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Reprinted from
ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Volume 253, Pages 670–684
June 30, 1975

ACTION OF COLCHICINE ON AXONAL FLOW AND PITUICYTES IN THE HYPOTHALAMOPITUITARY SYSTEM OF THE RAT *

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The newly accumulated knowledge of microtubules (MT) and their chemical composition has considerably increased our understanding of the mechanisms of the action of colchicine and its derivatives. Twenty years ago, at the time of writing a monograph on colchicine,[1] the main known actions of this alkaloid were on the mitotic spindle, and few facts indicated that intermitotic cells could be affected. For a period of less than ten years, however, the majority of workers have been studying the effects on intermitotic cells of the fixation of colchicine on the receptor site of the tubulin dimer.[2] This action and the mechanisms of assembly and disassembly of the tubulin monomers explain many effects of colchicine and of other microtubule poisons, such as the *Vinca* alkaloids, on such varied cell functions as mitosis, secretion (exocytosis), cytoplasmic flow, axoneme structure, and pigment cell movements. Several reviews on MT have been published recently.[3-9] The universal presence of MT in nearly all eucaryotic cells and the close chemical relationships of all tubulins[10] suggest that a general explanation of the action of colchicine and MT poisons on all cell changes linked with MT may be possible.

However, in the vast amount of published data, several observations do not agree well with the accepted opinion that colchicine, by combining with a specific receptor site on the tubulin dimer, prevents its assembly into MT.[11] Colchicine may also destroy already formed MT, as has been known since the first results were obtained on colchicine-mitosis[1, 3] and described in various cells such as neurons,[12] osteoclasts,[13] and Heliozoa.[14] This is often observed with high concentrations of the alkaloid, and may lead to the formation of intracytoplasmic fibrils, the chemical nature of which remains poorly defined.[12]

On the other hand, some MT structures appear morphologically resistant to colchicine, although functionally altered. This is the case with neurotubules which are not destroyed even when axonal flow, which for most authors is closely linked with neurotubule function, is impeded or arrested.[15]

The present paper arises from a series of experiments on the supraoptic and paraventricular nuclei of the rat's brain, from the neurons of which originate the hormones of the posterior lobe of the pituitary, and also some of the pituitary releasing hormones. One of us, after describing the ultrastructure of two types of neurons in the paraventricular nucleus,[16] used colchicine as a tool to increase the number of secretory granules in these neurons. A complete arrest of axonal transport of neurosecretion was observed, although the neuro-

* This work was supported by a grant (1971–1974) from the Belgian National Fund for Scientific Research and by grant no. 1120 from the Belgian National Fund for Medical Research.

tubules remained unchanged. Peculiar, elongated inclusions were observed in the neurosecretory axons.[17] In the pursuit of this series of experiments, the posterior lobe of the pituitary was studied after strong stimulation of antidiuretic hormone secretion by NaCl overloading of the drinking water, or after the administration of the diuretic furosemide (Lasix®). Quite unexpectedly, colchicine, in some of these experiments, appeared to have a stimulatory action on the formation of centrioles and cilia in the pituicytes.[18]

These paradoxical effects of colchicine will be presented and discussed briefly. They may be more than mere curiosities and may help us to understand better the functions of two types of microtubular structures, the neurotubules and the centrioles and associated cilia.

Materials and Methods

Animals. Male rats of the Sprague-Dawley strain were used in all experiments. Their average weight was 150 g.

Colchicine Administration. Under ether anesthesia, a dose of 200 μg of colchicine Merck diluted in 0.05 ml of distilled water was injected intracisternally. The animals were killed 8, 24, and 48 hours later. When injected intraperitoneally, 400 μg of colchicine in distilled water was given, and the animals were killed 8 hours later.

Furosemide. A total of 60 mg of furosemide (Lasix) was given in three intramuscular injections spaced every two hours. The animals were killed 8 hours after the first injection. In animals injected with colchicine, the first dose of furosemide was given immediately after the colchicine, and the animals were killed 8 hours later.

NaCl Overloading. The rats received for 15 days as drinking water a 2.5% solution of NaCl. These animals lost about 20 g of weight.

Diuresis. In these preliminary experiments, no precise estimation of the amount of urine secretion was made. The weight loss of the animals was considered to be a sufficient measure of water loss. After furosemide this reached about 30 g in eight hours.

Electron Microscope Techniques. Tissues were fixed by immersion in a 4% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4, for 30 min at room temperature. After washing for 24 hours in Millonig buffer with 0.5% sucrose, the fragments were postfixed for 30 min in 2% osmic acid. The nervous system was fixed by perfusion with about 200 ml of formaldehyde-glutaraldehyde solution, according to Karnovsky,[19] injected during 15 min under Nembutal anesthesia through an aortic canula. Blocks containing the supraoptic and paraventricular nuclei were dissected, fixed for one more hour in the same solution, washed in 0.1 M phosphate buffer, and postfixed in 1% osmic acid for two hours. Embedding was done in Epon, and the sections were stained by uranyl acetate-lead and examined with Siemens Elmiskop 1 and 101 microscopes.

Autoradiography. 50 μCi of ^{35}S-cysteine was injected intracisternally, and the animals were killed one and ten hours later. The brain and pituitary were fixed in formalin, serially cut, and coated with an Ilford K5 emulsion. Exposure time was 10–15 days.

Results

Axonal Flow

The hypothalamic-pituitary system lends itself well to a radioautographic study of the migration of neurosecretory granules from the neurons to the posterior lobe of the pituitary. After injection of ^{35}S-cysteine, the neurosecretion is strongly labeled, and its migration can be readily followed. In control animals, the posterior lobe is labeled about eight hours after a single injection of ^{35}S-cysteine.[17] After an intracisternal injection of colchicine the neurons remain labeled, indicating that there is no important disturbance of hormone synthesis, but no labeling of the posterior lobe could be evidenced, indicating the arrest of distal transport of neurosecretion.

The electron microscopy of these neurons shows that the amount of ribosomes and Golgi saccules in the cell body is normal, while the number of neurosecretory granules is increased. The neurotubules, which appear to arise from the axonal hillock, are comparable in number and size to those of normal cells. On the other hand, the axons show numerous swellings with accumulated neurosecretory granules (Figure 1). Neurotubules are, however, still visible in these regions, and no abnormal fibrillary material is present. Next to normal granules, with their dense content and strongly stained, fragile membrane, some elongated structures are found in all animals eight hours after colchicine injection, whether by intracisternal route or intraperitoneally.

These elongated inclusions have a dense content, somewhat similar to the neurosecretory granules (Figure 2). Their membrane, more or less discontinuous, is comparable to that of the normal granules. Some of these bodies show an internal fibrillar structure which, in well-oriented sections, appears to result from the close packing of tubules measuring about 20 nm in diameter (Figures 3, 4).

Centrioles and Cilia

It was known that in conditions of functional overloading, particularly in rats receiving drinking water with additional NaCl (2%), pituicytes were more active and could enter mitosis.[20, 21] It appeared interesting to study at the ultrastructural level the possible differential action of colchicine on spindle and axonal MT. A most unexpected result of these experiments was the discovery that such conditions stimulated the formation of centrioles and cilia.

Because NaCl overloading has a complex action, it was thought preferable to increase diuresis by injecting furosemide (Lasix). The results obtained, although not fundamentally different from those of NaCl overloading, are far more intense and will be described first.

In control animals, no centrioles or cilia have been observed in pituicytes. Although it is known that cilia may be found in neurons [22] and malignant glial cells,[23] in normal nervous tissue, apart from the ependymal cells, such organelles are exceedingly rare. On the contrary, in animals injected with furosemide and colchicine, remarkable changes were visible in the posterior lobe of the pituitary.† Numerous centrioles were found, sometimes with a quite normal struc-

† There are reasons to believe that similar changes may be found in other cells, but it appears preferable to limit this study to the posterior lobe.

FIGURE 1. Paraventricular nucleus. Rat, 48 hours after 200 μg of colchicine intracisternally. Accumulation of neurosecretory granules in a cell body (Cb) and in an axon (ax). Arrows indicate elongated formations among normal elementary neurosecretory granules.

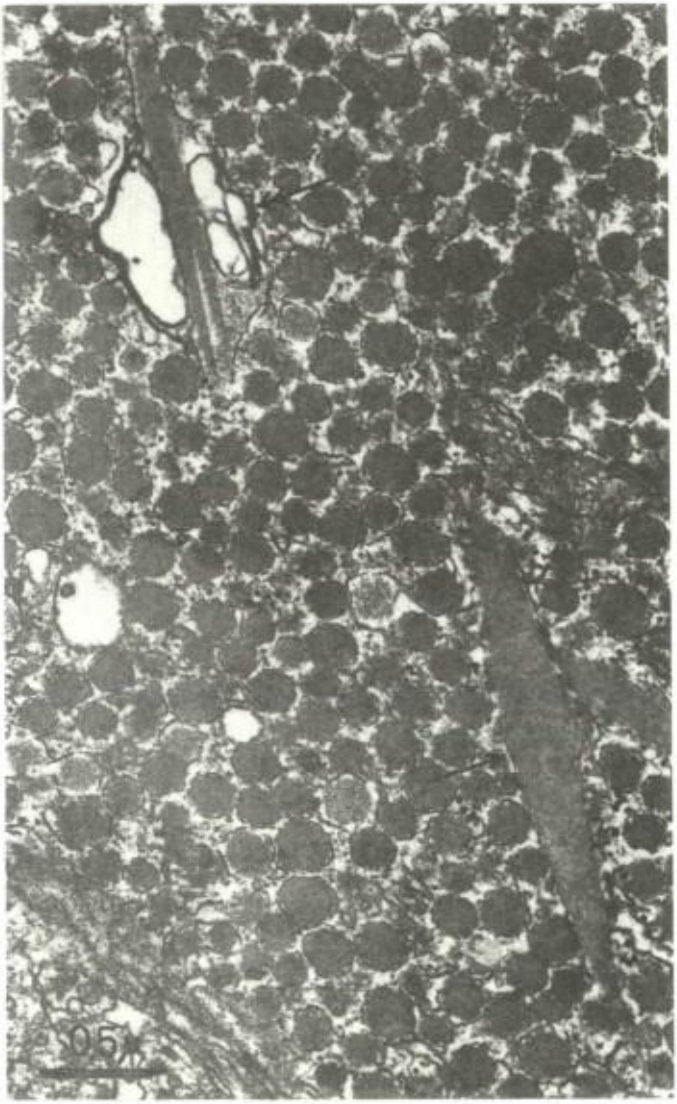

FIGURE 2. Paraventricular nucleus. Rat, 48 hours after 200 μg of colchicine intracisternally. Elongated formations (→) are seen among normal neurosecretory granules. Their content and membrane suggest fusion of elementary granules. Occasional myelin-like membranes are seen (⟶).

ture: nine groups of three tubules embedded in an electron-dense matrix (FIGURE 5). The centrioles often led to formation of cilia, with the centriolar tubules extending in a typical ciliary vesicle. These cilia were sometimes very long, and extended between the pituicytes (FIGURE 6). They were rarely of the 9 + 2 type; most were of the 9 + 0 type, often with irregularities of structure and with less than 9 groups of doublets (FIGURE 7, c). Sometimes, 8 + 1

cilia resulted from the curving of one of the doublets which came to occupy a central position (FIGURE 6, b) as described by Dahl [24] in the adenohypophysis. Some cilia appeared double. None showed any clearly visible basal plate, the transition between the centriolar structure of nine triplets and the nine doublets of the cilia taking place once the centrioles started to elongate. Striated rootlets were often associated with the basal bodies (FIGURE 7, b).

Evidence that the centrioles were newly formed was provided by the fact

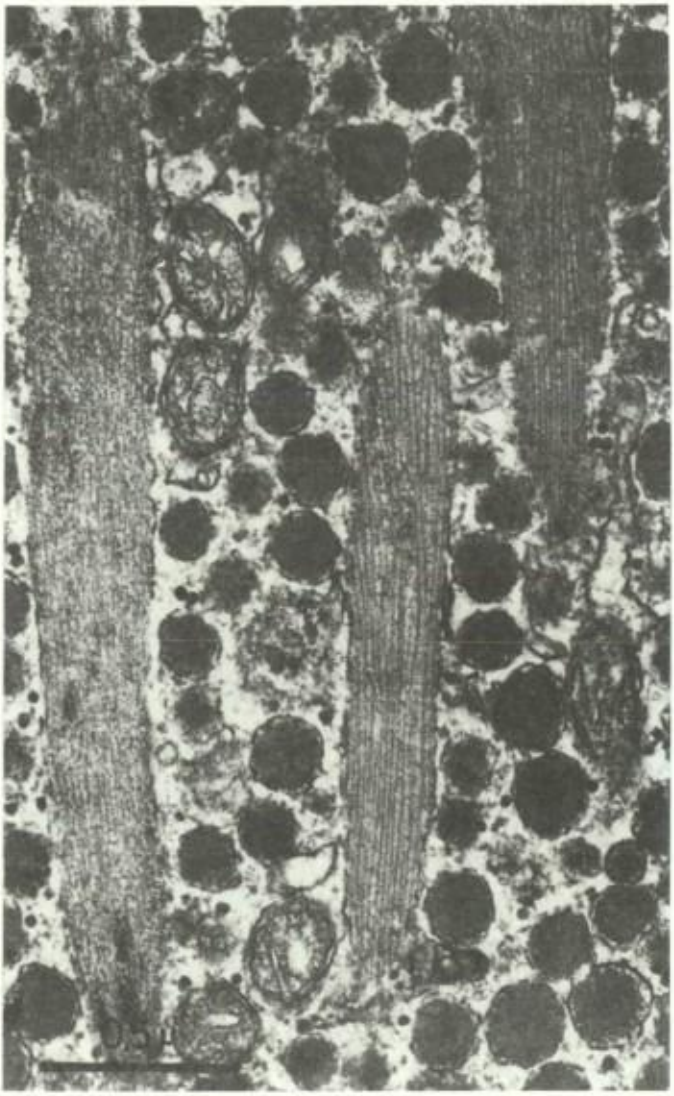

FIGURE 3. Paraventricular nucleus. Rat, 48 hours after 200 μg of colchicine intracisternally. Elongated bodies with a fibrillar content.

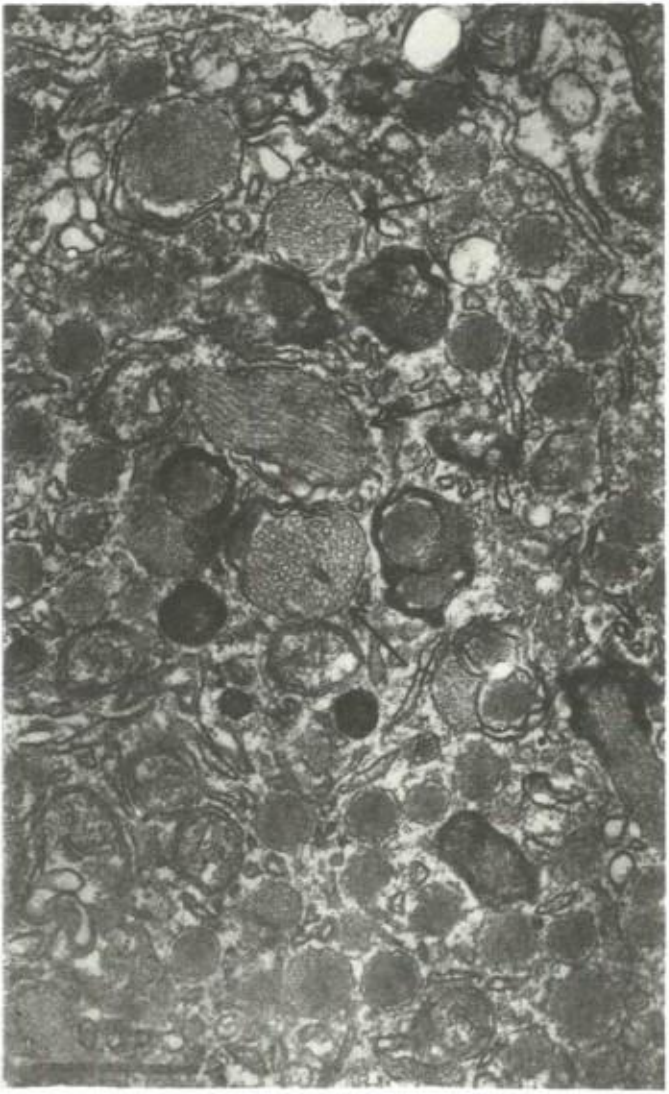

FIGURE 4. Posterior lobe of the pituitary. Rat injected with 400 μg colchicine and 60 mg furosemide intraperitoneally. Cross (→) and longitudinal (⟶) sections demonstrate the tubular content of some inclusions.

that in some pituicytes numerous centrioles could be observed in one section and that steps of their differentiation from a dense matrix were visible (FIGURES 5 & 7, a). Some of these centrioles did not have their full complement of microtubules while their ninefold symetry was already apparent, and some showed a U-shape, as a result of the absence of one or two groups of MT. These steps of centriologenesis are comparable to those described in the oviduct [25] and the respiratory epithelium.[26, 27]

The pituicytes were not the only cells affected by the combined action of colchicine and furosemide, and in the endothelial cells of the posterior lobe, one or two centrioles were often found, without, however, any cilia formation.

A comparison was made between the animals injected with furosemide and colchicine, colchicine alone, furosemide alone, or NaCl overloading with or without colchicine (TABLE 1). Colchicine was also administered either by the

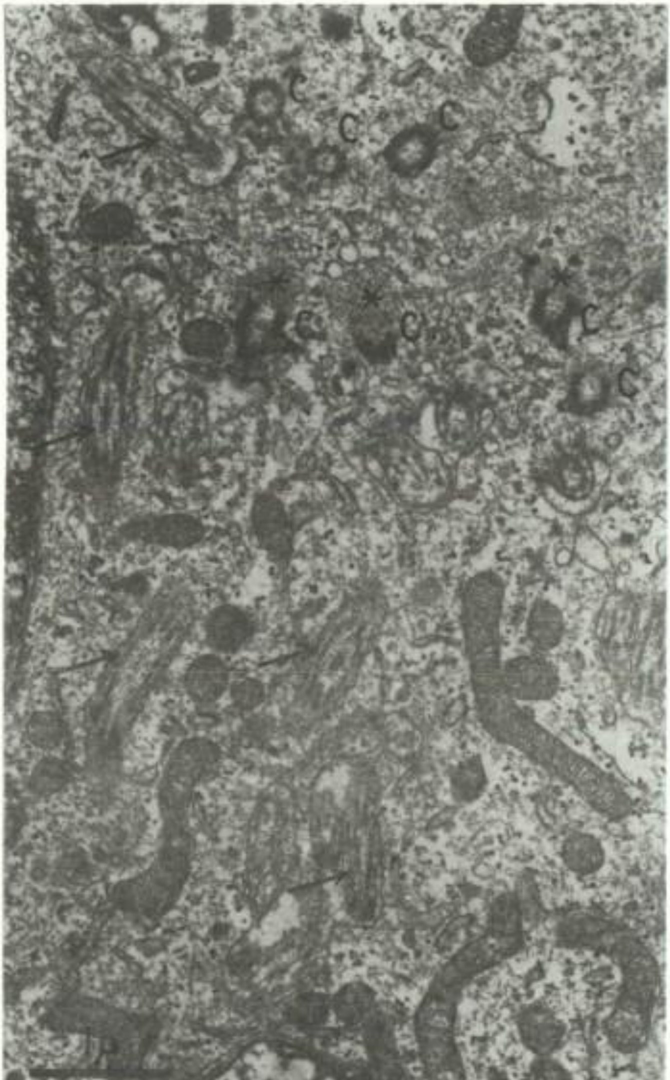

FIGURE 5. Pituicyte of a rat injected with 400 μg colchicine and 60 mg furosemide intraperitoneally. Numerous centrioles (C), sometimes U-shaped, differentiating from a granular matrix. (*) Numerous cilia in various spatial orientation (arrows).

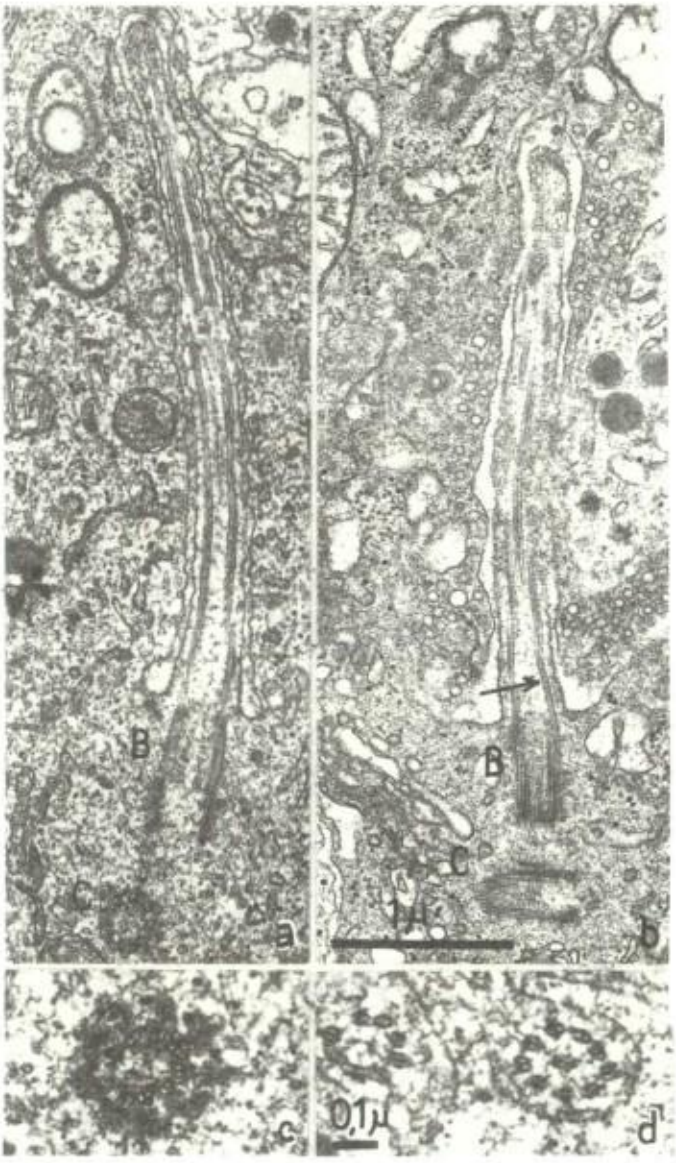

FIGURE 6. Pituicytes of a rat injected with 400 μg colchicine and 60 mg furosemide intraperitoneally. a & b: longitudinal section of cilia with basal bodies (B) and centrioles (C). b: one peripheral doublet appears to be displaced distally to the center of the cilium (arrow). c: centriole with pericentriolar bodies. The centriolar structure is incomplete; only 7 groups of tubules are visible. d: cilium with 9 + 2 groups of tubules.

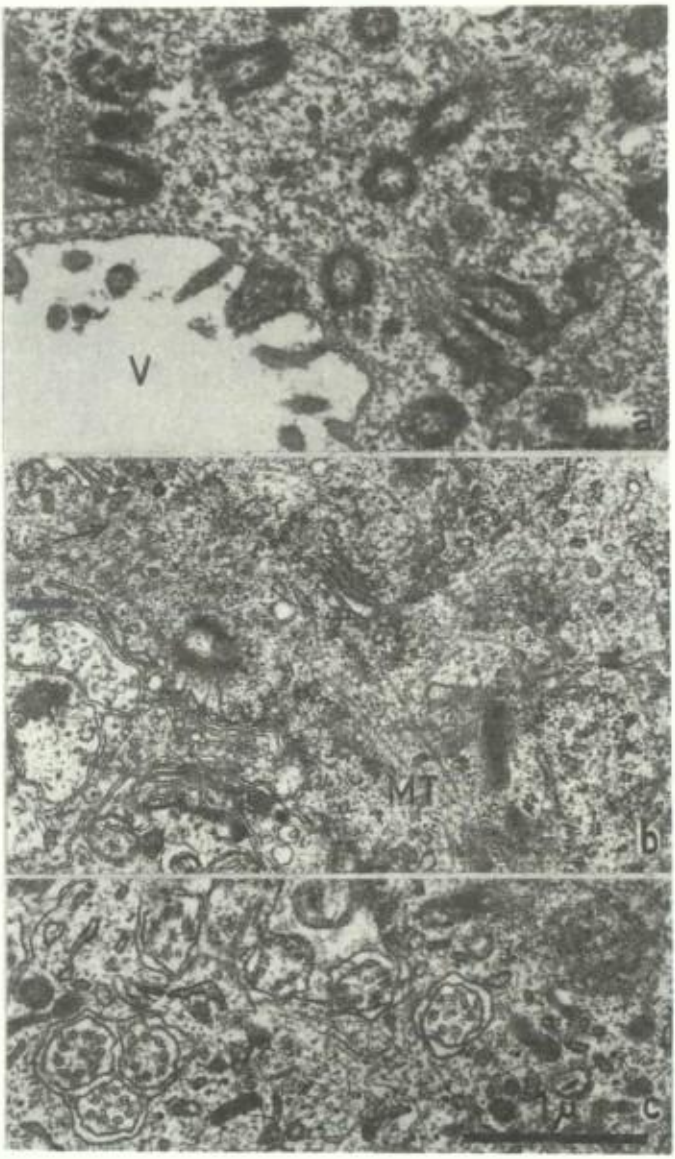

FIGURE 7. Pituicytes of a rat injected with 400 μg colchicine and 60 mg furosemide intraperitoneally. a: centrioles associated with a dense matrix. V: intracytoplasmic vacuole. b: centriole associated with striated rootlets (arrow) close to normal microtubules (MT). c: tuft of abnormal cilia.

intraperitoneal route or by intrathecal injection as in the experiments on axonal flow. These preliminary observations need further investigation of other modes of dehydration and the use of other MT poisons. It is apparent that the stimulation of pituicytes by NaCl overloading, resulting in their mitotic division, increases the number of centrioles and that colchicine alone has some action. The spectacular ciliogenesis, as illustrated by FIGURES 5 and 7(a), is found only as a result of the combined action of colchicine and furosemide, whereas this drug alone does not seem to have any action on pituicyte centrioles and cilia.

DISCUSSION

Two unexpected findings have been described after colchicine treatment: the formation of elongated intraaxonal bodies in conditions of inhibited axonal flow, and after additional dehydration (NaCl overloading and mainly furosemide administration), the proliferation of centrioles with formation of cilia. These two problems will be discussed briefly.

TABLE 1

RELATIVE INTENSITY OF CENTRIOLE AND CILIA MULTIPLICATION AND/OR ARRESTED MITOSES IN PITUICYTES

	Centrioles	Cilia	Mitoses
Colchicine	±	—	±
Furosemide	—	—	—
Colchicine + furosemide	+++	+++	±
NaCl overloading	±	—	±
NaCl overloading + colchicine	++	+	+++
Controls	—	—	—

Elongated Intraneuronal Structures

These have been found in the axons of neurosecretory cells and also, but in smaller numbers, in the perikaryon of these cells and occasionally in the axon terminals in the posterior lobe of the pituitary. They are associated with a considerable accumulation of neurosecretory granules, which is one of the most apparent evidences of stagnation of neurosecretion under the action of colchicine.

The elongated structures often have a dense content, resembling that of the neurosecretory granules, and they are limited by a heavily stained membrane that is quite similar to that of these granules. One could thus believe that the slowing down of the axonal flow favored the fusion of elementary granules into abnormally large bodies. These are nearly always oriented parallel to the axon, a fact difficult to understand if axonal flow is really arrested. Indeed, it has been shown by radioautography that the rapid axonal flow [17, 28-30] of neurosecretion is arrested, but it is known that slow centrifugal movements also exist in axons, affecting the axonal cytoplasm, the mitochondria, and probably the

neurotubules themselves.[31] It may be that the stagnant granules are fused, and the resulting large inclusions elongate by the continuous slow flow of the surrounding cytoplasm. This hypothesis clearly deserves further research.

Another problem is raised by the fact that the content of some of these long inclusions appears fibrillar, and on cross section, even presents an aspect of closely packed tubules (FIGURE 4). The aspect and size of these are clearly different from those of neurotubules, which, in these experiments, do not appear to be modified. However, it could be thought, as in other experiments where colchicine has been shown to transform MT into a fibrillar material,[12, 32, 33] that these fibrils do result from altered neurotubules. They could become wrapped up into membranes identical with those of the neurosecretory granules, these membranes appearing in all electron micrographs, even after the best fixations, to be loosely bound to the granules. Such fibrillar inclusions have been found in the axon terminals and close to the axonal hillock.

Last, some indications suggest that lysosomes may be involved in these processes, since some elongated structures with a dense content, similar to that of the lysosomes seen in the pericaryon and sometimes in the axon itself, have a multilamellar limiting membrane suggesting an engulfment of material.

These structures thus present many problems, the most interesting being probably the true dynamic conditions inside the axons when the rapid flow is arrested or considerably slowed down, conditions that may explain the shape and orientation of these bodies.

Centriole and Cilia Formation

The multiplication of centrioles in animals injected with a MT poison such as colchicine is surprising. Centrioles, cilia, and basal bodies, which are complex structures made of at least three proteins—tubulin, dynein,[34] and nexin[35]—are usually resistant to colchicine. It is known, however, that the regeneration of cilia may be inhibited by colchicine.[36]

In the literature on MT poisons, two papers seem to be of interest in relation to our findings. Stubblefield and Brinkley[37] described the paradoxical multiplication of centrioles in hamster cells cultivated *in vitro*, under the influence of colcemid (desacetyl-N-methyl-colchicine). This took place in the intermitotic cells two hours after colcemid treatment (0.06 μg/ml). Ciliary vesicles and buds were formed. If the cells were returned to a normal medium, the growth of the cilia was resumed, and they became visible in phase-contrast microscopy. In control cells about 4% of the centrioles showed some evidence of ciliogenesis, but this figure rose to 25% after colcemid. The ciliary growth after removal of colcemid parallels the reassembly of spindle MT. The authors considered that their findings indicated "a specific ultrastructural differentiation induced by colchicine."

The second paper is that of Milhaud and Pappas.[38] In the brain of the adult cat, they found that after six daily injections of pargylin (20 mg/kg), an inhibitor of monoamine oxidase, cilia formation was increased in various cells of the brain, principally glial cells. They suggest that this drug stimulated the multiplication of centrioles, leading to the formation of cilia of the $9 + 2$ type in nondividing cells. Many cilia may be present in the same cell, particularly in the habenula and the mamillary bodies. The cilia arise from typical basal bodies. The mode of action of pargylin on centrioles and cilia is obscure; it

brings an accumulation of serotonin as a consequence of the inhibition of monoamine-oxidase, particularly in the habenula. The authors mention some evidence that serotonin may affect the motility of cilia in Invertebrates, but there is no indication that it promotes ciliogenesis. It may be of interest to recall that melatonin, which is closely related to serotonin, is a MT poison.[39]

Furosemide, on the other hand, has no known actions on monoamine oxidases. Its diuretic action is effected through poisoning of the mitochondria in the renal tubules.[40] It is most probable that the stimulus to the pituicytes is functional, i.e., dehydration, as indicated by the presence of centrioles, a few cilia, and mitotic stimulation after NaCl overloading alone. No explanation, however, can be suggested for the action of colchicine, which, as in the experiments of Stubblefield and Brinkley,[37] appears to promote the assembly of tubulin into centrioles and cilia, contrary to what would be expected. Because the local concentration of the alkaloid in the pituitary is not known, it may be that the formation of centrioles and cilia, structures that are resistant to the alkaloid, takes place when some of the tubulin pool, uncombined with colchicine, is able to reassemble into MT. That the mechanism involved is probably far more complex is shown by the other reactions of the cytoplasm, such as the formation of ciliary vesicles by the Golgi apparatus and the differentiation of ciliary rootlets (FIGURE 7, b).

SUMMARY

Changes in the hypothalamo-pituitary tract and the pituicytes of the rat were studied after intrathecal and intraperitoneal injections of colchicine.

Radioautography with ^{35}S-cysteine demonstrates that intrathecal colchicine prevents the migration of neurosecretory granules from the supraoptic and paraventricular nuclei to the posterior lobe of the pituitary. This results in accumulations of neurosecretory granules and in the formation in the axons of elongated structures resembling neurosecretory products, although they sometimes have a fibrillary content. Neurotubules appear to remain intact in these conditions.

The stimulation of the posterior pituitary by dehydration, in particular after injection of the diuretic furosemide, leads to an increased activity of pituicytes. When colchicine is injected at the same time as furosemide, a considerable new formation of centrioles is observed in the pituicytes. These become associated with ciliary vesicles, and form numerous cilia of the $9 + 0$ type. An increased number of centrioles is also seen in the endothelial cells of the posterior lobe of the pituitary.

These apparently paradoxical results were briefly discussed in relation to the action of colchicine on neurotubules and axonal flow and to the limited data from the literature indicating a stimulation of cilia formation under the action of colchicine and other drugs.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. A. M. Hunninck-Couk for her devoted and skillful technical assistance and Mrs. D. Libert-Kaça for the careful typing of the manuscript. They are endebted to Dr. J. C. Heuson for kindly supplying

the rats and to the firm Hoechst Belgium S.A. for provinding samples of furosemide (Lasix®).

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