INTERACTION OF ESTROGEN AND PROGESTERONE IN CHICK OVIDUCT DEVELOPMENT

I. Antagonistic Effect of Progesterone on Estrogen-Induced Proliferation and Differentiation of Tubular Gland Cells

TAKAMI OKA and ROBERT T. SCHIMKE

From the Department of Pharmacology, Stanford University, School of Medicine, Stanford, California 94305

ABSTRACT

Daily administration of estrogen to immature female chicks results in marked oviduct growth and appearance of characteristic tubular gland cells which contain lysozyme. Although a rapid increase in total DNA and RNA content begins within 24 hr, cell specific protein, lysozyme, is first detectable after 3 days of estrogen. Progesterone administered concomitantly with estrogen antagonizes the estrogen-induced tissue growth as well as appearance of tubular gland cells and their specific products, lysozyme and ovalbumin. When the initiation of progesterone administration is delayed for progressively longer periods (days) during estrogen treatment, proportionally greater growth occurs with more lysozyme and tubular gland cells after 5 days of total treatment. Progesterone does not inhibit the estrogen-stimulated increase in uptake of α-aminoisobutyric acid and water by oviduct occurring within 24 hr or the estrogen-induced increase in total lipid, phospholipid, and phosphoprotein content of serum. The above results of progesterone antagonism can best be explained by the hypothesis that progesterone inhibits the initial proliferation of cells which become tubular gland cells but does not antagonize the subsequent cytodifferentiation leading to the synthesis of lysozyme and ovalbumin once such cell proliferation has occurred.

INTRODUCTION

The chick oviduct provides an excellent system for the study of hormonal regulation of growth, cytodifferentiation, and function of specific cell types. The growth of the oviduct is markedly stimulated by estrogen (1–3). In addition, the synthesis of specific cell products such as lysozyme and ovalbumin is induced by estrogen in the magnum portion of oviduct (4–8). Various effects of progesterone on the responses of the oviduct to estrogen have been described. Thus, Hertz et al. first reported that avidin synthesis is induced by progesterone in an estrogen-primed oviduct (9).

More recently O'Malley and his collaborators have shown that the above effect of progesterone can be demonstrated in an estrogen-stimulated minced oviduct and oviduct epithelial cells in culture (10–12). In contrast, several workers have reported that progesterone administered concomitantly with estrogen inhibits estrogen-induced growth (13–16), while a synergistic effect of progesterone has also been described (16–18).

In a recent communication (19) we described some aspects of an antagonistic effect of concomitant progesterone administration on the estrogen-induced cytodifferentiation of tubular gland cells, cells which are involved in synthesis of the egg white proteins, ovalbumin, and lysozyme. We proposed that progesterone antagonizes the proliferation of those cells destined to become tubular gland cells, cells that comprise a major portion of the estrogen-stimulated oviduct. Our results also suggested that once such proliferation takes place, the subsequent processes of cytodifferentiation are not inhibited by progesterone.

In the present paper, we report in more detail on the progesterone antagonism of estrogen-induced oviduct development. In addition, we show here that the antagonism produced by progesterone is tissue specific and noncompetitive with estrogen. In addition, progesterone does not inhibit the early (4–12 hr) effects of estrogen to increase the amino acid and water uptake into the oviduct.

MATERIALS AND METHODS

Hormones and Chemicals

17β-estradiol benzoate, progesterone, testosterone propionate, and hydrocortisone 21-phosphate were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Calf thymus DNA and yeast RNA were obtained from Worthington Biochemical Corp., Freehold, N.J. The strain of *Micrococcus lysodeikticus* was kindly provided by Dr. C. E. Nardini, Department of Medical Microbiology, Stanford Medical School.

Treatment of Animals

Newborn white leghorn female chicks obtained from Kimber Farms, Fremont, Calif., were maintained in electrically heated brooders and fed a Purina Laboratory Chow (micro-mixed) diet (Ralston Purina Co., St. Louis, Mo.). At the age of 4 days, the chicks, weighing about 50 g each, were given the hormone(s) dissolved in sesame oil or 0.15 M saline (in the case of hydrocortisone). The volume of injection was 0.1 ml per chick for each hormone; injections were given into the separate muscle mass of opposite limbs just below the knee when two hormones were administered.

Assays

At indicated times after hormone administration, chicks were sacrificed by decapitation, and oviducts were promptly dissected. The magnum part of the 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 (20). DNA was assayed by the diphenylamine reaction with calf thymus DNA as standard (21), and RNA was assayed by the orcinol method with yeast RNA as standard (22). Protein was estimated by the method of Lowry et al. (23) with crystalline bovine serum albumin as standard. Lysozyme was assayed by a modification of the method of Litwack (24). The activity was measured by following the decrease in absorbancy at 645 mμ at 25° C with Gilford Multiple Sample Absorbance Recorder Model 2000 (Gilford Instrument Company, Oberlin, Ohio) attached to a Beckman DU monochromator (Beckman Instruments Inc., Fullerton, Calif.). The reaction mixture consisted of 2.5 ml of 0.066 M potassium phosphate buffer, pH 6.2, containing 0.1% NaCl and *Micrococcus lysodeikticus* 75 mg dry weight/100 ml and enzyme solution. Activity is expressed as μg of lysozyme present and is based on the activity of purified egg white lysozyme (Calbiochem, Los Angeles, Calif.) as standard. Addition of oviduct extract to the purified lysozyme resulted in additive enzyme activity in all cases. Hence no activator or inhibitor of lysozyme could be demonstrated in oviduct extract.

Serum was collected from the jugular vein for the determination of total lipid, phospholipid, and phosphoprotein in serum. Total lipid and phospholipid were extracted from serum with a chloroform-methanol mixture (2:1, v:v), and the extract was washed with 0.05% sulfuric acid to remove nonlipid substances (25). Total lipid content in serum was estimated with palmitic acid as standard by measuring the amount of chromium potassium sulfate produced from potassium dichromate spectrophotometrically at 580 mμ in a Zeiss spectrophotometer, Model PMQ II (26). Phospholipid was estimated by measuring the phosphorus content in the chloroform-methanol extracts according to Fiske and SubbaRow (27). Phosphoprotein in serum was extracted and measured as described by Greengard et al. (28).

Counting Procedures

^{14}C-α-aminoisobutyric acid (SA, 5.1 mc/mmole) was obtained from New England Nuclear Corp., Boston, Mass. It was dissolved in 0.15 M NaCl, and 1

μc per chick was injected intramuscularly (breast muscle) in a volume of 0.1 ml 4 hr before hormone administration. At indicated times after hormone administration, oviduct and other tissues were dissected, placed in scintillation vials, and dried for 24 hr at 84° C in the oven. Dried tissues were weighed, soaked in 0.1 ml of distilled water, and then solubilized in 1 ml of NCS (Nuclear-Chicago Corporation, Des Plaines, Ill.) at 45° C for 4 hr. 10 ml of toluene scintillator containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]-benzene (Packard Instrument Co., Inc., Downers Grove, Ill.) was added and radioactivity was measured in a Packard liquid scintillation spectrometer model No. 3310 with counting efficiency of 53%. The background was 29 cpm.

Immunologic Procedures

Antibodies against lysozyme and ovalbumin were prepared in adult male albino rabbits as described by Leskowitz and Waksman (29). An 0.15 M NaCl solution (0.5 ml) containing 2.5 mg of purified lysozyme (Calbiochem, Los Angeles, Calif.) or ovalbumin (General Biochemicals, Div., Chagrin Falls, Ohio) was emulsified with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected into toe pads of rabbits. Acrylamide gel electrophoresis (30) of 20 μg of either purified lysozyme or purified ovalbumin preparation showed one major band for each protein. 2 weeks after immunization, sera were collected and were subjected to partial purification by ammonium sulfate fractionation. The globulin fraction precipitating between 30 and 33% ammonium sulfate saturation was dissolved in 0.01 M potassium phosphate buffer and 0.15 M NaCl mixture, pH 7.2, and was dialyzed against this solution for 36 hr (31). Control antiserum prepared from rabbits receiving injections of Freund's adjuvant-0.15 M NaCl mixture was treated in a similar fashion. Reactivity of antibody against lysozyme was assessed by the following experiment: Increasing amounts of anti-lysozyme serum were added to a constant amount of either purified lysozyme or lysozyme present in oviduct extracts. After incubation at 37° C for 30 min and then at 4° C for 24 hr, the resultant antibody-antigen complex was removed by centrifugation and the supernatant was assayed for enzyme activity. It was found that the loss in lysozyme activity was proportionately related to the amount of antibody added until the complete loss in enzyme activity was reached. Control antiserum failed to cause such a loss in lysozyme enzyme activity by the above assessment.

Ouchterlony agar diffusion tests were performed as outlined by Kabat and Mayer (32) with 1% agar (Noble Agar, Difco Laboratories, Detroit, Mich.) in 0.15 M NaCl adjusted to pH 8.0 with 1 M NaOH. Immunoelectrophoresis was performed according to the manual obtained from Buchler Instruments Inc., N. J. (33). The electrophoresis was performed in 0.01 M veronal buffer pH 8.7, ionic strength 0.075, for 2.5 hr with 2.5 mamp per slide with a total voltage of 70–90 volts. Oviduct extracts for immunologic analyses were made as follows: Oviducts were homogenized in 9 volumes (w/v) of 0.001 M $MgCl_2$, 0.001 M potassium phosphate buffer, pH 6.8, and were centrifuged for 10 min at 10,000 *g* in Servall RC2-B at 4° C and then for 60 min at 105,000 *g* in Spinco Model L ultracentrifuge at 4° C. The resulting supernatant was used for all studies.

For fluorescent antibody staining, the indirect method was used (34). 5-μ thick sections of the magnam were prepared as described below in the histological procedures. After successive treatment in toluene, the graded alcohols, and water, the sections were treated with the rabbit antiserum to lysozyme for 15 min at 25° C and then incubated for 30 min at 25° C with a fluorescein conjugate of a goat anti-rabbit globulin antiserum (Microbiological Associates Inc., Bethesda, Md.). The specificity of the staining was controlled by (1) adding anti-rabbit antibody conjugate to the sections without pretreatment with rabbit anti-lysozyme serum, (2) adding control rabbit serum instead of anti-lysozyme antiserum to the sections before incubation with the conjugate, and (3) adding a conjugate of normal goat globulin instead of goat anti-rabbit globulin. For microscopy, a Zeiss GFL research microscope, equipped with a basic camera body V, was used. The light source was a PEK short-arc mercury lamp which was operated by a PEK model 401 DC power supply (PEK Labs, Inc., Sunnyvale, Calif.). Photomicrographs were taken on Kodak Tri-X Pan film[1] (35).

Histological Procedures

For histological examination, portions of the magnum were fixed in 0.01 M sodium phosphate buffer, pH 7.2, containing 4% glutaraldehyde (36). Following dehydration in the graded alcohols and clearing in toluene, the tissues were embedded in paraffin. 5-μ thick sections were stained with Mayer's hematoxylin and eosin.

RESULTS

Effect of Estrogen and its Combination with Other Steroid Hormones on Oviduct Wet Weight

As shown in Fig. 1, daily administration of estrogen results in a marked increase in wet weight.

[1] The authors are grateful to Dr. A. H. Saunders, Department of Pathology, Stanford Medical School for assistance with the immunofluorescent studies.

Following an initial lag of 3 days, the increase is rapid, amounting to 500-fold in 10 days. Concomitant administration of progesterone with estrogen markedly inhibits the estrogen-stimulated increase in wet weight, although this treatment still increases the wet weight considerably. In accordance with the previous work of Breneman (18), and Zarrow et al. (37), testosterone is synergistic and hydrocortisone is somewhat antagonistic to estrogen in increasing the wet weight when administered with estrogen. Thus 10 days of daily administration of estrogen with testosterone or with hydrocortisone results in more than 700-fold and about 400-fold increases, respectively. Administration of testosterone or progesterone alone for 10 days results in 10- and 20-fold increases, respectively, whereas only a 2-fold increase occurs in oviducts of chicks receiving hydrocortisone or no hormone treatment.

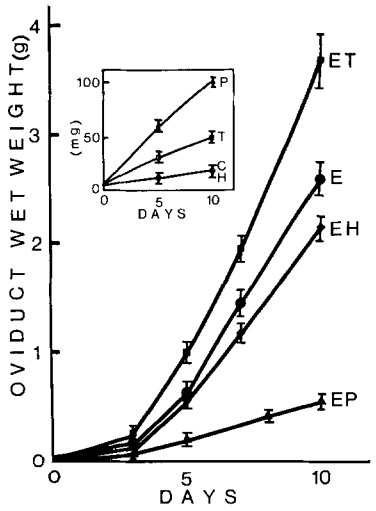

FIGURE 1 Changes in wet weight of the oviduct during daily treatment with various hormones. Chicks were treated with 17β-estradiol benzoate (*E*) alone or its combination with testosterone propionate (*ET*), progesterone (*EP*), and hydrocortisone 21-phosphate (*EH*) for 10 days. The insert shows the changes in wet weight of the oviduct from chicks treated with testosterone propionate (*T*), progesterone (*P*), hydrocortisone 21-phosphate (*H*) and control sesame oil (*C*) for 10 days, respectively. The dose of each hormone is 1 mg day per chick. Each point represents the means ± SEM of four chicks.

Effect of Estrogen and its Combination with Other Steroid Hormones on Lysozyme Content in Oviduct

As shown in Fig. 2, no lysozyme is detectable during the first 2 days of estrogen treatment. A barely measurable activity appears by the 3rd day. Thereafter the content of lysozyme increases rapidly such that the rate of its accumulation exceeds that of the increase in total wet weight between the 6th and 8th day of estrogen administration. Concomitant administration of progesterone with estrogen prevents the appearance of lysozyme throughout the 10-day treatment period. In an occasional experiment a very small amount of lysozyme activity is detected after 5 and 10 days of combined estrogen-progesterone treatment. Hydrocortisone administered with estrogen reduces slightly the estrogen-induced increase in lysozyme content, whereas the combined treatment of estrogen and testosterone results in greater increase in lysozyme content than estrogen treatment alone. There is no measurable lysozyme in the oviducts from untreated, progesterone-, hydrocortisone-, and testosterone-treated chicks.

Effect of Estrogen and its Combination with Progesterone on DNA, RNA and Total Protein Content in Oviduct

As shown in Table I, within 3 days of estrogen treatment, total DNA content increases markedly, indicating that extensive cell proliferation has occurred during the initial lag period in wet weight increase and before lysozyme appearance. The DNA concentration (milligrams/gram tissue) decreases during this period, due largely to the imbibition of water into the tissue provoked by estrogen (see following paragraph). With continued estrogen treatment, the concentration of DNA declines further although the total DNA continues to increase. The RNA concentration as well as total content, and the RNA/DNA ratio increase during the lag period. In contrast, the protein concentration does not increase markedly until 3 days after the estrogen treatment, at which time lysozyme begins to appear (Fig. 2). Thereafter the protein concentration increases more than threefold with a marked increase in total content by 10 days. Combined treatment with estrogen and progesterone results in a significant decrease in the estrogen-stimulated increase in total DNA, RNA, and protein content ($P = 0.01$). Likewise, the concentrations of DNA, RNA, and

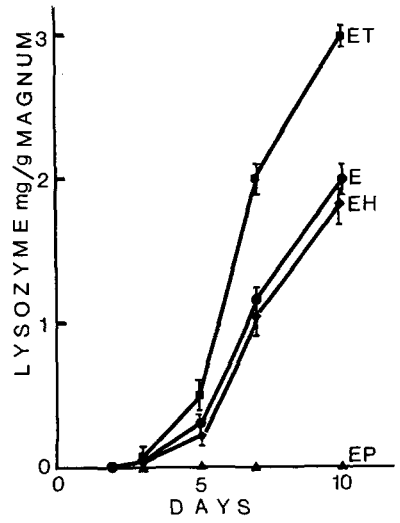

FIGURE 2 Changes in lysozyme activity in the magnum portion of the oviduct during daily treatment of various hormones. Chicks were treated with 17β-estradiol benzoate (*E*) alone or its combination with testosterone propionate (*ET*), progesterone (*EP*), and hydrocortisone 21-phosphate (*EH*) for 10 days. The dose of each hormone is 1 mg/day per chick. Enzyme activity is expressed as milligrams/gram wet weight magnum. Each point represents the means ± SEM of four chicks.

protein are lower during the 10-day period (*E* vs. *EP*).

Effect of Estrogen and its Combination with Progesterone on the Histology of the Magnum Portion of Oviduct

LOW MAGNIFICATION: The appearance of the magnum from chicks treated with estrogen for 5 days is presented in Fig. 3 *b*. In contrast to the untreated control (Fig. 3 *a*), the size of the magnum is markedly increased with an increase in inner epithelial layer and outer muscle layer (note the difference in magnification of the two photomicrographs). The most prominent difference, however, is found in the glandular portion of the magnum, that area between epithelial and serosal surfaces, which comprises the bulk of the entire magnum. This area is occupied almost entirely by numerous nests of cells containing eosinophilic granules. As shown in Fig. 3 *c*, concomitant administration of progesterone with estrogen for 5 days results in a markedly altered appearance. Thus, the expansion of the glandular portion is essentially absent and the characteristic nests of cells containing an eosinophilic substance are not present. There is, however, a considerable increase in size of the magnum compared to the

TABLE I

Changes in DNA, RNA, Protein and Lysozyme in the Magnum Portion of Oviduct

Treatment	DNA		RNA		RNA/DNA	Protein	
	μg/magnum	mg/g magnum wet weight	μg/magnum	mg/g magnum wet weight		mg/magnum	mg/g magnum wet weight
C5	33 ± 2	6.5 ± 0.1	35 ± 4	7.0 ± 0.1	1.1	0.4 ± 0.1	75 ± 1
C10	49 ± 3	6.4 ± 0.1	50 ± 2	7.1 ± 0.2	1.1	0.6 ± 0.1	70 ± 5
E3	171 ± 5	3.0 ± 0.2	518 ± 19	9.2 ± 0.1	3.1	5.7 ± 0.4	86 ± 4
E5	485 ± 7	2.6 ± 0.2	1695 ± 23	9.4 ± 0.2	3.6	19.8 ± 1.2	128 ± 5
E7	730 ± 10	2.4 ± 0.2	2390 ± 21	8.4 ± 0.1	3.5	42.3 ± 1.1	139 ± 7
E10	1010 ± 22	2.3 ± 0.2	3850 ± 20	8.0 ± 0.1	3.5	112.2 ± 1.8	195 ± 10
EP3	70 ± 2	2.1 ± 0.1	140 ± 7	4.2 ± 0.1	2.0	3.1 ± 0.2	80 ± 2
EP5	110 ± 6	2.0 ± 0.2	267 ± 4	4.8 ± 0.1	2.4	5.2 ± 0.2	99 ± 5
EP7	210 ± 8	1.9 ± 0.1	380 ± 15	4.0 ± 0.2	2.1	8.1 ± 0.1	90 ± 7
EP10	300 ± 8	1.8 ± 0.2	440 ± 20	3.9 ± 0.2	2.2	12.0 ± 0.2	102 ± 3

C5 and C10 ..... 5 and 10 days of control sesame oil administration, respectively.

E3, E5, E7, E10 ..... 3, 5, 7, and 10 days after daily administration of 17β-estradiol benzoate (1 mg/day per chick), respectively.

EP3, EP5, EP7, EP10 .... 3, 5, 7, and 10 days after daily administration of 17β-estradiol benzoate (1 mg/day per chick) and progesterone (1 mg/day per chick), respectively.

Each value represents the mean ± SEM of 4 chicks.

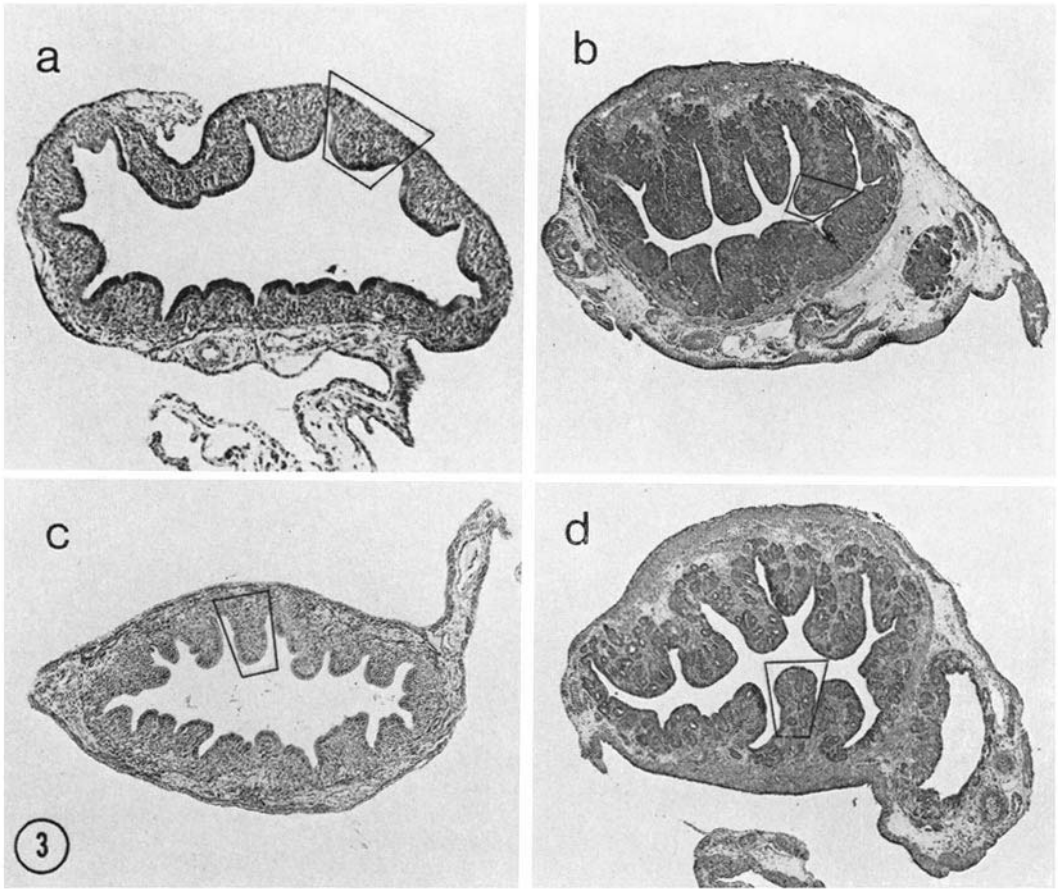

FIGURE 3 *a–d* Effect of estrogen and its combination with progesterone in the histology of the magnum portion of the oviduct: low magnification. 3 *a*. no hormone treatment. × 130. 3 *b*. 5 days of daily estrogen treatment. × 28. 3 *c*. 5 days of daily combined treatment with both estrogen and progesterone. × 41. 3 *d*. 2 days of daily estrogen treatment and 3 days of daily combined treatment with both estrogen and progesterone. × 31. 17β-estradiol benzoate and progesterone were administered at a dose of 1 mg/day per chick.

untreated control, although the increase is not so marked as that in the estrogen-stimulated magnum.

In Fig. 4, indicated regions of the sections in Fig. 3 have been magnified further, to show more detailed characteristics of the cell types. As shown in Fig. 4 *b*, tubular gland cells occupy essentially the entire section of the magnum of chicks treated with estrogen for 5 days, whereas only stromal cells are present in this region of the untreated gland (Fig. 4 *a*). Characteristic of tubular gland cells is a distinct, basal nucleus and deeply staining small granules in the apical region. The gland lumina are formed by the assembly of such tubular gland cells and are often filled with small granules.

Unlike the estrogen-treated magnum, tubular gland cells are absent when chicks are treated with both estrogen and progesterone for 5 days (Fig. 4 *c*). In addition, no densely eosin-staining granules are found in any portion of the magnum. Instead, stromal cells are abundant in this region as in the untreated gland (Fig. 4 *a*). Another noticeable feature of progesterone treatment is its effect on the variety and structure of cells in the lining epithelium of the magnum (Fig. 4 *c*). Thus, the epithelial cells are distinctly more columnar than those in the epithelium from chicks that have received estrogen alone (Fig. 4 *b*). In addition, various types of cells are evident, some with cilia,

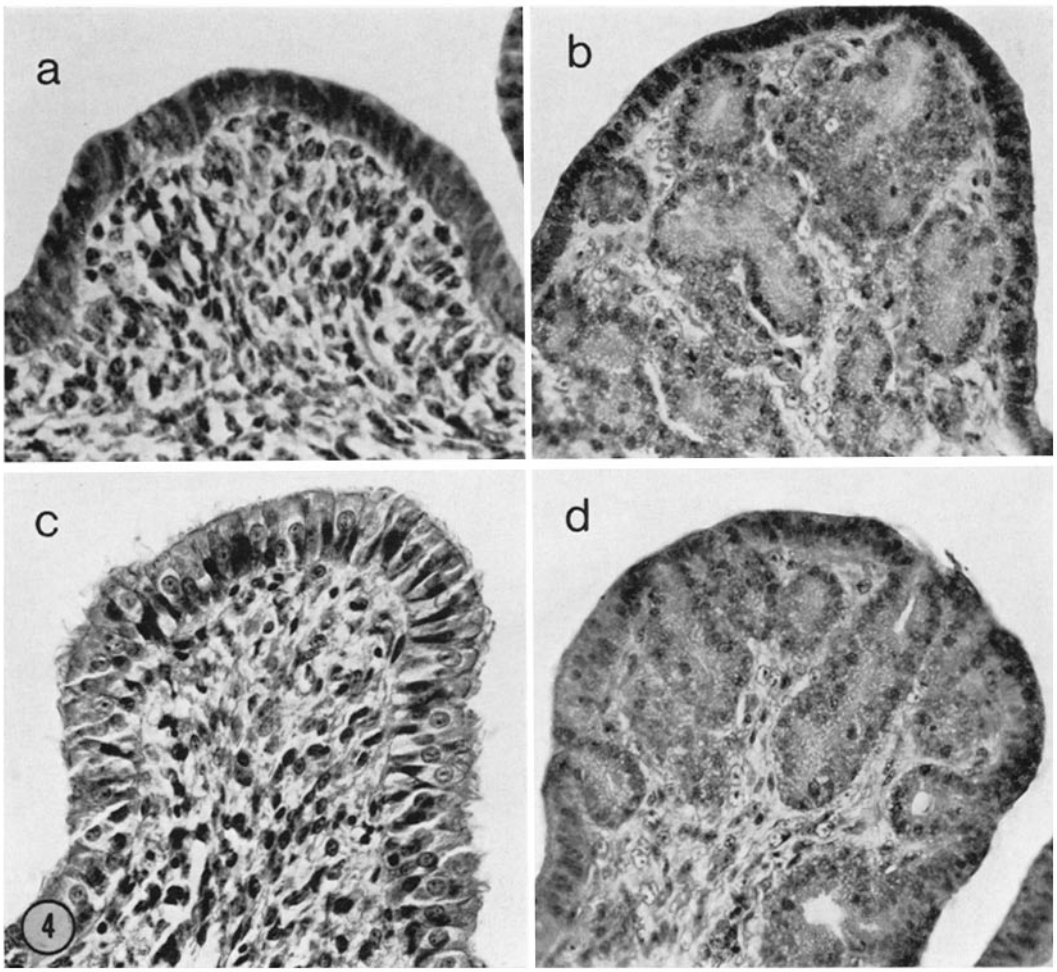

FIGURE 4 *a–d* Effect of estrogen and its combination with progesterone on histology of the magnum portion of the oviduct: high magnification. The indicated sections of the magnum presented in Fig. 3. 4 *a*. no hormone treatment. × 660. 4 *b*. 5 days of daily estrogen treatment. × 340. 4 *c*. 5 days of daily combined treatment with both estrogen and progesterone. × 420. 4 *d*. 2 days of daily estrogen treatment and 3 days of daily combined treatment with both estrogen and progesterone. × 340.

some with nuclei that are placed centrally, and others with basally placed nuclei. Thus, whereas progesterone essentially abolishes formation of one cell type, i.e. tubular gland cells, it enhances differentiation of other cell types, i.e. epithelial cells.

IMMUNOFLUORESCENT STAINING: Sections of the magnum from the chicks treated with estrogen for 5 days were subjected to fluorescent antibody staining to localize lysozyme. As shown in Fig. 5 *a*, lysozyme is localized specifically in tubular gland cells. No lysozyme is localized in the cells of the lining epithelium or of the serosal surface. In the magnum from untreated chicks or from chicks treated for 5 days with both estrogen and progesterone where no tubular gland cells are found histologically (Fig. 4 *c*), there is also no evidence of lysozyme by this technique. Kohler et al. also localized ovalbumin in the tubular gland cells of the estrogen-stimulated magnum by a similar technique (6).

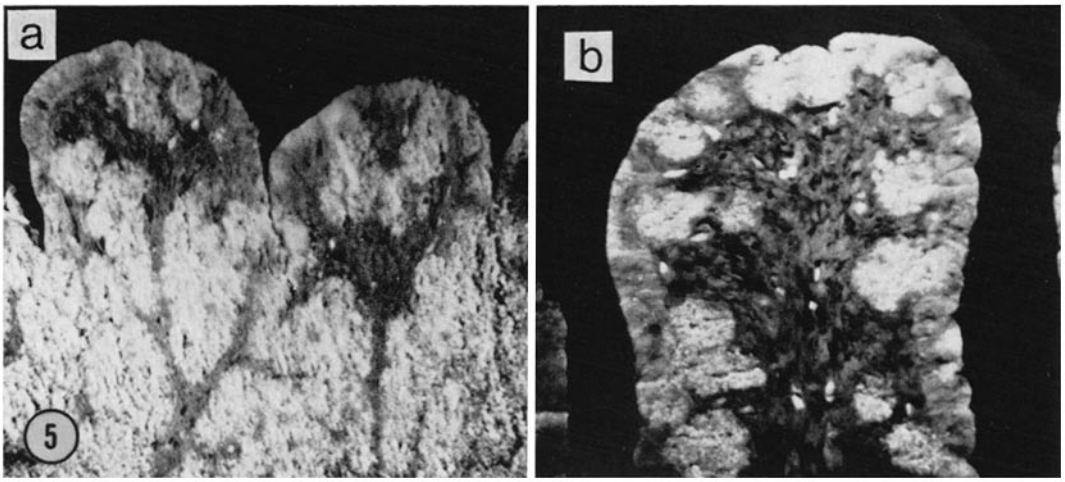

FIGURE 5 *a–b* Localization of lysozyme by immunofluorescent staining. Cross sections of the magnum of chicks treated with: 5 *a*. estrogen for 5 days. × 140. 5 *b*. estrogen for 2 days, and estrogen plus progesterone for 3 days. × 210. 17β-estradiol benzoate and progesterone were administered at a dose of 1 mg/day per chick.

Immunologic Analysis of Lysozyme and Ovalbumin in the Nontreated, Estrogen-Treated and Estrogen-Progesterone–Treated Oviduct

As shown in Figs. 6 and 7, immunologic analyses by both Ouchterlony double diffusion and immunoelectrophoresis techniques reveal that oviduct extracts prepared from chicks treated with estrogen for 5 days contain both ovalbumin and lysozyme. The immunoelectrophoresis pattern for ovalbumin shows two major bands, one which coincides with purified egg white ovalbumin in electrophoretic mobility and the other which does not migrate under the experimental conditions. Whether these two bands represent different yet immunologically reactive ovalbumins is unknown. In this connection, it is interesting to note that at least two reports (38, 39) show that three different ovalbumins have been isolated from hen oviduct, each having a different electrophoretic mobility due to the differences in degree of phosphorylation. The oviduct extracts prepared from the untreated chick or from the oviducts of chicks treated with estrogen plus progesterone for 5 days do not contain ovalbumin and lysozyme as detected by these methods (Fig. 6, 7).

Effect of Progesterone Administered at Various Times After the Onset of Estrogen Administration

From the difference in time of the onset of the estrogen-induced cell proliferation and of the onset of the appearance of lysozyme (Fig. 2 and Table I), it would appear that estrogen first stimulates the proliferation of those cells, which, within 2–3 days, become tubular gland cells and synthesize lysozyme and ovalbumin. To determine the point in time when progesterone antagonizes the effects of estrogen on this process, progesterone administrations were initiated from 0 to 5 days after the onset of estrogen administrations. 5 and 10 days after the initial estrogen administration changes in wet weight, total content, and concentration of DNA, RNA, protein and lysozyme in the magnum were examined for the effect of the delay in the onset of progesterone administration. As shown in Table II, even a 1 day delay in the onset of progesterone administration allows for estrogen to induce a detectable amount of lysozyme as well as a significant increase in the total content of DNA by the 5th and 10th day (*E*1 *EP*4 vs. *EP*5, *E*1 *EP*9 vs. *EP*10). As the onset of progesterone administration is delayed longer, the increase in total DNA, RNA, and protein content becomes proportionately greater. Further-

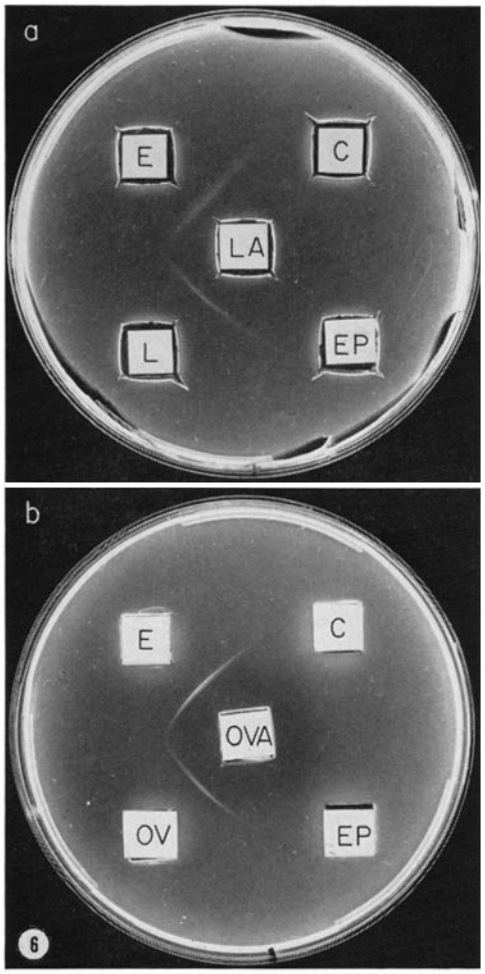

FIGURE 6 *a–b* Immunologic analysis of lysozyme and ovalbumin. Ouchterlony double diffusion. The center wells of Fig. 6 *a* and *b* contained 0.2 ml of lysozyme antibody (*LA*) and ovalbumin antibody (*OVA*), respectively. Side wells of each plate contained 0.2 ml of oviduct extracts (0.4 mg protein/ml) prepared from the chicks treated with 17β-estradiol benzoate (1 mg/day per chick) for 5 days (*E*), its combination with progesterone (1 mg/day per chick) for 5 days (*EP*), and sesame oil control (*C*) as described under Materials and Methods. Side wells of each plate also contained purified lysozyme (*L*) and ovalbumin (*OV*) as standard. The patterns were allowed to develop for 14 days at 25° C before photography.

more, the amount of lysozyme also increases progressively with the length of delay in the onset of progesterone administration, as depicted graphically in Fig. 8. The increase in total DNA, RNA, protein, and lysozyme contents correlates well with the increase in the delay in onset of progesterone administration during 5 days of estrogen treatment. This correlation also can be seen from a histological examination of the magnum from chicks treated for 2 days with estrogen followed by 3 days of combined estrogen and progesterone treatment (*E*2 *EP*3). As seen in Fig. 3 *d*, the magnum from *E*2-*EP*3-treated chicks shows a picture somewhat like the one of the magnum from *E*5-treated chicks. Thus, there is an increase in size of the magnum and an enlargement of the glandular portion, both of which, however, are less extensive than they are in the *E*5-treated magnum. The scattered nests of cells containing an eosinophilic substance are more closely associated with the epithelial layer, and the lumina formed by such cells are not completely filled with an eosinophilic substance as is the case in the *E*5-treated magnum. In addition, as shown in Fig. 4 *d*, such cells are not so columnar as those tubular gland cells in the *E*5-treated magnum (Fig. 4 *b*). Such tubular gland cells contain lysozyme as shown by immunofluorescent staining (Fig. 5 *b*). The central region of the tissue is, however, occupied by stromal cells, which appear similar to those present in the *EP*5 treated magnum (Fig. 4 *c*). Thus, the magnum from *E*2-*EP*3-treated chicks exhibits certain characteristics of both the *E*5- and the *EP*5-treated magnum. Such histological findings are consistent with the biochemical data presented above, indicating that during the period before the initiation of progesterone treatment estrogen stimulates the proliferation of cells which subsequently become tubular gland cells and synthesize lysozyme. Concomitant administration of progesterone prevents this proliferation. However, once such proliferation has occurred, the cytodifferentiation and lysozyme appearance are not prevented by progesterone administration.

Some Characteristics of Progesterone Antagonism to Estrogen

In order to examine whether the antagonistic effect of progesterone is competitive or noncompetitive, the effect of increasing dosages of estrogen on the magnum wet weight increase was studied under variable doses of progesterone. Results presented in Fig. 9 show that the inhibitory effect of progesterone is noncompetitive, since at least a four-fold increase in the dosage of estrogen is in-

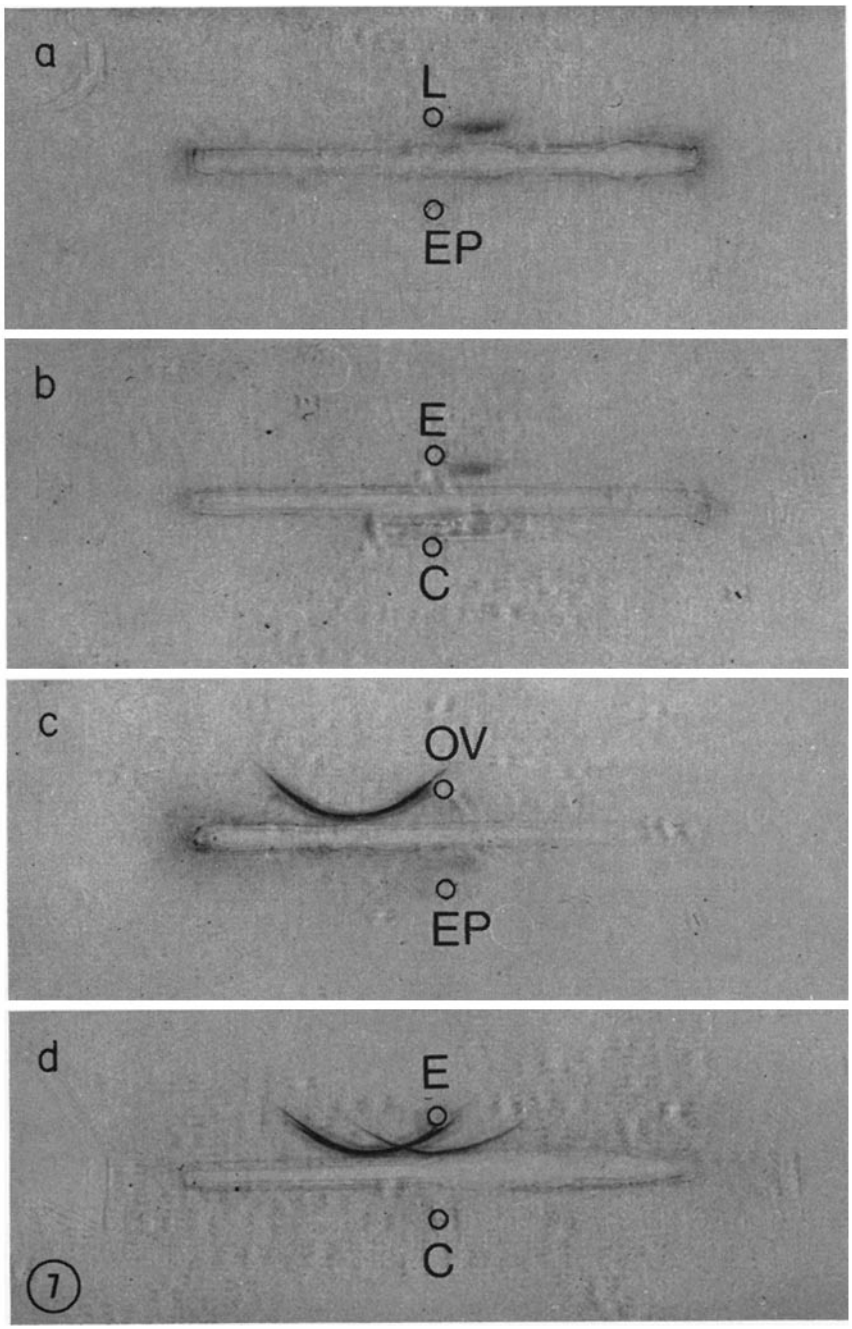

FIGURE 7 *a–d* Immunologic analysis of lysozyme and ovalbumin-immunoelectrophoresis. Center trough of Fig. 7 *a–d* contained 0.025 ml of lysozyme antibody and ovalbumin antibody, respectively. Aliquots of oviduct extracts prepared from the chicks treated with 17β-estradiol benzoate (1 mg/day per chick) for 5 days (*E*), its combination with progesterone (1 mg/day per chick) for 5 days (*EP*), and sesame oil control (*C*) were added to the side wells as indicated. Purified lysozyme (*L*) and ovalbumin (*OV*) were also added to the side well as standard. The patterns were allowed to develop for 24 hrs at 25° C, leached out, and stained with Ponceau S before photography.

TABLE II

Changes in DNA, RNA, Protein and Lysozyme in the Magnum Portion of Oviduct After 5 and 10 Days of Estrogen Treatment with Various Onset of Progesterone Administrations

Treatment	DNA		RNA		RNA/DNA	Protein		Lysozyme	
	μg/magnum	mg/g magnum wet weight	μg/magnum	mg/g magnum wet weight		mg/magnum	mg/g magnum wet weight	μg/magnum	mg/g magnum wet weight
Control	35 ± 4	6.4 ± 0.1	36 ± 6	7.0 ± 0.1	1.1	0.4 ± 0.1	60 ± 4	Undetectable	
E5	480 ± 9	2.6 ± 0.1	1730 ± 20	9.6 ± 0.3	3.6	20.0 ± 1.8	135 ± 5	66 ± 1.0	443 ± 10
E10	1150 ± 12	2.3 ± 0.1	3880 ± 22	8.2 ± 0.1	3.6	101.7 ± 2.5	215 ± 7	1500 ± 10.0	2040 ± 50
EP5	110 ± 4	2.0 ± 0.1	270 ± 7	5.1 ± 0.1	2.4	5.3 ± 0.2	99 ± 3	Undetectable	
E1 EP4	130 ± 4	2.3 ± 0.1	330 ± 7	5.7 ± 0.2	2.5	6.4 ± 1.2	108 ± 4	5 ± 1.0	80 ± 11
E2 EP3	190 ± 3	2.4 ± 0.2	480 ± 6	6.1 ± 0.2	2.7	9.3 ± 0.9	107 ± 1	12 ± 2.7	153 ± 38
E3 EP2	250 ± 1	2.5 ± 0.2	690 ± 4	7.1 ± 0.2	2.8	13.1 ± 0.4	133 ± 10	25 ± 3.6	260 ± 30
E4 EP1	310 ± 4	2.6 ± 0.2	960 ± 4	7.9 ± 0.1	3.0	16.3 ± 1.4	135 ± 23	45 ± 3.4	373 ± 15
EP10	269 ± 2	2.3 ± 0.2	456 ± 6	3.8 ± 0.1	1.7	12.7 ± 0.5	106 ± 3	Undetectable	
E1 EP9	275 ± 2	1.8 ± 0.2	503 ± 6	3.3 ± 0.2	1.8	17.6 ± 0.5	117 ± 4	33 ± 2.5	223 ± 20
E2 EP8	328 ± 2	1.6 ± 0.3	613 ± 5	3.4 ± 0.1	2.1	22.9 ± 1.4	127 ± 4	90 ± 3.2	479 ± 23
E3 EP7	531 ± 4	2.1 ± 0.2	1165 ± 7	4.6 ± 0.1	2.2	43.3 ± 2.2	181 ± 8	262 ± 5.8	1048 ± 70
E4 EP6	750 ± 3	2.5 ± 0.2	1645 ± 3	5.5 ± 0.2	2.2	58.5 ± 1.7	195 ± 6	375 ± 8.8	1250 ± 43
E5 EP5	993 ± 4	2.5 ± 0.1	2370 ± 7	5.9 ± 0.1	2.4	86.4 ± 3.0	216 ± 8	1083 ± 12.0	2708 ± 97

Control..... sesame oil treatment

E5 and E10..... 5 and 10 days 17β-estradiol benzoate (1 mg/day per chick), respectively.

EP5 and EP10..... 5 and 10 days 17β-estradiol benzoate (1 mg/day per chick) and progesterone (1 mg/day per chick), respectively.

E1 EP4 and E1 EP9..... 1 day 17β-estradiol benzoate (1 mg/day per chick), and 4 and 9 days 17β-estradiol benzoate and progesterone (1 mg/day per chick), respectively.

E2 EP3 and E2 EP8..... 2 days 17β-estradiol benzoate (1 mg/day per chick), and 3 and 8 days 17β-estradiol benzoate and progesterone (1 mg/day per chick), respectively.

E3 EP2 and E3 EP7..... 3 days 17β-estradiol benzoate (1 mg/day per chick), and 2 and 7 days 17β-estradiol benzoate and progesterone (1 mg/day per chick), respectively.

E4 EP1 and E4 EP6..... 4 days 17β-estradiol benzoate (1 mg day per chick), and 1 and 6 days 17β-estradiol benzoate and progesterone (1 mg/day per chick), respectively.

E5 EP5..... 5 days 17β-estradiol benzoate (1 mg/day per chick), and 5 days 17β-estradiol benzoate and progesterone (1 mg/day per chick).

Each value represents the mean ± SEM of 4 chicks.

effective in reversing the inhibitory effect of progesterone on the estrogen-stimulated increase in wet weight. It can also be seen that the degree of progesterone inhibition of the estrogen effect is approximately proportional to the dose of progesterone administered. With a maximum dose of progesterone (1 mg/day per chick), the increase in wet weight is essentially the same regardless of the dosage of estrogen administered ranging from 0.25 to 4 mg/day per chick.

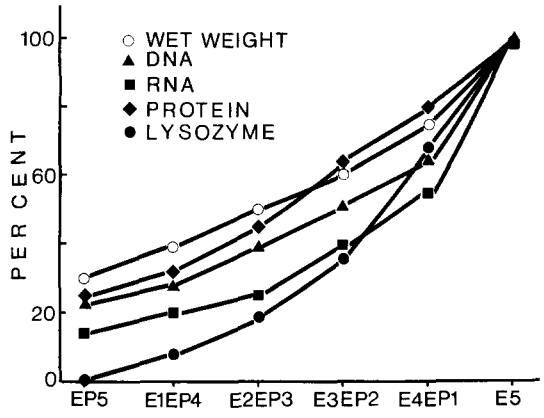

FIGURE 8 Effect of the delay in the onset of progesterone administration on estrogen-stimulated increase in total DNA, RNA, protein, and lysozyme of chick oviduct. Increases are described as percent of values obtained after 5 days of estrogen treatment. Details are given in Table II.

Estrogen treatment of the chick induces a marked lipemia and the appearance of a specific phosphoprotein in serum (40). As shown in Table III, specific phosphoprotein content as well as total lipid and phospholipid content in serum is increased markedly by estrogen administration. Concomitant administration of progesterone with estrogen does not inhibit this effect of estrogen. These findings confirm the report of Hertz et al. (13) and indicate that progesterone antagonism is rather specific for the oviduct.

In rat and rabbit uteri, one of the early changes induced by estrogen is the increased uptake of amino acids (41–43) and water (44). It might be possible that progesterone exerts its inhibitory effect by blocking one of these early changes caused by estrogen. The effect of estrogen and its combination with progesterone on the uptake of a nonmetabolizable amino acid, α-aminoisobutyric acid, was studied to test this possibility. As shown in Fig. 10, increased uptake of α-aminoisobutyric acid is noted as early as 2 hr and reaches the maximum around 12 hr after estrogen administration. This effect is sustained for over 24 hr. Progesterone, by itself, increases the uptake of α-aminoisobutyric acid to a lesser extent than estrogen. The combination of progesterone with estrogen results in the same degree of increase as found after estrogen administration. This effect of estrogen is relatively specific for oviduct since the kinetics of α-aminoisobutyric acid uptake in

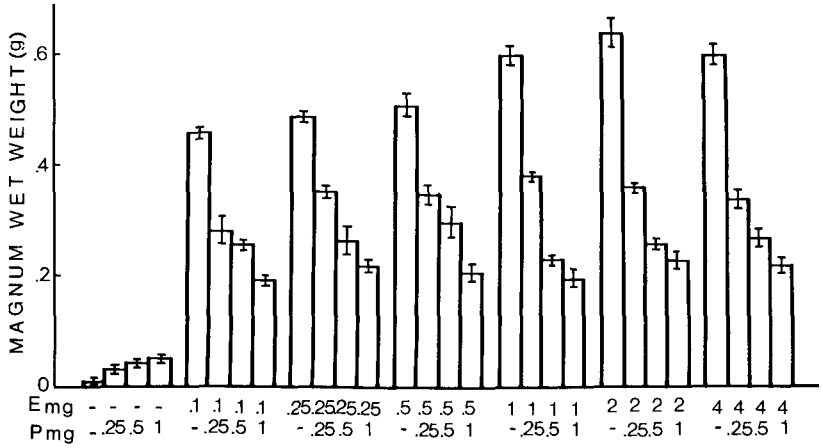

FIGURE 9 Effect of increasing dosage of estrogen (E) on the magnum wet weight under variable doses of progesterone (P). Chicks were treated with hormone(s) in the combination of doses indicated, daily for 5 days. Each column represents the means ± SEM of four chicks.

TABLE III

Effect of Progesterone on the Estrogen-Induced Increase in Total Lipid, Phospholipid and Phosphoprotein

	Total lipid, g/100 ml serum	Phospholipid phosphorus, mg/100 ml plasma	Phosphoprotein phosphorus, mg/100 ml plasma
Control	0.44	5.1	Undetectable
17β-estradiol benzoate*	1.81	15.1	9.9
17β-estradiol benzoate* + progesterone	1.99	16.1	9.8
Progesterone*	0.64	4.8	Undetectable

* Chicks were treated with 17β-estradiol benzoate (1 mg/day per chick), progesterone (1 mg/day per chick) and their combination for 5 days. Control chicks were given sesame oil for 5 days. Each value represents the mean of 2 determinations.

liver and plasma are not affected by either estrogen or progesterone over the 24-hr period. There is, however, a transient increase in α-aminoisobutyric acid uptake into the skeletal muscle during the first 4 hr of either estrogen or combined estrogen-progesterone treatment.

Fig. 11 shows the pattern of increase in wet weight and dry weight of the oviduct occurring within 24 hr following the administration of estrogen, progesterone, and their combination. The initial rise in wet weight is already notable at 4 hr and reaches its first peak at about 8 hr after estrogen administration. During this initial increase in wet weight, dry weight remains rather constant as shown in the insert in Fig. 11, indicating that there is an increase in the uptake of water by the oviduct. Following the slight decline in wet weight, there is a second, continuous rise in wet weight which is accompanied by an increase in dry weight after 16 hr of estrogen treatment. Concomitant administration of progesterone with estrogen does not affect either the estrogen-induced uptake of water or the increase in wet weight to any great extent. Progesterone administration alone induces similar changes but to a lesser degree than estrogen.

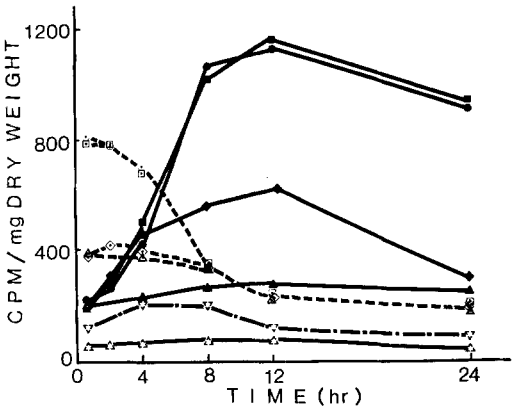

FIGURE 10 Effect of estrogen, progesterone, and their combination on uptake of α-aminoisobutyric acid by chick tissues. Chicks were given a single injection of either 17β-estradiol benzoate (*E*) 1 mg per chick, or progesterone (*P*) 1 mg per chick, or their combination (*EP*) 1 mg each per chick, or control sesame oil (*C*). At the indicated times after the hormone treatment, the level of α aminoisobutyric acid was determined for oviduct, liver, muscle, and serum. Radioactivity is expressed as counts per minute/milligram dry weight or counts per minute/microliter serum. Each point represents the mean of three values. (■——■) E oviduct, (●——●) EP oviduct, (◆——◆) P oviduct, (▲——▲) C oviduct; (⊡------⊡) E skeletal muscle, (⊙------⊙) EP skeletal muscle, (◇------◇) P skeletal muscle, (△------△) C skeletal muscle; (△—·—·—△) E, EP, P, C liver; (▽——▽) E, EP, P, C plasma.

DISCUSSION

The results described in this paper can be summarized as follows: Repeated administration of estrogen to the immature female chick results in rapid cell proliferation, formation of tubular gland cells, and appearance of specific egg white proteins such as lysozyme and ovalbumin in the oviduct. Concomitant administration of progesterone antagonizes the above characteristic effects of estrogen. Such an antagonistic effect of progesterone can best be explained by the following tentative hypothesis based on the data presented herein: Under the influence of estrogen the proliferation of various cell types in the magnum is stimulated, among which are those cells destined to become tubular gland cells and to synthesize lysozyme and ovalbumin as shown by histological and immunologic studies. Proliferation of such potential tubular gland cells appears to com-

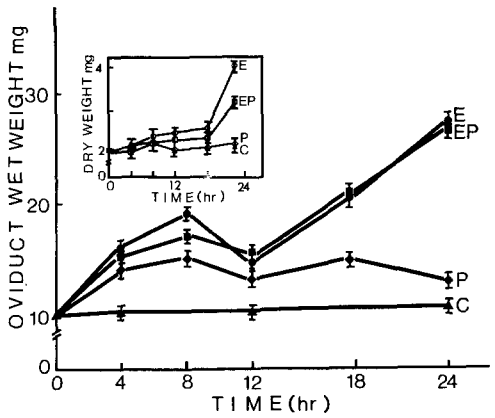

FIGURE 11 Effect of estrogen, progesterone, and their combination on total wet and dry weight of chick oviduct. Chicks were given a single injection of either 17β-estradiol benzoate (*E*) 1 mg per chick, or progesterone (*P*) 1 mg per chick, or their combination (*EP*) 1 mg each per chick, or control sesame oil (*C*). At indicated times after the hormone treatment, total wet and dry weights were determined. The insert shows the changes in dry weight. Each point represents the means ± SEM of four chicks.

mence before the appearance of lysozyme, which is indicated by the difference in the time course of the estrogen-induced increase in total DNA content and of the appearance of lysozyme (Fig. 2 and Table I). The proliferation of these cells is prevented by concomitant administration of progesterone as indicated by biochemical as well as histological findings. Our data also suggest that once such cell proliferation occurs, synthesis of lysozyme and ovalbumin is not inhibited by progesterone when administered with estrogen. Thus, as shown in Table II and Figs. 3 *d*, 4 *d*, 5 *c*, and 8, when estrogen is administered before initiation of progesterone treatment, tubular gland cells and lysozyme are present after 5 days, with the increase in quantity proportionate to the length of the delay in the onset of progesterone administration. Furthermore, progesterone appears to prevent the estrogen-induced appearance of lysozyme and ovalbumin by inhibiting the formation of potential tubular gland cells. On the basis of the foregoing discussion, we propose that estrogen-induced proliferation of a specific cell type, i.e. tubular gland cells, plays an important role in the subsequent events of differentiation of tubular gland cells characterized by the appearance of both lysozyme and ovalbumin. A similar proposal has been made by Stockdale and Topper (45), who have shown that in mouse mammary gland, DNA synthesis in the presence of insulin and hydrocortisone is necessary for prolactin to stimulate new casein synthesis. In addition, androgens appear to inhibit new casein synthesis by blocking the preceding insulin-mediated DNA synthesis (46). Thus, this effect of androgens appears to be comparable to the effect of progesterone described above.

Attempts to localize the primary site of the antagonistic effect of progesterone have been made. An antiestrogenic compound such as MER-21 has been shown to inhibit effects of estrogen in rat uterus by largely blocking the uptake of estrogen into the tissue (47). However, in chick oviduct, progesterone does not inhibit either the absorption of estrogen from the injection site or the uptake of estrogen into the oviduct.[2] Early changes including the increased permeability of the oviduct to α-aminoisobutyric acid and water are not affected by progesterone as shown in Figs. 10 and 11. This suggests that the antagonistic effect of progesterone on specific cell proliferation is probably not directly related to these early estrogen effects.

The effect of estrogen to increase permeability to amino acids (41–43) and water (44) has been also demonstrated in the mammalian uterus. However, many notable differences exist between chick oviduct and mammalian uterus. For example, rat uterus possesses a marked ability to accumulate estrogen due to the presence of a binding substance(s) as shown by Jensen and his coworkers (48, 49), and by Gorski and his collaborators (50, 51), whereas chick oviduct does not seem to possess such an apparent feature (52). This apparent lack of a binding substance might be one of the reasons why the minimum threshold dose of estrogen to cause a similar extent of increase in wet weight in 3–5 days is much higher in chick oviduct (1.6 mg/kg body weight) (2) than in rat uterus (1 μg/kg body weight) (53). Testosterone, which is synergistic to estrogen in chick oviduct, is one of the potent antiestrogenic compounds in rat uterus (54). Progesterone is a potent antagonist to estrogen at a dose of about 1:1 for the increase in wet weight in chick oviduct. On the other hand, in rat uterus, a ratio of 150–1500 of progesterone to estrogen is necessary for progesterone to cause a

[2] Unpublished observation: T. Oka and R. T. Schimke.

small decrease in the estrogen-stimulated increase in wet weight (55). The above characteristic differences are of interest from the standpoint of comparative endocrinology of the female genital tract of mammals and birds.

An antagonistic as well as a synergistic effect of combined treatment with progesterone and estrogen on the oviduct weight increase has been described by several workers (13–18). Dorfman and Dorfman (16) have reported that these conflicting data result from the difference in the intensity of estrogen stimulation used, in that at the near maximal dose of estrogen administered, the effect of estrogen is antagonized by progesterone, while at the minimal dose of estrogen, progesterone is synergistic to estrogen. In this paper, we have used the near maximal dose of estrogen (1 mg/day per chick) and have presented evidences of an antagonistic effect of progesterone on estrogen. This evidence confirms partially the report by Dorfman and Dorfman. However, we have found that dual effects of progesterone, that is, inhibitory and stimulative, can be seen even at the near maximal dose of estrogen, depending on the developmental stage of oviducts. Preliminary results[2] suggest that after the initial cell proliferation, synthesis of specific egg white proteins such as lysozyme requires continued estrogen administration. This estrogen requirement may be replaced in part by progesterone administration. In addition, estrogen plus progesterone treatment results in more increase in lysozyme content once cell proliferation has occurred. Thus, it would appear that the estrogen-induced cell proliferation and subsequent synthesis of lysozyme may be separate effects of estrogen, both of which are affected differently by progesterone administration. It is of considerable interest that progesterone, with its dual effects, may play an important regulatory role for the development and function of oviduct. Furthermore, progesterone, being a highly specific inhibitor of the estrogen-induced tubular gland cell proliferation, may be a useful tool for understanding hormonal regulation of the development and function of this specific cell type.

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