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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References and Notes

1. E. Robbins and N. K. Gonatas, *J. Histochem. Cytochem.* **12**, 704 (1964); H. Wisniewski, M. L. Shelanski, R. D. Terry, *J. Cell Biol.* **38**, 224 (1968).
2. K. G. Bensch and S. E. Malawista, *Nature* **218**, 1176 (1968); *J. Cell Biol.* **40**, 95 (1969); S. S. Schochet, Jr., P. W. Lampert, K. M. Earle, *J. Neuropathol.* **27**, 645 (1968).
3. R. Marantz, M. Ventilla, M. Shelanski, *Science* **165**, 498 (1969); R. Weisenberg and S. N. Timasheff, *Biophys. J.* **9**, A174 (1969).
4. R. C. Weisenberg, G. G. Borisy, E. W. Taylor, *Biochemistry* **7**, 4466 (1968).
5. W. W. Schlaepfer, *Fed. Proc.* **28**, 2771 (1969).
6. L. G. Tilney and K. R. Porter, *J. Cell Biol.* **34**, 327 (1967).
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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

nuclear fraction was obtained with all tissues; however, the AP nuclear fraction contained four to five times more estradiol after each incubation period. These results compare favorably with previous reports that the AP concentrates more estradiol per milligram of tissue than the hypothalamus (*3, 11*), and that nuclear uptake by the liver is lower than that by the AP (*12*).

In experiment 3, we studied the effect of 800*g* supernatant on uptake of estradiol by AP nuclear fraction. Freshly prepared AP nuclear pellet, equivalent to two and a half AP's per milliliter, was incubated at 23°C for 120 minutes with 22,000 dpm of ^{3}H-estradiol per milliliter of TMK or 800*g* supernatant. Uptake of estradiol by nuclear pellet when resuspended in 800*g* supernatant was 906 ± 49 dpm per 10 mg of AP, while uptake by nuclear pellet resuspended in TMK was 186 ± 28 dpm per 10 mg of AP. The significant ($P < .001$) reduction in uptake of estradiol by the AP nuclear fraction in the absence of AP supernatant fraction indicates that nuclear uptake of estradiol in the pituitary depends on cytoplasmic factors, as is the case for uterus (*7, 13*).

To investigate other factors affecting uptake of estradiol by AP nuclear fraction in vitro, we incubated two concentrations of AP homogenate with a fixed concentration of ^{3}H-estradiol at 23°C (experiment 4). Under these conditions, total uptake of estradiol into the AP nuclear fraction was related quantitatively to the concentration of AP; that is, a twofold increase in nuclear uptake occurred when AP content was increased twofold. Furthermore, the ratio of nuclear binding of estrogen per unit of AP was constant with this concentration of estradiol (57 pg/ml), and the total nuclear uptake of estrogen increased with incubation for 120 minutes at 23°C.

The effect of quantity of estradiol per unit of AP was studied in experiment 5 by incubating a fixed concentration of AP homogenate with two amounts of ^{3}H-estradiol (22,000 dpm equals 57 pg, and 220,000 dpm equals 570 pg). When the quantity of estradiol was changed, a direct relation between amount of estradiol available and nuclear uptake was not observed. A tenfold change in concentration of estradiol did not result in a tenfold change in nuclear uptake of estradiol at any of the temperatures studied. This may be accounted for partly by the fact that nuclear-binding capacity is limited, and saturation of nuclear receptors occurred when 220,000 dpm of ^{3}H-estradiol (570 pg) was added per milliliter. Even though the estrogen-binding capacity is no doubt limited by the number of nuclear receptor sites available, such an explanation does not completely account for the differences observed in nuclear uptake at different temperatures in experiment 5. Significant nuclear binding of estradiol resulted at all temperatures with both amounts of estradiol. However, maximum uptake of estradiol occurred at 23°C rather than at 37°C, which suggests that temperature-dependent processes in the estrogen-binding system, perhaps involving uptake or unloading phenomena (or both), regulate the amount of estradiol bound by AP nuclei.

Radioactivity extracted from AP nuclear fractions in experiment 5 was analyzed for ^{3}H-estradiol content by a reverse isotope dilution procedure (*14*). All radioactivity extracted from AP nuclear fractions after incubation at 4°, 23°, or 37°C was unaltered ^{3}H-

Table 1. Experiment 1: Uptake of estradiol by anterior pituitary (AP) nuclear fraction compared with that of corticosterone, progesterone, and testosterone. Experiment 2: Uptake of estradiol by the nuclear fraction of AP and homogenates of hypothalamus and liver; homogenate concentration was 50 mg per milliliter for each tissue. Experiment 4: Effect of AP concentration on uptake of estradiol by AP nuclear fraction. Experiment 5: Effect of estradiol concentration on uptake of estradiol by AP nuclear fraction at different incubation temperatures. Adult, 7-day ovariectomized rats were used. Uptake is expressed as the mean disintegrations per minute (dpm) ± the standard error of the mean; the number of incubations per point is in parentheses.

Steroid	Incubation time (min)	Incubation temperature (°C)	AP concentration (glands per milliliter)	Steroid concentration (dpm/ml)	Uptake by nuclear fraction*		
					AP	Hypothalamus	Liver
				Experiment 1			
Estradiol 17β-6,7-^{3}H	120	23	2.5	22,000	925 ± 59(7)		
Corticosterone-1,2-^{3}H	120	23	2.5	22,000	20 ± 1(4)		
Progesterone-7α-^{3}H	120	23	2.5	22,000	31 ± 8(8)		
Testosterone-1,2-^{3}H	120	23	2.5	22,000	17 ± 2(4)		
				Experiment 2			
Estradiol 17β-6,7-^{3}H	30	23	5.0	22,000	434 ± 19(6)	112 ± 6(4)	51 ± 11(4)
Estradiol 17β-6,7-^{3}H	60	23	5.0	22,000	805 ± 42(6)	177 ± 31(6)	117 ± 14(5)
Estradiol 17β-6,7-^{3}H	120	23	5.0	22,000	857 ± 29(5)	171 ± 11(4)	104 ± 21(4)
				Experiment 4			
Estradiol 17β-6,7-^{3}H	30	23	2.5	22,000	1,027 ± 114(5)		
Estradiol 17β-6,7-^{3}H	30	23	5.0	22,000	2,165 ± 94(6)		
Estradiol 17β-6,7-^{3}H	60	23	2.5	22,000	1,855 ± 171(5)		
Estradiol 17β-6,7-^{3}H	60	23	5.0	22,000	4,034 ± 208(6)		
Estradiol 17β-6,7-^{3}H	120	23	2.5	22,000	2,283 ± 174(7)		
Estradiol 17β-6,7-^{3}H	120	23	5.0	22,000	4,282 ± 144(5)		
				Experiment 5			
Estradiol 17β-6,7-^{3}H	60	37	2.5	22,000	223 ± 25(5)		
Estradiol 17β-6,7-^{3}H	60	37	2.5	220,000	1,230 ± 97(4)		
Estradiol 17β-6,7-^{3}H	60	23	2.5	22,000	738 ± 85(5)		
Estradiol 17β-6,7-^{3}H	60	23	2.5	220,000	1,947 ± 103(5)		
Estradiol 17β-6,7-^{3}H	120	4	2.5	22,000	554 ± 34(7)		
Estradiol 17β-6,7-^{3}H	120	4	2.5	220,000	1,090 ± 110(5)		

* Uptake for experiments 1, 2, and 5 is expressed as disintegrations per minute per 10 mg of tissue; uptake for experiment 4 is expressed as total disintegrations per minute in nuclear fraction.

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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References and Notes

1. E. M. Bogdanove, *Vitamins Hormones* **22**, 206 (1964).
2. ———, *Endocrinology* **73**, 696 (1963).
3. A. J. Eisenfeld and J. Axelrod, *ibid.* **79**, 38 (1966).
4. W. E. Stumpf, *Science* **162**, 1001 (1968); *Z. Zellforsch.* **92**, 23 (1968); ——— and L. J. Roth, *J. Histochem. Cytochem.* **14**, 274 (1966).
5. W. E. Stumpf and L. J. Roth, *Endocrinology* **83**, 777 (1968); R. J. B. King, J. Gordon, L. Martin, *J. Endocrinol.* **41**, 223 (1968).
6. T. H. Hamilton, *Science* **161**, 649 (1968).
7. P. I. Brecher, R. Vigersky, H. S. Wotiz, H. H. Wotiz, *Steroids* **10**, 635 (1967).
8. Tritium-labeled steroids were from New England Nuclear Corp. and were checked for radiochemical purity before use. The position of tritium label is given in Table 1, and the specific activities expressed as curies per millimole were: estradiol, 48; corticosterone, 25; progesterone, 20; and testosterone, 42.
9. Procedure for DNA and RNA was that of J. F. Scott, A. P. Fraccastoro, E. B. Taft [*J. Histochem. Cytochem.* **4**, 1 (1956)].
10. Microscopic examination of the washed nuclear pellet revealed little particulate material other than that adhering to the outer nuclear membrane.
11. J. Kato and C. A. Villee, *Endocrinology* **80**, 567 (1967); *ibid.*, p. 1133.
12. R. J. B. King, J. Gordon, D. R. Inman, *J. Endocrinol.* **32**, 9 (1965).
13. E. V. Jensen, T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut, E. R. DeSombre, *Proc. Nat. Acad. Sci. U.S.* **59**, 632 (1968); H. Rochefort and E. Baulieu, *Endocrinology* **84**, 108 (1969).
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)].
15. E. V. Jensen and I. H. Jacobsen, *Recent Progr. Hormone Res.* **18**, 387 (1962).
16. W. W. Byrnes and R. K. Meyer, *Endocrinology* **48**, 133 (1951); ———, *ibid.* **49**, 449 (1951); A. F. Parlow, *ibid.* **75**, 1 (1964).
17. J. L. McGuire and R. D. Lisk, *Proc. Nat. Acad. Sci. U.S.* **61**, 497 (1968).
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.
19. J. A. Robinson and W. W. Leavitt, in preparation.
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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