INTERACTION OF ESTROGEN AND PROGESTERONE IN CHICK OVIDUCT DEVELOPMENT

II. Effects of Estrogen and Progesterone on Tubular Gland Cell Function

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ABSTRACT

The effects of estrogen and progesterone on the function of chick oviduct tubular gland cells have been studied. Such function, as measured by the increase in specific cell products such as lysozyme and ovalbumin, requires the continuous presence of estrogen or progesterone. Withdrawal of hormone results in a rapid cessation of function and an involution of the oviduct accompanied by rapid decreases in total weight, lysozyme, and RNA. During such involution, tubular gland cells per se persist, as evidenced by a lack of comparable decrease in total DNA content and by histological demonstration of tubular gland cells. When estrogen administration is reinstituted, preexisting tubular gland cells rapidly synthesize ovalbumin and lysozyme without requiring new DNA synthesis. Administration of progesterone also stimulates the function of such cells. Furthermore, the effects of estrogen and progesterone are synergistic on the synthesis of lysozyme and ovalbumin, whereas progesterone antagonizes the estrogen-evoked formation of tubular gland cells. It is suggested that such complex interactions of estrogen and progesterone on oviduct development and function result from differences in responsiveness of the various cell types present in the tissue.

INTRODUCTION

Studies from several laboratories have shown that administration of estrogen to immature female chicks elicits characteristic responses in the oviduct including cell proliferation, cytodifferentiation, and the synthesis of specific cell products such as lysozyme and ovalbumin by tubular gland cells (1–6). The interaction of estrogen and progesterone in oviduct development and function appears to be complex. O'Malley and his coworkers have studied extensively the initiation of avidin synthesis by progesterone in epithelial goblet cells in the oviduct of estrogen-primed chicks (7–11). Recently we have reported a very different effect of progesterone, namely that the effect of estrogen to elicit the formation of tubular gland cells is antagonized by concomitant administration of progesterone (5, 6). We also found that once tubular gland cells are formed, proges-

terone does not prevent the subsequent function of this cell type.

In this paper, we have examined further the interaction of estrogen and progesterone in the oviduct, focusing mainly on their effects on tubular gland cell function once such cells exist. We find that the maintenance of such function requires the continuous presence of estrogen, as determined by the presence of lysozyme and ovalbumin. A similar finding has been reported by O'Malley et al. (2). Withdrawal of estrogen results in a cessation of function and an involution of the oviduct, although tubular gland cells persist. When estrogen administration is reinstituted, such preexisting cells rapidly synthesize lysozyme and ovalbumin without requiring new DNA synthesis. Most striking is the finding that the function of such cells can also be stimulated by progesterone which evokes little response in the immature oviduct. Furthermore, the effects of estrogen and progesterone appear to be synergistic, in contrast to their effects on the formation of tubular gland cells. These results, then, indicate that effects of estrogen and progesterone are complex and differ according to the cell type present during different stages of oviduct development.

MATERIALS AND METHODS

Hormones and Chemicals

Progesterone, testosterone, androstanedione, and hydrocortisone 21-phosphate were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Estradiol 17β-benzoate was kindly provided by Schering Corp., Bloomfield, N.J. Deoxycorticosterone was obtained from Ciba Pharmaceutical Co., Summit, N.J. Medroxyprogesterone acetate was purchased from The Upjohn Co., Kalamazoo, Mich. Norethindrone was purchased from Parke, Davis & Co., Detroit, Mich. Ribose, deoxyribose, and hydroxyurea were obtained from Calbiochem, Los Angeles, Calif.

Treatment of Animals

Newborn white leghorn female chicks were obtained from Kimber Farms, Fremont, Calif. Chicks were maintained in electrically heated brooders and fed a Purina Laboratory chow (micromixed) diet (Ralston Purina Co., St. Louis, Mo.) throughout the experiments. Chicks weighed about 50 g at 4 days and 250 g at 26 days after birth. At ages indicated in legends of the figures the chicks were given hormone injections into the muscle mass of the hind limb below the knee. Hormones were dissolved in sesame oil with the exception of hydrocortisone 21-phosphate and medroxyprogesterone which were dissolved in 0.15 M NaCl. Each hormone was injected in a volume of 0.1 ml per chick.

Assays

The magnum portion of oviduct was removed, weighed, and homogenized in a motor-driven glass homogenizer with 9 volumes (w/v) of ice-cold distilled water. DNA and RNA were extracted from the homogenate by the method of Schneider (12). DNA was assayed by the diphenylamine reaction with deoxyribose as standard (13), and RNA was assayed by the orcinol method with ribose as standard (14). Protein was estimated by the method of Lowry et al. (15) with crystalline bovine serum albumin as standard. Lysozyme was assayed by a modification of the method of Litwack (16). Activity was measured by continuous recording of the decrease in absorbancy at 645 mμ at 25°C with *Micrococcus lysodeikticus* as substrate with a Gilford Multiple Sample Absorbance Recorder Model 2000 (Gilford Instrument Company, Oberlin, Ohio) and a Beckman DU monochrometer (Beckman Instruments Inc., Fullerton, Calif.). Activity is expressed as micrograms of lysozyme present and is based on the activity of purified egg white lysozyme (Calbiochem) as standard.

Measurement of Extent of DNA Synthesis

The extent of DNA synthesis was estimated by the incorporation of thymidine-^{3}H into trichloroacetic acid-insoluble material. Thymidine-^{3}H (SA[1] 6.0 c/mmole, Schwarz Bio Research Inc., Orangeburg, N.Y.) was dissolved in 0.15 M NaCl and was injected into the breast muscles at a dose of 5 μc per chick in a volume of 0.1 ml. 30 min after the injection, the magnum was removed and homogenized as described above. An aliquot of homogenate was treated with an equal volume of cold 10% trichloroacetic acid, and the resulting precipitate was collected by centrifugation at 8000 *g* for 10 min at 2°. The pellet was washed 3 times with cold 5% trichloroacetic acid, 2 times with cold absolute ethanol, and 2 times with ether. The final residue was dissolved in 1 ml of NCS solubilizer (Nuclear-Chicago Corporation, Des Plaines, Ill.) at 35°C overnight and then placed in a scintillation vial for measurement of radioactivity. Treatment of the tissue homogenate which had been obtained after injection of thymidine-^{3}H with DNase (Worthington Biochemical Corp., Freehold, N.J.) at a concentration of 100 μg/ml in 0.001 M potassium phosphate buffer, pH 7.2, containing 0.001 M $MgCl_2$ for 30 min at 37° converted 95% of trichloroacetic acid-insoluble radioactivity into trichloroacetic acid-soluble material;

[1] Specific activity.

this indicates that the majority of thymidine-^{3}H was incorporated into DNA.

Measurement of Extent of Ovalbumin Synthesis

Since it has been shown that laying-hen oviduct minces synthesize ovalbumin in vitro at a rate qualitatively, if not quantitatively, similar to that in vivo (17), the extent of ovalbumin synthesis was estimated as follows. The magnum was removed with aseptic technique, placed in Hanks' salts solution (GIBCO,[2] Oakland, Calif.), and cut into small pieces (1–2 mg wet weight each). 20–25 tissue pieces were placed in a Petri dish (5 cm diameter; Falcon Plastic, Division of B-D Laboratories, Inc., Los Angeles, Calif.) with 4 ml of Medium 199 (GIBCO, Oakland, Calif.) modified to contain one-tenth the normal concentration of lysine (0.7 μg/ml). Penicillin G and streptomycin were added to a final concentration of 50 units/ml and 50 μg/ml, respectively. 15 min after incubation, 4,5-lysine-^{3}H (SA 291 c/mmole, Nuclear-Chicago) was added to a final concentration of 0.96 μc/ml, and incubations were continued. All incubations were done at 37°C in an atmosphere of 95% O_2–5% CO_2 with continuous shaking. 3 hr after the addition of lysine-^{3}H, incubations were stopped by cooling to 2°C and adding cyclohexamide and sodium azide to a final concentration of 1 and 10 mM, respectively. Tissues were blotted, weighed, and homogenized in a motor-driven glass homogenizer with 2 ml of 0.001 M potassium phosphate buffer, pH 6.8, containing 1 mM $MgCl_2$. 2 ml of tissue homogenates were sonicated for 5 sec at 4°C with a Sonifer Cell Disrupter (Heat Systems Co., Melville, N.Y.) at a setting of 2 with the microtip attachment, then passed through glass wool to remove connective tissues, and centrifuged at 105,000 *g* for 1 hr at 2°. To an aliquot of the 105,000 *g* supernatant was added antiovalbumin or control gamma globulin prepared as described previously (6). Immunoprecipitation reactions were always carried out in the antibody-excess region, and two different amounts of sample were employed to insure the quantitative precipitation of ovalbumin. After incubation at 37°C for 1 hr and then at 4° for 24 hr, the antibody-antigen precipitate was centrifuged and washed 3 times with 0.01 M potassium phosphate, pH 7.2, containing 0.15 M NaCl. The washed precipitate was then dissolved in 1 ml of NCS at 37° overnight and placed in a scintillation vial for measurement of radioactivity. In preliminary studies,[3] the rate of lysine-^{3}H incorporation into ovalbumin and total soluble protein was found to be linear with time for 3–4 hr under the conditions described above.

Radioactivity Measurements

Radioactivity was determined with a Packard Model No. 3310 Liquid Scintillation Spectrometer with a counting efficiencies of 28% for tritium and 75% for carbon 14. Background was 25–29 cpm. The scintillation fluid contained 4 g of 2,5-diphenyloxazole and 0.4 g of 1,4-bis(2-(5-phenyloxazolyl))-benzene (Packard Instrument Co., Inc., Downers Grove, Ill.) in 1 liter of toluene, and 10 ml of toluene-phosphor fluid was used in each vial.

Histological Procedures

Portions of magnum were fixed either in Bouin fixative (18) or in 4% glutaraldehyde buffered with 0.01 M sodium phosphate buffer, pH 7.2 (19), for 24 hr at 25°C. The tissues were embedded in paraffin after dehydration in a series of graded alcohol and clearing in toluene. Sections of 7 μ thickness were stained with Mayer's hematoxylin and eosin.

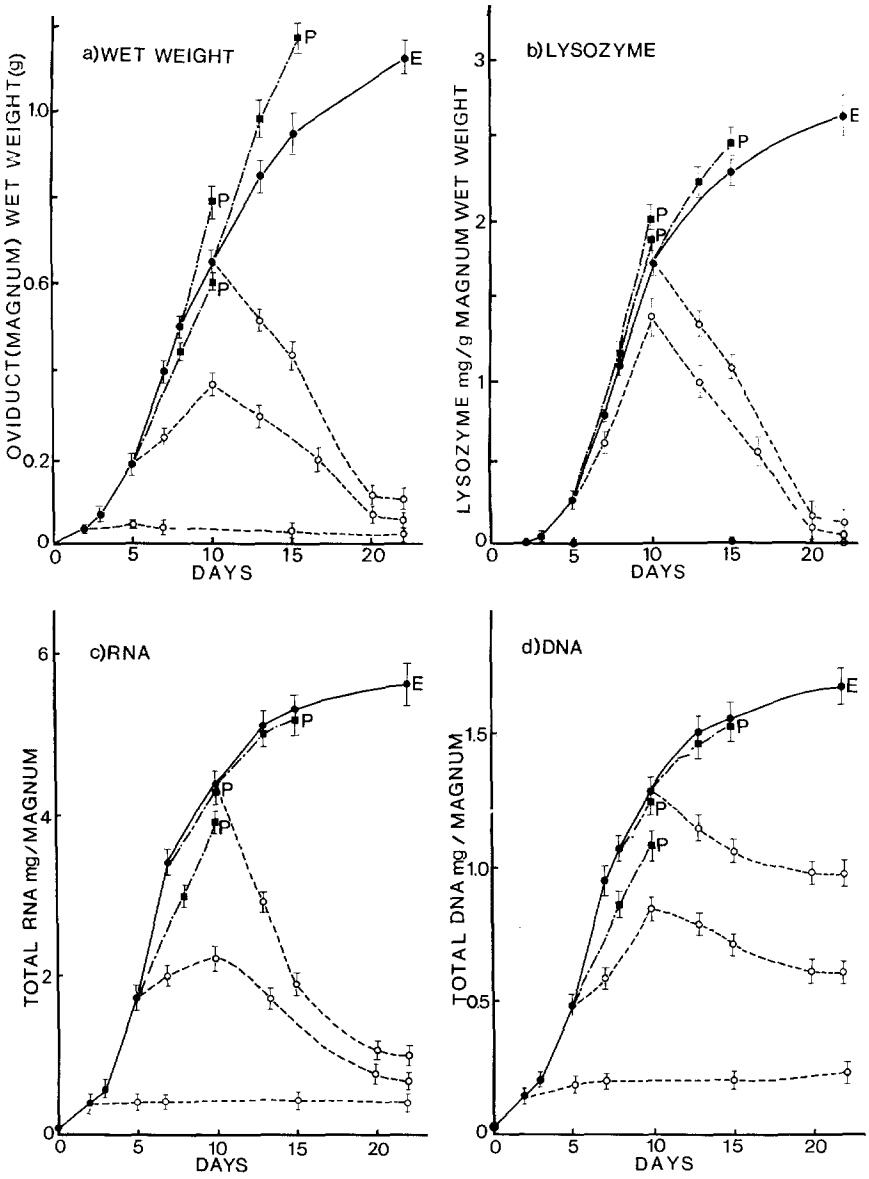
RESULTS

As shown in Fig. 1 *a*, daily administration of estrogen to immature female chicks markedly increases oviduct wet weight. The rate of increase is minimal in the first 2–3 days, reaches the maximum at 5–8 days, and thereafter gradually declines. Discontinuation of estrogen administration at day 2 results in no appreciable increase in tissue weight over the following period. Termination of estrogen administration on day 5 results in a slower rate of increase up to day 10, after which time tissue weight declines rapidly. When estrogen administration is discontinued on day 10, a rapid decrease in wet weight occurs in the subsequent days. Substitution of progesterone administration for estrogen, starting on day 5, 8, or 10 of estrogen treatment, results in a continued increase in wet weight to almost the same or an even greater extent than does estrogen administration.

Fig. 1 *b* shows changes in lysozyme content. Lysozyme is localized in tubular gland cells (6), and it can be used as a marker for the function of these cells. After its first detection on day 3, lysozyme content increases rapidly until day 10 following the daily administration of estrogen. Thereafter, it continues to increase at a reduced rate. When estrogen administration is discontinued on day 2, the day prior to the first appearance of lysozyme, no lysozyme is detected in the subsequent days. Estrogen withdrawal on day 5

[2] Grand Island Biological Company, Division of the Northamerican Mogul Product Co., Grand Island, N.Y.

[3] T. Oka and R. T. Schimke. Unpublished observation.



FIGURES 1 *a–d* Changes in magnum: (*a*) wet weight, (*b*) lysozyme content, (*c*) total RNA content, and (*d*) total DNA content. 4-day-old chicks were treated daily with a dose of 1 mg estradiol 17β-benzoate/chick (*E*). At the indicated days, estrogen administration was either stopped (O——O), or chicks were given progresterone (*P*) in place of estrogen daily at a dose of 1 mg/chick. Each point represents the means ± SEM of four chicks.

results in a slow, continued increase in lysozyme content until day 10, following which a rapid decrease occurs. When estrogen administration is stopped on day 10, there is a rapid decrease in lysozyme content in the following 10 days. Progesterone, administered in place of estrogen starting on day 5, 8, or 10, results in slightly greater accumulation of lysozyme than does continued estrogen administration.

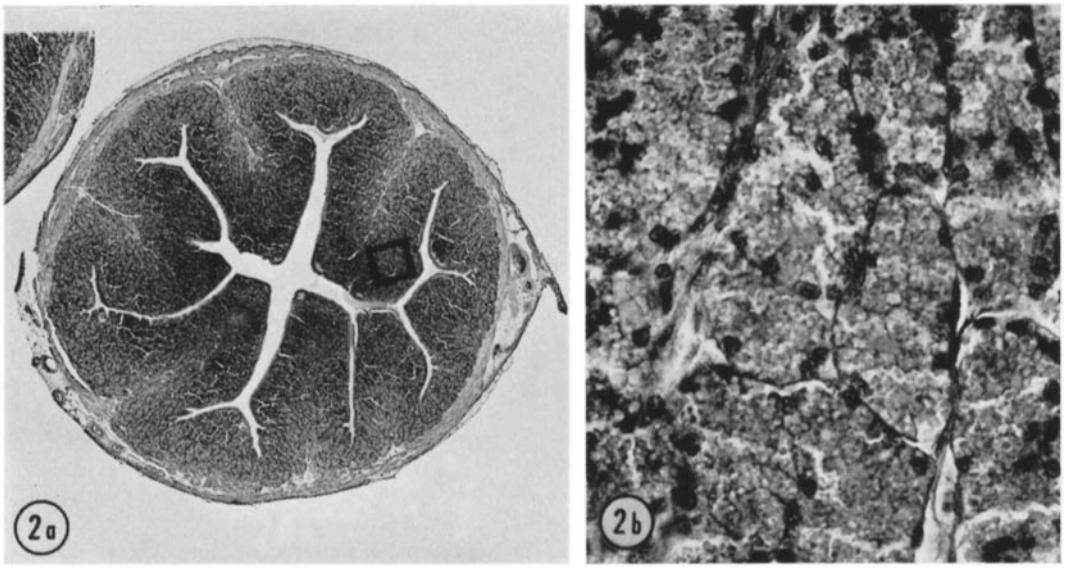
During daily administration of estrogen, total RNA content increases continuously with the maximal rate of increase between days 3 and 7

(Fig. 1 *c*). Cessation of estrogen administration on day 5 results in a continued increase in total RNA for approximately 5 days, following which a rapid decline commences. When estrogen administration is stopped on day 10, there is a rapid decline in total RNA in the following period. Administration of progesterone prevents the decrease in RNA content occurring after estrogen withdrawal.

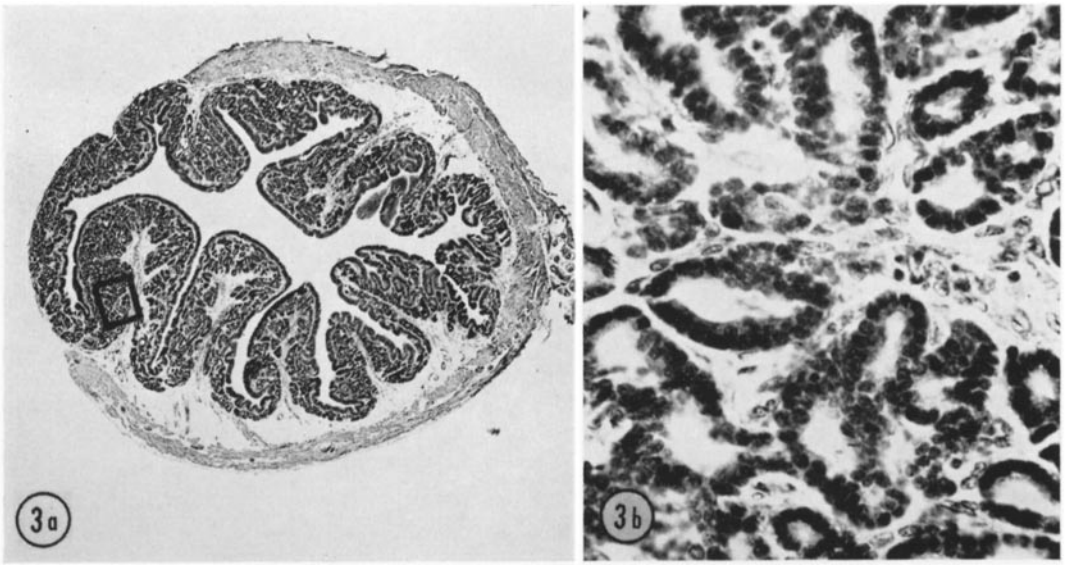
In contrast to the rapid decreases in tissue wet weight, lysozyme, and RNA content after withdrawal of estrogen, changes in total DNA content are somewhat different (Fig. 1 *d*). Thus, when estrogen administration is discontinued on day 10, total DNA content decreases only 24% in the following 12 days, whereas wet weight and lysozyme and RNA content decrease 76, 91, and 82%, respectively, in the same time period. These findings suggest that the function of tubular gland cells, as evidenced by changes in lysozyme and RNA content, is continuously dependent on the presence of hormone, whereas tubular gland cells per se, as evidenced by the lack of comparable change in total DNA content, remain in the tissue after estrogen withdrawal. This suggestion is substantiated by a comparison of the histological appearance of the oviduct of chicks treated with estrogen for 10 days with that of chicks treated with estrogen for 10 days followed by 12 days of estrogen withdrawal ("pretreated" chicks).

As shown in Fig. 2 *a*, a 10 day treatment of immature chicks with estrogen results in a marked enlargement of the magnum with characteristic tubular gland cells constituting the major portion of the tissue. Such cells have been shown to contain both lysozyme (6) and ovalbumin (20). Higher magnification of the area indicated in Fig. 2 *a* is shown in Fig. 2 *b*. These cells are literally engorged with large numbers of eosinophilic granules and form a characteristic acinar pattern with peripherally placed nuclei which are widely separated in the cells. Progesterone, administered in place of estrogen from day 5 to day 10 (Fig. 1), results in a histological appearance of tubular gland cells that is similar to that produced by a 10 day estrogen treatment. As described by Kohler et al. (4), ciliated cells and goblet cells with basally placed nuclei are found in the epithelial layer of the magnum of estrogen-treated chicks. In addition, progesterone administration appears to enhance the changes in the epithelial layer including more pronounced presence of goblet and ciliated cells.

The histological appearance of the magnum of pretreated chicks is shown in Figs. 3 *a* and *b*. In spite of extensive involution of the tissue as shown in Fig. 1, the magnum retains its original configuration including a well-defined outer muscle layer and an inner epithelium. The area between



FIGURES 2 *a* and *b* Histology of magnum of chicks treated daily with estrogen for 10 days. 2 *a*, × 18; 2 *b*, × 510.



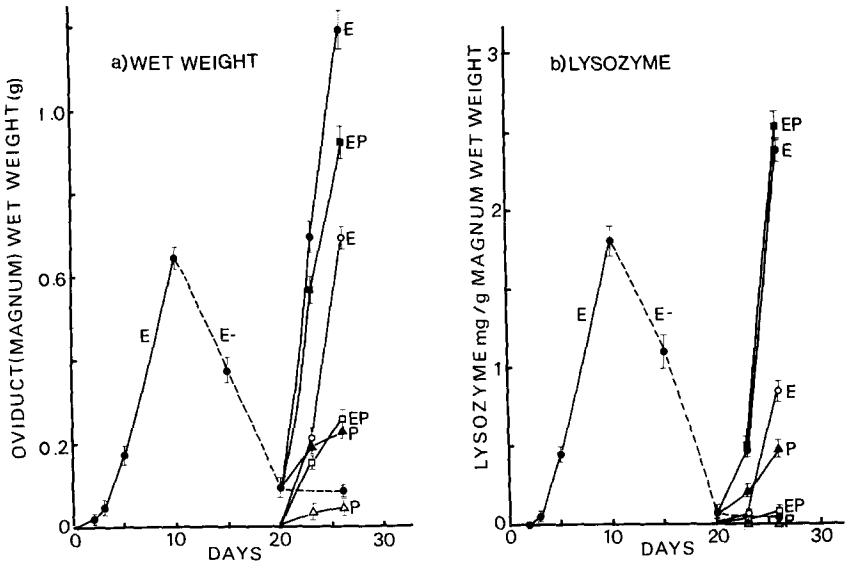
FIGURES 3 *a* and *b* Histology of magnum of chicks treated initially with estrogen for 10 days and then untreated for 12 subsequent days. 3 *a*, × 25; 3 *b*, × 360.

the epithelial and serosal surfaces is still occupied by tubular gland cells and accompanying stromal cells. Most tubular gland cells retain their acinar pattern, whereas others appear to form cell clumps. However, in contrast to Fig. 2 *b*, the cells are small in size and the eosinophilic granules which formally constituted the bulk of tubular gland cells, as well as the bulk of the magnum mass, are absent.

Thus, the histological findings are consistent with the biochemical data in demonstrating that tubular gland cell function requires the continuous presence of estrogen. Furthermore, it appears that progesterone administration can substitute for estrogen in the maintenance of the function of tubular gland cells. After hormone withdrawal, however, tubular gland cells per se persist, as evidenced by the disproportionate lack of decrease in DNA content compared to tissue weight, lysozyme, and RNA contents as well as by the histological findings.

The responsiveness of the pretreated oviduct, which contains nonfunctioning tubular gland cells, was examined with respect to combinations of estrogen and progesterone. For comparison, chicks which received no prior hormone treatment were treated in the same way. As shown in Figs. 4 *a* and *b*, during 6 days of estrogen treatment, the wet weight and lysozyme content of the pretreated oviduct increase rapidly, whereas the characteristic lag in the increase in both tissue weight and lysozyme content (6) was observed with the immature oviduct of previously untreated chicks. Furthermore, combined estrogen-progesterone treatment of pretreated chicks increases the tissue weight and lysozyme content to almost the same extent as does estrogen treatment. In contrast, such combined treatment of untreated chicks reduces the estrogen-induced increase in tissue weight and essentially abolishes the increase in lysozyme content as reported previously (6). 6 days of treatment with progesterone, which evokes little response in the oviduct of untreated chicks, increases both the tissue weight and lysozyme content of the pretreated oviduct.

Changes in DNA, RNA, and protein content during the above experiment are shown in Table I. In the pretreated oviduct, 6 days of estrogen treatment result in more than a sixfold increase in total DNA content. Progesterone administered alone for 6 days increases total DNA content only 1.5-fold and, when administered with estrogen, antagonizes the estrogen-induced increase in total DNA content. In the untreated oviduct, 6 days of treatment with estrogen increases total DNA content more than 22-fold, whereas treatment with progesterone increases total DNA content just over twofold. In addition, concomitant administration of progesterone with estrogen markedly



FIGURES 4 *a* and *b* Comparison of changes in (*a*) wet weight and (*b*) lysozyme content in magnum of immature and estrogen-pretreated chicks. One group of 26-day-old chicks, treated previously with estrogen (1 mg/hick/day) for 10 days and subsequently untreated for 12 days, was treated daily with either 1 mg of estradiol 17β-benzoate (*E*, ●——●), or progesterone (*P*, ▲——▲), or both (*EP*, ■——■) for 6 days. Another group of chicks of the same age that had received no prior hormone treatment were also treated with either estradiol 17β-benzoate (*E*, ○——○), or progesterone (*P*, △——△), or both (*EP*, □——□) for 6 days at the same dose of hormone as the pretreated chicks. Each point represents the means ± SEM of three chicks.

antagonizes the estrogen-induced increase in total DNA content, resulting in a sevenfold increase in 6 days. Total RNA and protein contents of the pretreated oviduct after 6 days of estrogen treatment increase about 13- and 19-fold, respectively, whereas in the untreated immature oviduct, such increases are much greater following 6 days of estrogen treatment. 6 days of treatment with progesterone result in a twofold increase in total RNA content of both pretreated and untreated oviducts, but the RNA/DNA ratio is somewhat higher in the pretreated oviduct than in the untreated oviduct. The increase in total protein content is also greater in the pretreated oviduct, amounting to a fivefold increase, whereas a threefold increase is found in the immature oviduct. Combined treatment with estrogen and progesterone increases both total RNA and protein contents about 12-fold in the pretreated oviduct, whereas this treatment markedly reduces the estrogen-induced increase in both total RNA and protein content in the untreated oviduct.

The results of Fig. 4 and Table I, then, demonstrate that the pretreated oviduct responds rapidly to estrogen, and furthermore, that it is also responsive to progesterone administration. In our previous reports (5, 6), we presented evidence indicating that accumulation of lysozyme in the immature oviduct first requires the formation of tubular gland cells, a process evoked by estrogen administration. Furthermore, concomitant administration of progesterone antagonizes this process and, therefore, prevents the estrogen-induced increase in lysozyme content (see also Figs. 4 *a* and *b*). That new DNA synthesis is required for the estrogen-induced increase in lysozyme in the immature oviduct is supported by the results of Table II. In these experiments, we have used hydroxyurea as an inhibitor of DNA synthesis. A considerable body of experimental evidence indicates that this compound inhibits DNA synthesis in various animal tissues by inhibiting nucleotide reductase, which converts ribonucleotides into deoxyribonucleotides (21–24). As shown in Table II, concomitant administration of hydroxyurea with estrogen inhibits the estrogen-stimulated incorporation of thymidine-^{3}H into DNA by 90% and, furthermore, antagonizes the estrogen-induced increase in wet weight, total DNA, and lysozyme content

TABLE I

Comparison of Changes in DNA, RNA, and Protein in the Magnum of the Immature and the Estrogen-Pretreated Chick During Treatment with Estrogen, Progesterone, and Estrogen-Progesterone

Treatment	DNA	DNA	RNA	RNA	RNA/DNA	Protein	Protein
	μg/magnum	*mg/g magnum wet weight*	*μg/magnum*	*mg/g magnum wet weight*		*mg/magnum*	*mg/g magnum wet weight*
Immature oviduct*							
Control	77 ± 5	6.4 ± 0.4	84 ± 8	7.0 ± 0.2	1.1	0.94 ± 0.3	78 ± 2
E 3	655 ± 10	3.1 ± 0.2	2030 ± 31	9.6 ± 0.2	3.1	18.9 ± 1.5	89 ± 4
E 6	1780 ± 35	2.6 ± 0.2	7150 ± 28	10.5 ± 0.3	4.0	119.0 ± 2.5	175 ± 6
EP 3	346 ± 9	2.3 ± 0.1	730 ± 15	4.7 ± 0.1	2.1	13.3 ± 1.5	89 ± 7
EP 6	560 ± 12	1.9 ± 0.2	1160 ± 22	4.4 ± 0.3	2.3	20.9 ± 1.7	103 ± 5
P 3	102 ± 4	6.0 ± 0.2	120 ± 7	7.2 ± 0.3	1.2	1.5 ± 0.2	92 ± 3
P 6	162 ± 5	6.0 ± 0.1	210 ± 8	7.8 ± 0.2	1.3	2.7 ± 0.4	99 ± 3
Pretreated oviduct‡							
Control	600 ± 18	6.5 ± 0.3	870 ± 25	9.4 ± 0.1	1.5	13.7 ± 1.2	148 ± 7
E 3	2900 ± 38	4.2 ± 0.1	10100 ± 59	14.6 ± 0.3	3.2	92.0 ± 2.1	133 ± 5
E 6	3240 ± 51	2.7 ± 0.3	12000 ± 78	12.4 ± 0.2	3.6	248.0 ± 2.8	207 ± 10
EP 3	2020 ± 60	3.7 ± 0.2	6000 ± 75	10.5 ± 0.3	2.8	75.5 ± 1.9	132 ± 9
EP 6	2720 ± 41	2.9 ± 0.2	10000 ± 67	10.9 ± 0.2	3.6	172.0 ± 2.2	186 ± 11
P 3	1050 ± 61	5.4 ± 0.3	2060 ± 51	10.6 ± 0.1	2.0	36.7 ± 1.4	137 ± 7
P 6	961 ± 55	4.0 ± 0.1	1930 ± 58	8.3 ± 0.2	2.2	72.6 ± 1.8	148 ± 12

* 26-day-old chicks received no prior hormone treatment.

‡ 26-day-old chicks received daily estrogen treatment (1 mg estradiol 17β-benzoate/chick) for 10 days and subsequently remained untreated for 12 days.

Control, no hormone treatment; E3 and E6, 3 and 6 days after daily treatment with estradiol 17β-benzoate (1 mg/day/chick); EP3 and EP6, 3 and 6 days after daily treatment with both estradiol 17β-benzoate (1 mg/day/chick) and progesterone (1 mg/day/chick); P3 and P6, 3 and 6 days after daily treatment with progesterone (1 mg/day/chick). Each value represents the mean ± SEM of four chicks.

when given continuously for 5 days. However, when hydroxyurea administration is started 2 days after the daily administration of estrogen, at a time when essentially no lysozyme is yet detectable (Fig. 1), then lysozyme activity is detectable after 5 days of total treatment. These results, then, indicate that in the immature oviduct the appearance of lysozyme requires DNA synthesis, presumably for the formation of tubular gland cells. When estrogen is administered for 2 days prior to inhibition of DNA synthesis by hydroxyurea, a certain number of potential tubular gland cells have been formed as evidenced by the increase in total DNA content. However, they do not function in lysozyme synthesis until sometime later. Concomitant administration of hydroxyurea does not inhibit the effect of estrogen to stimulate the function of tubular gland cells once such cells are formed. Thus the effect of hydroxyurea is analogous to that of progesterone, as described previously (5, 6).

The effect of hydroxyurea to prevent the estrogen-stimulated increase in lysozyme in the immature oviduct is to be contrasted to its effect on the pretreated oviduct (Table III). Administration of estrogen to pretreated chicks results in a twofold increase in oviduct wet weight, a sixfold increase in total lysozyme, and a 50-fold increase in thymidine-^{3}H incorporation into DNA as determined at 24 hr after the treatment. Hydroxyurea administration inhibits the estrogen-stimulated DNA synthesis by 90% but has essentially no effect on the increase in wet weight or lysozyme content. Progesterone administration produces comparable effects on increases in wet weight and total lysozyme content although its effect on DNA synthesis, as judged by the thymidine-^{3}H incorporation, is only 12-fold. The increases in wet weight and

TABLE II

Effect of Hydroxyurea on Changes Induced by Estrogen in the Immature Oviduct

Treatment*	Magnum wet weight	Total lysozyme in magnum	Total DNA in magnum	‡ DNA synthesis
	mg	*μg*	*μg*	*cpm/mg DNA*
Control	13 ± 2	undetectable	40 ± 5	40 ± 2
Estrogen 3 days	63 ± 7	3.2 ± 0.2	182 ± 6	
Estrogen 5 days	162 ± 11	59.5 ± 1.5	492 ± 15	450 ± 15
Estrogen + HU§ 5 days	33 ± 5	<0.9	95 ± 9	52 ± 5
Estrogen 2 days + estrogen + HU§ 3 days	103 ± 9	18.7 ± 0.8	139 ± 6	

* 4-day-old immature chicks were administered estradiol 17β-benzoate daily at a dose of 1 mg/chick.
‡ DNA synthesis was measured at 24 hr after the first treatment. Details are described in Materials and Methods.
§ Hydroxyurea (HU) was injected subcutaneously every 12 hr at a dose of 400 mg/100 g body weight. Each value represents the mean ± SEM of three chicks.

TABLE III

*Effect of Hydroxyurea on Changes Induced by Estrogen, Progesterone, and Their Combination in the "Pretreated" Magnum**

Treatment	Magnum wet weight	Total lysozyme in magnum	Ovalbumin‡ synthesis	Protein‡ synthesis	DNA§ synthesis
	mg	*μg*	*cpm × 10⁻³/g wet weight*		*cpm × 10/mg DNA*
Control	119 ± 3	8.0 ± 0.1	92, 88	1195, 1060	50 ± 3
Estrogen	233 ± 6	48.7 ± 0.5	8500, 10400	14400, 17650	2620 ± 28
Estrogen + HU‖	206 ± 9	47.2 ± 0.2	11675, 10900	25400, 23200	253 ± 11
Estrogen + HU**	191 ± 7	47.3 ± 0.1			135 ± 13
Progesterone	229 ± 5	44.7 ± 0.2	5050, 4600	11000, 10000	602 ± 19
Progesterone + HU‖	207 ± 7	46.7 ± 0.4	5310, 5400	10000, 9500	252 ± 15
Estrogen + progesterone	263 ± 7	50.6 ± 0.5	9800, 9680	20000, 19700	1497 ± 36
Estrogen + progesterone + HU‖	212 ± 8	48.7 ± 0.2	9850, 9950	18200, 19500	236 ± 13

* 4-day-old chicks were initially treated daily with a dose of 1 mg estradiol 17β-benzoate for 10 days and thereafter remained untreated for 12 days. Chicks were then treated with 1 mg of either estradiol 17β-benzoate, or progesterone, or both for 24 hr.
‡ The extent of ovalbumin and protein synthesis was measured in vitro as described in Materials and Methods. Protein synthesis was measured in 105,000 *g* supernatant fraction. Each value was obtained from two chicks.
§ The extent of DNA synthesis was measured at 24 hr after treatments as described in Materials and Methods. Each value represents the mean ± SEM of three chicks.
‖ Hydroxyurea (HU) was injected subcutaneously at a dose of 400 mg/100 g body weight at 0 and 12 hr after hormone treatment.
** Hydroxyurea (HU) was injected subcutaneously at a dose of 400 mg/100 g body weight at 0, 6, 12, and 18 hr after estrogen treatment.

lysozyme content produced by progesterone are not affected by hydroxyurea, although hydroxyurea inhibits the progesterone-induced DNA synthesis. Combined administration of estrogen and progesterone increases the wet weight and total lysozyme content somewhat more than does estrogen or progesterone separately. However, the estrogen-induced DNA synthesis is inhibited by concomitant administration of progesterone by 40%. Hydroxyurea inhibits DNA synthesis by

85% but has essentially no effect on the increase in wet weight and total lysozyme content resulting from the combined treatment. Also of interest are the effects of these hormones and their combination on ovalbumin synthesis. All of the hormone treatments increase markedly the capacity of oviduct minces to incorporate amino acids into ovalbumin as well as total soluble protein. In addition, a comparison of radioactivity incorporated into ovalbumin and total soluble protein indicates that ovalbumin is a major protein being synthesized in oviduct after the above hormone treatment, amounting to about 50% of total soluble protein. In these experiments, progesterone appears to be less active than estrogen in stimulating ovalbumin synthesis, whereas in other experiments progesterone has been found to be at least as active as estrogen during the first 12 hr period after the hormone treatment (see Fig. 7). Treatment with hydroxyurea does not inhibit such hormone-induced increases in ovalbumin and protein synthesis. These results, then, indicate that at 24 hr after the administration of estrogen, progesterone, or their combination to pretreated chicks, the synthesis of lysozyme and ovalbumin is already stimulated markedly and, furthermore, does not require new DNA synthesis.

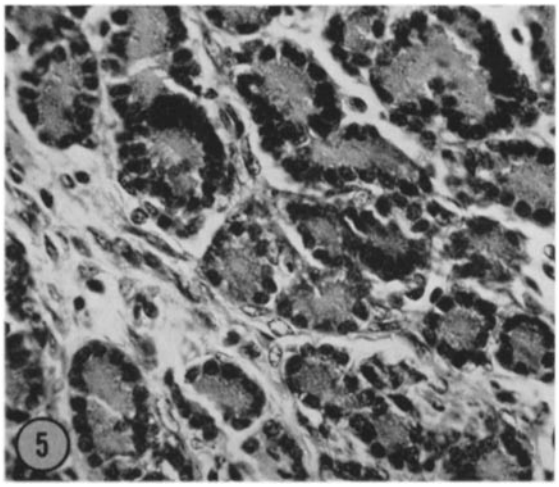


FIGURE 5 Histology of magnum of the pretreated chicks 12 hr after estrogen administration. 4-day-old chicks were initially treated daily with a dose of 1 mg of estradiol 17β-benzoate for 10 days and thereafter remained untreated for 12 days. The chicks were then given another injection of estrogen (1 mg/chick), and 12 hr later the tissue was fixed. × 360.

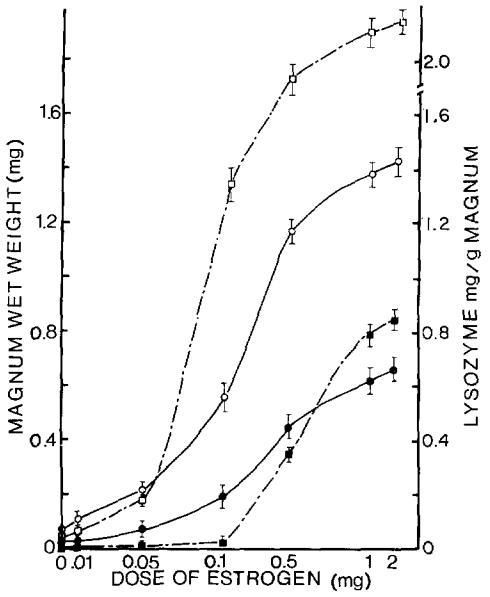


FIGURE 6 Comparison of dose-response relationship of estrogen and increase in wet weight and lysozyme activity in magnum of immature and estrogen-pretreated chicks. 26-day-old chicks were treated with the indicated dose of estradiol 17β-benzoate for 5 days. Another group of chicks of the same age which had received 10 days of estrogen treatment and thereafter was untreated for 12 days was also treated with the indicated dose of estradiol 17β-benzoate for 5 days. Results are presented as increase in magnum wet weight (immature oviduct, ●——●; pretreated oviduct, ○——○) and lysozyme content per gram of magnum wet weight (immature oviduct, ■-·-■; pretreated oviduct, □-·-□) after 5 days of daily treatment. Each point represents the means ± SEM of four chicks.

The rapid nature of the response of the pretreated oviduct to estrogen is also indicated by histological examination of the oviduct at 12 hr after the administration of estrogen to pretreated chicks. As shown in Fig. 5, essentially all of the tubular gland cells have increased cytoplasmic volumes, and characteristic eosinophilic granules are present in their apical region in contrast to control pretreated oviduct (Fig. 3 *b*). Similar changes in histology of the oviduct are seen when pretreated chicks are given progesterone or a combination of estrogen and progesterone for 12 hr.

The difference in responsiveness of the magnum between the immature oviduct and the pretreated oviduct which contains nonfunctioning tubular gland cells is further indicated by the differences

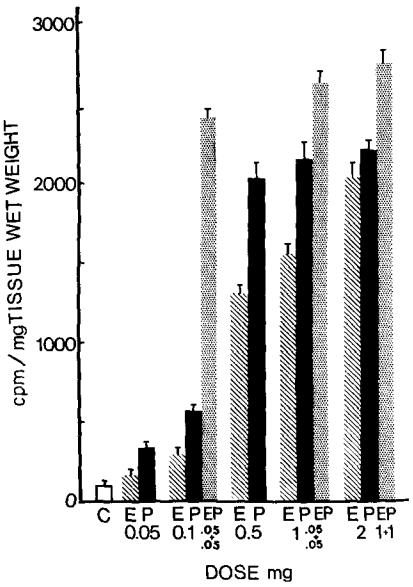


FIGURE 7 Comparison of dose-response relationship of estrogen, progesterone, and their combination with respect to ovalbumin synthesis in magnum of estrogen-pretreated chicks. 26-day-old chicks which had received 10 days of estrogen treatment and thereafter were untreated for 12 days were given the indicated dose of either estradiol 17β-benzoate (▨), or progesterone (▬), or both (▩) for 12 hr. Control chicks received sesame oil only (□). The extent of ovalbumin synthesis was measured in vitro as described in Materials and Methods. Each column represents the means ± SEM of three determinations.

in the dose of estrogen required to produce an increase in wet weight and lysozyme content. As shown in Fig. 6, the dose of estrogen necessary to elicit an increase in wet weight and lysozyme content is much less in the pretreated oviduct than in the immature oviduct. This is particularly apparent with respect to lysozyme content; a dose of 0.1 mg/day evokes essentially no lysozyme increase in the immature oviduct, whereas as little as 0.01 mg/day produces a significant increase in lysozyme in the pretreated oviduct.

The specificity of hormones evoking the rapid oviduct responses in pretreated chicks is evaluated in Table IV. Among the steroid hormones studied, only estrogen (estradiol 17β-benzoate) and progesterone are effective in inducing the increase in wet weight and total lysozyme content. Since progesterone is known to be a precursor of various steroid hormones in mammals including androgens and estrogens (25), the possibility arises that progesterone could be acting by metabolic conversion to an androgenic or estrogenic steroid(s) such as testosterone, estradiol-17β, estriol, or estrone. This possibility, however, would not seem likely since androstanedione, a steroid which is an intermediate on the pathway from progesterone to testosterone and thence to estradiol-17β (26), is inactive as is testosterone itself. Furthermore, two synthetic progestins, medroxyprogesterone and norethindrone, are also active. These results, then, suggest strongly that the pretreated oviduct is responsive to both estrogen and progesterone.

Some preliminary attempts have been made to clarify the mode of action of estrogen and progesterone, both of which stimulate the synthesis of lysozyme and ovalbumin in the pretreated oviduct. In order to examine whether their effects are additive or synergistic, the effect of various dosages of estrogen and progesterone and their combination has been studied on ovalbumin synthesis at 12 hr after the hormone treatment. Results shown in Fig. 7 indicate that the increase in ovalbumin synthesis becomes greater with increasing doses of both hormones. A dose of 1–2 mg appears to be the near maximal dose of both estrogen and protesterone. At all the dosages tested, however, pro-

TABLE IV

*Effect of Various Steroid Hormones on Wet Weight and Lysozyme Content in the Magnum of Pretreated Chicks**

Treatment‡	Magnum wet weight	Total lysozyme in magnum
	mg	*μg*
Control	208 ± 4	12.0 ± 0.8
Estradiol 17β-benzoate	366 ± 18	51.3 ± 0.7
Progesterone	367 ± 11	50.0 ± 0.5
Norethindrone	467 ± 35	59.5 ± 0.9
Medroxyprogesterone	311 ± 25	30.7 ± 0.3
Testosterone	233 ± 10	15.7 ± 0.7
Androstanedione	234 ± 20	13.8 ± 0.6
Hydrocortisone 21-phosphate	200 ± 3	11.4 ± 0.6
Deoxycorticosterone	170 ± 2	11.1 ± 0.2

* 4-day-old immature chicks were initially treated with 1 mg of estradiol 17β-benzoate daily for 12 days and remained untreated for 10 days.

‡ Each hormone was administered intramuscularly at a dose of 1 mg/chick.

Each value was obtained 24 hr after the hormone treatment and represents the mean ± SEM of three chicks.

gesterone appeared to be more potent than estrogen. Combined administration of both estrogen and progesterone at a dose of 0.05 mg, which produces the minimal increase, stimulates ovalbumin synthesis far greater than does separate administration of estrogen or progesterone at an equivalent dose of 0.1 mg. At the near maximal dose of the two hormones, combined treatment produces still greater increase, although the difference between combined and single hormone treatment becomes smaller. Such a synergistic effect suggests that estrogen and progesterone may stimulate ovalbumin synthesis by somewhat different mechanisms.

DISCUSSION

Our present conception of the effect of estrogen and/or progesterone on tubular gland cell formation and function is outlined in Fig. 8. Initiation of tubular gland cell proliferation from the surface epithelium commences within 24 hr of the initial estrogen treatment. However, such cells become functional in the synthesis of lysozyme and ovalbumin only after 2–3 days of estrogen treatment. Tubular gland cell function is dependent on the continuous presence of estrogen since its discontinuation results in a rapid cessation of function and oviduct involution which is accompanied by the loss of tissue weight and total lysozyme and RNA content. However, tubular gland cells per se persist during such involution.

The response of the oviduct containing nonfunctioning tubular gland cells (the pretreated oviduct) to estrogen treatment is to be contrasted with that of the immature oviduct. In the pretreated oviduct, estrogen evokes an increase in lysozyme and ovalbumin within 12 hr of treatment, a process which is not dependent on new DNA synthesis. In the immature oviduct, the appearance of lysozyme is detectable only after 2–3 days of treatment and is prevented by inhibition of DNA synthesis by hydroxyurea. In addition, a larger dose of estrogen appears to be necessary for an increase in lysozyme in the immature oviduct compared to the pretreated oviduct. These differences can be attributed to the fact that the pretreated oviduct contains tubular gland cells which are capable of responding rapidly to estrogen with the synthesis of lysozyme and ovalbumin. Preliminary data[3] indicate that an increase in lysozyme and ovalbumin synthesis occurs as early as 3–4 hr after estrogen treatment. In the immature oviduct, in contrast, tubular gland cells are absent, and therefore the synthesis of lysozyme and ovalbumin must be preceded by the formation of tubular gland cells, a process which is prevented by inhibition of DNA by hydroxyurea.

The effect of progesterone administration differs markedly at different stages of oviduct development. In the immature oviduct, progesterone administration results in no increase in lysozyme but antagonizes the effect of estrogen to induce the increase in lysozyme by preventing the formation of tubular gland cells when given concomitantly with estrogen. Once tubular gland cells are present, i.e. 4–5 days after estrogen treatment, progesterone can substitute for estrogen in maintaining the function of tubular gland cells. Kohler et al. (4) have reported that continued estrogen treatment stimulates the mitosis associated with tubular gland cells (glandular mitosis, see Fig. 8) in addition to the appearance of epithelial goblet and ciliated cells. We do not know if progesterone stimulates mitosis in tubular gland cells. Proges-

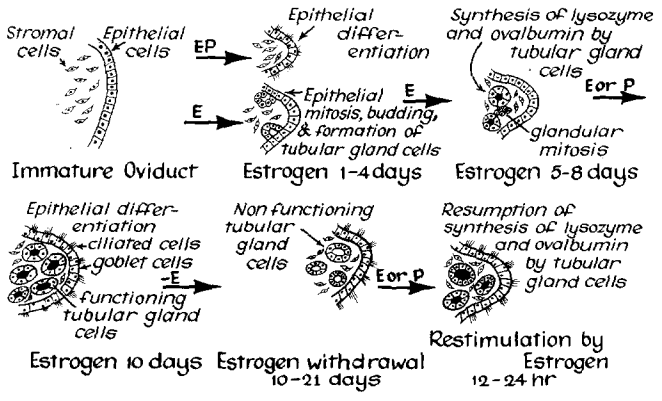


FIGURE 8 Summary of estrogen-progesterone interactions in the development and function of the tubular gland cells in chick oviduct. *E*, estrogen; *P*, progesterone; *EP*, estrogen plus progesterone.

terone appears to enhance morphological differentiation of surface epithelial cells when it is administered alone or with estrogen, as opposed to its antagonistic effect on the initiation of tubular gland cell formation.

Perhaps most striking is the finding that administration of progesterone stimulates the synthesis of lysozyme and ovalbumin in tubular gland cells which previously were not functioning (Fig. 8). Such effects of progesterone are to be compared and contrasted with the findings of O'Malley and his coworkers who have shown that progesterone stimulates avidin synthesis in goblet cells (7–11). Initiation of avidin synthesis by progesterone (26) does not require new DNA synthesis in goblet cells. This is similar to the effect of progesterone on lysozyme and ovalbumin synthesis in preexisting tubular gland cells. However, in avidin synthesis only progesterone and its analogues are effective (8), whereas in lysozyme and ovalbumin synthesis both estrogen and progesterone are effective. Such diverse effects of progesterone as well as estrogen on a single tissue suggest differences in competence of various cell types in that tissue to respond to a hormonal stimulus. Thus, in the study of this system (or of any system, for that matter) care must be taken in interpreting biochemical results where little attempt has been made to consider different responses among various cell types.

The effects of estrogen and progesterone in the oviduct illustrate two general types of hormonal responses commonly observed. The first response is characterized by cell proliferation leading to the acquisition of cells that engage in a specific function. Thus, estrogen evokes the appearance of new cell types, among which are tubular gland cells that can synthesize lysozyme and ovalbumin, goblet cells that can synthesize avidin under appropriate hormonal stimulation, as well as ciliated epithelial cells. The results with hydroxyurea in the immature oviduct (Table II) indicate that DNA synthesis is necessary for cells to acquire the ability to respond to estrogen with the synthesis of lysozyme. In this respect, then, the system is similar to that studied by Topper and his collaborators who have shown that, in cultured mouse mammary gland tissue, the effect of prolactin to accelerate casein synthesis requires new DNA synthesis and mitosis of epithelial cells, a process that is stimulated by insulin and requires the presence of hydrocortisone (27). In addition, androgens appear to inhibit new casein synthesis by inhibiting the insulin-mediated new DNA synthesis (28). Thus, this effect of androgens appears to be comparable to the effect of hydroxyurea and/or progesterone in the immature oviduct. The apparent requirement for DNA synthesis in the acquisition of the ability of oviduct epithelial cells to respond to estrogen with the synthesis of ovalbumin and lysozyme appears to be similar to that requirement as observed in a number of differentiating systems. It has been suggested (27, 29) that a cell can be programmed for a new set of cell functions, such as the synthesis of specific proteins, only at the time of DNA synthesis.

The second type of response involves the stimulation of function of preexisting cells as observed in the pretreated oviduct. Other examples exist in which the stimulation of synthesis of specific cell proteins does not appear to require new DNA synthesis but does require the continued presence of the hormone. Among such examples are the hydrocortisone-mediated increased rate of synthesis of tyrosine aminotransferase in rat liver (30) and in cultured hepatoma cells (31, 32) and the stimulation of avidin synthesis by progesterone in the estrogen-primed chick oviduct (7–11). In these cases the competent cells are already present, and the hormones can be considered to "switch on" cell function in previously programmed cells.

The interaction of estrogen and progesterone is obviously complex in oviduct development. With respect to the initiation of tubular gland cell formation, progesterone is antagonistic to estrogen. As previously reported (6), progesterone does not inhibit either the absorption of estrogen from the injection site or the uptake of estrogen into the tissue. One simple hypothesis that progesterone simply displaces estrogen from the specific receptor site(s) seems unlikely, inasmuch as increasing the dose of estrogen does not overcome the progesterone antagonism. In addition, the antagonism appears to be tissue specific since the estrogen-induced synthesis of phosvitin in liver is not inhibited by progesterone administration. At the present time, we do not know how progesterone produces its antagonistic effect. Clearly, the events leading to cell proliferation are complicated and may require a sequence of preceding events. Thus, progesterone may exert its inhibitory effect by preventing one of a number of events that occur prior to the increase in DNA synthesis which commences only 8–12 hr after estrogen administration.[3] Such events include stimulation of RNA synthesis[3] accompanied by increased RNA polymerase ac-

tivity (33), migration of circulating monocytes into the tissue (4), and increased uptake of water and amino acids, the latter of which are not inhibited by progesterone (6). One other possible mechanism of progesterone antagonism relates to its effect to produce epithelial differentiation. Thus, one might propose that progesterone induces undifferentiated epithelial progenitor cells into specifically differentiated epithelial cells, and that, once differentiated, such cells are no longer available for differentiation into tubular gland cells. It is also possible that progesterone antagonism may be mediated through complex effects on the metabolism of other hormones, since all the experimental evidences have been obtained from the intact chick. A report on the Corturnix quail by Brody and Zarrow (34) indicates that ovariectomy diminishes the effect of prolactin, but not that of progesterone, in stimulating the estrogen-induced oviduct growth.

In contrast to its antagonistic effect on tubular gland cell formation, progesterone can stimulate function once such cells exist. Several explanations can be envisaged for this effect. Since all the experiments have been performed in the intact chick, the possibility exists that progesterone is converted to an estrogenic steroid which is the active agent. We have no conclusive proof against such a theory, particularly since we do not know whether the hormone is active as itself, or as a metabolite. Two lines of evidence suggest that metabolic conversion from progesterone to estrogen does not account for the action of progesterone on tubular gland cell function. Thus, steroid compounds that one would presume to be intermediates as judged by studies in mammals (25), i.e. androstanedione and testosterone, are not effective in initiating the synthesis of lysozyme, whereas synthetic progestational steroids are equally active. Furthermore, the effects of estrogen and progesterone appear to be different in that progesterone stimulates mainly cell function, whereas estrogen stimulates both cell proliferation and function (Tables I and IV). In addition, progesterone is more potent than estrogen during a short period after hormone administration, i.e. 12 hr, while estrogen produces a greater increase in lysozyme and ovalbumin at later time intervals (Figs. 2 and 7; Table IV). Perhaps most striking is the apparent synergistic effect of estrogen and progesterone on ovalbumin synthesis (Fig. 7). Thus, whereas low doses of either estrogen or progesterone produce minimal effects on ovalbumin synthesis, a combination of the two hormones produces nearly maximal stimulation of ovalbumin synthesis. Such a finding suggests that the two hormones may be acting in a slightly different fashion to stimulate cell function. Further studies are in progress to clarify the mode of action of estrogen and progesterone and to identify the active form of each steroid hormone in this system.

The pretreated oviduct, with a large number of nonfunctioning tubular gland cells, is a good system for the study of hormonal regulation of specific protein synthesis. This system is characterized by the presence of a specific cell type which synthesizes well-characterized proteins, including lysozyme and ovalbumin. Ovalbumin amounts to as much as 50% of the total soluble proteins synthesized, and its synthesis is rapidly initiated by treatment with two different steroid hormones which may act through somewhat different mechanisms. The studies presented to date have been descriptions of over-all events and hopefully will form the basis of a better understanding of biochemical and macromolecular events in tubular gland cells as affected by estrogen and progesterone. Thus, dissection and comparison of their effects in this system may provide useful knowledge in understanding hormonal control of protein synthesis.

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