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Induction in vitro of Microtubular Crystals by Vinca Alkaloids

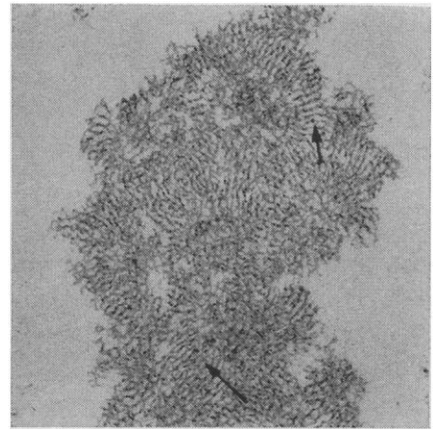
Abstract. *The addition of vinblastine or vincristine to solutions of pure microtubule protein or to supernatants from high-speed centrifugation of rabbit-brain homogenates results in the formation of a fine precipitate. Examination of this precipitate by electron microscopy reveals ordered structures with areas of ladder-like configuration.*

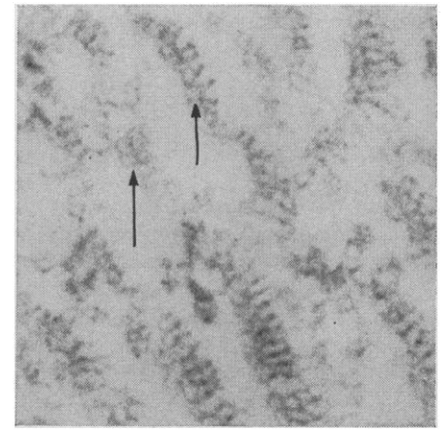
The vinca alkaloids, known for their metaphase-blocking antimitotic activity, induce a proliferation of 80- to 100-Å filaments (*1*) and the formation of microtubular crystals in the cytoplasm of various types of cells (*2*). The demonstration that a selective, quantitative precipitation of microtubule protein could be induced by vinblastine (*3*) suggested that similar filaments and crystals might be formed by the interaction of microtubule protein and the vinca alkaloids in vitro. We now report the formation of ordered structures from purified microtubule protein and supernatants obtained from high-speed centrifugation of rabbit-brain homogenates after the supernatants were incubated with vinblastine and vincristine.

Purified microtubule protein (colchicine-binding protein) was prepared from fresh pig brain (*4*). After reduction and alkylation the purified protein migrated as a single band on disc electrophoresis. Rabbit brains which had been freed of blood vessels and meninges were homogenized in 1.5 times the amount (weight to volume) of 1.15 percent KCl solution containing 3 m*M* $MgCl_2$ and 0.5 m*M* guanosine triphosphate (GTP) at *p*H 6.2 in a tissue grinder with a slowly rotating Teflon pestle at 4°C. The homogenate was centrifuged for 1 hour at 30,000*g* and the sediment was discarded. The GTP concentration was raised to 1 m*M*, and the *p*H was maintained at 6.2. The supernatant was then centrifuged at 100,000*g* for 1 hour. This high-speed supernatant was gently decanted and used for the experiments.

Portions of the supernatants and samples of the purified microtubule protein at concentrations of 1 to 2 mg/ml in 0.01*M* phosphate buffer containing 0.01*M* $MgCl_2$ and $10^{-4}M$ GTP at *p*H 6.5 were dialyzed against or incubated with $2 \times 10^{-4}M$ vinblastine or vincristine for 2 hours at 37°C. At the end of the incubation period a faint turbidity was noted in both the control and vinblastine-treated homogenates and also in the vinblastine-treated protein solution. The samples were then centrifuged at 30,000*g* for 30 minutes, and the sedimented pellets were fixed in 5 percent buffered glutaraldehyde at *p*H 6.5 followed by osmium tetroxide and embedded in Epon 812. Ultrathin sections were cut on the Reichert microtome, stained in uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 1. The microscope was calibrated against a standard carbon-replica calibration grid.

Sections of the samples treated with vinca alkaloid revealed large areas of ordered structures with a ladder-like arrangement in the case of the pure protein preparations (Fig. 1), and more widely dispersed ladder-like structures in the rabbit-brain supernatant (Fig. 2). The ladders in both cases have "rungs" of approximately 80 Å in diameter (Fig. 3). The distance be-





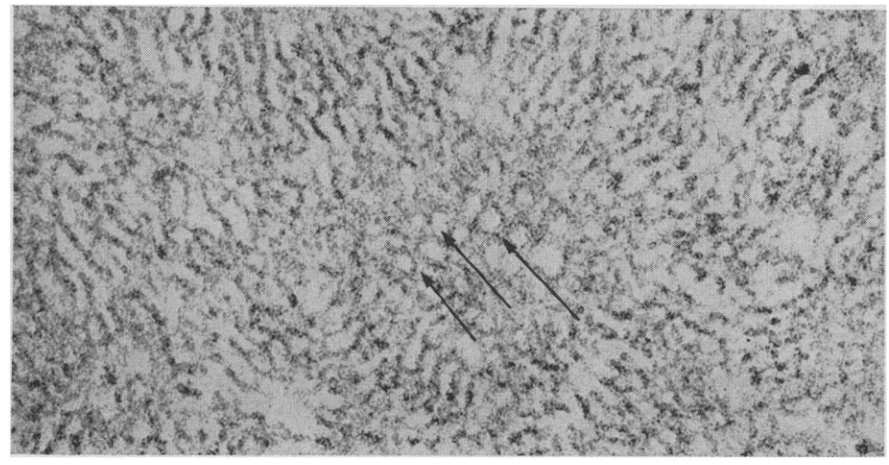


Fig. 1 (top left). Purified microtubule protein treated with $10^{-4}M$ vinblastine. Areas of ladder-like appearance (arrows) surround areas where circular profiles predominate (× 32,000). Fig. 2 (top right). Rabbit-brain supernatant treated with vinblastine. Dispersed "ladders" often appear to end in circular profiles (× 60,000). Fig. 3 (bottom). Purified microtubule protein treated with vinblastine showing detail of circular profiles (arrows) and of the 80-Å filamentous "rungs" arranged in an arciform pattern around them (× 115,000).

tween "rungs" (edge to edge) averages approximately 200 Å. In the structures formed from pure protein, the ladder-like regions tend to surround areas where circular profiles of 310 to 370 Å in diameter predominate (Fig. 3). Similar circular profiles, often appearing to be in continuity with a pair of "rungs," are seen in the rabbit-brain supernatants (Fig. 2). Vinblastine treatment of purified microtubule protein at 4°C resulted in structures identical to those formed at 37°C. No filamentous or ordered areas were seen in the control samples.

The structures reported here are probably closely related to the filaments and crystals observed in vivo in response to vinca alkaloid treatment (*1*, *2*). The filamentous "rungs" are of a diameter similar to the filaments seen in vivo (*1*). The tubular cross sections are larger than those seen in the majority of intracellular microtubular crystals reported—360 compared to 280 Å (*2*). However, in one study on crystals in nerve induced by vinca alkaloid, circular profiles with a diameter varying from 300 to 360 Å were reported (*5*). Microtubules of 310 to 360 Å in diameter have been observed in Actinospherium after treatment at 4°C (*6*), a condition which—like the treatment with vinca alkaloids—results in the breakdown of normal cytoplasmic microtubules. The crystals are somewhat less well ordered than the intracellular crystals, a condition which may simply reflect the loss of cytoplasmic constraints.

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Estradiol: Specific Binding by Pituitary Nuclear Fraction in vitro

Abstract. *The nuclear fraction of a homogenate of anterior pituitary has a marked binding affinity for estradiol but not for other hormonal steroids. Characteristics of the uptake of estradiol by pituitary nuclear fraction are like those reported for the uterus. Study in vitro will be useful in elucidating how direct estrogen feedback control of anterior pituitary function is mediated.*

Estrogen feedback control of anterior pituitary function is well documented, but the mechanism by which estrogen regulates synthesis and release of pituitary hormone is unknown (*1*). Attempts to elucidate the estrogenic actions within the hypothalamo-pituitary complex have been hampered by the difficulty of distinguishing between indirect (hypothalamic) and direct (pituitary) estrogenic effects on pituitary function in vivo (*2*).

Information derived from studies in vivo has shown that uterus, vagina, pituitary, and hypothalamus preferentially concentrate and bind estrogen (*3*). Using high-resolution autoradiography after special dry-mount tissue processing, Stumpf (*4*) has demonstrated that radioactivity from physiologic doses of ^{3}H-estradiol in vivo is concentrated rapidly within nuclei of hypothalamic and pituitary cells. Vaginal and uterine nuclei also concentrate estrogen (*5*), and there is evidence that specific intranuclear receptors are the sites of primary estrogen action (*6*). This prompted us to examine in vitro the estrogen-binding system of anterior pituitary tissue, using a working hypothesis that nuclear uptake and binding is the primary event by which estrogen directly regulates pituitary function. We describe here the specificity and quantitative nature of estrogen-binding by the nuclear fraction of anterior pituitary homogenate. The data substantiate observations in vivo that specific estrogen uptake and binding occurs in the pituitary nuclear fraction.

The system was essentially the same as that used by Brecher *et al.* (*7*) for the study of estrogen-binding by rat uterine nuclei. Charles River rats (225 to 275 g) were ovariectomized 1 week before use. Animals were killed by decapitation, and the anterior pituitary gland (AP) minus the posterior lobe was rapidly removed, weighed, and homogenized with a Teflon-glass grinder in cold TMK medium (0.01*M* tris, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, *p*H 7.2 at 25°C). The homogenate, either 2.5 or 5.0 glands per milliliter of TMK, was incubated with shaking in 10-ml beakers. Steroid of high specific activity (*8*) was added at time 0 in 4 μl of 50 percent aqueous ethanol. After incubation, homogenates were transferred to cold centrifuge tubes, and the incubation beakers were rinsed with cold TMK. The combined homogenate and beaker rinse was centrifuged at 800*g* for 10 minutes in the cold. The pellet was then washed twice with cold TMK by suspension and centrifugation at 800*g*. The washed nuclear pellet resulting from this procedure was analyzed for DNA and RNA (*9*); good recovery of relatively clean nuclei was obtained as indicated by the recovery of 94 percent DNA and only 43 percent RNA in the washed pellet (*10*). Radioactivity was extracted from the nuclear pellet by shaking with ethanol, chloroform and ether (1:1), and ether, respectively. The organic phases were combined and evaporated to dryness in counting vials, and the residue was dissolved in Liquifluor and counted to 1 percent error at 60 percent efficiency in a liquid scintillation spectrometer (Beckman LC 150). Uptake of radioactivity by the nuclear fraction was usually expressed as disintegrations per minute (dpm) per 10 mg of tissue weight.

In experiment 1, ^{3}H-labeled estradiol, corticosterone, progesterone, and testosterone were each incubated in the same volume of AP homogenate at 23°C. Under these conditions, the AP nuclear fraction had a marked binding affinity for ^{3}H-estradiol only, which demonstrates the specificity of the AP nuclear binding system for estradiol.

Estrogen-binding by the nuclear fraction in pituitary, hypothalamus, and liver homogenates was compared in experiment 2. Equivalent amounts of each tissue were incubated with a fixed concentration of ^{3}H-estradiol. Significant uptake of estradiol into the