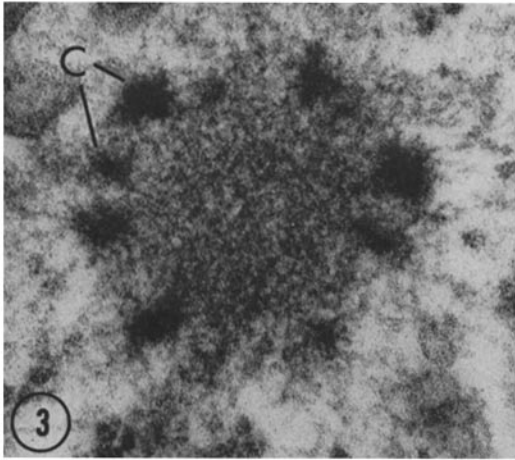
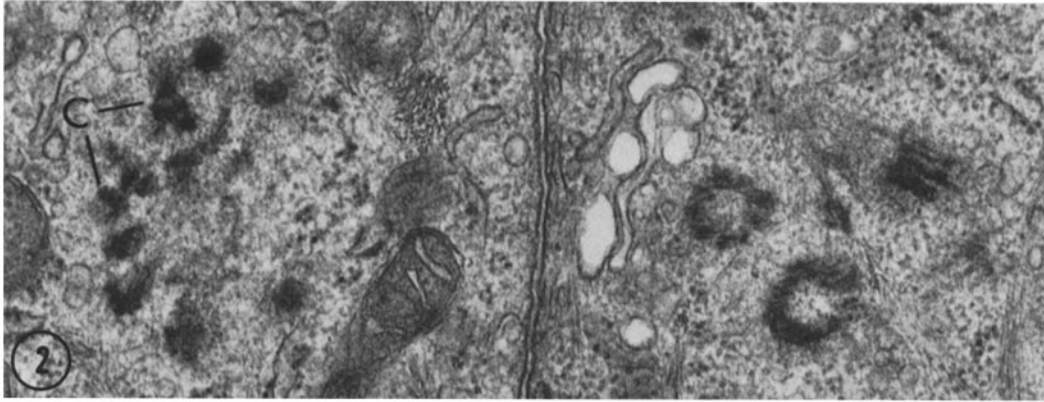
amounts found in the soluble fractions (S), colchicine binding in extracted pellets was negligible for all ages. It would appear that any possible tubulin aggregates in the developing ciliated cell, possibly in the form of the more labile fibrogranular centriole precursors, have been solubilized, thus increasing the activity of the soluble fraction. Further evidence that phosphate buffer tends to disrupt aggregated microtubular structures (i.e., centriole precursors) is derived from the inability to find any of these structures in either the low- or high-speed pellets prepared for electron microscopy. It is also of interest to note that even "free" centrioles (i.e., Fig. 5), whose stability should be greater than that of the precursors, were difficult to locate in the large number of blocks sectioned.

Tris-sucrose buffer, used as a homogenization medium, produced extracts of low- and high-speed pellets whose colchicine-binding activity was substantially higher than that of the preceding buffer (Fig. 8). With Tris-sucrose it was also possible to obtain pellets for electron microscopy in which some centriole precursors (Fig. 9) as well as many free centrioles were present. This buffer, because it apparently preserved a particulate, colchicine-binding cellular constituent, had considerable advantage and was used for all subsequent experiments. Further advantage of this buffer is that, when used with sucrose, it stabilizes colchicine-binding activity (18). These data have been confirmed by our experiments since we have observed that protein extracts, obtained from oviducts homogenized in sucrose-containing buffer, can be stored at 4°C for at least 24 h without any significant loss in colchicine-binding activity.

Using Tris buffer with sucrose, the soluble fraction is shown to contain less than 70% of the total tubulin (Fig. 8). Approximately 30% has been preserved in a polymerized or aggregated form and can be recovered in extracts of the particulate fractions. There is relatively little change in tubulin distribution between the soluble and particulate fractions as a function of age. Particulate-associated tubulin is found not only in newborn, but in adult mouse oviduct homogenates as well.

Quantitative Determination of Tubulin in Soluble and Particulate Fractions

The specific activity of bound colchicine (counts per minute per milligram protein) was determined in particulate and soluble fractions obtained from mouse oviducts homogenized in Tris-sucrose buffer (Fig. 10). There is an increase in activity



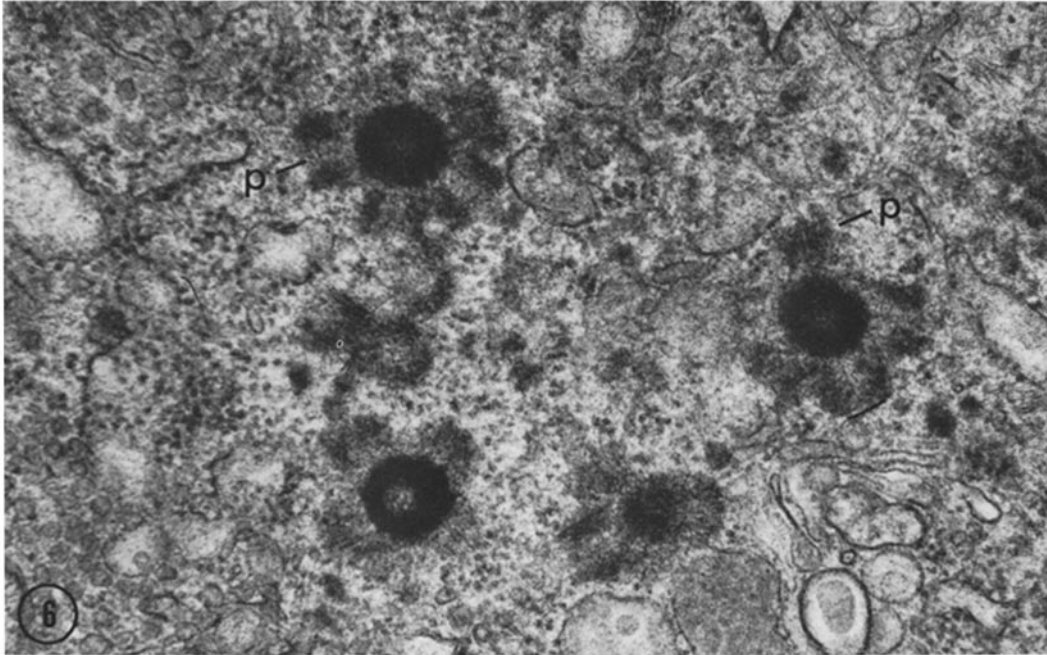


FIGURE 6 Centriole precursors, consisting of condensation forms surrounded by pro-centrioles (*p*), can be seen in this cell from the oviduct of a 5-day old mouse. $\times 53,000$.

during that period of oviduct development when centriole formation and ciliary growth are at its maximum, in both the soluble and particulate fractions. The adult oviduct still has considerable colchicine-binding activity although ciliogenesis has been completed.

Quantitative polyacrylamide gel electrophoresis was also used for the determination of tubulin concentrations (percent) in the soluble fractions of oviducts homogenized in Tris-sucrose buffer. Gels obtained from the soluble fractions of oviducts at various ages after birth yielded multiple bands on scanning (Fig. 11). Oviduct tubulin was identified

by using porcine brain tubulin as a marker for the migration distance. Its band corresponded to 50,000–54,000 mol wt when coelectrophoresed with proteins of known molecular weights. Variations in the size of tubulin bands in gel scans were used to reflect changes in tubulin concentration. Since gel scans of purified brain tubulin show a linear relationship between the relative area occupied by the tubulin peak and protein concentration, this method is valid for quantitative estimations of tubulin concentration (percent) in soluble fractions from homogenized mouse oviducts. For each age, the tubulin concentration (percent) ob-

FIGURE 2 Portions of two cells in the oviduct of a 5-day old mouse in which small condensation forms (*C*) can be seen at the left and free centrioles at the right. $\times 46,000$.

FIGURE 3 A large fibrogranular mass, an example of a centriole precursor, is present in this section of a cell from a 5-day old mouse oviduct. The center consists of filamentous material and is surrounded by small condensation forms (*C*). $\times 95,000$.

FIGURE 4 An asymmetrical aggregate of condensed centriole precursor material (*C*) is surrounded by developing centrioles in a cell from a 6-day old oviduct. $\times 65,000$.

FIGURE 5 A cell from the oviduct of an 8-day old mouse. Fibrogranular masses (*f*), a condensation form (*C*), and many free centrioles (arrows) can be seen in the cytoplasm. $\times 36,000$.

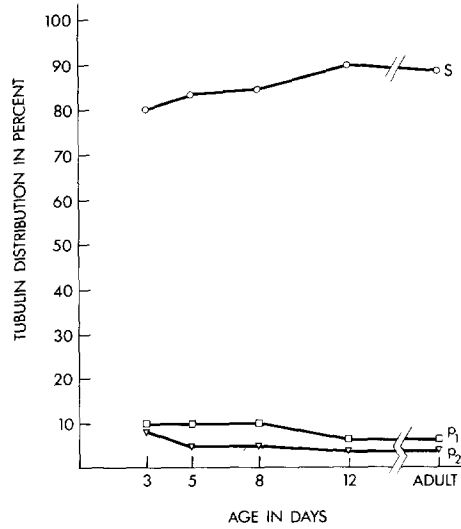


FIGURE 7 Distribution of total colchicine-binding activity between the soluble (S), KCl-extracted low-speed (p_1) and high-speed (p_2) pellets from mouse oviducts, at various ages, homogenized in phosphate buffer.

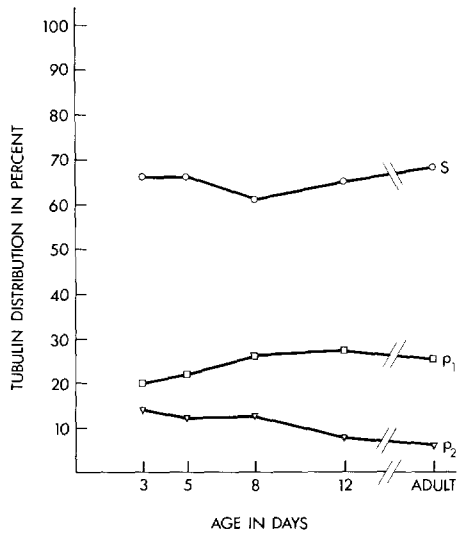


FIGURE 8 Distribution of total colchicine-binding activity between the soluble (S), KCl-extracted low-speed (p_1) and high-speed (p_2) pellets from mouse oviducts, at various ages, homogenized in Tris-sucrose buffer.

tained from the oviduct soluble fractions represents an average of four determinations with a 4% variation (Fig. 11).

Using the relationship between the specific activity of colchicine binding in the KCl extracts of the particulate fractions and that of the soluble

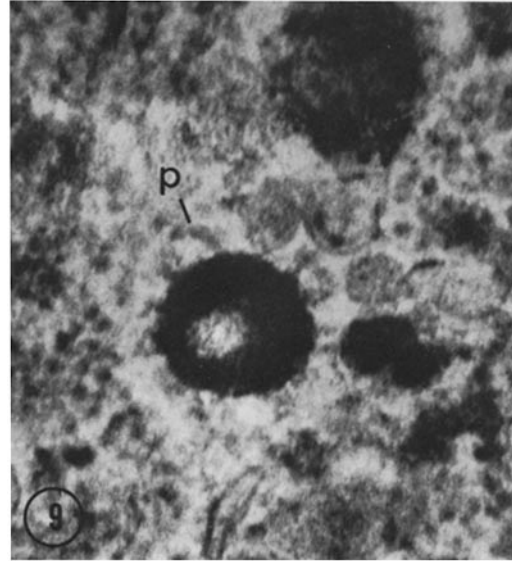


FIGURE 9 Centriole precursor in the high-speed pellet from a preparation of 5-day old oviducts homogenized in Tris-sucrose buffer. Note the suggestion of procentrioles (p) surrounding the condensation form. $\times 98,000$.

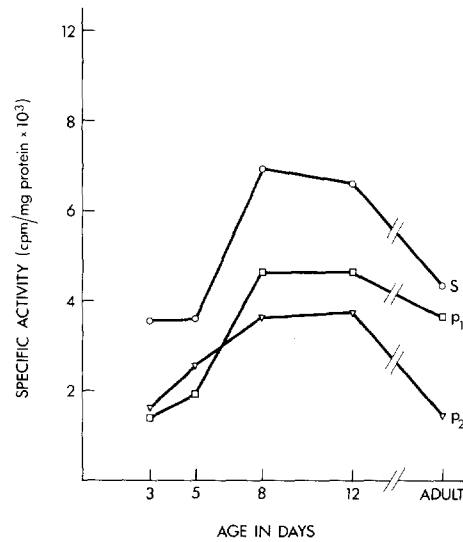


FIGURE 10 Changes with age in colchicine-binding specific activity in each fraction from oviducts homogenized in Tris-sucrose buffer; Soluble (S), KCl-extracted low-speed (p_1) and high-speed (p_2) pellets.

fractions, as well as the tubulin concentrations (percent) of the soluble fractions obtained from the gel scans, the concentration (percent) of tubulin in the particulate fractions could be calculated (see Materials and Methods). The concentration (per-

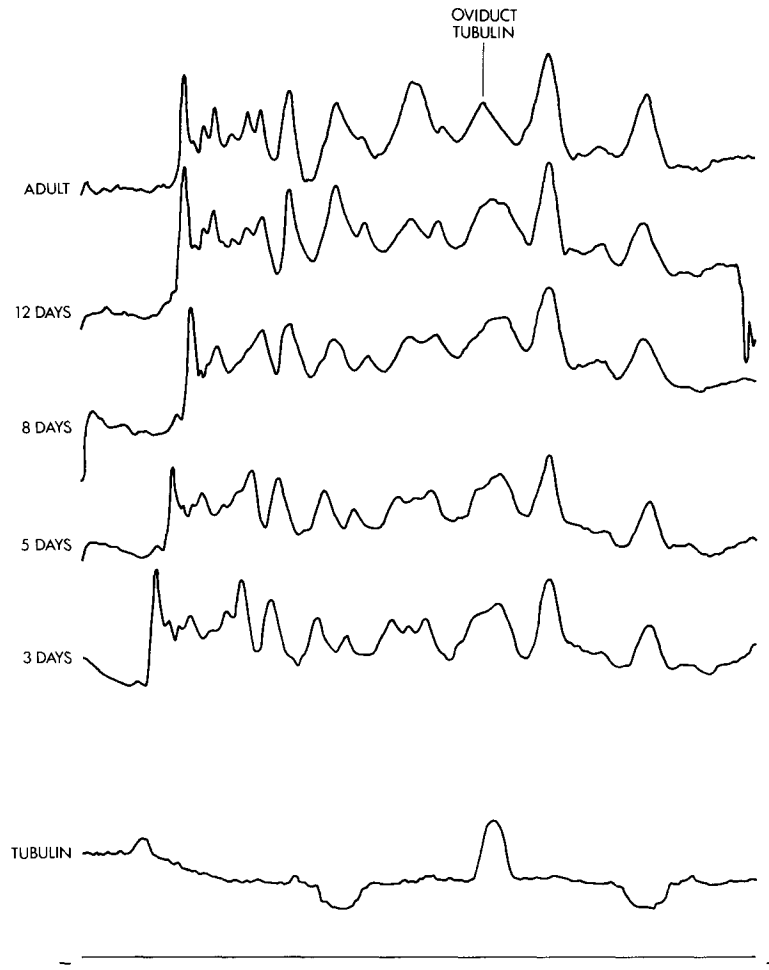


FIGURE 11 Densitometer tracings from oviduct tubulin gels obtained for different ages. Purified porcine brain tubulin (bottom tracing) was used as a standard for migration distance (left to right) determinations.

cent) of tubulin in the particulate fractions of oviduct homogenates during development appears in Fig. 12, together with the concentration (percent) of tubulin in the soluble fractions. Here, a rise in tubulin concentration corresponds to the morphological events in which centriole formation and ciliogenesis are also at a maximum during the 3–12-day period. It is of further interest to note that there is agreement between the results obtained from colchicine binding (Fig. 10) and gel scans (Fig. 11) for the soluble fractions. This verifies that colchicine binding is an accurate reflection of the concentration (percent) of microtubule protein present in oviduct, and that the affinity of tubulin for colchicine remains fairly constant during ciliogenesis and growth. Tubulin concentration in the particulate fractions parti-

tions itself between the low- and high-speed pellets (Fig. 12). A considerable portion of colchicine binding protein, therefore, remains in the combined particulate (p_1 and p_2) fractions.

Electron Microscopy of the Pellets

Oviduct cellular constituents partitioned themselves after homogenization in the following manner: (a) The low-speed pellet consisted primarily of nuclei, large mitochondria, and cilia, with and without basal bodies. (b) The high-speed pellet contained the microsomal fraction, small mitochondria, lysosomes, cilia, free centrioles, and some centriole precursors (Fig. 9). KCl-extracted pellets still contained intact cilia, demonstrating that the microtubule protein measured in these preparations came from structures other than cilia.

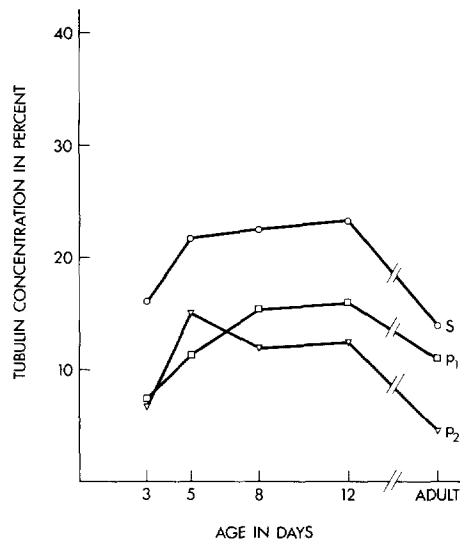


FIGURE 12 Tubulin concentration as a function of age for the soluble (S) and particulate fractions (p_1 , p_2) from oviducts homogenized in Tris-sucrose buffer.

DISCUSSION

We have shown that colchicine-binding material, characterized as microtubule protein, is present in both the soluble and particulate fractions of homogenized newborn and adult mouse oviducts. In addition, we have also demonstrated that in these fractions microtubule protein concentrations are at a maximum during that period in development when the epithelial cells are most actively forming centrioles and cilia. This rapid period of ciliated cell differentiation occurs between the ages of 5 and 12 days. In order for the cell to ready itself for this event, microtubule protein concentration increases substantially from 3 to 5 days after birth. Tubulin concentration in the soluble fraction increases by 6% in this 2-day preliminary synthetic period, and in the combined particulate fractions by 13%. Once organellar morphogenesis begins, a plateau level is maintained between 8 and 12 days of development which provides a steady supply of microtubule protein. Tubulin concentration decreases, this event being correlated with the gradual decrease in centriole and cilium formation. In the adult the concentration is then maintained at about the 3-day level. It is of considerable interest to note that although centrioles and cilia are no longer being formed in the adult, microtubule protein is maintained at this level. Thus, tubulin in the adult accounts for 14% of the total cell protein in the soluble and 16% in the combined particulate

fractions. This large tubulin pool in the adult cannot be accounted for by the breakdown of basal bodies or cilia since the KCl-extracted particulate fractions still contained intact organelles when examined by electron microscopy.

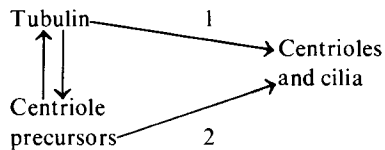
Few quantitative measurements are available for other ciliary microtubule pools. Raff and Kaumeyer (27) have measured a small amount of microtubule protein (0.37% of total cell protein) in sea urchin embryos which, they suggest, is used for the assembly of tubulin-containing structures. Other experiments on the utilization of tubulin protein pools during ciliogenesis in sea urchin embryos demonstrate that such proteins, when labeled before cilium formation has begun, appear in these organelles (35). It has also been reported that during oral replacement in *Tetrahymena* 94% of the tubulin comes from a small preexisting pool (41). Finally, regeneration experiments of flagella in *Chlamydomonas* (29) and of cilia in sea urchin embryos (3), in the presence of protein synthesis inhibitors, also indicate an available microtubule protein pool. Thus, the larger tubulin pool present in adult oviduct is difficult to explain since this is no longer a developing system. These amounts, however, are comparable to the concentrations present in adult chick, where 12% of the total brain protein is tubulin (5).

Recent results of Weisenberg (39) on sea urchin eggs during artificial activation can be compared to the data obtained in this study. He found that the breakdown of "tubulin-containing structures" (TCS) in *Spisula* eggs could be correlated with the utilization of the resulting microtubule protein in the formation of the mitotic spindle of the activated eggs. These results suggest that there is storage of tubulin which can be used for organelle assembly. The TCS are analogous to the centriole precursor structures in the mouse oviduct and were found in the particulate fractions of homogenized sea urchin eggs. Because the TCS are unstable, as are the oviduct centriole precursors, they were distributed between the low- and high-speed pellets when identified by colchicine binding.

Our results show that tubulin concentration changes not only in the soluble, but also in the particulate fractions during centriole and cilium formation. This colchicine-binding component is at a maximum in the particulate fractions during the most active period of organelle proliferation. The presence of considerable amounts of colchicine-binding activity in the particulate fractions suggests that they have a component high in

microtubule protein. If the electron-dense precursor structures consist of aggregates of microtubule protein, then part of the extensive colchicine-binding activity found in the particulate fractions of oviduct homogenates at the appropriate ages would be derived from these precursors. The present data support this view since no other structures have been observed in the pellets by electron microscopy which could account for high colchicine binding in the particulate fractions. We cannot account for the colchicine-binding activity remaining in the adult particulate fractions at this time. The centriole precursors, however, vary considerably in size and density. This could explain the distribution of colchicine-binding activity in the particulate fractions in the newborns. Some of the activity in the soluble fractions could be derived from the breakdown of cytoplasmic microtubules during homogenization as well as from the very labile fibrogranular precursors.

The view that assembled microtubules and microtubular structures exist in a dynamic equilibrium with tubulin subunits in the cell has gained wide acceptance (21). It can therefore be argued that, in mouse oviduct, tubulin pools exist in equilibrium with polymerized labile centriole precursors simultaneously as these precursors provide "building material" to the more stable organelles, in this manner:



At this time it is not possible to distinguish between pathways 1 and 2.

Centrioles, once they begin to function as basal bodies, provide assembly sites for the ciliary axonemes either from precursor structures (33) or from soluble tubulin pools. In this regard it is worth emphasizing that the most rapid increase in tubulin concentration (3–5 days) occurs just before maximal centriole proliferation (5–8 days) and that tubulin concentration is maintained at a maximum during the entire period of organelle proliferation (5–12 days), which declines after approximately 12 days.

These observations will be further elucidated when purified preparations of tubulin from mouse oviduct homogenates are obtained. Further studies are also in progress on the physical-chemical

factors controlling stability of the labile centriole precursors during homogenization and isolation procedures.

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