INTERACTION OF ESTROGEN AND PROGESTERONE IN CHICK OVIDUCT DEVELOPMENT

III. Tubular Gland Cell Cytodifferentiation

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ABSTRACT

Administration of estrogen (E) to immature chicks triggers the cytodifferentiation of tubular gland cells in the magnum portion of the oviduct epithelium; these cells synthesize the major egg-white protein, ovalbumin. Electron microscopy and immunoprecipitation of ovalbumin from oviduct explants labeled with radioactive amino acids in tissue culture were used to follow and measure the degree of tubular gland cell cytodifferentiation. Ovalbumin is undetectable in the unstimulated chick oviduct and in oviducts of chicks treated with progesterone (P) for up to 5 days. Ovalbumin synthesis is first detected 24 hr after E administration, and by 5 days it accounts for 35% of the soluble protein being synthesized. Tubular gland cells begin to synthesize ovalbumin before gland formation which commences after 36 hr of E treatment. When E + P are administered together there is initially a synergistic effect on ovalbumin synthesis, however, after 2 days ovalbumin synthesis slows and by 5 days there is only 1/20th as much ovalbumin per magnum as in the E-treated controls. Whereas the magnum wet weight doubles about every 21 hr with E alone, growth stops after 3 days of E + P treatment. Histological and ultrastructural observations show that the partially differentiated tubular gland cells resulting from E + P treatment never invade the stroma and form definitive glands, as they would with E alone. Instead, these cells appear to transform into other cell types—some with cilia and some with unusual flocculent granules. We present a model of tubular gland cell cytodifferentiation and suggest that a distinct protodifferentiated stage exists. P appears to interfere with the normal transition from the protodifferentiated state to the mature tubular gland cell.

INTRODUCTION

The chick oviduct provides an excellent system for the study of hormonal regulation of growth, cytodifferentiation, and function of specific cell types (1–3). The growth of the oviduct is markedly stimulated by estrogen (4–7). A large portion of this growth can be accounted for by the appearance of tubular glands in the magnum portion of the oviduct. The cells of these glands synthesize the majority of the egg-white proteins including ovalbumin (8), lysozyme (7), and probably conalbumin.[1] We have been studying the cytodifferentiation of the cells which make up these glands; they have historically been called tubular gland cells. Before the administration of estrogen to new-

[1] R. Palmiter, unpublished observation.

born pullets, no tubular gland cells are recognizable in the magnum portion of the oviduct, as judged by either biochemical or ultrastructural criteria. However, some of the epithelial cells are competent to differentiate directly into tubular gland cells or to divide and produce daughter cells which can do so; these cells will be called progenitor tubular gland cells.

It has been reported that progesterone interferes with the normal estrogen-mediated cytodifferentiation of tubular gland cells (3, 7). Our working model at the start of this study was that progesterone completely inhibited the induction of egg-white protein synthesis by preventing the formation of tubular gland cells from a progenitor cell type (3). It was a surprise, then, when Dr. Wrenn observed, with the electron microscope, cells with secretory granules (presumably containing ovalbumin) in the oviduct epithelium after 36 hr of estrogen-plus-progesterone treatment. We therefore decided to reinvestigate the effects of progesterone on estrogen-mediated cytodifferentiation of tubular gland cells by using methods more sensitive than those used in prior studies to measure specific protein synthesis (3, 7). We found that tubular gland cell cytodifferentiation does commence and proceed to an intermediate stage, which we call the protodifferentiated stage. Protodifferentiated tubular gland cells are defined as cells in the surface epithelium which have not yet formed glands but are synthesizing low levels of secretory proteins (ovalbumin) and can potentially differentiate in directions other than into mature tubular gland cells. With estrogen plus progesterone treatment the protodifferentiated tubular gland cells never invade the stroma or form glands. Instead, they appear to transform into other cell types.

MATERIALS AND METHODS

Materials

Newborn, white Leghorn pullets were obtained from Kimber Farms, Fremont, Calif. Chicks 4–6 days old were injected daily with either 1 mg estradiol-17β benzoate, 1 mg of progesterone, or 1 mg each of the two hormones as previously described (7). The estradiol-17β benzoate was a gift from Schering Corp., Bloomfield, N.J.

Purified ovalbumin-^{14}C was prepared from oviduct tissue which had been incubated in culture with amino acids–^{14}C as previously described (9). The preparation contained 340,000 cpm/mg ovalbumin.

Conalbumin was obtained from egg white by the method of Warner (10) and then further purified by diethylamino ethyl (DEAE)–cellulose chromatography using the buffer system proposed by Mandeles (11). The final preparation was pure as judged by acrylamide gel electrophoresis in Tris-glycine, pH 8.5 (12), or 0.1% sodium dodecyl sulfate (13). A solution containing 1 A_{280} unit/ml was assumed to equal 1 mg protein/ml.

Anti-ovalbumin and anti–bovine serum albumin (control) γ-globulin fractions were prepared from rabbit serum as previously described (9). Both γ-globulin preparations used in these experiments contained 17.7 A_{280} units/ml.

Toluene-based scintillator fluid contained 5 g of 2,5-diphenyloxazole and 0.3 g dimethyl 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene/liter toluene. "NCS" solubilizer is a product of Amersham-Searle Corp., Arlington Heights, Ill.

Methods

INCUBATION OF OVIDUCT TISSUE: The magnum portion of the oviduct was isolated, and cut into small pieces (about 1–3 mg each). Both the dissection and incubation were done in Medium 199 (Grand Island Biological Co., Grand Island, N.Y.), diluted 1:50 with Hanks' salt solution containing penicillin and streptomycin and buffered with $NaHCO_3$ (9). 10–20 pieces of tissue were incubated in 2 ml of medium in plastic Petri dishes. After 30 min of incubation at 37°C with continuous shaking and gassing with 95% O_2 and 5% CO_2, the cultures were labeled with 10 μCi/ml of an amino acid–^{3}H mixture, algal profile (Schwarz Bio Research Inc., Orangeburg, N.Y.), for 4 hr.

PREPARATION OF HOMOGENATES: 2% homogenates (w/v) were made in 10 mM sodium phosphate and 15 mM sodium chloride, pH 7.5. The homogenate was filtered, sonicated, and centrifuged as previously described (9).

DETERMINATION OF RADIOACTIVITY IN TOTAL SOLUBLE PROTEIN AND OVALBUMIN: Total soluble protein radioactivity was determined by precipitating and washing a sample of the supernatant with 5% trichloroacetic acid. The precipitate was dissolved with NCS and counted in toluene-based scintillator fluid at 55% efficiency for ^{3}H using a Packard 3320 Scintillation Spectrometer (9).

The techniques used for the immunoprecipitation of ovalbumin have been described (9). In the present experiments each assay tube contained the following components added in the order given: 100 μl of anti-ovalbumin γ-globulin (1.8 A_{280} units), 25 μl conalbumin (30 μg), 20 μl sodium desoxycholate (10% solution in H_2O), and 100 μl of radioactive supernatant fraction. The supernatant fraction was diluted when necessary to keep the ovalbumin content below the equivalence point of the antibody prepara-

tion. Control tubes contained 100 μl of anti–bovine serum albumin γ-globulin instead of anti-ovalbumin γ-globulin. After 1 hr incubation at room temperature, 5 μg of unlabeled ovalbumin were added to samples which contained little endogenous ovalbumin. The precipitates were collected, washed, and the radioactivity was determined as described (9).

DETERMINATION OF TOTAL SOLUBLE PROTEIN CONTENT AND OVALBUMIN CONTENT: Total soluble protein was determined by the method of Lowry et al. (14). Ovalbumin content was determined using a reaction mixture similar to that used for determining radioactive ovalbumin (above) except that unlabeled ovalbumin was not added. The precipitates which formed were collected and washed as described (9), dissolved in 200 μl of 1.0 N NaOH, and then the antigen-antibody protein was determined (14). A standard curve of the amount of protein precipitated by increasing amounts of ovalbumin was established. It was similar to that described by Kabat and Mayer (15). At the equivalence point 100 μl of this antibody preparation precipitated 32 μg of ovalbumin, and the antibody protein–to–antigen protein ratio was 10. All sample sizes were adjusted so that 0–15 μg of ovalbumin precipitated; in this range the antibody–to–antigen ratio was 13–15. Thus, after determining the total antibody-antigen protein precipitated, these values were divided by 14 to arrive at the ovalbumin content. The protein precipitated by control γ-globulin was used to determine background (see Fig. 3).

SODIUM DODECYL SULFATE ACRYLAMIDE GEL ELECTROPHORESIS: The electrophoresis of antibody-antigen precipitates and trichloroacetic acid precipitates has been described (9).

LIGHT AND ELECTRON MICROSCOPY: Chicks were sacrificed at various times following the beginning of hormone injections. The oviducts were removed and placed immediately in 2% glutaraldehyde–4% paraformaldehyde in 70 mM Sorenson's phosphate buffer, pH 7.4, at room temperature (modified from Karnovsky [16]). The magnum was cut into pieces about 1 mm long. These pieces remained in the primary fixative for 1–1.5 hr, were rinsed with 70 mM Sorenson's buffer, and were postfixed in 1% osmium tetroxide in 140 mM Veronal buffer, pH 7.4 and 4°C for 1 hr. Following fixation, the tissues were dehydrated in a series of cold alcohols and gradually brought to room temperature in absolute alcohol. The specimens were then cleared with propylene oxide, infiltrated with, and embedded in, Epon 812, and cured to hardness.

For light microscopy, 1-μ sections were cut on an LKB ultrotome and stained with a 1:1 mixture of 1% Azure II and 1% methylene blue in 1% sodium borate (17). For electron microscopy, silver sections were also cut on the LKB ultrotome and stained with uranyl acetate (in 50% alcohol) and with lead citrate. The sections were examined in a Hitachi HU-11E-1 microscope.

RESULTS

Growth

Fig. 1 and Table I show that during the first 3 days of hormone treatment the wet weight of the oviduct magnum increases about 8-fold whether estrogen alone or estrogen plus progesterone is administered to chicks. Measurements of DNA content (7) reveal, however, that at 3 days there are about twice as many cells with estrogen treatment compared to those with estrogen-plus-progesterone treatment. After the third day, the wet weight continues to increase with estrogen but ceases to increase if both hormones are administered. A log plot of the estrogen-stimulated growth (not shown) indicates that the wet weight doubles every 21 hr between the first and fourth day of hormone treatment. When progesterone is administered alone

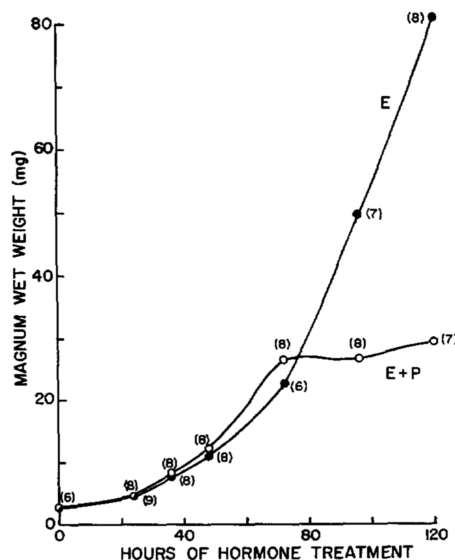


FIGURE 1 Effect of estrogen (*E*) or the combination of estrogen plus progesterone (E + P) on oviduct magnum growth. 5-day old chicks were injected daily with 1 mg of estradiol-17β benzoate or 1 mg estradiol-17β benzoate plus 1 mg progesterone. The magnum portion of the oviduct was removed at the indicated times, cleaned of adhering mesentery, blotted, and weighed. The numbers in parentheses indicate the number of chicks used for each point.

there is no significant increase in the wet weight of the oviduct magnum (Table II).

Ovalbumin Concentration

Since ovalbumin is the major protein synthesized by the differentiated magnum, accounting for over 50% of the soluble protein synthesized (3, 18), the presence of this protein can be used as a primary criterion, as well as a sensitive test, for differentiation. A rapid and sensitive immunological method was used to measure ovalbumin content in the supernatant fraction of magnum homogenates (9). The sensitivity of the immunological assay for ovalbumin in these experiments was about 10^5 ovalbumin molecules/cell.[2]

The concentration of ovalbumin in the magnum at various times after the initiation of hormone treatment is presented in Fig. 2. With estrogen alone ovalbumin is not detectable until 24–36 hr of hormone treatment. After this time the ovalbumin concentration doubles every 9 hr for the next 60 hr. If progesterone is administered along with estrogen, ovalbumin is detected earlier and the ovalbumin concentration is higher for the first 48 hr of treatment than in estrogen-treated chicks. After 48 hr of estrogen-plus-progesterone treatment the rate of ovalbumin accumulation declines substantially. After 5 days of estrogen treatment there are nearly 2 mg of ovalbumin per magnum, whereas with estrogen plus progesterone there is only 0.1 mg of ovalbumin per magnum (calculated from Table I). The rise in soluble protein concentration (Table I) between 3 and 5 days of estrogen treatment is due almost entirely to the accumulation of ovalbumin. Ovalbumin constitutes 35% of the total soluble protein at that time.

[2] Fig. 2 shows that the limiting amount of ovalbumin that can be detected is about 0.1 μg/mg wet weight, which is equal to about 10^5 molecules/cell.

0.1 μg ovalbumin/mg wet wt $\times$ 1 mg wet wt/7 μg DNA $\times$ 2.5 $\times$ 10^{-6} μg DNA/cell $\times$ 6 $\times$ 10^{23} molecules/mole $\times$ 1 mole ovalbumin/4.2 $\times$ 10^{10} μg ovalbumin = 10^5 molecules/cell

The values for DNA content per milligram wet weight and DNA content per cell are from references (7) and (19), respectively. The sensitivity could be increased about 10-fold using a micro Lowry determination (20).

Ovalbumin Synthesis

To estimate the rate of ovalbumin synthesis, oviduct magnums were incubated in radioactive amino acids for 4 hr before preparation of the tissue homogenates. The radioactivity precipitated immunologically by anti-ovalbumin relative to total radioactivity in supernatant proteins was used to express the degree to which the tissue was specialized in secretory protein synthesis. This expression avoids the problem of not knowing the specific activity of the radioactive amino acids within the tissue after various hormonal treatments. Table I (column 8) shows that the rate of isotope incorporation into total soluble protein declines with increasing duration of hormone treatment. This decline is most likely due to the increased magnum thickness which may limit the diffusion of nutrients into the tissue.

Fig. 3 shows that the relative rate of ovalbumin synthesis is higher during the first 60 hr with estrogen plus progesterone treatment than with estrogen treatment alone. This higher relative rate of synthesis presumably accounts for the more rapid accumulation of ovalbumin (Fig. 2) during this same time. After 60 hr the relative rate of synthesis with estrogen alone exceeds the rate with estrogen plus progesterone.

Progesterone alone does not stimulate ovalbumin synthesis (Table II). The small amount of radioactivity that appeared to be specifically precipitated (50 cpm out of 30,000) was not ovalbumin as judged by the migration of the precipitated radioactivity on sodium dodecyl sulfate acrylamide gels. The radioactivity was dispersed throughout the gel and was apparently nonspecifically trapped in the precipitate.

To demonstrate the specificity of the immunoprecipitation reaction, purified ovalbumin-^{14}C was added to samples of the tritiated supernatant proteins, and the ovalbumin was precipitated with excess anti-ovalbumin γ-globulin. The precipitate was washed, solubilized, and electrophoresed on sodium dodecyl sulfate acrylamide gels. Fig. 4 shows that most of the ^{3}H radioactivity migrated with the authentic ovalbumin-^{14}C. There is a low background amount of radioactivity in most of the fractions which is corrected for in the determination of ovalbumin radioactivity (Fig. 3 and Table I) by using control γ-globulin. It was imperative to add unlabeled conalbumin, since, without it, a peak of radioactivity which had the

TABLE I

The Effect of Estrogen or Estrogen Plus Progesterone on Protein Synthesis in Chick Oviduct Magnum

The experimental details are given in the companion figures and Methods. In brief, the magnums from the indicated number of chicks were incubated with radioactive amino acids for 4 hr. Then a homogenate was prepared and centrifuged. Samples of the supernatant were used to determine soluble protein content, ovalbumin content, total soluble trichloroacetic acid (TCA)-precipitable radioactivity, and ovalbumin radioactivity.

	No. of chicks	Hormones administered*	Duration of hormone treatment	Average magnum wet weight	Soluble protein‡	Ovalbumin§	% of soluble protein as ovalbumin	Total TCA-precipitable radioactivity ‖	Radioactivity precipitable with anti-ovalbumin γ-globulin‖	Radioactivity precipitable with control γ-globulin‖	% of soluble radioactivity precipitable with antibody**
Column	1	2	3	4	5	6	7	8	9	10	11
			hr	*mg*	*μg/mg wet wt*	*μg/mg wet wt*		*cpm/mg wet wt*	*cpm/mg wet wt*	*cpm/mg wet wt*	
	6	None		2.9	24.5	0	0	33,500	195	80	0.33
	9	Estrogen	24	4.5	43.0	0	0	70,200	550	102	0.64
	8	"	36	7.7	42.5	0.17	0.40	47,800	700	75	1.30
	8	"	48	10.9	42.5	0.46	1.10	36,200	760	85	1.85
	6	"	72	24.6	40.0	3.50	8.70	22,200	1780	45	7.80
	7	"	96	49.5	46.5	9.80	21.20	10,940	2050	20	18.50
	8	"	120	81.0	68.0	24.00	35.20	13,120	4860	30	36.80
	8	Estrogen + progesterone	24	4.5	39.0	0.18	0.46	84,000	1030	230	0.95
	8	"	36	8.1	48.0	0.24	0.50	56,000	1310	140	2.10
	8	"	48	12.4	45.5	1.05	2.30	37,000	1400	120	3.45
	8	"	72	26.6	41.5	1.30	3.10	18,800	900	45	4.55
	8	"	96	26.6	37.5	1.87	5.00	13,500	1065	40	7.55
	7	"	120	29.4	44.0	4.20	9.50	17,100	2530	40	14.40

* 1 mg of estradiol benzoate or progesterone was administered every 24 hr.

‡ Supernatant after centrifugation at 350,000 g for 30 min. Protein was determined by the Lowry method using BSA as standard.

§ Based on the protein precipitable by anti-ovalbumin γ-globulin minus that precipitable by control γ-globulin (equal to 0.85 μg) divided by 14 (the approximate antibody-to-antigen ratio).

‖ After washing the precipitate twice the sample was dissolved in 100 μl NCS and counted in 10 ml toluene scintillator fluid; counting efficiency was 54–55%.

** The value in column 9 minus the value in column 10 divided by the value in column 8.

same molecular weight as conalbumin was observed in some samples. Addition of excess conalbumin prevents the immunological precipitation of labeled conalbumin.

Fig. 4 demonstrates that ovalbumin synthesis is detectable at 24 hr, before it is measurable using the quantitative precipitin assay (Fig. 2). By making the oviduct magnum proteins radioactive the sensitivity of the ovalbumin immunoprecipitation assay is increased to about 10^4 molecules/cell. Electrophoresis of antibody precipitates from the early (24–48 hr) estrogen-plus-progesterone–treated chicks revealed even more tritium radioactivity with an apparent molecular weight similar to ovalbumin than in the estrogen-treated samples (figure not shown), in agreement with the data in Table I and Fig. 3.

Sodium dodecyl sulfate acrylamide gel electrophoresis separates proteins essentially according to their monomeric molecular weight (13). The slightly lower molecular weight of the newly synthesized ovalbumin, relative to ovalbumin-^{14}C, ob-

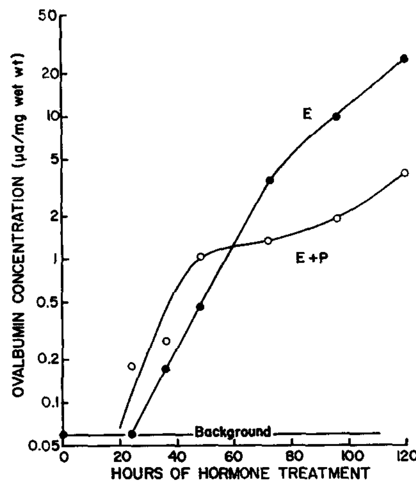


FIGURE 2 Effect of estrogen (E) or estrogen plus progesterone ($E + P$) on ovalbumin concentration in oviduct magnum. Chicks were injected daily with the indicated hormones as described in Fig. 1. The magnums from five chicks were homogenized and the homogenate was then centrifuged as described in Methods. Ovalbumin was determined in the supernatant using an anti-ovalbumin antibody as described in Methods. "Background" indicates the limit of the assay and is the value obtained when control γ-globulin was used.

served at day 1 and day 2 with either estrogen (Fig. 4) or estrogen plus progesterone (not shown) may be due to the absence of a heptapeptide, a carbohydrate moiety of 1500 daltons, and/or phosphate groups (21).

Comparison of Protein Synthesis in Unstimulated and Estrogen-Stimulated Magnum Tissue

Previous experiments have demonstrated that the proteins synthesized in culture are nearly the same as those synthesized in vivo (9). Thus, the analysis of proteins synthesized in culture provides a valid representation of the developmental state. Fig. 5 shows the molecular weight distribution of the soluble proteins being synthesized in unstimulated as well as 4-day estrogen-stimulated magnum tissue. Two prominent protein species are being synthesized in the oviduct after 4 days of estrogen treatment. The first peak (fraction 7) corresponds to conalbumin and the second, larger peak (fraction 14–15) corresponds to ovalbumin.

Is Ovalbumin Being Synthesized in the Unstimulated Oviduct?

Table I shows a low, but questionable, amount of ovalbumin synthesis in magnum tissue from unstimulated chicks. To check the validity of those measurements another experiment was performed. This time, older unstimulated chicks were used and the immunologically precipitated proteins were electrophoresed on a sodium dodecyl sulfate acrylamide gel to ascertain whether the radioactive proteins migrated like ovalbumin or whether they had heterogeneous migration rates. Magnums from 23-day old chicks were incubated in an amino acid–^{3}H mixture as before and a supernatant fraction was prepared. Samples of the supernatant, equivalent to 4.5 mg wet weight, containing 175 μg of protein and 145,000 cpm, were precipitated with trichloroacetic acid or anti-ovalbumin γ-

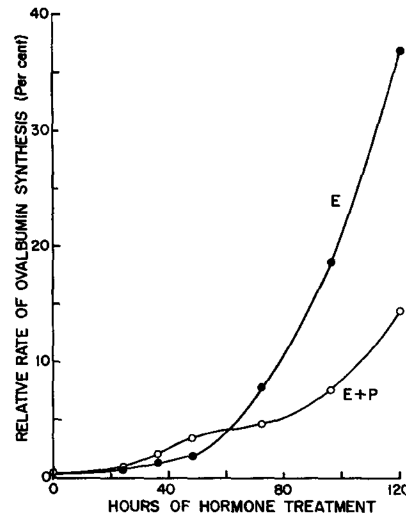


FIGURE 3 Effect of estrogen (E) or estrogen plus progesterone ($E + P$) on ovalbumin synthesis. Chicks were injected as described in Fig. 1, and at the times indicated the oviduct magnums from five chicks were pooled and incubated for 4 hr with an amino acid–^{3}H mixture (10 μCi/ml) in 1:50 Medium 199. The tissue was then blotted, weighed, homogenized, and centrifuged as described in Methods. Total acid-precipitable radioactivity was determined from one sample while ovalbumin radioactivity was determined from two other samples (see Table I for raw data). The extent of tissue specialization in ovalbumin synthesis is presented on the ordinate as the per cent of total soluble protein radioactivity which is also precipitable by anti-ovalbumin γ-globulin.

Table II

Effect of Progesterone Alone on Ovalbumin Synthesis

Each group of chicks (five per group) was administered progesterone (1 mg/chick every 24 hr) for the indicated times. The oviduct magnums were removed and cultured as described in Table 1 and Methods. The tissue was incubated with 10 μCi/ml of an amino acid–^{3}H mixture in 2 ml of media for 4 hr. Isotope incorporation into total soluble protein and ovalbumin was determined as described in Methods. Counting efficiency was 55%.

Duration of treatment	Average wet weight	Total soluble protein synthesis a	Radioactivity precipitable with anti-BSA γ-globulin b	Radioactivity precipitable with anti-ovalbumin γ-globulin c	Per cent ovalbumin $\frac{(c-b)}{a}$
hr	*mg*	*cpm/mg wet wt*	*cpm/mg*	*cpm/mg*	
44	3.2	31,100	130	179	0.16
118	2.6	30,300	114	164	0.16

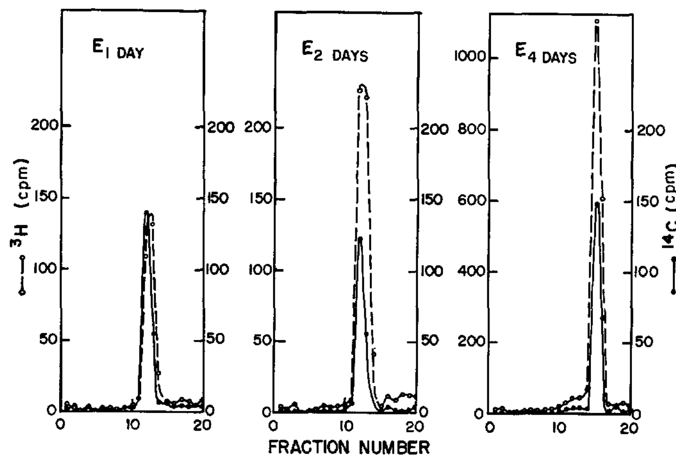


Figure 4 Electrophoresis of ovalbumin-^{3}H precipitated immunologically from oviduct magnum homogenates along with authentic ovalbumin-^{14}C. Pieces of magnum from chicks were incubated with 10 μC/ml of an amino acid–^{3}H mixture as in Fig. 3. The soluble protein fraction was prepared (see Methods) and a sample of the supernatant was incubated with anti-ovalbumin γ-globulin, sodium desoxycholate, and conalbumin for 1 hr at room temperature. Then about 2 μg of ovalbumin-^{14}C, purified as described in a prior paper (9), was added, and the reaction was allowed to proceed another hour. The precipitate was collected, washed, solubilized, and electrophoresed on sodium dodecyl sulfate acrylamide gels as described (9). ^{3}H counting efficiency was 35%; ^{14}C was 60%. Tritium radioactivity was corrected for ^{14}C spillover. ----, ^{3}H; ——, ^{14}C.

globulin. Fig. 5 shows that less than 0.01% of the radioactivity in the samples which was precipitated by anti-ovalbumin migrates like ovalbumin on sodium dodecyl sulfate acrylamide gels. Thus, we estimate that less than 0.01% of 175 μg of protein is ovalbumin, which equals 0.02 μg/4.5 mg wet wt (0.005 μg/mg tissue). Using the formula shown[2] we calculate that the unstimulated oviduct magnum contains less than 5000 ovalbumin molecules per cell.

Microscopy

Some aspects of the histological and ultrastructural changes which occur in the chick oviduct after either primary (1) or secondary estrogen stimulation (18) have been published; therefore, only those microscopic observations which pertain to the differences between the estrogen and estrogen-plus-progesterone treatments will be presented. An ultrastructural study of the early effects

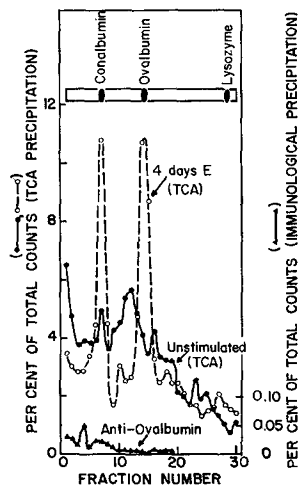


FIGURE 5 Comparison of the proteins synthesized in the oviduct magnum from unstimulated and 4-day estrogen-stimulated chicks. The magnums from eight unstimulated and three 4-day estrogen-stimulated chicks were incubated with 10 μCi/ml of an amino acid-^{3}H mixture for 4 hr. The soluble protein fraction was then prepared as described in Methods, and a sample was precipitated and washed with 5% trichloroacetic acid (TCA). The precipitated proteins were solubilized and electrophoresed on sodium dodecyl sulfate acrylamide gels as described (9). Another gel (top of the figure) contained three purified oviduct proteins as molecular weight markers: conalbumin, 76,000 daltons; ovalbumin, 42,000 daltons and lysozyme, 14,000 daltons. This gel was stained with Buffalo black. On a fourth gel (bottom curve) the precipitate formed by adding anti-ovalbumin γ-globulin, conalbumin, sodium desoxycholate, and 5 μg of cold ovalbumin to a sample of the *unstimulated* magnum supernatant was electrophoresed. The precipitate was collected and prepared for electrophoresis as described in Fig. 4. The trichloroacetic acid (TCA)-precipitable radioactivity is expressed as the per cent of the total gel radioactivity in each fraction. The gel of unstimulated magnum proteins contained 145,000 cpm; the gel of the 4-day stimulated magnum contained 7200 cpm. The immunologically precipitated radioactivity is expressed as the per cent of the total soluble protein radioactivity (145,000 cpm) found in each fraction after precipitation with anti-ovalbumin γ-globulin.

(up to 48 hr) of estrogen on tubular gland cell morphogenesis will be presented elsewhere.[3]

The surface epithelium of the unstimulated chick oviduct consists of columnar cells containing sparse endoplasmic reticulum, few polysomes, and no apparent secretory granules (1, 2). By 36 hr of either estrogen (Fig. 6) or estrogen-plus-progesterone (Fig. 7) stimulation, the epithelial cells show a pronounced endoplasmic reticulum and many polysomes. The epithelium is uneven with small evaginations of the lumen into the epithelium and of the epithelium into the stroma beneath; these evaginations are the sites where glands will form.[3] The stomal cells are closely packed in several layers directly beneath the epithelium, but are more loosely arranged farther away.

There is one notable difference between the two treatments at 36 hr. With estrogen-plus-progesterone treatment (Fig. 7 *b*) the secretory granules near the apex of most of the epithelial cells are larger and more numerous than those in the estrogen-treated oviduct (Fig. 6 *b*). The granules presumably contain egg-white proteins such as ovalbumin (8). If so, the quantity and size of the granules are consistent with the biochemical determinations of ovalbumin in oviduct magnums at 36 hr for the two types of treatment.

Mitotic cells with both secretory granules and condensed chromosomes are occasionally seen after either treatment (Fig. 8), indicating that partially differentiated cells can still divide.

A comparison of the oviduct magnums after 48 hr of either treatment reveals major differences in the gross morphology and the ultrastructure of the epithelial cells. First, with estrogen (Fig. 9 *a*), but not with both hormones (Fig. 10 *a*), numerous glands have formed. They are tubular structures, composed of epithelial cells, which have migrated into the underlying stroma. The glands extend several cell diameters below the surface epithelium. The longitudinal section shown in Fig. 9 *a* passes just beneath the surface epithelium and therefore passes through those glands which protrude into the stroma. Fig. 9 *b* shows the ultrastructure of one of these glands. A second difference between the two treatments is that in the estrogen-plus-progesterone–treated tissue a new type of membrane-bounded granule is apparent (Fig. 10 *b*). Compared to dense secretory granules, these granules are generally larger and contain a more flocculent material, either filamentous or granular. In one section a cell may display only dense granules, only flocculent granules sometimes with a small dense patch of material within, or a combination of both types (Fig. 10 *b*).

With both treatments the endoplasmic reticulum

[3] J. T. Wrenn, in preparation.

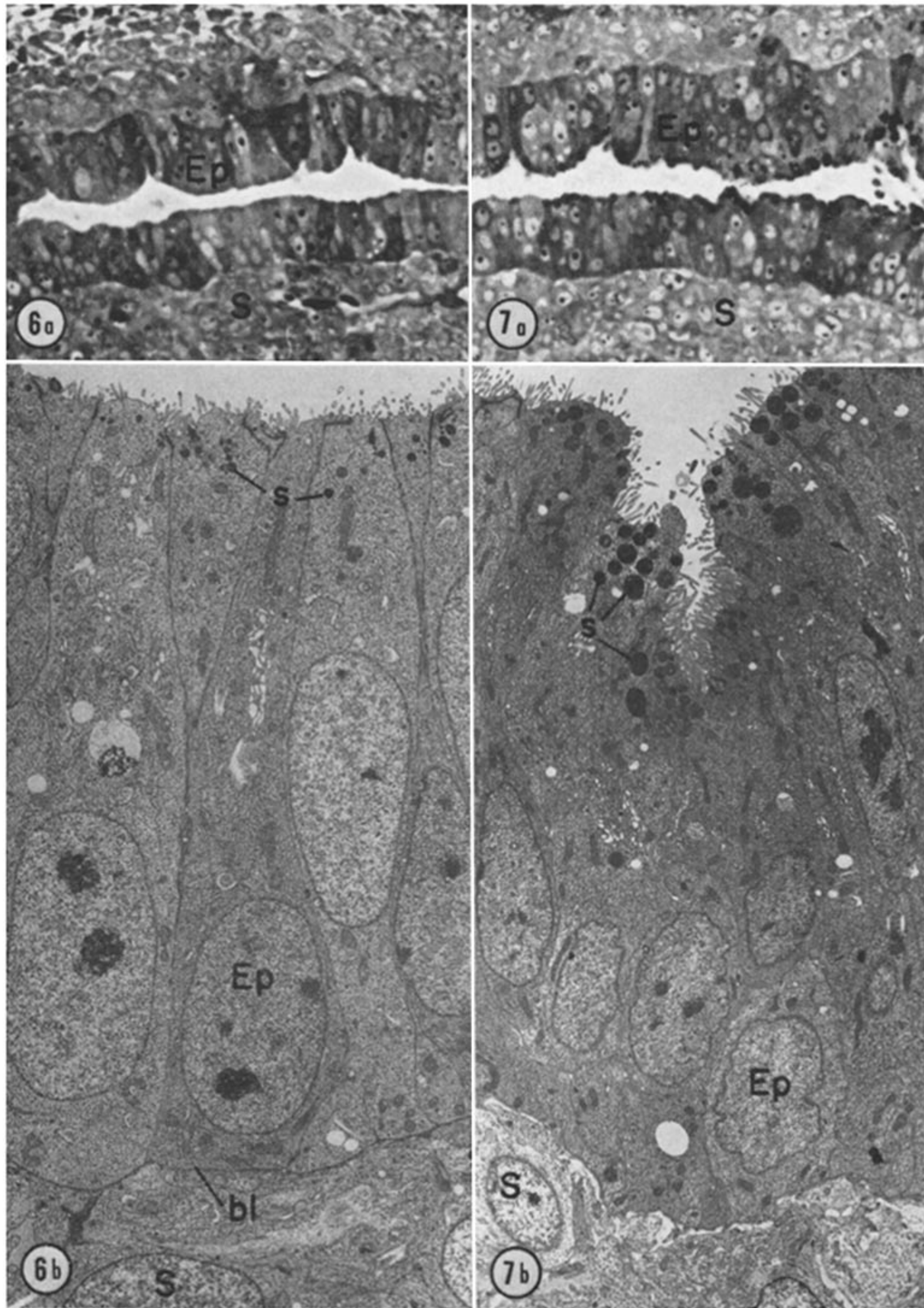


FIGURE 6 Light (Fig. 6 *a*) and electron (Fig. 6 *b*) micrographs of chick oviduct, in longitudinal section, after 36 hr of estrogen (E) treatment. A columnar epithelium (*Ep*) is separated from adjacent stromal cells (*S*) by a basement lamina (*bl*). Depressions in the luminal surface of the epithelium indicate the beginnings of gland formation. Note small secretory granules (*s*) at the apex of the epithelial cells. Fig. 6 *a*, × 600; Fig. 6 *b*, × 4000.

FIGURE 7 Oviduct after 36 hr of estrogen-plus-progesterone treatment (E + P). Secretory granules (*s*) are apparent at the apex of the epithelial cells even in light micrographs (Fig. 7 *a*). The cells around a surface evagination, which marks the beginning of gland formation, are shown in the electron micrograph (Fig. 7 *b*). The stroma and epithelium are generally juxtaposed. Fig. 7 *a*, × 600; Fig. 7 *b*, × 4000.

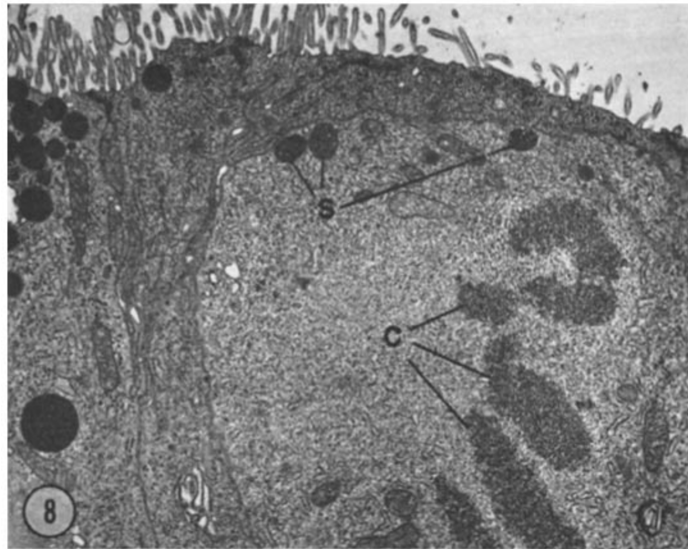


FIGURE 8 A mitotic epithelial cell after 36 hr of E + P treatment. Note the inclusion of both secretory granules (*s*) and condensed chromosomes (*c*) within the same cell. × 8200.

and Golgi complexes are more highly developed at 48 hr than before (Figs. 9 *b* and 10 *b*). The secretory granules in the epithelial cells of estrogen-stimulated oviducts are larger (up to 1.0 μ) and more numerous at 48 hr than at 36 hr and hence are apparent even in light micrographs (Fig. 9 *a*). A few less dense granules, sometimes with irregular outlines, are also seen. These are probably condensing vacuoles that were fixed during the process of development into dense secretory granules, and they should not be confused with the flocculent granules observed with estrogen-plus-progesterone treatment.

In addition to the changes in epithelial cells, by 48 hr the stroma of the estrogen-plus-progesterone–treated magnum is markedly different from that observed in the estrogen-treated controls. With both hormones, the epithelial and stromal cells are not juxtaposed, as they are with estrogen alone. Instead, there are large intercellular spaces filled not only with collagen but also with a flocculent material (compare Figs. 9 *b* and 10 *b*).

By 72 hr of treatment with estrogen alone (not illustrated) there are more and larger glands, more granules, and more dilated endoplasmic reticulum than there were 24 hr earlier. With estrogen plus progesterone definitive tubular glands have not developed (Fig. 11). The stroma is very edematous. The light granules first observed at 48 hr are more numerous and are easily observed in light micrographs (Fig. 11). In addition, cells with multiple cilia or multiple centrioles (probably presumptive basal bodies from which cilia will soon develop) occur frequently in the epithelium of estrogen-plus-progesterone–treated oviduct and rarely in oviducts responding to estrogen alone. Cells with presumptive basal bodies may also have secretory granules of several types at their apices (Fig. 12).

After 5 days of stimulation with estrogen alone, tubular glands are enlarged and their lumina are filled with an electron-opaque secretion apparently discharged by secretory granules, the membranes of which have fused with the apical cell membrane (Fig. 13). The endoplasmic reticulum of the gland cells is very dilated. In a cross-section of a gland, the granules are of different densities. Probably some are condensing vacuoles, some are discharging into the lumen at another level and therefore have the same density as the lumen, and some are the usual electron-opaque secretory granules. Within a single granule of any type the material is relatively homogeneous.

Treatment with estrogen plus progesterone for 5 days still does not produce tubular glands. Light micrographs show many ciliated cells (7) interspersed among large, lightly staining cells along the lumen of the oviduct. Ultrastructurally (Fig. 14), the light cells contain many more flocculent granules than were seen earlier. The material within these granules still appears heterogeneous:

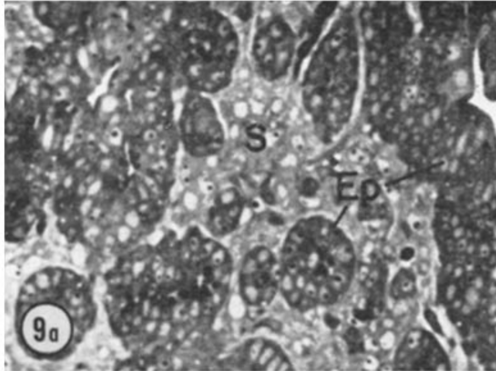


FIGURE 9 Gland formation in chick oviduct after 48 hr of E treatment. Fig. 9 *a*, a tangential section just beneath the surface epithelium, shows the many tubular glands which protrude into the stroma (*S*). Fig. 9 *b*, a cross-section through a gland, shows well-developed Golgi complex (*g*), endoplasmic reticulum (*er*), and condensing vacuoles (*cv*). *Ep*, epithelium; *S*, stroma; *s*, secretory granule. Fig. 9 *a*, × 600; Fig. 9 *b*, × 4700.

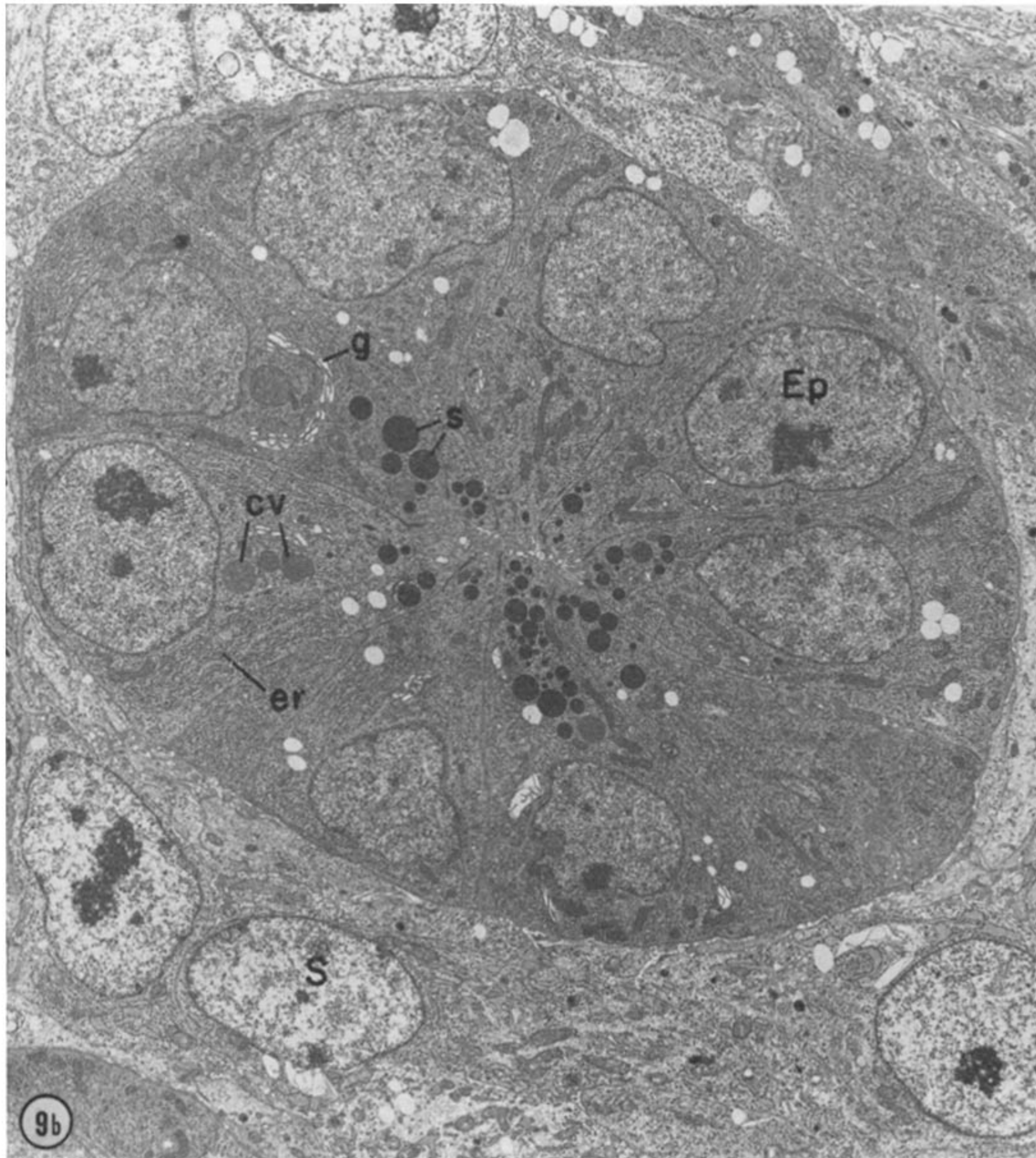
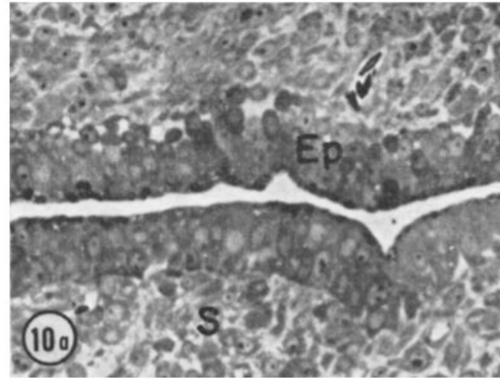
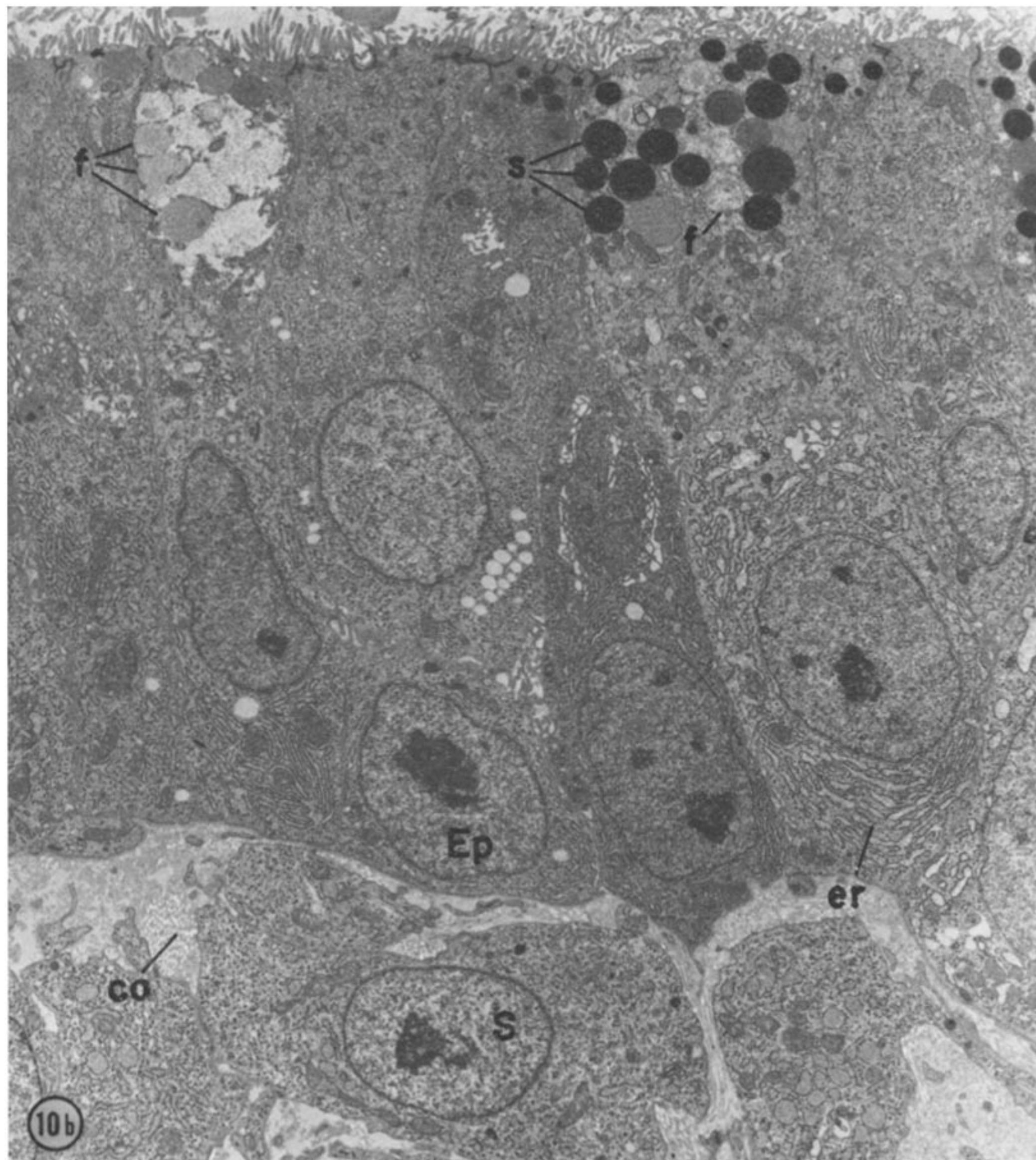


FIGURE 10 Absence of gland formation after 48 hr of E + P treatment (Fig. 10 *a*). The epithelial cells (Fig. 10 *b*) contain an assortment of granules: dense secretory granules (*s*), flocculent granules (*f*), and intermediate types. The stromal and epithelial cells are no longer juxtaposed. Bundles of collagen (*co*) are apparent in the gap between the cell layers. *Ep*, epithelium; *er*, endoplasmic reticulum; *S*, stroma. Fig. 10 *a*, × 600; Fig. 10 *b*, × 5000.





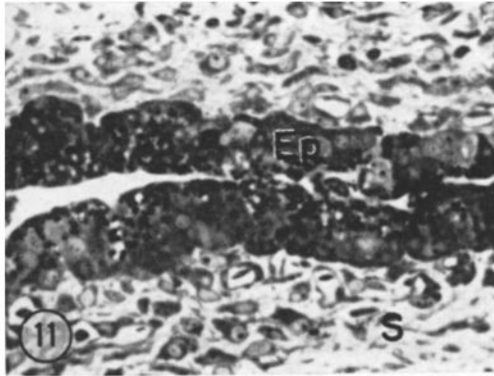


FIGURE 11 Oviduct after 3 days of E + P treatment. Note the accumulation of many flocculent granules (light spots) at the apex of the epithelial cells. Also, the stroma is less dense than after 2 days of treatment. *Ep*, epithelium; *S*, stroma. × 600.

it may be filamentous or granular and may contain a patch of dense substance. There are a few dense secretory granules along the lumen, but far fewer than there were at 3 days of estrogen-plus-progesterone treatment.

By 5 days of both treatments, the stromal cells are loosely arranged around the epithelial cells.

DISCUSSION

Effects of Estrogen Alone

The administration of estrogen to chicks evokes progressive morphological and biochemical changes in the oviduct magnum, leading to a tissue specialized for egg-white protein synthesis. This process is not observed naturally until the chick gonads mature at about 3–4 months of age (22). Whether estrogen acts directly on the oviduct magnum and whether it acts alone to produce the observed changes are unknown; however, estrogen clearly provides the stimulus which triggers the cytodifferentiation of tubular gland cells.

The biochemical, histological, and ultrastructural results reported here on the effects of estrogen on the immature chick oviduct are in substantial agreement with the observations of O'Malley and collaborators (1, 23). The major difference is that, in contrast to the results of O'Malley and McGuire (23), who reported 0.5 ± 0.3 mg ovalbumin/g wet weight, we have been unable to detect ovalbumin or its synthesis in unstimulated oviduct (Fig. 5). As discussed in the Results section, the sensitivity of our ovalbumin content assay is about 0.06 mg/g and our ovalbumin synthesis assay is about 10 times more sensitive. Thus, within the limits of our assays, we do not think that the progenitor cells of the chick oviduct are synthesizing ovalbumin before hormonal stimulation.

Many of the ultrastructural changes in oviduct cells occurring during the development of tubular glands are similar to changes seen during the cytodifferentiation of other cell types which secrete large amounts of protein. The increase in the amount of rough endoplasmic reticulum, the enlargement of the Golgi complexes, and the formation of condens-

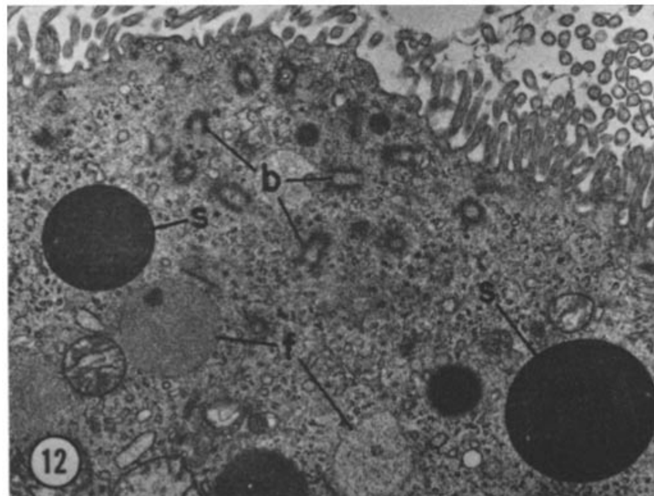
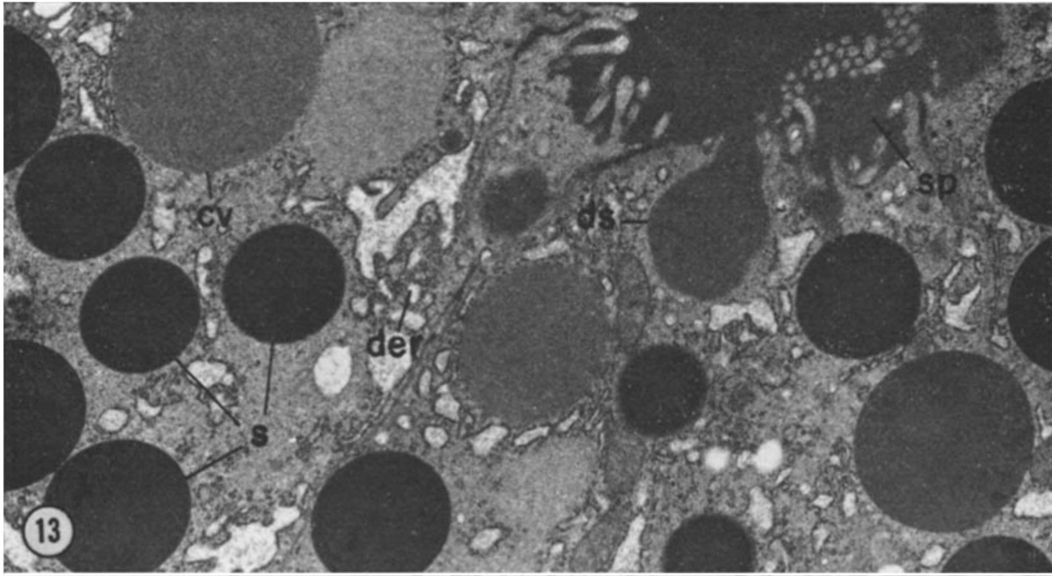


FIGURE 12 Apex of an oviduct cell (3 days E + P) which may be transforming. Note inclusion of both basal bodies (*b*), the primordia of cilia, and several kinds of secretory granules (*s* and *f*) within the same cell. × 12,000.



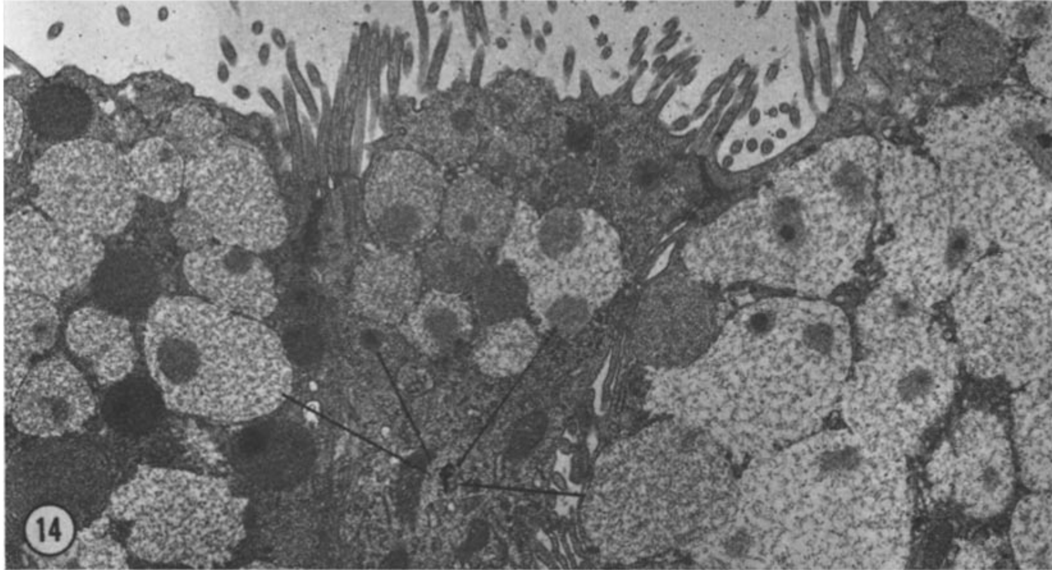


FIGURE 13 Apices of actively secreting tubular gland cells after 5 days of E treatment. A discharging secretory granule (*ds*) can be seen fused with the lumen which already contains secreted protein (*sp*). Large secretory granules (*s*), condensing vacuoles (*cv*), and dilated endoplasmic reticulum (*der*) are prominent features. × 10,700.

FIGURE 14 Apices of epithelial cells after 5 days of E + P treatment. Many kinds of flocculent granules have accumulated. A single granule often contains material of different density. *f*, flocculent granules. × 12,800.

ing vacuoles and secretory granules are events which also occur during the differentiation of cells such as those of the mammary epithelium (24) and the pancreas (25). However, there appear to be some significant developmental differences between the formation of tubular glands in the oviduct magnum and the development of pancreatic acini or mammary alveoli. Cytodifferentiation, i.e. the synthesis of specific secretory proteins, commences in the tubular gland cells before the morphogenetic

formation of definitive glands, whereas the opposite appears to be the case in those systems.[4] Also, partially differentiated tubular gland cells are frequently observed in mitosis (Fig. 8).

It is thought that secretory granules contain ovalbumin. Immunofluorescent antibody techniques have shown that the cells of well-developed tubular glands contain ovalbumin (8). The synthesis and content of ovalbumin as measured by immunoprecipitation methods (Figs. 2–4) correlate well with the appearance of secretory granules within epithelial cells, even before they have evaginated to form tubular glands. By 5 days of estrogen treatment, ovalbumin alone constitutes about 35% of the soluble protein of the magnum. Concomitant with the induction of secretory protein synthesis, changes in oviduct RNA populations have been reported (23, 26, 27).

After tubular gland cells begin to evaginate, some cells still in the surface epithelium (that is, not involved in a glandular structure) begin to show signs of differentiation into other cell types (1). An electron-lucent cell type is occasionally observed with cilia by 5–6 days of estrogen. Later, goblet cells with small granules begin to appear. These cells are electron-opaque and synthesize the biotin-binding protein avidin (28). Progesterone administration to estrogen-primed cells promotes the rapid differentiation of these goblet cells (29).

Effects of Progesterone Alone

Progesterone, in contrast to estrogen, has little effect on growth. During 6 days of progesterone treatment the dry weight (2) and DNA content (7) barely double, whereas with estrogen a 20-fold increase is observed in these parameters (7). Although little growth occurs, Cox and Sauerwein (2) have observed several different cell types by electron microscopy after 2–5 days of progesterone treatment. Cells with diffuse granules "suggestive of mucose-secreting droplets" are seen along with cells containing small dense granules. "Light" cells and cells with cilia are also observed (2, 7). These findings suggest that progesterone alone can promote several kinds of cytodifferentiation. Our electron micrographs of oviduct tissue from chicks treated with progesterone alone for 36 hr (not shown) do not reveal any secretory granules similar to those seen with either estrogen or estrogen plus progesterone (Figs. 6 and 7). Furthermore, we are unable to detect ovalbumin synthesis after either 2 or 5 days of progesterone treatment (Table II). Thus, progesterone does not appear to promote the differentiation of tubular gland cells.

Effects of Estrogen Plus Progesterone

The combination of estrogen plus progesterone has four pronounced effects on the development of the oviduct magnum when compared to those observed with estrogen alone. First, growth commences normally but virtually stops after 3 days of estrogen-plus-progesterone treatment. Second, the synthesis and accumulation of ovalbumin is enhanced by the combination of hormones during the first 2 days and thereafter becomes slower than in the estrogen-mediated controls. Third, no definitive tubular glands ever form with estrogen-plus-progesterone treatment, although an initial step in gland formation, luminal evagination of partially differentiated tubular gland cells, is observed (Fig. 7 *b*). Fourth, instead of forming glands, the partially differentiated tubular gland cells appear to transform into other cell types.

Tubular Gland Cell Cytodifferentiation

Fig. 15 illustrates a hypothetical scheme for cytodifferentiation and modulation of tubular gland cells. After administration of estradiol-17β benzoate to newborn pullets, there is a period lasting about 24 hr before ovalbumin can be detected in the progenitor cells. We have designated the period, between the time when ovalbumin synthesis commences and the time when the tubular gland cells have finished their morphogenetic movements and become organized into glands, as the protodifferentiated stage. Protodifferentiated tubular gland cells are characterized by low levels of egg-white protein synthesis—observed ultrastructurally as the slow accumulation of small but progressively larger secretory granules in the apex of the cells. Condensing vacuoles are rare and there is no evidence of secretion. These cells are in the surface epithelium and can still divide (Fig. 8). The direction of differentiation is still flexible, since progesterone can change potential tubular gland cells to other cell types. This stage appears to be analogous in some respects to the protodifferentiated stage during the development of the pancreas, which has been observed by Rutter et al. (30, 31).

[4] N. K. Wessells, F. Stockdale, personal communication.

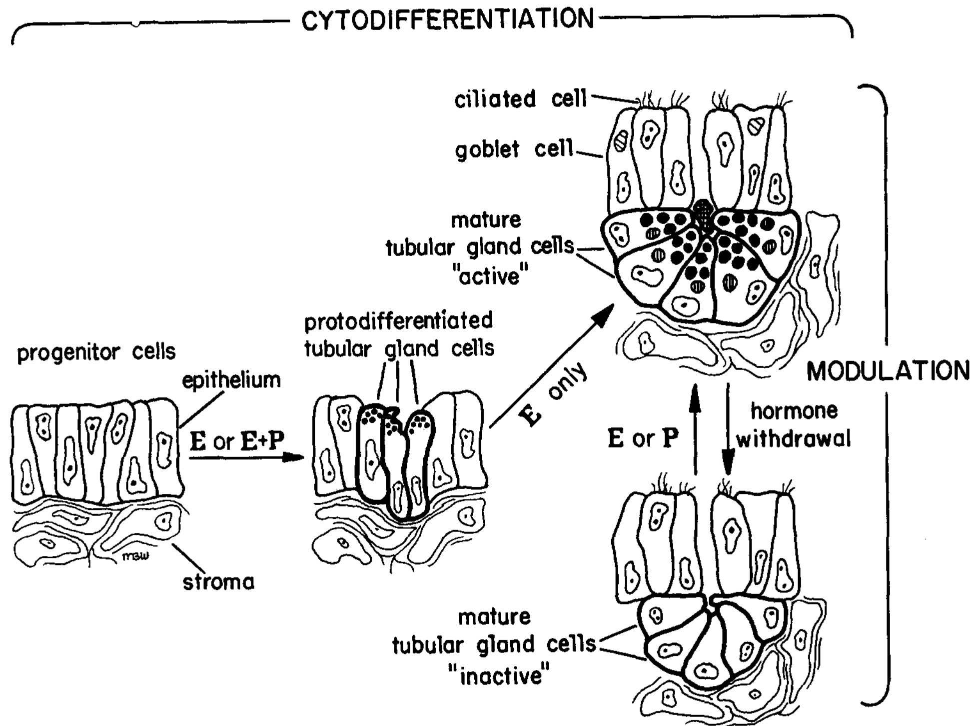


FIGURE 15 Model of hormone-induced cytodifferentiation and modulation of tubular gland cells. On the left are depicted the progenitor cells (epithelium) of the various cell types that will develop in response to estrogen (*E*). The cells are illustrated with nuclei and nucleoli. They overlie a stromal layer consisting of fibroblast-like cells and collagen. After hormonal stimulation with either E or E + P, protodifferentiated tubular gland cells (bold outline) are recognizable due to their accumulation of small secretory granules. With continued E stimulation these cells migrate from the surface epithelium and form glands (bold outline). The mature tubular gland cells contain large secretory granules (solid) and condensing vacuoles (striped). Secretory material is apparent within the lumen. Goblet cells and ciliated cells differentiate in the surface epithelium. Withdrawal of E results in the loss of secretory granules and the cells become smaller. Either E or P can stimulate these "inactive" mature tubular gland cells to become "active" again.

The "active" mature tubular gland cells are in a glandular structure and are characterized by secretory protein synthesis and secretion. Ultrastructurally, the endoplasmic reticulum appears dilated, the Golgi complex is engorged, and condensing vacuoles are numerous. This cell type is stable to hormone withdrawal; the gland cells are retained although they cease synthesizing secretory proteins. When "inactive" mature tubular gland cells are activated again by a secondary hormone stimulation, the ultrastructural changes which ensue do not reveal an intermediate stage which resembles protodifferentiation (18).

Progesterone may interfere with normal estrogen-mediated tubular gland cell cytodifferentiation in at least two general ways. One possibility is that, in the presence of both hormones, progenitor tubular gland cells differentiate in several directions at once due to conflicting signals. At first they appear to respond predominantly to the estrogen signals and come to look like protodifferentiated tubular gland cells. Later they may respond more strongly to progesterone signals and begin to differentiate in other directions, although they retain some of their original program, i.e., they continue to synthesize some ovalbumin.

Another possibility is that progesterone may interfere with epithelial-stromal cell interactions which may be necessary for differentiation of mature tubular gland cells. Many morphogenetic

movements of epithelial cells depend upon a dense contiguous mesoderm during the differentiation of the overlying epithelia (32–37). The very diffuse mesoderm, and the gaps observed between epithelial and mesodermal cells in estrogen-plus-progesterone–treated oviducts at the time when tubular glands normally form, lend credence to the possibility that with progesterone the mesodermal cells do not elicit suitable signals for gland formation. Furthermore, if not arranged in normal, functional groups, tubular gland cells may not be able to retain their early differentiated condition, and as a consequence they may transform into other cell types.

We cannot be sure that the protodifferentiated tubular gland cells which appear after 36 hr of estrogen-plus-progesterone treatment actually transform into the other cell types observed at later times. However, since growth has essentially stopped by 3 days, cell death is not observed, and protodifferentiated tubular gland cells like those seen at 36 hr become increasingly difficult to find; it seems likely that many of the cells are transforming. Fig. 12 shows a cell with both secretory granules and multiple centrioles, possibly indicating a transformation of a potential protein-secreting cell into a ciliated cell type. Furthermore, we do not know the composition of the flocculent granules which appear in most of the epithelial cells after 2 days of estrogen-plus-progesterone treatment. These granules may be modified dense granules, i.e. they may contain the same egg-white proteins as dense granules, or they may be newly synthesized granules with an entirely different protein composition than the dense granules. Fig. 3 shows that ovalbumin synthesis continues at a relatively higher rate at 5 days than at 36 hr, but dense granules are difficult to find at 5 days, whereas they were prevalent at 36 hr. If ovalbumin is sequestered in granules at all, this inverse correlation between synthesis and number of dense granules suggests that some of the flocculent granules probably contain ovalbumin "packaged" in an unusual manner.

In conclusion, progesterone, acting alone, is incapable of promoting tubular gland cell cytodifferentiation. However, acting along with estrogen, it may have a synergistic effect through the protodifferentiated stage; then it appears to block mature tubular gland formation. In contrast to these findings, progesterone alone can activate "inactive" mature tubular gland cells (3, 18). Thus, although progesterone is incapable of promoting the cytodifferentiation of tubular gland cells, once those cells are formed and are located in glands it can stimulate them to synthesize secretory proteins.

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REFERENCES

1. Kohler, P. O., P. M. Grimley, and B. W. O'Malley. 1969. *J. Cell Biol.* **40**:8.
2. Cox, R. F., and H. Sauerwein. 1970. *Cell Res.* **61**:79.
3. Oka, T., and R. T. Schimke. 1969. *J. Cell Biol.* **43**:123.
4. Munro, S. S., and T. L. Kosin. 1943. *Poultry Sci.* **22**:330.
5. Dorfman, R. I., and A. S. Dorfman. 1948. *Endocrinology.* **42**:102.
6. Lorenz, F. W. 1954. *Vitamins and Hormones.* **12**:235.
7. Oka, T., and R. T. Schimke. 1969. *J. Cell Biol.* **41**:816.
8. Kohler, P. O., P. M. Grimley, and B. W. O'Malley. 1968. Science (*Washington*). **160**: 86.
9. Palmiter, R. D., T. Oka, and R. T. Schimke. 1971. *J. Biol. Chem.* **246**:724.
10. Warner, R. C. 1954. *In* The Proteins. N. Neurath and K. Bailey, editors. Academic Press, Inc., New York. Vol. 2.
11. Mandeles, S. 1960. *J. Chromatogr.* **3**:256.
12. Davis, B. J. 1964. *Ann. N.Y. Acad. Sci.* **121**:404.
13. Weber, K., and M. Osborn. 1969. *J. Biol. Chem.* **244**:4406.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193**:265.
15. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. Charles C. Thomas Publisher, Springfield, Ill. 25.
16. Karnovsky, M. J. 1965. *J. Cell Biol.* **27**(2):137A. (Abstr.)
17. Richardson, K. C., L. Jarett, and E. H. Finke. 1960. *Stain Technol.* **35**:313.
18. Palmiter, R. D., A. K. Christensen, and R. T. Schimke. 1970. *J. Biol. Chem.* **245**:833.

19. Sober, H. A. 1968. Handbook of Biochemistry. Chemical Rubber Company, Cleveland, Ohio. H57.
20. Rutter, W. J. 1967. *In* Methods in Developmental Biology. F. H. Wilt and N. K. Wessells, editors. Thomas Y. Crowell Company, New York. 677.
21. Neuberger, A., and R. D. Marshall. 1966. *Glycoproteins.* **5**:299.
22. Brant, J. W. A., and A. V. Nalbandov. 1956. *Poultry Sci.* **35**:692.
23. O'Malley, B. W. and W. J. McGuire. 1968. *Proc. Nat. Acad. Sci. U. S. A.* **60**:152.
24. Sekhri, K. K., D. R. Pitelka, and K. B. DeOme. 1967. *J. Nat. Cancer Inst.* **39**:459.
25. Kallman, F., and C. Grobstein. 1964. *J. Cell Biol.* **20**:399.
26. Hahn, W. E., R. B. Church, A. Grobman, and L. Wilmot. 1968. *Gen. Comp. Endocrinol.* **10**:438.
27. Dingman, C. W., A. Aronow, S. L. Bunting, A. C. Peacock, and B. W. O'Malley. 1969. *Biochemistry.* **8**:489.
28. O'Malley, B. W., and W. J. McGuire. 1968. *J. Clin. Invest.* **47**:654.
29. O'Malley, B. W., A. R. Means, and M. Rubin. 1970. *In* The Sex Steroids: Molecular Mechanisms. K. McKerns, editor. Appleton-Century-Crofts Inc., New York.
30. Rutter, W. J., W. D. Ball, W. S. Bradshaw, W. R. Clark, and T. G. Sanders. 1967. *Exp. Biol. Med.* **1**:110.
31. Wessells, N. K., and W. J. Rutter. 1969. *Sci. Amer.* **220**:36.
32. Wessells, N. K. 1965. *Develop. Biol.* **12**:131.
33. Wessells, N. K. 1964. *Proc. Nat. Acad. Sci. U. S. A.* **52**:252.
34. Grobstein, C., and J. Cohen. 1965. *Science (Washington).* **150**:626.
35. Spooner, B. S., and N. K. Wessells. 1970. *J. Exp. Zool.* **175**:445.
36. Golosow, N., and C. Grobstein. 1962. *Develop. Biol.* **4**:242.
37. Grobstein, C. 1955. *J. Exp. Zool.* **130**:319.