

# Mass Isolation of Calf Thymus Centrosomes: Identification of a Specific Configuration

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**Abstract.** Centrosomes from calf thymocytes were isolated using a simple preparative procedure that provides large yields of free organelles. A comparative study with centrosomes isolated from human cultured lymphoblasts has led to the discovery of important

differences in the structure of the two isolates and in their capacity to nucleate microtubules from purified tubulin. The possibility that the centrosomal structure depends upon the growth state of cells is discussed.

THE elucidation of the molecular basis of centrosome structure and functions is a major challenge in cell biology. These remarkable organelles are endowed with a variety of functions. They are capable of nucleating microtubules in interphasic and mitotic cells (for review see Bornens and Karsenti, 1984; Vorobjev and Nadehzhina, 1987; see also Mitchison and Kirschner, 1984). As the major microtubule-organizing center in most animal cells, they are centrally involved in cell responses to external stimuli (Schliwa et al., 1982), and more generally in the determination of cell polarity associated with cell motility (Koonce et al., 1984; Tassin et al., 1985) or with other cellular activities (Rogers et al., 1985). They are apparently capable of organizing complex cytoplasmic events (Picard et al., 1988; Raff and Glover, 1989).

Furthermore, centrosomes are replicative organelles that duplicate only once at each cell cycle. The reciprocal relationships between the cell and the centrosome cycles are not understood yet, despite recent advances in this direction (Nagano et al., 1981; Sluder et al., 1986; Freeman and Glover, 1986; Picard et al., 1988; Bailly et al., 1989). Clearly, our understanding of centrosome functions would greatly benefit from the identification and structural study of centrosome specific proteins. Very few of these proteins have as yet been identified (Kuriyama and Borisy, 1985; Gosti-Testu et al., 1986; Whitfield et al., 1988). For example, the proteins involved in the in vitro centrosome-specific function of nucleating microtubules are totally unknown.

Recent advances in the isolation of centrosomes have opened the way to experimental studies on this potentially important organelle (Mitchison and Kirschner, 1984; Karsenti et al., 1984; Bornens et al., 1987). The identification of centrosomal components is, however, hampered by the low amount of proteins obtained in centrosome preparations

and by the complexity of their protein composition. A way to overcome these difficulties is to use immunological probes (Gosti-Testu et al., 1986).

Centrosomes obtained through these procedures come from cycling cultured cells, a situation that brings additional complexity. The investigation of the structure-function relationships of centrosomes would obviously benefit from the isolation of centrosomes from normal quiescent cells. Aiming at this goal, we also wanted to scale up the preparation of centrosomes and therefore to attempt using tissue rather than cultured cells as starting material. Here we report the successful isolation of large numbers of centrosomes from calf thymus. We also compare these centrosomes to those isolated from human lymphoblasts for their structure, protein content and microtubule nucleating activity. We report important differences between these two isolates with regard to structure and nucleating functions.

## Materials and Methods

### Materials

All chemicals were from Sigma Chemical Co. (St. Louis, MO).

### Tissue

Fresh thymuses from 3-mo-old calves were collected in a local slaughterhouse within 20 min of slaughter and kept on ice during transportation to the laboratory. Isolated thymocytes (next section) were obtained within 1 h of removal of the thymus.

### Centrosome Preparation

All operations were done at 4°C. The protocol was adapted from previous work (Bornens et al., 1987) with the intention of simplifying it.

(a) The 70 g of tissue was mechanically dissociated in 450 ml PBS by pressing it through an 0.4-mm-mesh stainless steel sieve. The cell suspen-

sion containing  $\sim 7 \times 10^{10}$  thymocytes was filtered through a 0.25-mm-mesh sieve and centrifuged at 32 g during 5 min to remove heavy contaminants (usually collagen fibers).

(b) The supernatant was filtered through a nylon mesh and centrifuged at 310 g for 8 min in 50-ml conical tubes. Cells were resuspended in 400 ml PBS, sedimented once again, and finally resuspended in 20 ml of PBS to give a concentration of  $1.5\text{--}2 \times 10^9$  cells/ml (55–60% of the cells were lost during washing steps).

(c) The 1-ml aliquots of this cell suspension were successively dispersed in 2.5 liters of lysis buffer; 1 mM Tris (pH 8.0) containing 0.1%  $\beta$ -mercaptoethanol, 0.5% NP40,  $5 \times 10^{-4}$  M  $\text{MgCl}_2$ , 1 mg/ml iniprol,  $10^4$  U/liter leupeptin, 1 mM PMSF, and 1 mg/ml pepstatin (added extemporaneously) and magnetically stirred for 10 min. Optimal lysis was obtained with a concentration of  $10^7$  cells per ml. Most of the swollen chromatin stuck to the magnetic bar. The rest was sedimented at 2,400 g during 10 min.

(d) The supernatant was filtered through a nylon mesh; the filtrate was made 10 mM Pipes (pH 7.2) and 1 mM EDTA by addition of  $10\times$  solutions and centrifuged at 24,000 g during 1 h (in six 250-ml bottles on a centrifuge [Kontron Analytique, Velizy, France]).

(e) The tiny pellets of crude centrosomes were resuspended in 50 ml of supernatant, dispersed by several passages through a 22.5-gauge needle, and centrifuged at 198 g for 10 min to eliminate aggregates.

(f) The supernatant was overlaid on three discontinuous sucrose gradients set in three 30-ml tubes (Corex, Corning Medical, Le Vesinet, France), with 1 ml of 70% sucrose (wt/wt), successive 2-ml layers of 50%/40%/30%/20% (wt/wt) sucrose prepared in 10 mM  $\text{K}^+$  Pipes (pH 7.2), 1 mM EDTA, 0.1%  $\beta$ -mercaptoethanol, and 0.1% Triton X-100 with 1 mg/ml pepstatin and 1 mg/ml leupeptin, and run at 25,000 g for 1 h.

(g) The gradients were collected from the top. 1-ml fractions were assayed as described by Mitchison and Kirschner (1984) after sedimentation on a glass coverslip and fixation in methanol at  $-20^\circ\text{C}$ . Immunofluorescence was carried out using an anti- $\beta$ -tubulin mAb (Amersham, France SA, Les Ulis). A rhodamine-conjugated goat anti-mouse antibody was used as a secondary antibody (Cappel, West Chester, PA). When the rabbit anticontrosome serum 0013 (Manoury, 1978; Gosti-Testu et al., 1986) was used, the secondary antibody was a fluorescein-labeled sheep antirabbit from Institut Pasteur Production (Garches, France).

Enriched fractions were further purified on a discontinuous sucrose gradient in SW 50.1 (Beckman Instruments, Gagny, France) tubes made of 0.7 ml of sucrose 70% (wt/wt), 0.6 ml of sucrose 50% (wt/wt), and 0.6 ml of sucrose 40% (wt/wt) prepared in the same buffer as the previous gradient. They were run at 120,000 g for 1 h. 150- $\mu\text{l}$  fractions were collected from the bottom and analyzed as described.

## Electron Microscopy

Isolated centrosomes were sedimented onto 12-mm round coverslips and processed as described elsewhere (Bornens et al., 1987), except that a 20-min treatment with 0.5% tannic acid (Simionescu and Simionescu, 1976) was carried out between glutaraldehyde and osmic fixations.

For the observation of in situ centrosomes, thymocytes were resuspended in RPMI 1640 medium complemented with 7% FCS and sedimented at 1 g for 1 h at  $37^\circ\text{C}$  on poly-lysine-coated coverslips. After a PBS wash, cells were fixed for 40 min with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixed with osmium tetroxide, dehydrated in ethanol, and embedded in Epon.

In all cases, sections parallel to the coverslips were observed in an electron microscope (model 201; Philips Electronics Instruments, Rahway, NJ) after contrasting with uranyl acetate and lead citrate.

## Microtubule Nucleation

The nucleating activity of isolated centrosomes was assayed as described by Mitchison and Kirschner (1984). In some assays, the centrosomes were not fixed in methanol for immunofluorescence study but fixed with glutaraldehyde and further processed for EM.

## Protein Analysis

Protein determination was done according to Lowry (1951). One-dimensional SDS-PAGE was performed according to Laemmli (1970) using a 6–15% gradient of polyacrylamide. Immunoblotting experiments were achieved according to Towbin et al. (1979) and reacted with serum 0013 as described elsewhere (Gosti-Testu et al., 1986).

## Results

### Yield of the Preparation

As shown in Fig. 1 A, centrosomes usually sedimented in one major peak (fractions 5–7) on the low-speed sucrose gradient, close to the theoretical interface between the 40 and 50% sucrose containing layers. The centrosome concentration ranged from 4 to  $9 \times 10^8$ /ml. Aggregated centrosomes, representing roughly one-third of the preparation, sedimented further down to the bottom of the gradients. These fractions were discarded in the reported experiments. The yield of the preparation at that step was  $\sim 20\%$ , when compared with the number of lysed cells.

Individual fractions were further purified on a high speed discontinuous gradient, as shown in Fig. 1 B. Centrosomes were usually concentrated in a single fraction corresponding to  $\sim 60\%$  sucrose. This purification step led to a decrease of the yield of  $\sim 50\%$ .

### The Structure of Thymus Centrosomes

Immunofluorescent staining of preparations with antitubulin revealed that calf thymus centrosomes were isolated as pairs of tubulin-containing dots, likely to correspond to the two centrioles (Fig. 2 A). These were compared with centrosomes isolated from human lymphoblasts: on average, the two tubulin-containing dots were closer to each other in the case of thymus centrosomes (Fig. 2, B and C). A quantitative analysis of the inter-centriolar distance was carried out on 100 centrosomes in each case (Fig. 2 D): calf thymus centrosomes showed a quite narrow distribution, 76% of them having an intercentriolar distance  $\leq 0.67 \mu\text{m}$ . This was in marked contrast with the distance between centrioles of KE37 centrosomes, wherein values were very dispersed, and in which 98% of cases had a distance  $> 0.67 \mu\text{m}$ . The principal mode was centered on an intercentriolar distance of  $0.9 \mu\text{m}$ . It is noteworthy that  $> 15\%$  of the calf thymus centrosomes had an inter-centriolar distance between 0.45 and  $0.5 \mu\text{m}$ . As centrioles displayed a diameter between 0.15 and  $0.2 \mu\text{m}$ , such short intercentriolar distances estimated by immunofluorescence suggested a very close association of the two centrioles. This prompted us to undertake an ultrastructural

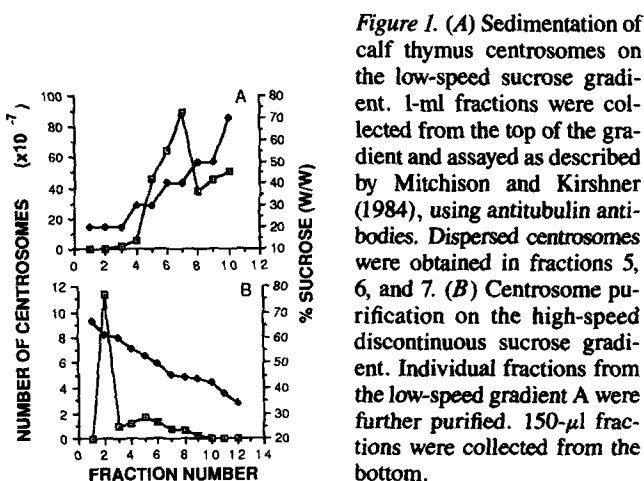
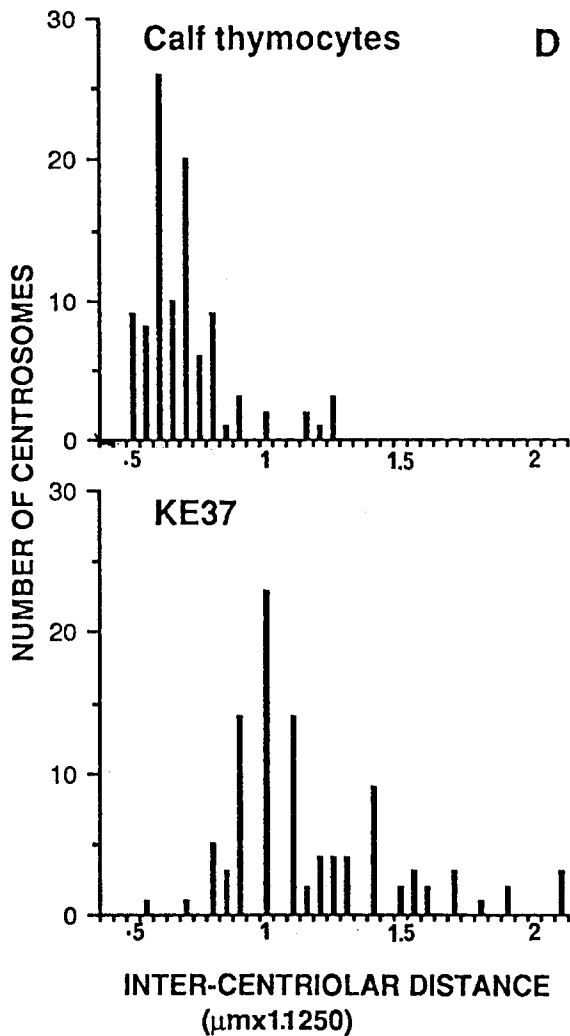
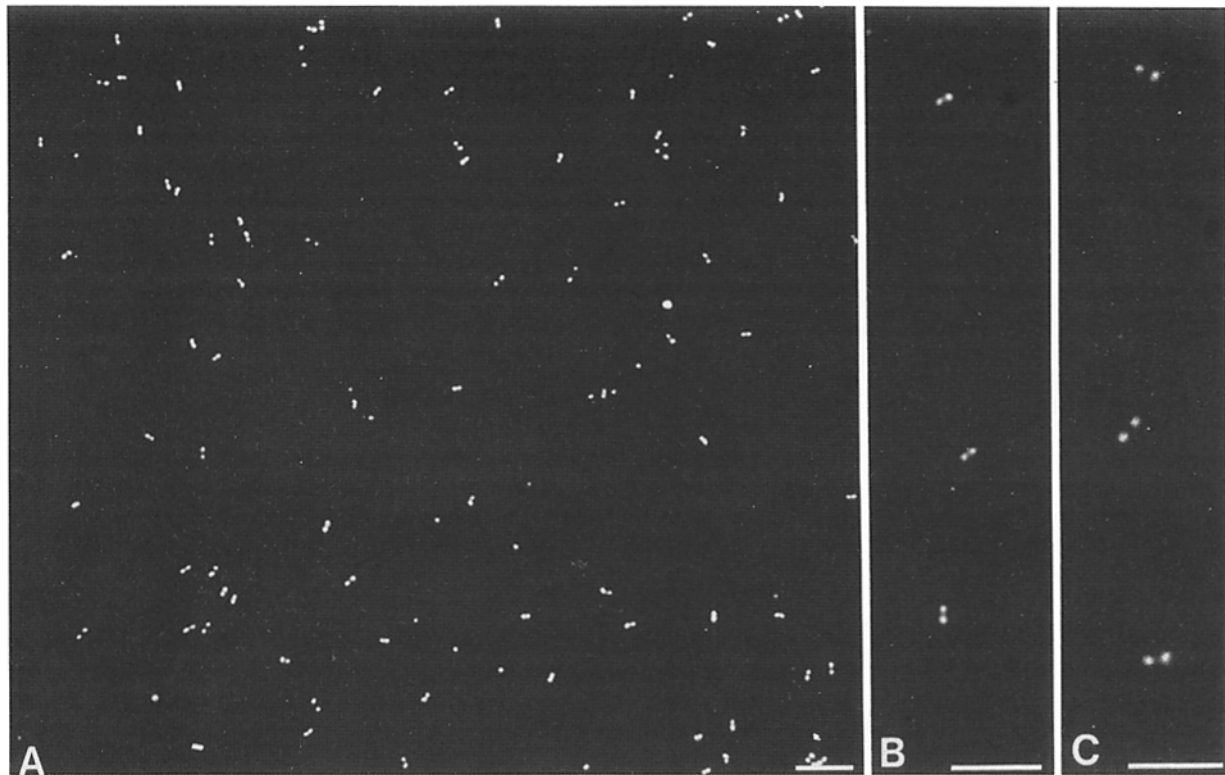
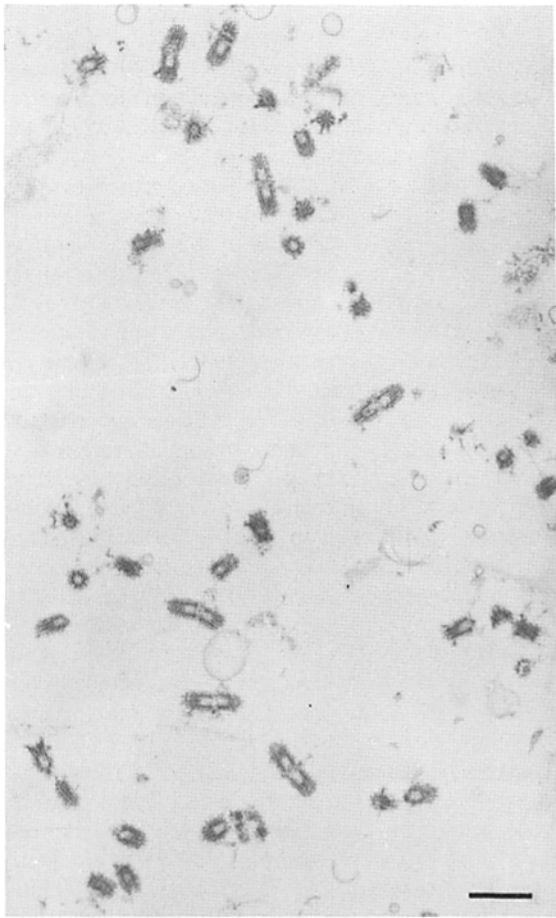


Figure 1. (A) Sedimentation of calf thymus centrosomes on the low-speed sucrose gradient. 1-ml fractions were collected from the top of the gradient and assayed as described by Mitchison and Kirschner (1984), using antitubulin antibodies. Dispersed centrosomes were obtained in fractions 5, 6, and 7. (B) Centrosome purification on the high-speed discontinuous sucrose gradient. Individual fractions from the low-speed gradient A were further purified. 150- $\mu\text{l}$  fractions were collected from the bottom.



*Figure 2. (A) Aspect of a preparation of centrosomes from calf thymus, as revealed by antitubulin antibodies. Centrosomes are isolated as pairs of centrioles. (B) Higher magnification showing that the centrioles in a centrosome are close to each other. For comparison, centrosomes from human lymphoblasts are shown in C. They were stained with the same antibody, observed on a Eichert Polyvar epifluorescence microscope with an 100-objective. Images were recorded on the same TMax 400 Kodak film (Eastman Kodak, Rochester, NY) for both preparations. (D) Histograms of the inter-centriolar distances in calf thymus centrosomes are compared to those of human lymphoblast centrosomes. 24 × 36 mm TMax negatives (×225) were projected on a screen (×50). Intercentriolar distances (measured between the centers of the tubulin-containing dots) were performed on 100 centrosomes in each case. Note the clear differences between both distributions. Bar, 10 μm in A, 5 μm in B and C.*

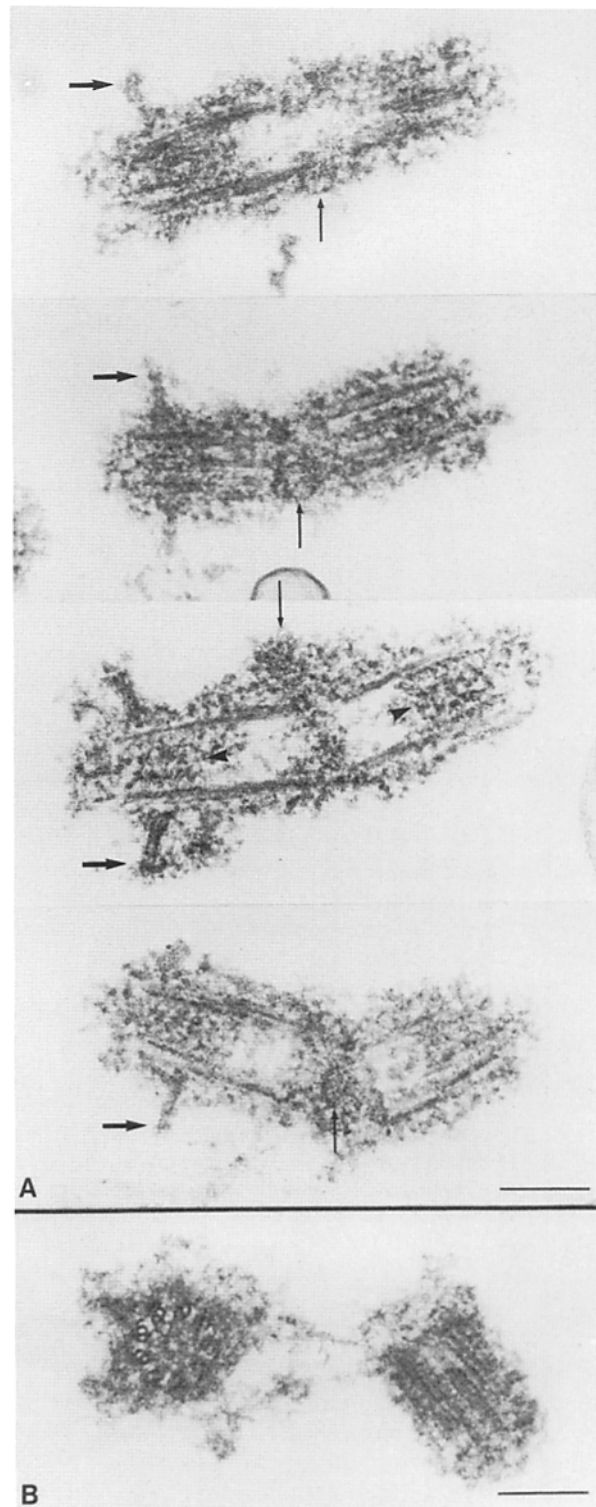


**Figure 3.** Field at low magnification of calf thymus centrosome preparations after high-speed sucrose gradient purification. Sections are parallel to the coverslip, on which isolated centrosomes have been sedimented. Note the homogeneity of the preparation and the characteristic bar shape of most of the centrosomes. Contaminants are pieces of membranes that sometimes appear associated with individual centrosomes. Bar, 1  $\mu\text{m}$ .

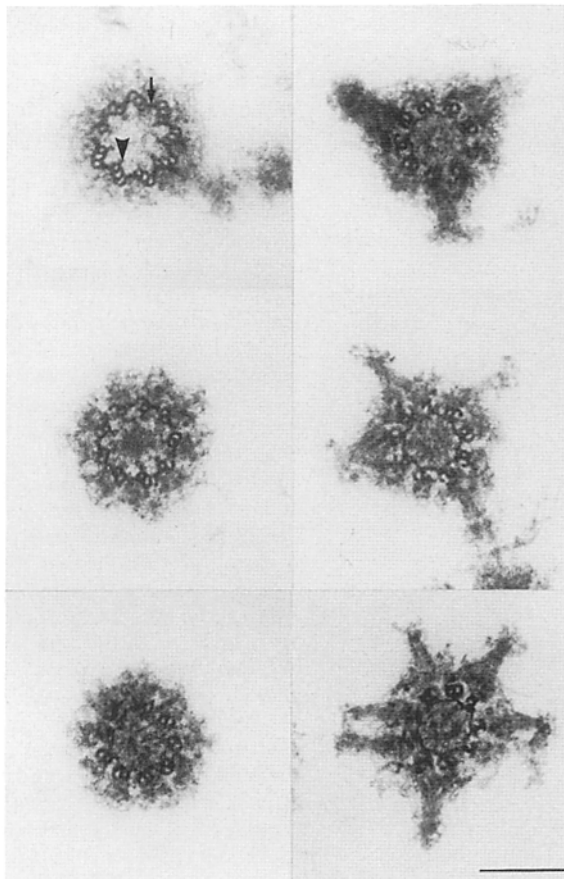
study. For this purpose, centrosomes were sedimented on a coverslip before processing for EM. This procedure allowed a reliable estimation of the purity of the preparations and favored the orientation of centrosomes. Most of the centrosomes were parallel to the coverslip, as they had a bar shape due to the colinear organization of the two centrioles (Figs. 3 and 4).

The features of this new type of centrosome follow. The two centrioles in a centrosome were symmetrically positioned with respect to a center: they were apparently stuck to each other by their proximal ends, i.e., by the ends having an empty lumen. A granular material seemed to fill the narrow space between both centrioles. Most often, a sort of nodule could be observed embedded in the intervening material, or slightly off-side (Fig. 4). This structure was more evident in centrosomes with disoriented centrioles. Comparison with centrosomes from KE37 cells suggested that this structure could be a folded form of the intercentriolar link.

The centrioles themselves were apparently similar to those of KE37 cells, with triplets of microtubules at the proximal end and doublets at the distal one and they had similar dimensions (Fig. 5). A link between tubule A of a triplet and



**Figure 4.** Centrosomes from calf thymus have a stereotypic organization (A). Centrioles are apparently attached together by the proximal ends. Their distal extremities are filled with an electron-dense material in which an axial hub can be seen in favorable sections (*arrowheads*). A globular domain can be seen at the junction between centrioles (*thin arrows*). Most often off-side, it looks like a hinge. The pericentriolar material is not abundant. Thin radial arms are observed associated with only one centriole in a centrosome (*fat arrows*). A centrosome from human lymphoblasts KE37 is shown for comparison in B. Note that the centrioles are linked by thin fibrils. Bars, 0.2  $\mu\text{m}$ .



**Figure 5.** Transverse sections of calf thymus centrosomes. Sections at the proximal end (judged by the empty lumen) of one or the other centriole in a pair (*top left corner*) demonstrate the typical triplet organization, with inter-triplet links from tubules *A* and *C* (*arrow*) and luminal projections associated with *A*-tubules (*arrow-head*). Sections at the distal end of the centriole bearing radial arms (*right*) showed nine doublets in the centriolar structure. Arms were never more than six and their distribution was often not radially symmetrical. Sections with a nine-doublet centriole having a filled lumen, whose position within the centrosome could not be ascertained, are shown on the left. They display a thin rim of pericentriolar material, possibly made of domains organized according to a ninefold symmetry. Bar, 0.2  $\mu\text{m}$ .

tubule *C* of the adjacent triplet was observed at the proximal end, provided centrosomes were fixed with tannic acid. The distal end of each centriole was filled about three-fifths of its length by a material apparently organized about an axis, as in human centrosomes (Bornens et al., 1987).

The pericentriolar material was not abundant and clearly distinguished one centriole from the other: radial arms were present at the distal end of one centriole only (Fig. 4). Arm length was not different from what was observed on centrosomes from KE 37 cells (0.1  $\mu\text{m}$ ) whereas the width was significantly smaller. Transverse sections in the plane of the arms never revealed more, and often less, than six arms (Fig. 5). Their radial distribution was not always symmetrical.

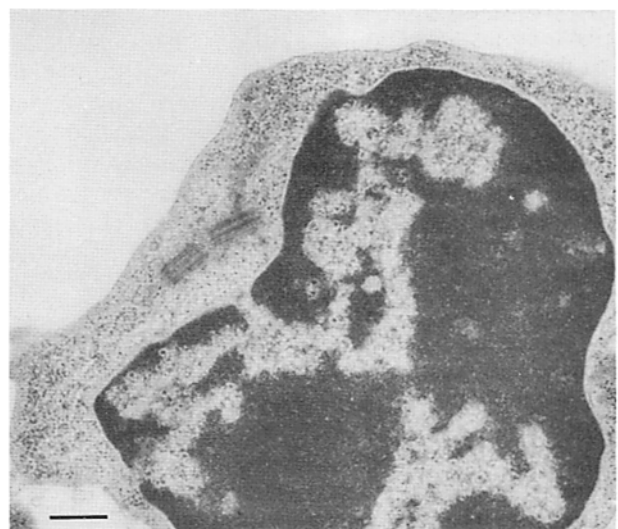
We checked that the peculiar configuration of calf thymus centrosomes was not the result of the isolation procedure. A similar configuration was indeed observed *in situ*. An example is shown in Fig. 6.

### Protein Content

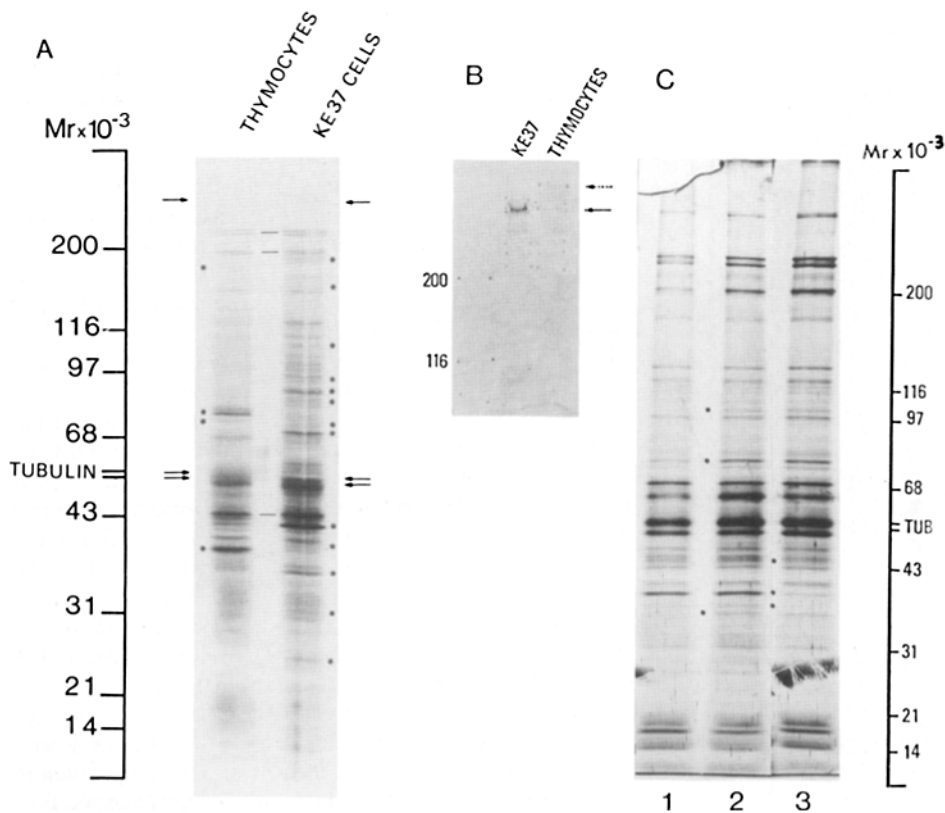
The total protein content was  $3.8 \times 10^{-2} \pm 0.49$  pg/centrosome before the second gradient sedimentation (six determinations), compared with the figure of  $2-3 \times 10^{-2}$  pg for centrosomes from human lymphoblasts.

The one-dimensional protein profile of centrosomes from calf thymus displayed an overall similarity with that of centrosomes from KE 37 cells, as judged by the migration of the polypeptides (Fig. 7 *A*) and the silver staining (from yellow to brown). Some of the common bands have been identified. They are the tubulins and a high molecular weight polypeptide (>300 kD), which has been identified as a pericentriolar protein in human centrosomes (Gosti-Testu et al., 1986). Its presence in calf centrosomes was established by Western blotting (Fig. 7 *B*) and by immunocytochemical means (see Fig. 8 *B* and 9 *A*). Three polypeptides identified by specific antibodies (not shown) have been consistently found associated with both preparations, apparently in similar amount: they are the spectrins, the myosin heavy chain, and the actin.

Quite a few polypeptide bands (indicated by asterisks in Fig. 7 *A*) were represented only in one or the other type of preparation. Two polypeptides of molecular masses of 37 and 78 kD were particularly abundant in thymus centrosome preparations. The 78-kD polypeptide, as other minor bands (indicated with dots between lanes 1 and 2 in Fig. 7 *C*), were dissociated by 0.15 M NaCl, demonstrating that they were not integral proteins of thymus centrosomes. These proteins could be artefactually adsorbed on centrosomes during the isolation procedure because of their insolubility at low ionic strength. Their association with centrosomes was maintained after further purification at low ionic strength on the high-speed sucrose gradient (compare lanes 2 and 3). By contrast, the 37-kD polypeptide and few minor bands (indicated by dots between lanes 2 and 3 in Fig. 7 *C*) were not dissociated by salt but were separated from centrosomes during the second gradient purification. These proteins are likely to belong to particulate contaminants that cannot be separated from the centrosomes during the low-speed gradient sedimentation.



**Figure 6.** Ultrathin section of calf thymocyte observed by EM. The colinear organization of the two centrioles is visible *in situ*. Bar, 0.5  $\mu\text{m}$ .



somes (arrow) but its affinity for serum 0013 or/and its amount is much lower. The possibility that the antigen is composed also of a higher polypeptide is indicated by the dotted arrow. (C) One-dimensional electrophoretic analysis of centrosomal proteins from calf thymus ( $2.5 \times 10^7$  centrosomes in each case) after the low- (lane 2) and high-speed gradient (lane 3). Lane 1 corresponds to salt-treated centrosomes after the low-speed gradient. Protein pattern modifications are indicated with dark dots (see text).

Figure 7. (A) One-dimensional electrophoretic analysis of centrosomal proteins from calf thymus and from human lymphoblasts on a 6-15% gradient SDS-PAGE. Identified polypeptides have been indicated, with arrows for the centriolar tubulins and the high-molecular weight pericentriolar protein, and with short lines between the two slots for spectrins, the heavy chain myosin, and the actin (from top to bottom), which are consistently associated with centrosome preparations (see text). Asterisks, bands that are highly represented or are absent in the other centrosomal preparation. (B) Identification of 0013 serum specific antigen in centrosome preparations from KE37 cells and from calf thymus. Proteins from  $2 \times 10^7$  and  $7 \times 10^7$  centrosomes respectively were analyzed on a 4% acrylamide-SDS gel containing urea. The dots on the filter mark proteins from the samples, and the molecular weight standards, stained with Ponceau red before immunoreaction with rabbit serum 0013. This antigen is present in calf thymocyte centro-

### Microtubule Nucleation

The capacity of calf thymus centrosomes to nucleate microtubules in vitro from purified tubulin was compared with that of centrosomes from KE 37 cells (Fig. 8). A significant difference was observed: on average, calf thymus centrosomes nucleated  $\sim 20$  microtubules, which is two to four times less than that obtained with centrosomes from KE 37 cells (compare Fig. 8, A and C). Moreover, the few microtubules nucleated by thymus centrosomes were often collapsed in two bundles. The length of microtubules was similar in both cases.

Given the peculiar structure of calf thymus centrosomes, we were eager to know where microtubules were growing from. EM revealed that they show biased growth of microtubules off the distal ends of centrioles (Fig. 9 A). The situation was obviously different with centrosomes from KE 37 cells, which nucleated numerous microtubules from the pericentriolar material (Fig. 9 B).

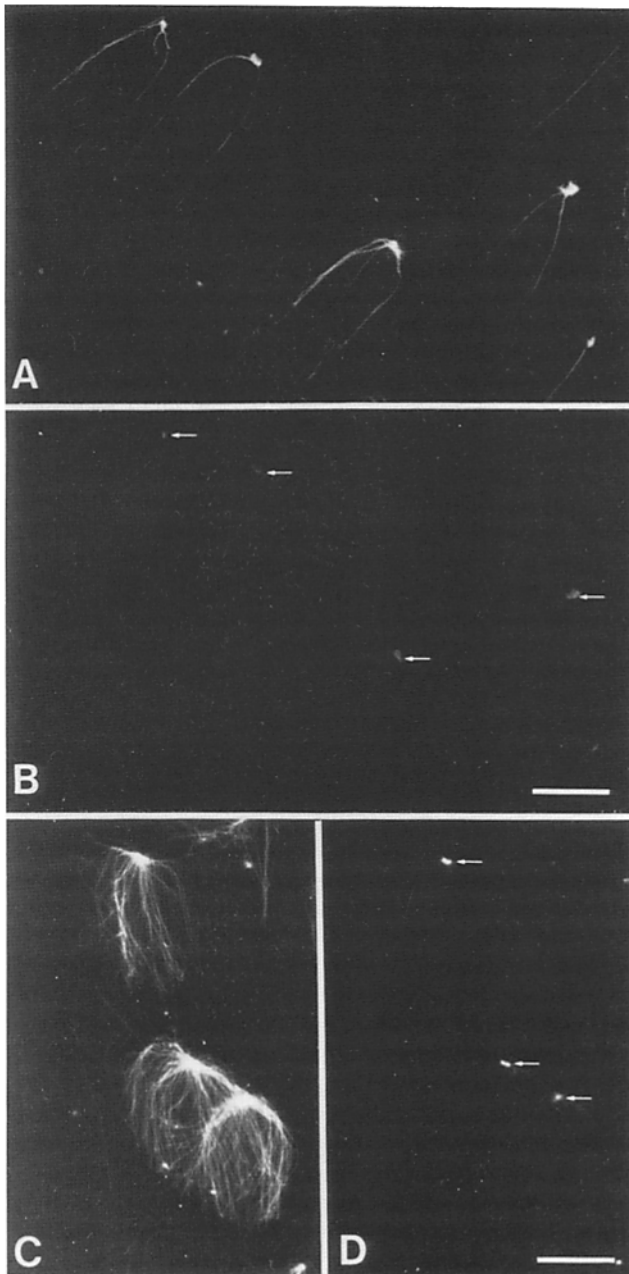
### Discussion

We report here the successful development of a simple isolation procedure of centrosomes from calf thymus. This procedure has also led to the discovery of an unexpected organization of centrosomes in thymocytes.

Among tissues, thymus was an obvious candidate for centrosome preparation as it contains junctionless cells with the

lowest known cytoplasmic-to-nuclear ratio. Calf thymus has turned out to be a quite favorable material as it provides, at low cost, a considerable number of cells. We systematically attempted to simplify the procedure previously developed for human lymphoblasts (Bornens et al., 1987). Pretreatments of cells with nocodazole and cytochalasin D were found to be unnecessary. The cells could be lysed directly after PBS washes without decrease in the yield or in the purity of the preparations. The lysis step could be shortened to 10 min and the DNase treatment could be suppressed. The large volume of lysis buffer used in the original procedure can become difficult to handle when large numbers of cells are lysed. Attempts to increase the cell concentration to higher than  $10^7$  cells/ml were unsuccessful, however, resulting in severe aggregation of the centrosomal material. Finally, the starting material is so abundant that one is not too concerned by the yield obtained. It was consistently close to 20%, assuming one centrosome per lysed cell, after the low-speed gradient, and decreased on average to 10% after purification on the high-speed gradient.

Total protein content of centrosomes from calf thymus after the low speed sucrose gradient purification was significantly higher ( $\sim 50\%$ ) to that of human centrosomes. From the electron microscopic examination of the preparations, it appeared that the more frequent contaminations were pieces of plasma membrane. The subsequent high-speed gradient purification eliminated most of the large pieces of membrane



**Figure 8.** Centrosomes isolated from calf thymus (*A* and *B*) nucleate less microtubules from purified tubulin than the centrosomes isolated from human lymphoblasts (*C* and *D*), and in a manner strikingly different. A double immunofluorescence was carried out using a mixture of a anti-tubulin mAb and the rabbit anti-pericentriolar material serum 0013. Mixed rhodamine-conjugated goat anti-mouse and fluorescein-labeled sheep anti-rabbit secondary antibodies were used. (*A* and *C*) Antitubulin. (*B* and *D*) Anticentrosome (arrows point to the centrosomes); the bovine centrosomes react poorly with the serum 0013. Bars, 10  $\mu\text{m}$ .

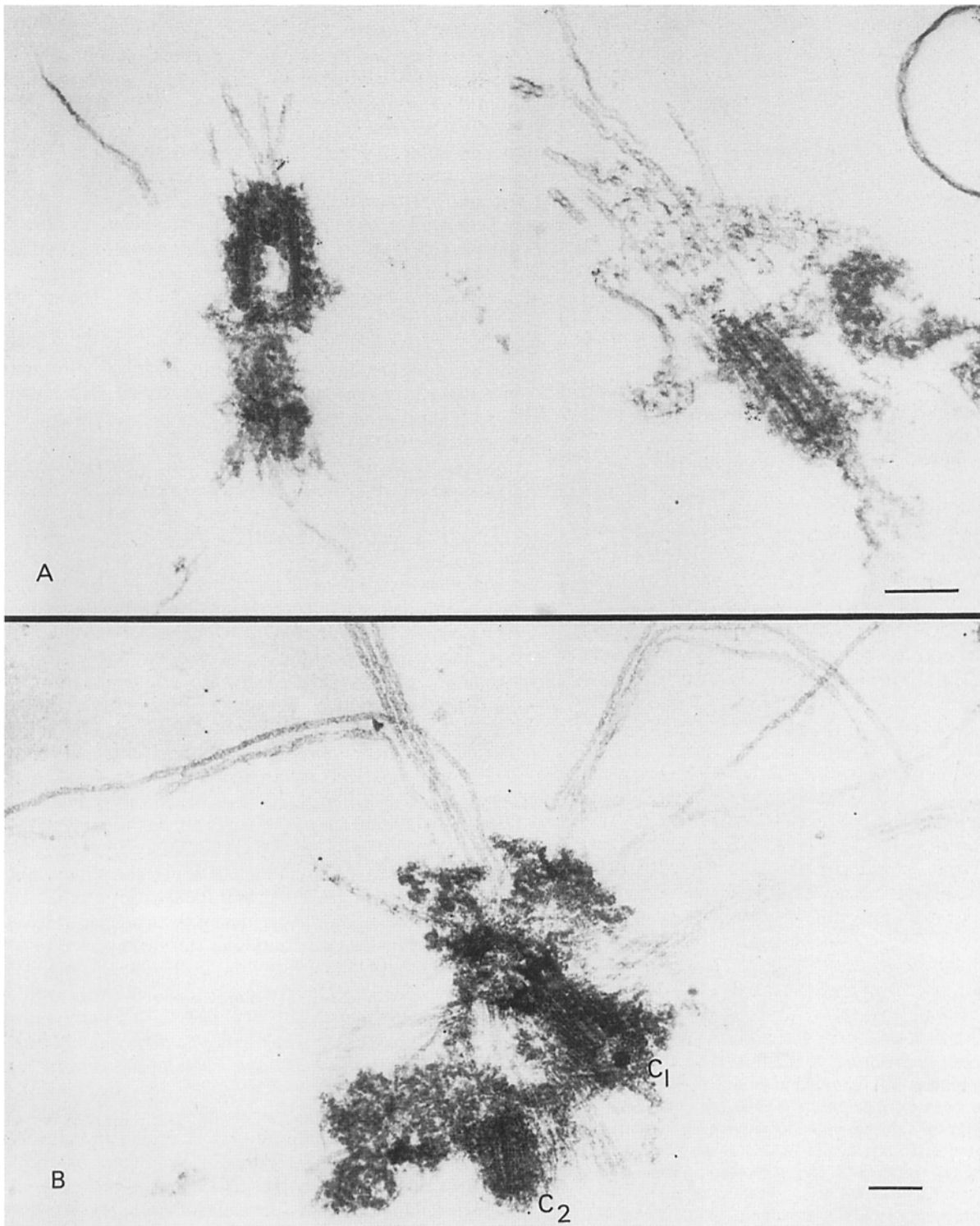
(see Fig. 3). However, the total protein content was not dramatically modified as judged by silver staining after side-by-side electrophoresis of proteins from equal numbers of centrosomes before and after the second gradient (Fig. 7 *C*, lanes 2 and 3). Moreover, spectrin and myosin, which have been identified in the preparations, were not specifically

eliminated by the high-speed gradient. These proteins, which were also present together with actin in the human centrosome preparations, might represent mere contaminations or constitutive associations as centrosomes are dissociated from their environment in an unknown manner during cell lysis. In the preparations from calf thymus, small pieces of membrane sometimes seemed to be linked to the centrosomes, although serial sectioning will be necessary to establish this point unambiguously.

Comparative protein analysis of centrosomes from calf thymus and from human lymphoblasts revealed important similarities, suggesting that the specific enrichment of centrosomes was the same in both cases. An extensive comparison of the two-dimensional patterns of both preparations and of each preparation with the respective total cellular proteins will be necessary, however, to identify genuine centrosomal proteins. The protein pattern of calf centrosome preparations was complex, with the majority of the polypeptides in the range of 40–70 kD. Several polypeptide bands present in centrosomes from the low-speed gradient, some of them in significant amount, were weakly associated with the centrosomes or belonged to contaminants as they were largely eliminated either by 0.15 NaCl or by high-speed gradient purification.

Tubulins were indeed observed but it was difficult to estimate their relative abundance from silver staining, which is rather erratic for these proteins (compare for example Fig. 7 *A*, lane *thymocytes*, with Fig. 7 *C*). Few high-molecular weight components were present in both preparations. An unexpected similarity between both preparations concerned the presence of a high-molecular mass protein (>300 kD) that had been identified as a pericentriolar protein in human centrosomes with the rabbit serum 0013 (Gosti-Testu et al., 1986). It was shown to react with isolated calf centrosomes by Western blotting on low porosity gels and by immunofluorescence (Fig. 7 *B* and Fig. 8 *B*). The affinity for serum 0013 or the amount of this high-molecular weight protein was, however, much lower in calf than in human centrosomes. Ultrastructural localization by immunogold techniques revealed also a pericentriolar localization in calf thymus (see Fig. 9 *A*). The serum used to identify this centrosomal component was shown previously to possess a specificity restricted to primate centrosomes (Maunoury, 1978). This study on bovine centrosomes has provided evidence for a possibly larger conservation than previously suspected for this centrosomal antigen.

Ultrastructural studies of centrosomes in situ have always been confronted with the problem of orientating ultrathin sections with respect to the complex three-dimensional organization of this organelle. Isolated centrosomes can be oriented by sedimentation on a coverslip before being processed for EM. This allowed us for example to demonstrate the structural basis of the paired configuration of centrosomes (Bornens et al., 1987). In this work, we observed a further aspect of the paired configuration of centrosomes: the link between both centrioles can have a cell-specific structure. This link is very short and compact in calf thymocytes, as if it was in a folded form. This brings close together the proximal ends of both centrioles, resulting in a linear configuration of the centrosome. We thus were also able to establish unambiguously the occurrence of differentiation among the two centrioles in a centrosome, one of them only having



**Figure 9.** Biased growth of microtubules off the distal ends of the centrioles of centrosomes isolated from calf thymus (*A*) compared with the aster-like growth of microtubules from the pericentriolar material of centrosomes isolated from human lymphoblasts (*B*). Sections are parallel to the coverslip, on which centrosomes were sedimented after the nucleation experiment with purified tubulin. The gold particles in *A* correspond to immunostaining with the rabbit 0013 serum, which was carried out in some experiments after sedimentation of the asters nucleated on centrosomes. Bars, 0.2  $\mu\text{m}$ .

radial arms. The number of radial arms was never more, and often less, than six, contrasting with the most common mode of nine arms radially organized about the centriole in human centrosomes (Bornens et al., 1987).

We demonstrated that the few microtubules nucleated by calf thymus centrosomes grew from the distal ends of both centrioles, suggesting a low nucleating activity of the pericentriolar material or the absence of such an activity. The



growth rate was apparently quite similar to that observed with human centrosomes, an observation that is consistent with plus end assembly (Bergen et al., 1980). This is also what could be inferred for tubulin assembly at the distal end of centrioles, a pattern similar to axoneme growth on basal bodies (Bergen et al., 1980). Biased assembly of microtubules off the distal ends of centrioles might be for some reasons (low nucleating activity of the pericentriolar material, artificial conditions) favored in vitro and by-pass the physiological mechanism of assembly. We attempted to localize the microtubule network in situ: immunofluorescence staining with antitubulin revealed, as in small lymphocytes, an aster of few microtubules surrounding the nucleus, without apparent directional bias. Triton extraction of thymocytes before processing for EM, carried out in an effort to identify the origin of these microtubules at the centrosome level, failed so far to provide convincing pictures, as the number of microtubules is quite small in these cells. On this aspect at least, in vivo and in vitro observations do not conflict: centrosomes from calf thymus nucleate quite a small number of microtubules.

Several questions are raised by the linear configuration of these centrosomes. If we eliminate the unlikely possibility that it is a bovine-specific configuration, one is left with the conclusion that it is a thymus-specific one. This kind of configuration is rarely observed except in some plant organisms where centrosomes could be permanently organized in this way, even during duplication (Heath and Greenwood, 1970; Moser and Kreitner, 1970). In animal cells, this configuration is observed during the assembly of basal bodies, or their transport to the apical pole of ciliated cells (Anderson and Brenner, 1971; Lemullois et al., 1987). Thus, besides the mode of microtubule nucleation, this configuration is the second feature of thymus centrosomes that is reminiscent of basal bodies. Unlike epithelial or fibroblastic G0 cells in culture, which possess a primary cilium associated with their centrosome and unlike many cells in the organism that possess a permanent primary cilium, lymphoid cells never display a primary cilium. Our data suggest, however, that some features of the centrosome might be modified at some stage during thymocyte differentiation. The functional relevance of the existence of a centrosomal-associated primary cilium is unknown (Roth et al., 1988). A close link with the cell cycle control has been proposed in the past (Tucker et al., 1979a,b). Thymus is a remarkable tissue for T lymphocyte differentiation, in which thymocytes are severely selected for a viable phenotype. Although the selective mechanism is not completely understood, it is established that the majority (~85%) of thymocytes are double positive for the two coreceptors of T cells, and are arrested in G0-G1 (Rothenberg and Lugo, 1985). Most of the isolated thymus centrosomes therefore come from these particular cells. Contrary to many examples of terminal differentiation in which the cell cycle is irreversibly blocked, a characteristic feature of thymic differentiation is that cell cycle is only temporarily blocked, the mature T cells being capable of active proliferation during the immune response. The linear configuration reported in this work could correspond to the freezing of a transient state in the cell cycle.

Two independent approaches can be used to investigate the functional meaning of the linear configuration of thymus centrosomes. The first one is to examine the in situ situation,

trying to correlate the centrosome configuration with the progress of T cell maturation and with the proliferative state. The second one is to directly assay the parthenogenetic activity of isolated centrosomes in *Xenopus* eggs as recently achieved for human centrosomes from unsynchronized (Klotz et al., 1990) and synchronized cells (Tournier et al., 1989). Experiments are under way in both directions in our laboratory.

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## References

- Anderson, R. G. W., and R. M. Brenner. 1971. The formation of basal bodies (centrioles) in the rhesus monkey. *J. Cell Biol.* 50:10-34.
- Bailly, E., M. Dorée, P. Nurse, and M. Bornens. 1989. p34<sup>cdc2</sup> is located in both nucleus and cytoplasm; part is centrosomal associated at G2/M and enters vesicles at anaphase. *EMBO (Eur. Mol. Biol. Organ.) J.* 8: In press.
- Bergen, L. G., R. Kuriyama, and G. G. Borisy. 1980. Polarity of microtubules nucleated by centrosomes and chromosomes of chinese hamster ovary cells in vitro. *J. Cell Biol.* 84:151-159.
- Bornens, M., and E. Karsenti. 1984. The Centrosome. Membrane: Structure and Functions. Vol. 6. E. E. Bittar, editor. John Wiley & Sons, Inc. 100-171.
- Bornens, M., M. Paintrand, J. Berges, M. C. Marty, and E. Karsenti. 1987. Structural and chemical characterization of isolated centrosomes. *Cell Motil. Cytoskeleton.* 8:238-249.
- Freeman, M., C. Nüsslein-Volhard, and D. M. Glover. 1986. The dissociation of nuclear and centrosomal division in gnu, a mutation causing giant nuclei in *Drosophila*. *Cell.* 46:457-468.
- Gosti-Testu, F., M. C. Marty, J. Berges, R. Maunoury, and M. Bornens. 1986. Identification of centrosomal proteins in a human lymphoblastic cell line. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2545-2550.
- Heath, I. B., and A. D. Greenwood. 1970. Centriole replication and nuclear division in *Saprolegnia*. *J. Gen. Microbiol.* 62:139-148.
- Karsenti, E., J. Newport, R. Hubble, and M. W. Kirschner. 1984. Interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into *Xenopus* eggs. *J. Cell Biol.* 98:1730-1745.
- Klotz, C., M. C. Dabauvalle, M. Paintrand, T. Weber, M. Bornens, and E. Karsenti. 1990. Pathogenesis in *Xenopus* eggs requires centrosomal integrity. *J. Cell Biol.* 110: In press.
- Koonce, M. P., R. A. Cloney, and M. W. Berns. 1984. Laser irradiation of centrosomes in newt eosinophils: evidence of a centriole role in motility. *J. Cell Biol.* 98:1999-2010.
- Kuriyama, R., and G. G. Borisy. 1985. Identification of molecular components of the centrosphere in the mitotic spindle of sea urchin eggs. *J. Cell Biol.* 101:524-530.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature (Lond.)* 227:680-685.
- Lemullois, M., C. Klotz, and D. Sandoz. 1987. Immunocytochemical localization of myosin during ciliogenesis of quail oviduct. *Eur. J. Cell Biol.* 453:429-437.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Maunoury, R. 1978. Localisation immunocytochimique de la centrosphère de cellules tumorales humaines par utilisation d'anticorps naurels de lapin. *Compt. Rend. Acad. Sci. Paris.* 286:503-506.
- Mitchison, T., and M. Kirschner. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature (Lond.)* 312:232-237.
- Moser, J. W., and G. L. Kreiner. 1970. Centrosome structure in *Anthoceros laevis* and *Marchantia polymorpha*. *J. Cell Biol.* 44:454-458.
- Nagano, H., S. Hirai, K. Okana, and S. Ikegami. 1981. A chromosomal cleavage of fertilized starfish eggs in the presence of Aphidicolin. *Dev. Biol.* 85:409-415.
- Picard, A., M. C. Harricane, J. C. Labbé, and M. Dorée. 1988. Germinal vesicle components are not required for the cell cycle oscillator of the early starfish embryo. *Dev. Biol.* 128:121-128.
- Raff, J. W., and D. M. Glover. 1989. Centrosomes, and not nuclei, initiate pole cell formation in *Drosophila* embryos. *Cell.* 57:611-619.
- Rogers, K. A., N. C. McKee, and U. I. Kalnins. 1985. Preferential orientation

- of centrioles towards the heart in endothelial cells of major blood vessels is reestablished after reversal of a segment. *Proc. Natl. Acad. Sci. USA.* 82:3272-3276.
- Roth, K. E., C. L. Rieder, and S. S. Bowser. 1988. Flexible-Substratum technique for viewing cells from the side: some in vitro properties of primary (9 + 0) cilia in cultured kidney epithelia. *J. Cell Sci.* 89:457-466.
- Rothenberg, E., and J. P. Lugo. 1985. Differentiation and cell division in the mammalian thymus. *Dev. Biol.* 112:1-17.
- Schliwa, M., K. B. Pryzwansky, and U. Euteneuer. 1982. Centrosome splitting in neutrophils: an unusual phenomenon related to cell activation and motility. *Cell.* 81:705-717.
- Simionescu, N., and M. Simionescu. 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy: I-procedure and evidence for mordanting effect. *J. Cell Biol.* 70:608-621.
- Sluder, G., F. J. Miller, and C. L. Rieder. 1986. The reproduction of centrosomes. Nuclear versus cytoplasmic controls. *J. Cell Biol.* 103:1873-1881.
- Tassin, A. M., B. Maro, and M. Bornens. 1985. Fate of microtubule-organizing centers during myogenesis in vitro. *J. Cell Biol.* 100:35-46.
- Tournier, F., E. Karsenti, and M. Bornens. 1989. Parthenogenesis in *Xenopus* eggs induced by centrosomes from human synchronized cells. *Dev. Biol.* In press.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Tucker, R. W., C. D. Scher, and C. D. Stiles. 1979. Centriole deciliation associated with the early response of 3T3 cells to growth factors but not to SV40. *Cell.* 18:1065-1072.
- Tucker, R. W., A. B. Pardee, and K. Fujiwara. 1979b. Centriole ciliation is related to quiescence and DNA synthesis in 3T3 cells. *Cell.* 17:527-535.
- Vorobjev, I. A., and E. S. Nadehzhina. 1987. The centrosome and its role in the organization of microtubules. *Int. Rev. Cytol.* 106:227-284.
- Whitfield, W. G. F., S. E. Millar, H. Saumweber, M. Frasch, and D. M. Glover. 1988. Cloning of a gene encoding an antigen associated with the centrosome in *Drosophila*. *J. Cell Sci.* 89:467-480.