

*THE BINDING OF ESTRADIOL IN THE UTERUS:  
A MECHANISM FOR DEREPRESSION OF RNA SYNTHESIS*

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*Communicated by E. L. Tatum, August 17, 1964*

Several hormones stimulate the synthesis of RNA in specific target organs.<sup>1-13</sup> Studies on the effect of growth hormone on liver,<sup>1</sup> and estradiol on the uterus,<sup>2, 3</sup> show that the activation of RNA synthesis is an early metabolic event which precedes the synthesis of proteins (enzymes) and the increment in cytoplasmic mass. Short pulse labeling and fractionation studies<sup>5,6</sup> reveal that the stimulatory process originates in the nucleus. The system is sensitive to inhibition by actinomycin D. Moreover, the morphological manifestations of the hormone effect are dependent on the initial stimulation of RNA synthesis and are prevented by inhibition of DNA-dependent RNA synthesis.<sup>4</sup>

The manner in which hormones select target sites and activate the synthesis of

RNA in these organs is not known. It is evident that target organs sequester the appropriate hormone from the systemic circulation; some organs concentrate a given hormone to a greater extent than others. For example, estradiol is rapidly taken up by the uterus and the vagina.<sup>14</sup> The chemical identity of the hormone is not altered in these tissues. Although quantitative studies are lacking to permit a precise statement of the number of hormone molecules necessary to elicit a maximal response per unit cell, it is known that most hormones are effective when given in very minute concentrations, in the order of a fraction of a microgram or picogram. The facts that (a) the hormone localizes in specific organs and (b) it is concentrated by these organs from the systemic circulation even when the amount of the circulating hormone is very low suggest the probable existence of specific "receptor" substances in target cells capable of recognizing a particular hormone.

This communication describes the subcellular localization of circulating estradiol-17 $\beta$ -6, 7- $H^3$  in the ovariectomized rat uterus. A macromolecular fraction has been obtained with which the hormone associates *in vivo*. When obtained from ovariectomized rat uteri, this fraction is an inhibitor of RNA polymerase, while the same fraction from animals given estradiol-17 $\beta$  has no inhibitory influence. Preincubation of the fraction with the steroid *in vitro* reverses its inhibitory effect on the enzyme activity. The data suggest a dual role of this fraction as (a) a "receptor" for fixation of the hormone in uterine cells and (b) a repressor of RNA polymerase in the absence of the hormone, a property lost by its combination with the hormone.

*Materials and Methods.*—*Radioactive compounds:* Estradiol-17 $\beta$ -6, 7- $H^3$  with specific activity of 41.3 curies/mM and cortisone-1, 2- $H^3$  with specific activity of 721 mc/mM were obtained from New England Nuclear Corporation. Radiochromatographic analysis of the steroids at the time of shipment showed the migration of radioactivity in a single peak. Each batch of steroid was used within 3 weeks of delivery. ATP-C<sup>14</sup> was obtained from Schwarz BioResearch, Inc.

*RNA polymerase assay:* The enzyme was a purified preparation of *E. coli* (courtesy of Dr. J. Hurwitz) and was assayed by standard procedure described elsewhere.<sup>15</sup>

*Tissue preparation:* Adult female rats of the Holtzman strain weighing between 180 and 200 gm and ovariectomized for at least 2 weeks were used. Vaginal smears were taken on all animals to assure complete anestrus before they were placed on experiment. Animals were sacrificed by decapitation and uteri removed, dissected free of connective tissue and fat, and cut into small pieces in cold sucrose (0.88 *M*) or buffer solution (TMK-tris, 10<sup>-2</sup> *M*, pH 7.4; MgCl<sub>2</sub>, 1.5  $\times$  10<sup>-3</sup> *M*; KCl, 10<sup>-2</sup> *M*). The tissue was homogenized in a glass hand homogenizer and filtered through cheesecloth. This homogenate is referred to as the total extract, which was subjected to further fractionation as described in the footnotes of the tables and in the figure captions.

*Results.*—*Subcellular localization of estradiol-17 $\beta$ -6, 7- $H^3$  in the uterus:* Subcutaneous doses of 0.3  $\mu$ g of estradiol-6, 7- $H^3$  were given to ovariectomized animals twice daily for 3 days. On the fourth day a final dose of 0.3  $\mu$ g was injected intravenously, and 1 hour later the uteri were removed and homogenized in 0.88 *M* sucrose. The distribution of radioactivity (due to tritiated estradiol) in various fractions is shown in Table 1. The counts are localized principally in a heavy fraction (*N*) sedimenting at 1,500 *g* in 10 min and in the supernatant (*S*) obtained

TABLE 1  
LOCALIZATION OF ESTRADIOL-17 $\beta$ -6, 7-H<sup>3</sup> IN SUBCELLULAR FRACTION OF  
OVARIECTOMIZED RAT UTERUS

Fraction	Expt. 1		Expt. 2		Expt. 3*	
	TCA insoluble, cpm	% of total	TCA insoluble, cpm	% of total	TCA insoluble, cpm	% of total
1. <i>N</i> (1,500 g/10 min)	3,080	28	4,550	28	14,655	39
2. <i>M</i> (20,000 g/30 min)	800	7	1,150	6	1,215	3
3. <i>R</i> (105,000 g/75 min) (pellet)	1,500	14	1,600	10		
4. <i>S</i> (105,000 g/75 min) (supernatant)	5,500	51	9,200	56	21,960	58

0.3  $\mu$ g of estradiol-17 $\beta$ -6, 7-H<sup>3</sup> (45  $\mu$ c) is administered subcutaneously to two ovariectomized rats twice daily for 3 days. Uteri are removed on 4th day 1 hr after an intravenous injection of 0.3  $\mu$ g of labeled estradiol and homogenized in 0.88 *M* sucrose. Subfractions are treated with cold TCA. The precipitates are dissolved in 97% formic acid, and two aliquots of each are counted for radioactivity.

\* 10<sup>-3</sup>*M* MgCl<sub>2</sub> added to the homogenizing medium.

TABLE 2  
DISTRIBUTION OF ESTRADIOL-17 $\beta$ -6, 7-H<sup>3</sup> IN  
THE SUBCELLULAR FRACTIONS OF  
OVARIECTOMIZED RAT UTERUS  
FOLLOWING *in vitro* ADDITION

Fraction	Cpm $\times 10^{-2}$
1. Homogenate	6,380
2. <i>N</i>	2,200
3. <i>R</i> + <i>M</i>	210
4. <i>S</i>	3,260

Four ovariectomized rat uteri are homogenized in Tris 10<sup>-2</sup> *M* pH 7.4 containing 1.5  $\times 10^{-3}$  *M* MgCl<sub>2</sub> and 10<sup>-2</sup> *M* KCl. 10  $\mu$ l of estradiol-17 $\beta$ -6, 7-H<sup>3</sup> (0.01  $\mu$ g, 1.5  $\mu$ c) is added to the homogenate with stirring. *N* is the pellet obtained at 750 *g* in 10 min, *R* + *M* the pellet at 105,000 *g* for 30 min, and *S* the supernatant fraction.

TABLE 3  
NATURE OF COUNTS IN THE 105,000 *g*  
SUPERNATANT

	Cpm	Per cent of initial
1. No treatment	1,750	100
2. TCA precipitate	280	16
3. pH 5 precipitate	301	17
4. Alcohol-ether (3:1) insoluble (one extraction)	110	6

One-ml aliquots of the supernatant (fraction *S* in Table 1) are treated with cold TCA or acidified with HCl to pH 5. The precipitate is filtered on a Millipore membrane filter (pore size 0.45  $\mu$ ). The filter is dried and counted in a liquid scintillation counter. Another 1-ml aliquot is mixed with 5 ml of alcohol-ether (3:1). The precipitate is dissolved in hyamine hydroxide and counted.

after centrifugation at 105,000 *g* for 75 min. The radioactivity of the intermediate fractions is low and becomes still lower if the uteri are homogenized in 0.88 *M* sucrose containing 10<sup>-3</sup> *M* MgCl<sub>2</sub> (expt. 3, Table 1). Similar distribution patterns are obtained if the estradiol-6, 7-H<sup>3</sup> is added *in vitro* to ovariectomized rat uteri at the time of homogenization (Table 2). Sixteen to seventeen per cent of the estradiol radioactivity is precipitable with TCA or by acidification to pH 5 (Table 3). Almost all the estradiol present in this fraction is extractable with alcohol-ether. It appears, therefore, that a substantial portion of the estradiol present in the 105,000 supernatant of uterine cells is linked to macromolecular entities. The linkage is not covalent and lipid solvents can extract the steroid from its combination with the macromolecular fractions.

*Fractionation of the supernatant:* After centrifugation at 105,000 *g*, the supernatant was fractionated through a column of Sephadex G-100. Most of the radioactivity of this fraction was identified with the first peak of the eluate material having an absorbance at 280 *m* $\mu$  (Fig. 1). This fraction is referred to hereafter as the G-100-P.1 fraction. When estradiol is added *in vitro* to a homogenate of ovariectomized rat uteri and the homogenate processed in the same manner as in Figure 1, a peak of estradiol radioactivity appears associated with G-100-P.1 fraction. The free steroid (present in excess) is eluted at a later stage (Fig. 2).

The fixation of systemically administered estradiol in a nontarget organ such as the lung is low. Identical fractionation of the 105,000 *g* supernatant from this organ does not show the migration of estradiol-17 $\beta$ -6, 7-H<sup>3</sup> with the G-100-P.1 fraction

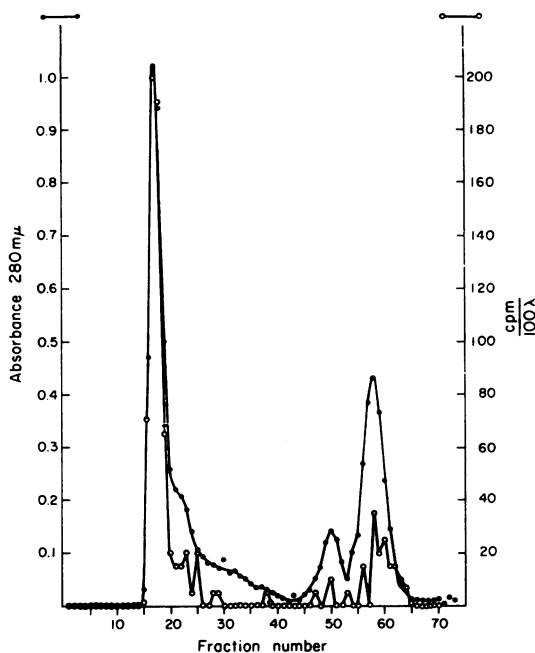


FIG. 1.—Fractionation of 105,000 *g* supernatant. Four ovariectomized rats were given 0.3  $\mu\text{g}$  of estradiol-17 $\beta$ -6, 7- $\text{H}^3$  (45  $\mu\text{c}$ ) once daily for 3 days. Uteri were removed on the 4th day 1 hr after an intravenous injection of 0.3  $\mu\text{g}$  of radioactive estradiol. The tissues were homogenized in TMK buffer pH 7.4 (Tris  $10^{-2}$  *M*, KCl  $10^{-2}$  *M*, and  $\text{MgCl}_2$   $1.5 \times 10^{-3}$  *M*). Supernatant fraction obtained after separation of particulate fractions at 750 *g* (10 min) and 105,000 *g* (45 min) is fractionated through a column of Sephadex G-100 (2 cm diam. and 45 cm long). Each eluate fraction measures 2.3 ml.

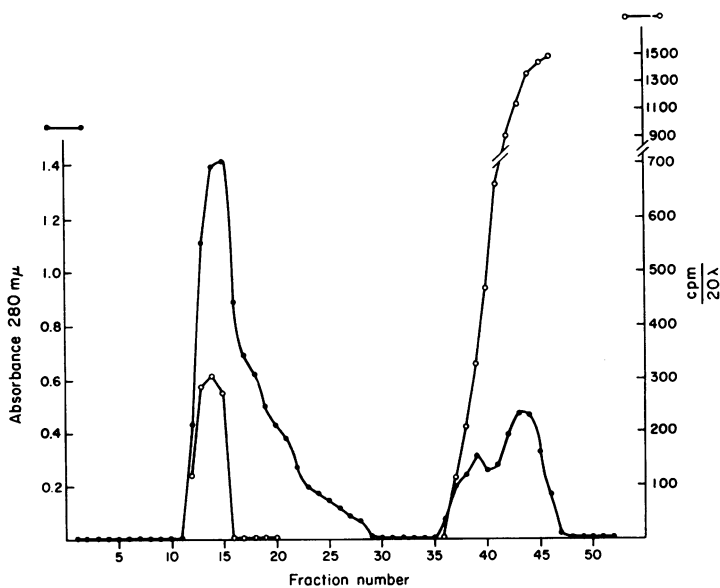


FIG. 2.—Identification of estradiol  $\beta$ -6, 7- $\text{H}^3$  with the 105,000 *g* supernatant subfraction. Twenty-five ovariectomized rat uteri were homogenized in TMK buffer to which 0.5  $\mu\text{g}$  (75  $\mu\text{c}$ ) of estradiol 17 $\beta$ -6, 7- $\text{H}^3$  was added *in vitro*. Seven-ml fractions were collected during its passage through Sephadex G-100 column used for experiment in Fig. 1.

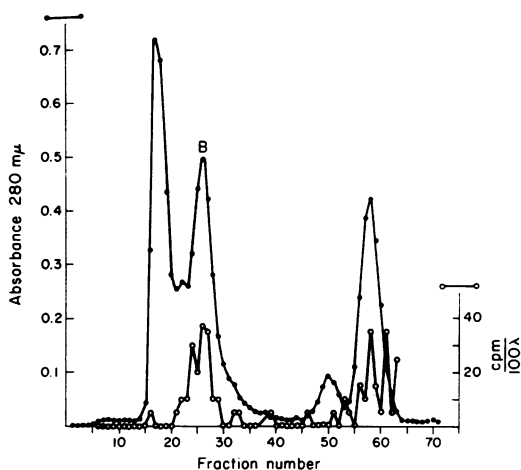


FIG. 3.—Fractionation of 105,000 *g* supernatant from a “nontarget” organ. Lungs from an animal given estradiol were extracted as described under Fig. 1. The 105,000 *g* supernatant had low radioactivity, to which was added  $10^{-4}$   $\mu\text{g}$  of estradiol-17 $\beta$ -6, 7- $\text{H}^3$  (10,000 cpm). Figure shows the fractions obtained through Sephadex G-100 column used for the experiment in Fig. 1.

even after an *in vitro* loading of the supernatant with labeled estradiol (Fig. 3). The “bound” estradiol elutes with the albumin-hemoglobin of blood origin (Fig. 3, peak B). It appears, therefore, that the G-100-P.1 fraction from uterus contains one (or more) components which bind estradiol, *in vivo* and *in vitro*, and that macromolecules with these properties are not present in the lung preparation.

*Properties of G-100-P.1:* The G-100-P.1 fraction of ovariectomized rat uteri has been tested for its capacity to bind estradiol-17 $\beta$  *in vitro* and for the specificity of its steroid affinity. Varying concentrations of labeled steroids (estradiol-17 $\beta$ -6, 7- $\text{H}^3$  and cortisone-1, 2- $\text{H}^3$ ) were incubated with 0.2 ml G-100-P.1 (OD 1.65 per ml at 280  $\text{m}\mu$ ) at 37°C for 15 min. TCA was added to a final concentration of 5 per cent. The steroid coprecipitated with the fraction was counted on a membrane filter. Parallel steroid blanks without the macromolecular fraction were run for each concentration of steroid to determine adsorption of free steroid on the membrane filter. The net counts per minute are determined by subtracting the value obtained for the steroid blanks from the G-100-P.1 plus steroid series (Table 4). Estradiol coprecipitates with the G-100-P.1 fraction in increasing amounts from a concentration of  $6 \times 10^{-5}$   $\mu\text{g}$  in the medium to  $2 \times 10^{-2}$   $\mu\text{g}$ . With cortisone-1, 2- $\text{H}^3$ , an input concentration of  $6 \times 10^{-2}$   $\mu\text{g}$  fails to show binding in the TCA precipitate. The specific activity of this concentration of cortisone is well above that of binding concentrations of the tritiated estradiol. Only when the concentration of cortisone is increased to 0.125  $\mu\text{g}$ , 10,000-fold greater than the minimal binding concentration for estradiol, can precipitable counts be discerned. It is apparent that the G-100-P.1 fraction possesses greater capacity for fixation of estradiol than of cortisone.

*G-100-P.1 and RNA synthesis:* The influence of the G-100-P.1 fraction on RNA polymerase activity is shown in Table 5. The polymerase is a purified preparation from *E. coli* prepared by Dr. J. Hurwitz. Calf thymus DNA has been employed as the template in the assay. The G-100-P.1 fraction inhibits the incorporation of

TABLE 4  
SPECIFICITY OF STEROID BINDING WITH UTERINE FRACTION G-100-P.1

Estradiol-6, 7-H <sup>3</sup> added to 0.2 ml*		Postincubation TCA, ppt (net cpm)	Cortisone-1, 2-H <sup>3</sup> added to 0.2 ml*		Postincubation TCA, ppt (net cpm)
(μg)	(μc)		(μg)	(μc)	
0.02	0.3	16,528	0.125	0.2	245
0.01	0.15	9,300	0.062	0.1	0
0.006	0.1	5,512	0.0125	0.02	0
0.002	0.033	3,222	$6.2 \times 10^{-3}$	$10 \times 10^{-3}$	0
0.001	0.016	1,159	$3.1 \times 10^{-3}$	$5 \times 10^{-3}$	0
$5 \times 10^{-4}$	$7.5 \times 10^{-3}$	987	$1.25 \times 10^{-3}$	$2 \times 10^{-3}$	0
$2 \times 10^{-4}$	$3 \times 10^{-3}$	949	$1.25 \times 10^{-4}$	$2 \times 10^{-4}$	0
$1 \times 10^{-4}$	$1.5 \times 10^{-3}$	640	$6.25 \times 10^{-5}$	$10 \times 10^{-5}$	0
$6.6 \times 10^{-5}$	$1 \times 10^{-3}$	450	$3.1 \times 10^{-5}$	$5 \times 10^{-5}$	0
$2 \times 10^{-5}$	$3.3 \times 10^{-4}$	7	$1.25 \times 10^{-5}$	$2 \times 10^{-5}$	0
$5 \times 10^{-6}$	$7.5 \times 10^{-5}$	11	$1.25 \times 10^{-6}$	$2 \times 10^{-6}$	0
$2 \times 10^{-6}$	$3 \times 10^{-5}$	0			
$1 \times 10^{-6}$	$1.5 \times 10^{-5}$	0			
$6.6 \times 10^{-7}$	$1 \times 10^{-5}$	0			
$2.2 \times 10^{-7}$	$3.3 \times 10^{-6}$	0			

\* 0.2 ml G-100-P.1 (optical density 1.65 per ml at 280 mμ) incubated for 15 min at 37°C with labeled steroid. After addition of 5% TCA, preparation filtered on Millipore membrane filter (0.45 μ pore size). A blank for each steroid concentration (without G-100-P.1) treated similarly to assess the extent of adsorption of free steroid to membrane. Net cpm derived by subtracting counts adsorbed on membrane of steroid blank from figure obtained for G-100-P.1 plus steroid at same concentration.

TABLE 5  
INFLUENCE OF UTERUS PREPARATION G-100-P.1 FROM OVARIECTOMIZED RATS  
ON RNA POLYMERASE

	G-100-P.1 (ml)	Cpm (100 λ)	Total nucleotide incorporated (mμmoles)
1. Complete system	—	1,350	1.04
2. Plus G-100-P.1	0.1	1,190	0.92
3. " "	0.2	834	0.64
4. " "	0.3	544	0.41
5. Minus RNA polymerase	0.3	54	0.1

Reaction mixture contained in 0.5 ml 80 μmoles each of GTP, CTP, UTP, 136 μmoles of ATP-C<sup>14</sup> ( $6.4 \times 10^6$  cpm per μmole), 2 μmole MnCl<sub>2</sub>, 1 μmole of 2-mercaptoethanol, 25 μmoles of potassium maleate buffer pH 7.5, 8 μmoles of deoxyribonucleotides as calf thymus DNA,  $5 \times 10^{-5}$  M spermine tetrahydrochloride, and 1.6 units of purified *E. coli* RNA polymerase.

ribonucleotide triphosphates into polyribonucleotides in the system. A parallel preparation from lung tissue has no inhibitory action (Table 6). The polymerase-inhibiting activity of the uterine G-100-P.1 fraction is destroyed by heating for 10 min at 80°C (Table 7). The G-100-P.1 fraction from uteri of estrogen-treated ovariectomized rats does not inhibit the enzyme activity (Table 7). The inhibition caused by the G-100-P.1 fraction from ovariectomized animals is reversed by preincubation with estradiol (Table 7) but not by cortisone.

*Discussion and Conclusions.*—The systemic administration of tritiated estradiol to ovariectomized rats results in the localization of the hormone principally in two subcellular compartments. Between 30 and 50 per cent of the radioactivity is associated with the subcellular components that sediment at low speed. In most tissues this fraction is composed of nuclei, cell membranes, unbroken cells, and heavy particulate matter.

A substantial portion of the hormone is present also in the supernatant after centrifugation at 105,000 *g*. The data indicate that the estradiol in this fraction is in association with macromolecular entities. A partial purification has been attempted to identify the specific "receptor" substance(s) for estradiol in the 105,000 *g* supernatant. The G-100-P.1 fraction has characteristics which suggest the presence of one or more components having the capacity to bind estradiol-17β

TABLE 6  
EFFECT OF G-100-P.1 FROM UTERI AND LUNGS OF OVARIECTOMIZED RATS  
ON RNA POLYMERASE ACTIVITY

	G-100-P.1 (ml)	Cpm (100 $\lambda$ )	Nucleotide incorporated (ATP) (m $\mu$ moles)
1. Complete system	—	960	0.88
2. Plus TMK buffer	0.3	1,200	1.08
3. Plus uterus G-100-P.1 in TMK buffer	0.3	310	0.276
4. Plus lung G-100-P.1	0.3	890	0.816

The reaction mixture (total volume 0.6 ml) contained the same amounts of constituents as in Table 6. The optical density (absorbance) of uterus G-100-P.1 at 280 m $\mu$  was 1.65 per ml and that of lung G-100-P.1 was 1.87 per ml.

TABLE 7  
SPECIFICITY AND REVERSAL OF POLYMERASE-INHIBITING ACTIVITY  
OF UTERINE G-100-P.1 FRACTION

System	Cpm/0.1 ml	ATP incorporated (m $\mu$ moles)
Expt. I		
1. Control—complete system	490	0.46
2. + G-100-P.1	305	0.28
3. " preheated 80°/10 min	725	0.68
4. " preincub./DNA	478	0.44
5. " + estradiol 0.03 $\mu$ g	490	0.46
6. " + " 0.015 $\mu$ g	557	0.57
7. " of " -treated rat	725	0.68
Expt. II		
1. Control—complete system	1,681	1.21
2. + G-100-P.1	1,060	0.76
3. " + 0.03 $\mu$ g estradiol	1,149	0.83
4. " + 0.03 $\mu$ g preincub.	1,249	0.90
5. " + 0.015 $\mu$ g estradiol	1,465	1.06
6. " + 0.015 $\mu$ g preincub.	1,391	1.05
Expt. III		
1. Control—complete system	1,335	0.48
2. + G-100-P.1	1,035	0.37
3. " + estradiol 0.01 $\mu$ g	1,190	0.42
4. " + " 0.006 $\mu$ g	1,321	0.47
Expt. IV		
1. Control—complete system	720	0.67
2. + G-100-P.1	407	0.38
3. " + cortisone 0.06 $\mu$ g	379	0.34
4. " preheated 80°/10 min	705	0.65
5. " of estradiol-treated rat	661	0.61

Assay system same as described under Table 6. Reaction volume 0.6 ml in expts. I, II, IV, and 0.3 ml in III. The specific activity of ATP-C<sup>14</sup> in expts. I and IV was 6,400 cpm/m $\mu$  and 8,300 cpm/m $\mu$ -mole in expts. II and III.

*in vivo* and *in vitro*. Cortisone, a steroid hormone with no known direct biological effect on the rat uterus, is not bound by the G-100-P.1 fraction except in very high concentration.

In addition, the G-100-P.1 fraction prepared from ovariectomized rat uteri inhibits *E. coli* RNA polymerase activity. The inhibitory effect is lost if the preparation is preheated at 80°C for 10 min. The inhibitory factor is thus heat-labile. Heating destroys also the ability to bind the steroid. The G-100-P.1 fraction prepared from the uteri of ovariectomized rats which received estradiol systemically 15 min or 1 hr before removal of the tissues has no inhibitory effect on the enzyme activity. Preincubation with estradiol at 37°C for 10 min reverses the inhibitory capacity of the G-100-P.1 fraction from ovariectomized rat uteri at least partially. Preincubation with cortisone does not result in reversal. These results suggest the existence of a repressor of RNA polymerase in the G-100-P.1 fraction of the ovariec-

tomized rat uterus, sensitive to derepression by binding estradiol *in vivo* or *in vitro*. The same fraction contains a component(s) having affinity for the specific steroid, responsible probably for the uptake of the hormone by the tissue. It is possible, although not proved, that the two functions, i.e., that of "receptor" and "repressor," are inherent in the same molecule. It is obvious that the repression of RNA synthesis *in situ* would be occurring in the nucleus. The fraction described here has been extracted from the supernatant and is *a priori* cytoplasmic. Its relation with the nucleus is not yet established. Tables 1 and 2 show the fixation of the steroid in the heavier fraction (containing the nuclei), which implies the presence of G-100-P.1 or similar "receptor" substances, binding the hormone in this fraction. In the absence of the hormone, the repressor would block the transcription of the genetic information and keep the hormone-sensitive tissue in an atrophied state. The uptake of the hormone by the tissue by means of the "receptor" function would simultaneously release the repression of RNA synthesis, a system proposed lucidly by Jacob and Monod<sup>16</sup> for regulation of cellular metabolism in bacterial cells.

This work was made possible by a grant (M-64.015) from The Population Council to G.P.T. We wish to thank Dr. B. B. Dikshit and Dr. K. L. Wig for encouragement, and Dr. A. E. Mirsky for helpful discussions. Miss H. Stanek, Mrs. R. Caputo, and Mrs. M. Bonevardi rendered technical assistance in various aspects of this work. We wish also to thank Mrs. E. W. Bodine for editorial assistance, and Misses Hella Heydecke and Mariagnes McCabe for typing of the manuscript.

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