estradiol. This is further evidence for the specificity of the AP nuclear-binding system for estradiol and is consistent with data from studies in vivo (*11, 15*) which have shown that radioactivity localized in estrogen-sensitive tissues after parenteral administration of ^{3}H-estradiol is mostly unaltered estradiol.

Since in our study all radioactivity extracted from AP nuclear fractions was estradiol, and in view of Stumpf's autoradiographic evidence for intranuclear estradiol uptake by AP cells in vivo, we conclude that nuclear uptake and binding of unaltered estradiol may represent a major event in direct estrogen feedback control of anterior pituitary function. Although the physiologic importance of specific estradiol binding by the AP nuclear fraction is unknown, its significance may be related to the amount of estradiol found in the AP under conditions of known physiologic effect. The daily dose of injected estradiol required for physiologic effect on AP gonadotropic function in the ovariectomized rat is between 5 and 400 ng (*16*). The amount of estradiol concentrated by the AP can be estimated from the amount of radioactivity found in the AP shortly after injection of physiologic doses of labeled estradiol.

We have estimated the amount of estradiol bound by the AP in vivo from data presented elsewhere (*3, 11, 17*), and radioactivity equivalent to 0.3 to 24.6 pg of estradiol (*18*) is localized in the AP within 60 minutes in vivo. In comparison, the amount of estradiol bound by AP nuclear fraction in vivo was calculated from the results in experiment 5; between 0.6 and 5.1 pg of estradiol per AP was bound to the nuclear fraction after incubation for 60 minutes. The ranges in the amount of estradiol taken up per AP in vivo and that bound by AP nuclear fraction in vitro are in excellent agreement. Clearly, therefore, the in vitro system originally developed for the study of estrogen-binding by uterine nuclei can be used to reveal much about other target tissues such as the pituitary.

Our results establish that uptake and binding of estradiol by the pituitary nuclear fraction is very much like that reported for the uterus (*7*). The fact that the estrogen-binding systems of uterus and pituitary are similar may provide some insight as to the mechanism of estrogen action in pituitary cells. The RNA content and the ratio of RNA to DNA of the pituitary gland are increased by estrogen treatment in vivo and decreased after ovariectomy (*19*). Therefore, we suggest that direct estrogen control of pituitary function is by regulation of nuclear RNA synthesis as is the case for the uterus (*6*).

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9. Procedure for DNA and RNA was that of J. F. Scott, A. P. Fraccastoro, E. B. Taft [*J. Histochem. Cytochem.* **4**, 1 (1956)].
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14. Authentic estradiol-17β (30 μg) was added as carrier to extracts of AP nuclear pellets containing a known amount of radioactivity. The disintegrations per minute per microgram of estradiol at the start was the specific activity before purification. Samples plus carrier were first purified as free estradiol by thin-layer chromatography (TLC) with chloroform and methanol (19:1) as the solvent system. The free estradiol zone was acetylated, further purified by TLC with chloroform and acetone (9:1), and the estradiol diacetate zone recovered. Determination of the disintegrations per minute recovered as estradiol diacetate was done in duplicate, and determination of the micrograms of estradiol diacetate recovered was done in triplicate by gas-liquid chromatography. The specific activity after purification was the disintegrations per minute per microgram of estradiol diacetate recovered. The ^{3}H-estradiol content of each sample was calculated from the ratio of specific activities before and after purification. The method satisfied the usual criteria for establishing radiochemical purity of steroids as set forth by L. R. Axelrod, C. Matthijssen, J. W. Goldzieher, J. E. Pulliam [*Acta Endocrinol.*, suppl. 99 (1965)].
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18. Our estimates were based on data from Eisenfeld and Axelrod (*3*), Kato and Villee (*11*), and McGuire and Lisk (*17*). The reported disintegrations per minute per milligram of AP was converted to disintegrations per minute per AP based on an assumed weight of 10 mg for AP. Disintegrations per minute were then converted to picograms of estradiol according to the original specific activity of the ^{3}H-estradiol used. Data from McGuire and Lisk were reported on the basis of AP dry weight. This was converted to fresh weight by assuming that dry weight equals 30 percent of the fresh weight. Our estimates may be high because it was assumed that all radioactivity present in the AP was estradiol. This assumption may not be valid because radioactivity from blood and metabolites may contribute to the total disintegrations per minute from which we made these estimates.
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Vinblastine-Induced Precipitation of Microtubule Protein

Abstract. *Vinblastine quantitatively precipitates a protein from supernatants obtained from high-speed centrifugation of homogenates of HeLa cells and of pig brain. This protein migrates as a single band on gel electrophoresis, has a mobility identical to that of purified microtubule protein, and—like microtubule protein—binds colchicine. The precipitation is partially inhibited by 0.9 percent NaCl.*

Microtubules are composed of a 6*S*, 120,000-dalton protein which binds guanosine triphosphate (GTP) and colchicine (*1*). It has been postulated that other metaphase-blocking antimitotic agents, including the vinca alkaloids, act by binding to the same protein subunit. Both colchicine and vinblastine induce a tremendous increase in the number of 100-Å filaments present in the cytoplasm of neurons and other cells (*2*). However, there may be certain differences between their mechanisms of action. Studies of the competition of vinblastine for the colchicine-binding site have shown an increase, rather than a diminution, of colchicine binding in the presence of vinblastine, suggesting that separate sites exist for each of these compounds (*3*). Vinblastine also induces the formation of intracellular crystals which have not been observed in colchicine-treated cells (*4*). These crystals are composed of microtubule protein (*5*). We now report the precipitation of microtubule protein by

vinblastine from solutions of purified microtubule protein and from supernatants obtained by high-speed centrifugation of brain and of HeLa cell homogenates.

Purified microtubule protein (colchicine-binding protein) was obtained from pig brain by the method of Weisenberg *et al.* (*6*). The high-speed supernatant was prepared by homogenization of pig brain with an equal volume of a 0.01*M* phosphate buffer containing 0.01*M* $MgCl_2$ and 0.24*M* sucrose at *p*H 6.5. Heavy, particulate material was removed by centrifugation at 20,000 rev/min for 10 minutes, and the supernatant was then centrifuged at 50,000 rev/min for 1 hour. The HeLa cell supernatant was prepared from a homogenate of 3×10^8 cells that had been treated with hypotonic buffer. The nuclei were removed by brief centrifugation at 1000*g*, and the homogenate was centrifuged at 50,000 rev/min for 1 hour. All operations were carried out at 4°C.

Tritiated colchicine binding was assayed by filtration through filter paper (Whatman No. 81) impregnated with DEAE (dimethylaminoethyl) (*6*), and radioactivity was determined by liquid-scintillation counting.

Sedimentation velocities were determined with a Spinco model E analytical ultracentrifuge. Polyacrylamide-gel electrophoresis was carried out in 5 percent polyacrylamide gels containing 8*M* urea and 0.01 percent sodium dodecyl sulfate (SDS) at *p*H 8.7. Samples were applied to 8*M* urea at *p*H 6.7 with 0.01 percent SDS. The gels were fixed for 12 hours in 20 percent sulfosalicylic acid and then stained for 2 hours in 0.5 percent amido black in 7 percent acetic acid.

The addition of $10^{-3}M$ vinblastine to purified microtubule protein, or to the supernatants from HeLa cells or pig brain, resulted in the rapid appearance of a fine precipitate. The respective precipitates were centrifuged at 100,000*g* for 30 minutes, and the sedimented pellets were subjected to electrophoresis on polyacrylamide gels. Only a single band was detected in each case, and the mobilities were identical (Fig. 1).

The percentage of microtubule protein precipitated was estimated from the distribution of bound ^{3}H-colchicine in centrifuged and uncentrifuged samples. Portions of the samples were incubated with 1 μc of ^{3}H-colchicine and $10^{-3}M$ vinblastine for 1 hour at 37°C. Precipitates were removed by centrifugation at 200,000*g* for 20 minutes. The radioactivity (count/min) remaining in the supernatant was then compared to that in an identical uncentrifuged sample to determine the percentage of colchicine-binding activity that precipitated. Some of the pellets were resuspended with the aid of a glass homogenizer, and bound colchicine was assayed by column chromatography (count/min). In each case the number of counts equaled the number of counts removed from solution by centrifugation.

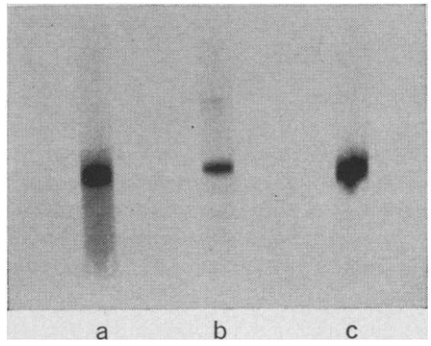

Fig. 1. Comparison of the vinblastine-induced precipitates of (a) purified microtubule protein (*4*) and of supernatants obtained from high-speed centrifugation of (b) HeLa cell and of (c) brain homogenates on 10-cm, 5 percent polyacrylamide gels containing 8*M* urea and 0.01 percent SDS at *p*H 8.7.

Incubation of solutions (0.5 to 2.0 mg/ml) of purified microtubule protein at *p*H 6.5 in the presence of 0.01*M* $MgCl_2$ caused the removal of 99 percent of colchicine-binding activity in centrifuged when compared to uncentrifuged sample. Similar treatment of portions of brain or HeLa cell supernatants resulted in only a 40 to 70 percent decrease in soluble activity in the spun sample. However, if these samples were dialyzed against an 0.01*M* phosphate buffer at *p*H 6.5 containing 0.01*M* $MgCl_2$ for 5 hours at 4°C and then incubated with vinblastine and tritiated colchicine, 99 percent of the bound tritiated material was precipitated. Precipitation was partially inhibited in solutions of pure protein by 0.9 percent NaCl. Control studies where incubation was carried out with colchicine alone showed only a 3 to 5 percent decrease in radioactivity (count/min) in centrifuged as compared to uncentrifuged samples.

The precipitation also occurs at lower temperatures; if the sample is first incubated with vinblastine alone at 4°C for 1 hour, centrifuged in the manner described above, and then incubated for 1 hour with tritiated colchicine at 37°C, there is 99 percent less activity in solution compared to that in an uncentrifuged control processed in the same manner. The precipitate can be dissolved by dialysis against vinblastine-free buffer for 5 hours.

Analytical ultracentrifuge studies on purified protein at concentrations of 5 to 10 mg/ml after incubation with $10^{-3}M$ vinblastine showed that the usual 6*S* peak disappeared with the concomitant appearance of a very small peak at approximately 25*S*. Most of the protein sedimented to the bottom of the cell very rapidly.

It therefore appears that microtubule protein may be quantitatively purified from supernatants obtained by high-speed centrifugation of homogenates of either brain or HeLa cells by the addition of vinblastine. This method provides an ideal approach to the problem of the synthesis and turnover of microtubule protein in cells and tissues. Both the mechanism by which the precipitation occurs and the relation of this phenomenon to the antimitotic effects of vinblastine remain obscure. However, this effect may be closely related to the mechanism of formation of microtubule crystals in vinblastine-treated cells.

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