THE EFFECTS OF ESTRADIOL AND PROGESTERONE
ON THE GROWTH AND DIFFERENTIATION
OF THE QUAIL OVIDUCT

by
CONNIE BOOGAARD
B.A., California State, Fullerton

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in the Department of
ZOOLOGY

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
May, 1975
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date May 30, 1975
ABSTRACT

The effects of estradiol and progesterone treatment on the growth and differentiation of the quail oviduct have been investigated, in terms of morphology, growth, and biochemical differentiation.

Histologically, estradiol induced the formation of tubular gland cells by 5 days of treatment, and epithelial differentiation by 10 days of treatment. Progesterone treatment appeared to enhance epithelial differentiation slightly.

The treatment also enhanced the growth of the oviduct, as indicated by increases in size, weight, and the content of the tissue constituents, protein, RNA and DNA.

Estradiol treatment also induced the synthesis of two specific proteins, ovalbumin and lysozyme. However, estradiol treatment failed to induce synthesis of the protein avidin. Only progesterone induced the synthesis of this protein.

Withdrawal from treatment for ten days caused a loss of cells and of tissue constituents. This loss was greater in those birds withdrawn from estrogen plus progesterone treatment than in those withdrawn from estrogen treatment. In some cases, specific proteins were also absent in oviducts from birds withdrawn from treatment.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>ii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>iv</td>
</tr>
<tr>
<td>List of Plates</td>
<td>v</td>
</tr>
<tr>
<td>Introduction to Literature Review</td>
<td>1</td>
</tr>
<tr>
<td>Literature Review</td>
<td>2</td>
</tr>
<tr>
<td>Introduction</td>
<td>16</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>17</td>
</tr>
<tr>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>Histology</td>
<td>21</td>
</tr>
<tr>
<td>Assays of Total Growth</td>
<td>46</td>
</tr>
<tr>
<td>Assays of Specific Proteins</td>
<td>59</td>
</tr>
<tr>
<td>Summary of Results</td>
<td>70</td>
</tr>
<tr>
<td>Discussion, Outline</td>
<td>76</td>
</tr>
<tr>
<td>Discussion of the Results of this Experimentation</td>
<td>77</td>
</tr>
<tr>
<td>Discussion of the Response as a Phenomenon of Induction</td>
<td>80</td>
</tr>
<tr>
<td>The Nature of the Cellular Response to Estrogen</td>
<td>82</td>
</tr>
<tr>
<td>The Nature of the Cellular Response to Progesterone</td>
<td>95</td>
</tr>
<tr>
<td>Events a General Model Will Have to Explain</td>
<td>101</td>
</tr>
<tr>
<td>Comparison of this Induction Phenomenon to Embryonic Induction</td>
<td>102</td>
</tr>
<tr>
<td>Conclusion</td>
<td>105</td>
</tr>
<tr>
<td>References</td>
<td>107</td>
</tr>
<tr>
<td>Appendices</td>
<td>121</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Proposed Time Sequence of Estrogenic Responses, with Influences of Progesterone</td>
<td>11</td>
</tr>
<tr>
<td>2A.</td>
<td>Diagram of the Chick Oviduct</td>
<td>12</td>
</tr>
<tr>
<td>2B.</td>
<td>Illustration of a Cross-Section through the Oviduct Magnum after Treatment with Estrogen and Progesterone</td>
<td>12</td>
</tr>
<tr>
<td>3.</td>
<td>The Variation in Wet Weight as a Function of Treatment</td>
<td>49</td>
</tr>
<tr>
<td>4.</td>
<td>The Variation in Protein Content as a Function of Treatment</td>
<td>49</td>
</tr>
<tr>
<td>5.</td>
<td>The Variation in RNA Content as a Function of Treatment</td>
<td>51</td>
</tr>
<tr>
<td>6.</td>
<td>The Variation in DNA Content as a Function of Treatment</td>
<td>51</td>
</tr>
<tr>
<td>7.</td>
<td>Elution Profile of Ovalbumin on Sephadex G-200</td>
<td>61</td>
</tr>
<tr>
<td>8.</td>
<td>The Variation in Lysozyme Concentration as a Function of Treatment</td>
<td>66</td>
</tr>
<tr>
<td>9.</td>
<td>Proposed Control of Ovalbumin Synthesis</td>
<td>84</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. The Content and Concentration of Various Tissue Components of the Oviduct at Different Stages of Treatment</td>
<td>53</td>
</tr>
<tr>
<td>II. Summary of the Weights, and the Presence or Absence of Specific Proteins in the Oviduct Magna of Birds Withdrawn from Treatment</td>
<td>68</td>
</tr>
</tbody>
</table>
# LIST OF PLATES

<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>la and lb.</td>
<td>Cross-section of an Oviduct from a 10-day old bird which had received control treatment for 5 days.</td>
<td>24</td>
</tr>
<tr>
<td>lc.</td>
<td>Cross-section of an oviduct from a 15-day old bird which had received control treatment for 10 days.</td>
<td>24</td>
</tr>
<tr>
<td>2a and 2b.</td>
<td>Cross-section of an oviduct from a 20-day old bird which had received control treatment for 15 days.</td>
<td>26</td>
</tr>
<tr>
<td>3a and 3b.</td>
<td>Cross-section of an oviduct from a 30-day old bird which had received control treatment for 15 days, followed by 10 days without treatment.</td>
<td>28</td>
</tr>
<tr>
<td>4a and 4b.</td>
<td>Cross-section of an oviduct magnum from a bird treated for 5 days with estradiol.</td>
<td>32</td>
</tr>
<tr>
<td>5a and 5b.</td>
<td>Cross-section of an oviduct magnum from a bird treated for 10 days with estradiol.</td>
<td>34</td>
</tr>
<tr>
<td>6a and 6b.</td>
<td>Cross-section of an oviduct magnum from a bird treated for 15 days with estradiol.</td>
<td>36</td>
</tr>
<tr>
<td>7a and 7b.</td>
<td>Cross-section of an oviduct magnum from a bird treated for 10 days with estradiol and 5 days with progesterone.</td>
<td>38</td>
</tr>
<tr>
<td>8a and 8b.</td>
<td>Cross-section of an oviduct magnum from a bird withdrawn from estrogen plus progesterone treatment for 10 days.</td>
<td>42</td>
</tr>
<tr>
<td>9a, b, c.</td>
<td>Cross-sections of oviduct magna from birds withdrawn from estrogen plus progesterone treatment for 10 days.</td>
<td>44</td>
</tr>
<tr>
<td>10a.</td>
<td>Ouchterlony plate demonstrating the reaction of anti-chick ovalbumin with chick ovalbumin, quail ovalbumin, and magnum extract from birds withdrawn from estrogen treatment.</td>
<td>63</td>
</tr>
<tr>
<td>10b.</td>
<td>Ouchterlony plate demonstrating the reaction of anti-chick ovalbumin with chick ovalbumin, quail ovalbumin, and serial dilutions of a magnum extract from a bird treated for 10 days with estrogen.</td>
<td>63</td>
</tr>
</tbody>
</table>
10c. Ouchterlony plate demonstrating the reaction of anti-chick ovalbumin with chick ovalbumin, quail ovalbumin, and a magnum extract from a bird withdrawn from estrogen plus progesterone treatment................................. 63
INTRODUCTION

The mature oviduct of a sexually active chicken comprises a tube nearly 80 cm in length, whose function it is to secrete the mainly proteinaceous material that will be included in the oviposited egg, as well as the shell that surrounds it. Since this is, then, a secretory organ, it lends itself nicely to studies which require both a morphological and a biochemical marker for differentiation, and has been studied extensively in this context.

Recently, a great deal of research has attempted to establish in detail the response of the chick oviduct to estradiol and progesterone. Work on that system has indicated that treatment of an immature chick with estradiol causes dramatic growth and differentiation of the oviduct tissue. It was further reported that withdrawal of estrogen after such treatment results in the loss of functioning of the newly-differentiated cells of the oviduct, but that the cells themselves remain. That is, the cells are stable to a loss of hormone. This cell stability is a very marked divergence from the situation in mammals, where this is known not to occur. It is possible that this characteristic is unique to fowl, or to the avian group. However, brief initial reports had indicated that in quail, the cells of the oviduct are lost during the lengthy resting period (54), thus indicating that the stability of the chick oviduct cells in the absence of hormone is characteristic of that species. A difference as marked as the life or death of the cells in the absence of hormone is surprising in species of the same order, and so it was decided to investigate in greater detail the response of the quail oviduct to steroid hormones. This initial study was therefore undertaken to establish the major responses of the quail oviduct to steroid hormones, in order to serve as a base for further experimentation.
Historical Literature

The oviduct of avian species, like its counterpart, the mammalian genital tract, has long been known to be under the control of sex hormones. As early as 1928, Riddle and Tange (1) demonstrated that estrogenic extracts from the ovary or placenta of sows would induce growth of the oviduct of immature pigeons, and noticed that, per unit dose, this growth was not as marked as that observed in the uterus of mammals. Two years later, in 1930, Juhn and Gustavson (2) performed the same type of experiment using a human placental extract on fowls, and observed a 10-fold increase in oviduct weight. Histological examination demonstrated that both growth and differentiation took place: the mucosal folds increased in size and the tubular gland cells developed. Increased vascularization was also noted, and it was possible to induce these same effects in the rudimentary Müllerian ducts of males and females at any stage of post-embryonic development (3, 4). This response has also been demonstrated in sparrows (5), and in fowl and turkey given synthetic estrogens orally (6), as well as in chicks given estradiol (7).

In 1944, Hertz and Sebrell (8) noted that a dietary deficiency of pantothenic acid, a folic acid precursor, inhibited the response of the oviduct to estrogen and in 1949 (9) it was demonstrated that a purine analog could do the same thing. Both these treatments are now known to interfere with DNA synthesis, indicating that such synthesis is necessary for the growth response.

Gradually it became of interest to many researchers to investigate the effects of the different sex steroids singly and in combination with each
other. In 1940, two groups investigated the effects of hormones other than estrogens. Witschi and Fugo (10) demonstrated that estrogens stimulate the oviducts and right rudimentary ducts of starlings, and that progesterone did not contribute to the response, although testosterone could. In this species, the females have paired vasa deferentia which respond to androgens, and are known to secrete considerable amounts of testosterone in the breeding season. The authors therefore thought it probable that testosterone contributed to the normal development of the oviduct, at least in this species. Androgen has also been shown to augment the oviducal response to estrogen in chicks (11), and in night herons (12). In this latter case, however, it was also shown that only estrogens can cause hyperplasia (12).

Subsequently, a marked dichotomy of results appeared, some indicating synergism and some antagonism in the oviducal response to estrogen in combination with other steroids. In 1947, Hertz et al (13) noted that progesterone was antagonistic to the oviducal estrogenic response; that is, a marked inhibition was noted when the two were given simultaneously, although progesterone did not have an antagonistic effect on the response of serum lipemia and calcemia to estrogen, indicating that the antagonism was specific to the oviduct. This inhibition by progesterone of the estrogen-induced hypertrophy of the oviduct of immature chicks was noted also by Phillips et al in 1952 (14).

On the other hand, in 1951, Mason (15) demonstrated that progesterone alone caused no weight increase in the chick oviduct, but when given with estrogen, caused a marked hypertrophy, which was greater than that caused by estrogen alone. He postulated that progesterone was necessary for normal
functioning of the oviduct. In 1956, Breneman (16) noted the same effect of progesterone, and found the synergistic effect for testosterone as well. However, he also noted that antagonism was observed with low doses of progesterone. These findings were supported by those of Mason in 1952 (15), which demonstrated that estrogen and progesterone may act synergistically or antagonistically in chick oviduct, depending on the relative ratios of both. Estrogen always caused an increase in weight, whereas progesterone alone had no such effect, but the greatest increases were always found when the two were combined in unequal amounts.

In 1950, Hertz (17) noted that estrogen caused an increase in serum biotin level, and that an anti-biotin factor present in the egg-white was made in the oviduct under the influence of the fully functional ovary. Chicks that had been castrated and treated with estrogen produced no anti-biotin, whereas those treated with both estrogen and progesterone did, thus demonstrating that progesterone was functional in causing secretion. This effect on secretion was also noted by Brant and Nalbandov in 1952 (18), who demonstrated that although estrogen alone caused growth of the oviduct, there were few secretory granules. Again, androgen or progesterone alone had no effect, but in combination with estrogen, these steroids caused an increase in size greater than that resulting from estrogen alone, and secretory granules were abundant. Brant and Nalbandov concluded that the simultaneous presence of two hormones was essential for albumin granule production, but did not demonstrate which steroid was normally the active second hormone.

In 1956 (19), they continued their studies by demonstrating that progesterone and testosterone act synergistically with estrogen in the secretion of albumin. They saw no antagonistic effect, but in these experiments the estrogen was
administered by pellet implantation a few days before progesterone or testosterone was given. (The significance of this will be apparent later.) By implanting a string of beads into the oviduct, they demonstrated that none of the hormones alone could cause secretion of the albumin. When the combination of estrogen plus progesterone or estrogen plus testosterone was used, a large secretion was apparent. This was more pronounced with estrogen plus progesterone than with estrogen plus testosterone. They concluded that the full secretory potential of the oviduct requires 2 hormones, although the second may be either progesterone or testosterone.

Estrogen and testosterone acted synergistically to cause nitrogen retention (20), which was not accomplished by either steroid alone. Similarly, it was demonstrated that estrogen and progesterone caused a rise in the free riboflavin content of the magnum (21), an increase which did not occur in response to estrogen or progesterone alone. It therefore appeared that both hormones are essential to some responses.

However, the controversy over whether progesterone acted antagonistically or synergistically with estrogen continued. In 1954, Tullner et al (22) showed that both progesterone and allopregnan-21-ol-3,20-dione acetate could antagonize estrogen. This latter compound, a non-corticoid, was the greatest inhibitor. They also demonstrated that such corticoids as desoxycorticosterone inhibited the estrogenic response of the oviduct. Since the allopregnan-21-ol-3,20-dione acetate and desoxycorticosterone did not act as progestagens in any of the assays used, the authors concluded that "the inhibition of the oviduct response in estrogen-treated chicks may be clearly dissociated from other biological actions of progesterone". They thus separated the anti-
estrogenic effects from the progestational properties of progesterone.

In 1963, Dorfman and Dorfman (23) reported that the antagonistic or synergistic effect of progesterone depended on both the concentration ratio and the dose. These results supported those of Mason in 1952 (15), and Breneman in 1956 (16). Progesterone was found to be synergistic to low doses of estrogen, but antagonistic to high doses of estrogen. Pregne-5-ene-3,20-dione acetate was synergistic with estrogen, but had no effect alone. Desoxycorticosterone was also synergistic with estrogen, although it was inhibitory at low doses. The authors noted that it had been suggested (22) that the antagonistic effect of progesterone was independent of its progestational properties. Having observed the identical action of corticoids, they suggested that the synergistic effect of progesterone on the estrogenic response may also be independent of its progestational properties.

Such a suggestion raises the question of why progesterone would be expected to act as a progestagen in a species which does not become pregnant; this is not necessarily a rhetorical question when the amount of discussion still devoted to this phenomenon is considered (24). Definite progestational effects of progesterone in this non-mammalian system have yet to be demonstrated. At any rate, Dorfman and Dorfman concluded that the type of response to progesterone, whether antagonistic or synergistic, will depend on the intensity of the estrogenic response rather than on the ratio of the two hormones to each other (24). Thus, at a maximal estrogen response, progesterone appeared to be antagonistic, whereas at minimal estrogen responses, it appeared to be synergistic.

A comparison of these early studies with each other is hindered by a number of problems which appear quite clearly in hindsight. The very earliest
studies used crude estrogenic extracts, demonstrating only that they contained estrogen, and not excluding the possibility that they contained progesterone or other hormones as well. Of the studies which immediately followed those, estrogens were chosen by somewhat random methods, since the biologically active steroid was not known. Some studies therefore utilized alphaestradiol, which is relatively inactive; others used estrone, still others used stilboestrol or one of its derivatives. These all have different potencies, and it is known that their effects, especially in combination with progesterone, will vary. The actual dose of active agent therefore differed in these studies. More recently, in studies concerning combination experiments, the method in which the two hormones were administered is also relevant, since pellet implantation would have a different effect on the serum estrogen level than injection. Because of the same consideration, the time course of injections would be important, in experiments where the hormones were injected. However, in spite of these problems, some tangible conclusions can still be drawn from this work: (1) estrogen stimulated the growth and differentiation of the oviduct; (2) a second steroid acted to increase secretion; (3) the effect of the second hormone on the response to estrogen would depend on the relative doses of the two hormones, and on the state of the oviduct at the time of administration of the second steroid.

Most of these early studies, then, were concerned with the overall growth response of the oviduct to estrogens alone, or estrogens and other hormones in combinations. Due to the relatively unrefined nature of the techniques available at the time, the effects of these hormones on specific protein synthesis were not investigated. However, one protein had been known to exist due to its biological activity of binding to the vitamin biotin. When
included in the diet of animals or the growth medium of micro-organisms such as yeast, the protein caused death to the organism. With this ability to assay for a specific protein, researchers could investigate the effects of various hormones on the production of this protein. Such studies occurred concomitantly with those hormone studies already mentioned, and should be seen in that light.

Avidin was first named by Eakin et al (25) as the protein factor in egg-white that caused egg-white injury to adult hens and rats fed on a diet of raw egg-white. Addition of biotin to the diet prevented or cured egg-white injury (26). Biotin accumulated in the yolk of the egg (27), under estrogenic control (28), which may explain, in retrospect, why the avidin does not cause injury to the embryo.

In 1942, Hertz and Sebrell (29) demonstrated that avidin was present in the egg-white of turkey, duck, goose and hen eggs, in the oviducts of hens and frogs, and in the egg-jelly of two species of Rana. They suggested that avidin was a secretory product of oviducts of birds and amphibians, and that it may play an important part in embryonic development (29). Although no further reports have elucidated its role in embryonic development, avidin has recently been shown to be present also in oviducts of pigeons (30) and lizards (31).

In 1943, Frapps et al (32) demonstrated that avidin was found in all levels of the magnum of chick oviduct, but not in the magna of non-laying hens. They concluded that avidin production was associated, either directly or indirectly, with the complete reproductive function of the ovary. They continued their studies by investigating the effects of hormones on avidin
production (33). Using immature chicks, they demonstrated that the production of avidin depended on the synergistic action of stilboestrol and progesterone, although they occasionally found a positive response to stilboestrol alone. Since at that time the identity of the hormones secreted by the bird ovary was in doubt, and since in lower vertebrates such as amphibians testosterone could also induce ovulation, they concluded only that the production of avidin required some other steroid in addition to estrogen. This other steroid, they postulated, could have been of extra-ovarian origin (32). In light of later developments in this area (24), that conclusion showed amazing insight.

In 1944 (34), Hertz et al demonstrated that both testosterone and desoxy-corticosterone acetate, as well as progesterone, could induce avidin production in chicks that had been pre-treated with estrogen. They recognized the interchangeability of these steroids in other function, such as the maintenance of life in adrenalectomized animals and the precipitation of endometrial bleeding in the monkey. They therefore questioned which of these agents might play the active role in inducing avidin synthesis in vivo (34).

In 1949 (35), Hertz et al further demonstrated that stilboestrol treatment caused a 5-fold increase in the biotin activity in the serum, but no avidin formation occurred in the oviduct. Since administration of estrogen and progesterone caused both an increase of biotin activity in the serum and of avidin content in the oviduct, they thought that avidin remained in the tissue of the oviduct, and did not circulate (17).

More recently, the system was looked at in greater detail, due to the increased sensitivity of some techniques, and the development of new ones. Most of these later studies centered around the changes in total or specific
protein content of the oviduct due to hormone administration.

In 1960, Brown and Jackson (36) reported that there were no striking differences in the compositions of the organ in different age groups, although the oviduct of the broody hen had a slightly lower protein content. In 1961, Kalman and Opsahl demonstrated that estrogen caused an increased incorporation of C-14-leucine into protein (37). In 1965, Oades and Brown (38) performed electrophoresis of the water-soluble proteins of the oviduct magnum after estrogen treatment, and demonstrated a relative increase in albumin A2 and A3 and the post-albumins in the treated oviduct. There was a lower content of water-soluble magnum proteins in broody hens and in hens treated with estrogen only, than in hens treated with LH or estrogen plus LH. However, there was a large increase in the water-soluble magnum proteins in oviducts from hens treated with estrogen plus progesterone or estrogen plus testosterone. This upheld the earlier work which demonstrated that a second steroid, when given with estrogen, acted to increase secretion. An increase in alkaline phosphatase activity was demonstrated in 1965 by Pande et al (39), and an increase in oviducal glycogen in 1969 by Cecil et al (40), as a result of estrogen action.

Recent History

Even more recently, a number of studies (41-53) have investigated extensively the response of the oviduct to estrogen and progesterone, both during primary and secondary stimulation. The results of these investigations form a comprehensive picture of the oviducal response.

The events of primary stimulation are summarized in Figure 1 and Figure 2. Estradiol causes the initial dispersion of the stroma of the tissue, resulting
Figure 1. Proposed Time Sequence of Estrogenic Responses, with Influences of Progesterone

Estrogen → Stroma

Hyperemia → Water Imbibition → Invasion by cells from blood → Stromal swelling → Dispersion

Epiestheliun

Quiescent Cells → Active Cells

Progenitor Tubular Gland Cells

E, P, or EP

E (lengthy treatment), P, or EP (P enhances)

Further DNA Synthesis, to Tubular Gland Cells

P blocks

P, or EP

Cell-Specific Protein Synthesis; Ovalbumin, Lysozyme, Conalbumin, Ovomucoid

Non-Functioning Cells

Hormone

Hormone

E = Estrogen; P = Progesterone; EP = Estrogen + Progesterone

See text for explanation.
Fig. 2a  Chick Oviduct

Infundibulum  Magnum  Isthmus  Shell gland  Vagina
(contains egg-white proteins)

Fig. 2b  Magnum (cross section)  Adapted from Oka & Schimke (Ref. 48)

Stroma  Undifferentiated epithelium  Ciliated cells
Immature + E  or Progesterone  Goblet secretory cells
Tubular gland cells, containing egg-white proteins
in hyperemia and water imbibition (41). Perhaps as a result of the hyperemia, mononuclear cells from the blood invade the stroma, giving rise to the possibility of cell-cell interactions (41). Concomitantly, or soon thereafter, the epithelial cells lining the lumen differentiate (41) as far as a protodifferentiated state (42), in which indentations into the stroma appear, but are not yet completely formed (43). These protodifferentiated tubular gland cells contain secretory granules, and ovalbumin is present in the oviduct magnum (43). At this stage, microfilaments become essential in the budding off of these cells to form tubular gland cells (44). Either estrogen, or estrogen and progesterone together, can induce the differentiation of the oviduct magnum to this stage; however, only estrogen can continue the differentiation of these protodifferentiated cells to tubular gland cells (43). The presence of progesterone together with estrogen abolishes the subsequent differentiation of these protodifferentiated cells to tubular gland cells (43), and leads instead to epithelial differentiation of ciliated cells and goblet cells (43, 45, 46, 47).

The process of budding off to form tubular gland cells continues for a number of days, depending on dose. After it is complete, the remaining epithelial cells differentiate to become either ciliated cells or secretory goblet cells (41, 45, 48). Progesterone enhances this type of differentiation (47). If progesterone is given with estrogen, so that tubular gland cell differentiation is arrested at the protodifferentiated stage, then cells can be seen in the electron microscope which contain both secretory granules and cilia (43). As the tubular gland cells differentiate, ovalbumin concentration increases 300-fold in the course of 15 days of treatment (49). The concentration of lysozyme, another protein made in the tubular gland cells, also increases in
this period (45, 48).

The effect of progesterone on this process is a complex one, depending primarily on the time at which it is administered. Progesterone will inhibit all the processes that require DNA synthesis (45, 48), and so will abolish the growth of the oviduct if it is given concomitantly with estrogen from the beginning of treatment (45, 48). This reduction is the result of abolishing the tubular gland cell differentiation, since these cells form the vast bulk of the differentiated oviduct magnum. However, if the onset of progesterone treatment is delayed somewhat, so that estrogen has had an opportunity to induce tubular gland cells, then those cells that have differentiated will continue synthesizing ovalbumin and lysozyme, even after progesterone treatment has begun (45, 48). Indeed, progesterone has a synergistic effect on the functioning of the tubular gland cells (45, 48).

Progesterone has the opposite effect on the differentiation of the epithelial ciliated cells and secretory goblet cells: it enhances their differentiation greatly (47). In addition, progesterone has an action on this tissue which estrogen does not have: the induction of the synthesis of the protein avidin in the epithelial goblet cells (50). This protein is present in highest amounts when progesterone is administered part-way through estrogen treatment: either too long or too short a pre-treatment with estrogen is inhibitory (49).

Upon estrogen withdrawal, a decrease in all tissue components is seen. This is less marked in the case of DNA content (48), but with time the cells are also lost (51, 52), contrary to earlier reports (45, 48). Thus, maintenance of the differentiated state of the oviduct requires continued stimulation by the hormone. Readministration of estrogen to chicks withdrawn from
treatment results in a quicker, greater response with a much shorter time lag in induction of specific protein synthesis (48, 53). The remaining tubular gland cells resume functioning, accounting for the shorter time lag (48).

Estrogen and progesterone both stimulate specific protein synthesis, but the response to progesterone is less than that to estrogen (48, 53). When both are given together, the response is greater than the response to either alone, indicating that the two hormones act synergistically in enhancing protein synthesis (48), although they act antagonistically with regard to cell division. It has therefore been suggested that cell proliferation and cell functioning are stimulated by estrogen in different manners, such that progesterone interferes with the one process, but not the other (45, 48).

The oviduct represents an excellent example of tissue growth and differentiation in response to steroid stimulation. It has biochemical markers that can be used to measure the effect of either estrogen or progesterone, and the considerable growth produces large tissue masses for biochemical analysis. It represents a tissue which responds to two inducers, where the actions of both are known (at least in part), and where the action of the second depends to a large extent on the action of the first. The oviduct therefore represents an excellent system in which to study the biochemical processes and possible control mechanisms of steroid-induced gene activity in differentiation. Two major laboratories have been investigating these processes: one directed by Palmiter, investigating the readministration of estrogen to chicks withdrawn from treatment, and one directed by O'Malley, investigating the role of progesterone in inducing avidin synthesis.
INTRODUCTION

The response of the chick oviduct to estrogen and progesterone has therefore been established in detail. The work on that system indicated that treatment of an immature chick with estradiol caused dramatic growth and differentiation of the oviduct tissue. However, one of the most striking differences reported was that, contrary to the case in mammals, the cells of the chick oviduct were not lost upon withdrawal from hormone treatment. In another species (Cortunix cortunix japonica) of the same order (Gallus), early reports had indicated that the cells of the oviduct were lost during the lengthy resting period (54, 55). This has also been more recently confirmed (56). Although this is now known to be the case in chicks as well (51, 52), at the time this study was begun, it had not yet been demonstrated.

Since a difference as marked as the life or death of the cells in the absence of hormone is a striking difference in species of the same order, it was decided to investigate in greater detail the major responses of the quail oviduct to estrogen and progesterone.

This initial study then, was designed to be an introductory and exploratory base for further studies. It therefore attempted to establish the quail's response to estrogen and progesterone in terms comparable to the chick system already studied. Thus, I decided to investigate the effects of estrogen and progesterone on the histologic structure of the oviduct, with particular reference to gland cell formation and epithelial cell differentiation. In addition, I investigated the effects of these steroids on the growth of the organ, as measured by wet weight, protein, RNA and DNA content, as well as their effects on specific protein synthesis, with particular reference to ovalbumin, lysozyme and avidin synthesis.
MATERIALS AND METHODS

Treatment of the Animals

Japanese quail of the strain *Coturnix coturnix japonica* were obtained from the Poultry Science Department, U. B. C., on the first day after hatching. They were maintained in electrically heated brooders, fed on 28% Turkey Starter, and were kept on a schedule of 8 hours light, 16 hours darkness. At five days of age, a 15-day injection plan was begun. Each bird received 0.2 mg daily of estradiol in 0.1 ml of sesame oil subcutaneously in the back of the neck. Control birds received the vehicle only. On the tenth day of treatment the experimental animals were divided into two groups, one of which received progesterone (0.2 mg in 0.1 ml of sesame oil daily), while the other continued to receive estradiol for the remaining 5 days of treatment. This treatment was then followed by ten days of hormone withdrawal. Five to nine animals from each group were sacrificed by ether-anaesthesia on the first, fifth, tenth, fifteenth, and twenty-fifth days of treatment, and their oviducts were excised and fixed in glutaraldehyde; or weighed and then frozen at -70°C, to be homogenized later for the assays. When tissue was excised for the cell-specific protein assays, only the magnum portion of the oviduct was used; in control animals this was estimated by using only the upper half of the oviduct.

Total Growth Assays

Five to nine oviducts from each of the indicated time groups were thawed and reweighed, and homogenized in distilled water by four 45-second bursts of a Sorvall Omni-Mixer, at a speed setting of 5, interrupted by one-minute cooling periods.
DNA and RNA were extracted from the homogenate by the method of Schneider (57). DNA was assayed by the diphenylamine reaction (58), with calf thymus DNA (Calbiochem) as the standard. RNA was assayed by the orcinol reaction (59), with yeast RNA (Calbiochem) as the standard. Protein was estimated by the method of Lowry et al (60), with crystalline bovine serum albumin as standard.

Specific Protein Assays

Oviduct magna were thawed and weighed, and homogenized in 0.01 M sodium phosphate buffer, pH 7.0, with 0.015 M NaCl, to give a 2% (w/v) homogenate, for the assays of ovalbumin, lysozyme, and avidin. The homogenates were centrifuged in a Beckman preparative ultracentrifuge for 60 minutes at 105,000 x g, and the supernatant was recovered.

Quail ovalbumin was partially purified from quail egg-white by the method described by Kabat and Meyer (61) for the purification of ovalbumin from chicken egg-white. Egg-white was diluted with an equal volume of distilled water, and a volume of 100% saturated ammonium sulphate solution equal to the total volume was added, to bring the mixture to 50% saturation. After 2 hours at room temperature, the precipitate from this was removed by centrifugation, and the supernatant adjusted to pH 4.6 with 0.2 M H$_2$SO$_4$. Saturated ammonium sulphate was then added by the drop with stirring until a permanent opalescence was reached. The mixture was allowed to stand for 2 days, and the crystals collected by centrifugation. The ovalbumin thus obtained was recrystallized twice from 50% saturated ammonium sulphate, recovered by centrifugation, and dried in a dessicator. It was later dissolved in homogenizing buffer, applied to a Sephadex G-200 column, 60 cm x 1½ cm, and eluted with 0.02 M Tris-HCl, pH 7.0:
Immunological cross-reactivity of quail and chick ovalbumin (Calbiochem) and the presence or absence of ovalbumin in the homogenates of oviducts from various stages of hormonal treatment was established by the use of Ouchterlony micro-diffusion tests. This was performed as described by Work and Work (62) with 1% agar in phosphate-buffered saline (8.9%), pH 6.9. The slides were washed in saline and distilled water, then dried and stained with amido black.

Lysozyme was assayed by the method of Litwack (63). The activity was measured by the change in transmittance at 645 μm at 25°C, in a Unicam SP 1800 Spectrophotometer. The reaction mixture consisted of 1 ml of 0.066 M potassium phosphate buffer, pH 6.2, with 0.1% NaCl; and Micrococcus lysodeikticus, 20 mg dry weight/100 ml of buffer solution. 0.1 ml of enzyme solution or oviduct extract was added. The reaction mixture was read against a blank of distilled water. Activity is expressed as micrograms of lysozyme present, and is based on the activity of purified chicken egg-white lysozyme (Calbiochem) as standard. The lower limit of sensitivity of this assay was 2 μgm/ml.

Avidin was assayed by the method of Korenman and O'Malley (64). The assay is based on the binding of avidin to C14-carbonyl biotin (45 mCi/m mole; Amersham Searle). To 0.5 ml of C14-biotin (1.35 x 10^-3 μg/ml) was added 0.1 ml of avidin standard (0.54 units/mg, Nutritional Biochemicals) of concentrations varying between 0.5 μg/ml to 2.0 μg/ml, dissolved in 0.2 M ammonium carbonate, or 0.1 ml of oviduct extract. After 15 minutes at room temperature, 1 ml of ammonium carbonate containing 10 mg bentonite was added. After an additional 5 minutes, the mixture was transferred to a 3 ml plastic Luer-Lok syringe (B-D), with a Swinnex filter holder (Millipore) attached to the end. Pressure was then applied with the plunger, and the mixture was filtered through the Millipore filter (0.45 μm pore size). The filter was washed twice
with about 1.8 ml of 0.2 M ammonium carbonate, then removed and transferred to a scintillation vial, dissolved in 10 ml of Bray's solution (65) and counted for 10 minutes in an Isocap 300 liquid scintillation counter (Nuclear Chicago) on Channel 5. Background was approximately 30 cpm, and efficiency was about 85%.

Histology

The freshly excised tissue was fixed in Bouin's or in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for at least 24 hours, then dehydrated through a series of alcohols (50-100%), and three changes of acetone. The tissue was embedded in JB-4 Plastic from Polysciences, Inc. (butoxy-ethanol-glycol methacrylate) as follows: three changes (15 minutes each) in Solution A; final polymerization in Solution A and B (42:1), overnight at room temperature. One to two micron sections were cut using glass knives, and stained at 45°C with Harris's hematoxylin and eosin. The thin plastic sections afforded only little contrast, and so were photographed using a phase contrast objective, thus giving the resultant pictures an unusual and often fluid appearance.
RESULTS

I. Histology

A. Control Animals

The oviducts from control birds appear to undergo progressive changes during the treatment period, primarily in the stroma. These include changes in the pattern of cell arrangement, in nuclear morphology, in size and in vascularization.

As can be seen in oviducts from birds which had served as controls for 5 days (refer to Plate 1), the stromal cells were very densely packed against the epithelium. At the base of each future mucosal fold, the cells were much less densely packed together, and there appeared to be greater intercellular space. The epithelium was a simple columnar epithelium. Each individual future mucosal fold therefore had the appearance of a primordium, such as a limb bud.

By fifteen days of control treatment, this pattern of stromal cell arrangement had changed (refer to Plate 2). The stromal cells had dispersed noticeably, and were no longer densely packed against the epithelium. They appeared to be uniformly distributed throughout the length and width of the mucosal fold. However, the cells in the center of the folds appear to have nuclei which are longer and thinner than those closer to the epithelium. These long, thin nuclei appeared to be oriented along the vertical axis of the fold. This gives an overall appearance of streaming, as if the cells were being sprayed out from a fountainhead at the center and base of each fold. Between the folds, near the periphery of the oviduct, the long thin nuclei are horizontally oriented, so that the streaming appeared to be continuous.
from the base and center of one fold to the base and center of the next. At
the very periphery of the oviduct, all of the long thin nuclei are horizontally
oriented, thus giving the impression that a stream of cells oriented in a
circular manner surrounds the oviduct, with perpendicularly oriented interrup­tions in the streaming at regular intervals. These interruptions form the
base and center of the folds.

The major differences, then, between the oviducts from five- and fifteen-
day controls, are the size and the dense packing of the stromal cells against
the epithelium in the five-day control oviducts. By fifteen days, the size
of the oviducts had approximately doubled, the mucosal folds had enlarged
somewhat, and the stromal cells had dispersed throughout the area of the folds.
The epithelium remained a simple columnar epithelium.

By 25 days of treatment, or 30 days of life, vascularization can be seen
to have increased (refer to Plate 3). Blood vessels were present only in the
mesenteries and at the very periphery of the oviduct in the five-day controls,
and at the very base of the mucosal folds in the ten- and fifteen-day controls.
In the oviducts from the twenty-five-day control birds, capillaries could be
seen extending from the base of the folds up into the center of the folds.
Thus vascularization, when it occurs, follows the "stream" of the cell pattern.

The changes which occur in the control oviducts therefore resemble those
of any embryonic primoridum in response to its inducer (70). This includes
the initial stromal packing against the epithelium, the stromal cell rearrange­ment, and the beginning of vascularization.

These observations are at variance with those of Fertuck and Newstead
(66), who have reported that tubular gland cells are present in the oviduct by
30 days of age. However, Oishi and Lauber (67, 68) have demonstrated that, in
Plate 1a. Cross-section of an oviduct from a 10-day-old bird, which had received control treatment for 5 days. x 168. Note arrangement of stromal cells (S) densely packed against epithelium (E), and vascularization (BV) in mesenteries surrounding oviduct. Fixed in Bouin's and embedded in wax; 5 microns.

Plate 1b. Same, x 680. Note simple columnar epithelium (E), with cells packed together closely enough to appear pseudo-stratified.

Plate 1c. Cross-section of an oviduct from a 15-day-old bird which had received control treatment for 10 days. x 672. The same arrangement of epithelial cells and stromal cells as described above appears at this time. Fixed in glutaraldehyde and embedded in plastic. 1 micron.
Plate 2a. Cross-section of an oviduct from a 20-day-old bird, which had received control treatment for 15 days. x 168. Note dispersion of stromal cells (S), throughout area of mucosal folds, vascularization (BV) at the base of the folds, simple columnar epithelium (E). Fixed in glutaraldehyde, and embedded in plastic. 1 micron.

Plate 2b. Same, x 672. Note orientation of stromal cell nuclei at center of folds along the vertical axis of the fold. Epithelium (E); stroma (S), and blood cells (BC).
Plate 3a. Cross-section of an oviduct from a 30-day-old bird, which had received control treatment for 15 days, followed by no treatment for 10 days. x 168. Note same arrangement of stromal cells (S) as in 15-day control birds (Plate 2). Fixed in glutaraldehyde and embedded in plastic. 1 micron.

Plate 3b. Same, x 672. Note appearance of blood vessels (BV) in the mucosal folds.
the short day length used in this study (8L/16D), gonadal growth is adversely
affected (relative to 24L/0D) by reducing, via the pineal, gonadotrophin
secretion (67, 68, 69; see also 164). Eroschenko and Wilson have also
demonstrated that regression of adult quail oviducts and gonads can be induced
by this short day length, relative to 16L/8D (56). The study by Fertuck and
Newstead (66) did not indicate the day length used; however, the discrepancy
between those results and these could be explained by a different day length.

The changes described here have also been reported by Kohler et al (41)
to be the initial results of estrogen treatment on the chick oviduct. Thus
it would appear that estrogen is present in the immature quail at a level
capable of inducing the early changes in the stroma, but not differentiation
of the tubular gland cells.

B. Treated Animals

Very definite changes took place within five days of estrogen treatment,
as can be seen in Plate 4. The oviduct had grown tremendously in size, and
the mucosal folds enlarged greatly to fill most of the lumen. These folds
were filled with tubular gland cells containing eosinophilic granules. The
glands, which secrete the egg-white proteins (50, 66, 43, 71), arise by budding
off of the epithelium, and the glands open into the lumen. Points of entry
into the lumen could be seen in the mucosal folds.

The simple columnar epithelium appeared to be very slightly ciliated in
some places, but no other distinct evidence of differentiation was seen in the
epithelium. Occasional ciliated cells also occur in mammalian uteri, even in
the absence of hormone (72).

By 10 days of treatment (refer to Plate 5), the oviduct size was greatly
increased. Tubular gland cells contain a great abundance of granules. These granules stain with varying intensity, and it has been suggested (66) that this reflects varying concentrations of the egg-white proteins. Nucleated blood cells were present as well, and epithelial differentiation had occurred. The pseudo-stratified columnar epithelium contained some cells which had intensely-stained, basally-situated nuclei, and others that had pale, centrally-placed nuclei. Cilia were apparent continuously along the epithelium, so that it was not possible to determine from these sections alone which cells they belonged to. However, it is likely they belonged to the cells with centrally-placed nuclei, since this is the case in chick oviduct, and since the other cells are thought to be secretory. In all epithelial cells, the nuclei were rounded and elongated.

By 15 days of estrogen treatment (refer to Plate 6), the basally-situated nuclei of the secretory epithelial cells were no longer elongated, but appeared to be pushed against the basement membrane. In some cases these cells seemed to be involved in the process of secretion. The centrally-placed nuclei of ciliated cells were rounded, and the cilia were much more prominent.

Oviducts treated for 10 days with estrogen and then 5 days with progesterone (refer to Plate 7) occasionally contained many unstained areas, suggesting that secretion of some of the granules into the lumen had occurred. The granules were so abundant and so darkly-staining that it was difficult to locate the nuclei of the tubular gland cells. In some oviducts both the ciliated cells with central nuclei and the secretory cells with basal nuclei had intensely-stained, compressed nuclei. In other oviducts, only the basal nuclei of the secretory cells were compressed, while the central nuclei of
Plate 4a. Cross-section of an oviduct magnum from a bird treated for 5 days with estrogen. x 168. Note the tubular glands (TG), vascularization (BV). Fixed in glutaraldehyde and embedded in plastic. 1 micron.

Plate 4b. Same, x 800. Note occasional ciliated cells (C), points of entry of tubular glands into the lumen (arrows), and presence of eosinophilic granules (G) in tubular gland cells.
Plate 5a. Cross-section of an oviduct magnum from a bird treated for 10 days with estrogen. x 168. Note abundance of glands and granules, and increase in size and vascularization. Fixed in glutaraldehyde and embedded in plastic. 1 micron.

Plate 5b. Same, x 800. Note vascularization (BV) near epithelium (E), abundance of cilia (C) on epithelium, and presence of two cell types in epithelium. Granules in tubular gland cells stain with many different intensities.
Plate 6a. Cross-section of an oviduct magnum from a bird treated for 15 days with estrogen. x 168. Compare with Plate 4a for an indication of increase in size over the treatment period. Fixed in glutaraldehyde and embedded in plastic. 1 micron.

Plate 6b. Same, x 1010. Note darkly-staining basal nuclei of goblet cells (C) and profuse cilia (C) of epithelium. Granules of the tubular gland cells appear to stain with same intensity.
Plate 7a. Cross-section of oviduct magnum from a bird treated for 10 days with estrogen, followed by 5 days of progesterone. x 168. Note unstained areas. Fixed in glutaraldehyde and embedded in plastic. 1 micron.

Plate 7b. Same, x 672. Note compressed appearance of nuclei and darker-staining cytoplasm of ciliated cells (C), and goblet cells (GC) in process of secretion. Lumen = L.
the ciliated cells were not. Others have also reported that the ciliated cells become extremely compressed between the secretory cells, because the latter become engorged with secretory products (56). Cilia appeared in some cases to be quite long and dense, and darkly-staining.

C. Animals Withdrawn from Hormone Treatment

Oviducts of animals withdrawn from estrogen plus progesterone treatment were smaller than those withdrawn from estrogen treatment. Of the oviducts withdrawn from estrogen plus progesterone treatment (refer to Plates 8 and 9), some were larger than others, although there were few if any histological differences between these two sizes of oviducts. Vascularization was still evident, although somewhat lessened, and there were fewer and smaller tubular gland cells. There were no distinct granules in any of the tubular gland cells, but some contained an eosinophilic material, whereas others appeared empty, in that their centers were entirely unstained. There appeared to be more single cells in the stroma which were not organized into tubular gland cells. The pseudo-stratified epithelium appeared to be in a differentiated state: it was profusely ciliated, and contained cells with central, light nuclei, and some with basally-situated, darkly-staining nuclei. The nuclei of all the epithelial cells were rounded and elongated, rather than the dark, compressed nuclei of the estrogen plus progesterone treated oviduct epithelium. There were no evidences of secretions in any of the epithelial cells (refer to Plate 8b).

Those oviducts withdrawn from estrogen plus progesterone treatment which were larger, appeared histologically to be quite similar to the smaller ones just described (refer to Plate 9). These showed greater vascularization,
and in addition had large areas of the mucosal folds which contained neither cells nor blood vessels. They also contained many tubular glands with unstained centers. Although the general appearance of these seemed less healthy than the smaller oviducts, it should be pointed out that these were fixed in Bouin's rather than glutaraldehyde. Bouin's is generally a poorer fixative (76).

Oviducts from animals withdrawn from estrogen treatment were all larger than those withdrawn from estrogen plus progesterone treatment. These had still greater vascularization than those withdrawn from progesterone treatment. In addition, there were large unstained areas in the center of the folds, as well as some tubular glands which were empty-appearing. These oviducts had the same general appearance, but contained many more tubular glands, and fewer tubular glands with unstained centers. Epithelial differentiation in these oviducts withdrawn from estrogen treatment did not differ significantly from that described for oviducts withdrawn from estrogen plus progesterone treatment.

It is evident from these histological results that the quail oviduct exhibits the three characteristics common to developing systems: growth, morphogenetic movement, and cell differentiation. These changes compare to those reported to occur in the chick oviduct as a result of estrogen and progesterone treatment. Estrogen is known to cause, initially, changes in the stroma, dispersing stromal cells, and to cause water imbibition and hyperemia (41). The vascularization of the region is followed by blood cell invasion of the stroma (41). After 2 to 3 days, tubular gland cells begin to bud off from the epithelium (41, 45, 48). A proto-differentiated state (42) has been
Plate 8a. Cross-section of an oviduct magnum from a bird treated for 10 days with estrogen, 5 days with progesterone, and then withdrawn from treatment for 10 days. x 550. Note empty-appearing tubular gland (arrows), blood cells (BC) and generally unorganized appearance of stroma. Epithelium retains cilia (C). Fixed in glutaraldehyde and embedded in plastic. 1 micron.

Plate 8b. Same, x 1606. Ciliated (C) pseudo-stratified epithelium (E) remains differentiated into two cell types. Although basally-situated, darkly-staining nuclei are a characteristic of secretory goblet cells, no evidence of secretion can be seen. Nuclei are elongated, and no longer compressed. Note invagination of epithelium (arrow) which may have been former opening of a tubular gland cell into lumen (L).
Plate 9a. Cross-section of an oviduct magnum from a bird treated for 10 days with estrogen, 5 days with progesterone, and then withdrawn from treatment for 10 days. x 168. This oviduct was one of the smaller ones from the group. Note empty-appearing tubular glands (arrow), vascularization (BV) and presence of some tubular gland cells with eosinophilic centers. Fixed in glutaraldehyde, embedded in plastic. 1 micron.

Plate 9b. Same, x 168. This was one of the larger oviducts from the group. Legend as above. Note difference in size, in comparison to 9a. Fixed in Bouin's, embedded in plastic. 1 micron.

Plate 9c. Same as 9b, x 550. Note ciliated cells (C) in pseudo-stratified epithelium, and epithelial invagination (arrow), perhaps representing former site of entry of tubular gland into lumen (L).
noted by Palmiter and Wrenn (43), with incomplete indentations of the epithelial cells into the stroma. These proto-differentiated cells continue to form tubular gland cells when estrogen is present (43). Microfilaments are essential for this process of budding (44), which is later accompanied by glandular mitosis as well (45). Budding continues for a number of days, and when it is complete, the luminal epithelial cells differentiate to form ciliated cells and goblet secretory cells (43, 45, 46, 47). The former contain centrally-located, palely-staining nuclei, and the latter contain dark, apically-situated nuclei (41, 45) as reported above for quail.

Progesterone treatment has been shown by Oka and Schimke to prevent the differentiation of tubular gland cells (45). However, that work demonstrated that the effects of progesterone depended on the stage of differentiation at which it was administered (45). Thus, if progesterone was given concomitantly with estrogen from the beginning of treatment, it abolished the tubular gland cell differentiation and therefore the growth of the oviduct. However, if the onset of progesterone treatment was delayed until later stages of estrogen treatment, it had less inhibitory effect, since it did not interfere with the tubular gland cells once they were differentiated (45). This latter fact would explain why progesterone in the present study demonstrated no antagonism: it is begun only after prolonged estrogen treatment, at a time when tubular gland cell differentiation may already be complete.

Progesterone is known to enhance epithelial differentiation in chicks (47, 48). This is the case even if estrogen is not given first (47). This result was not indicated in the present work with quail, perhaps because prolonged estrogen treatment has the same effect (41, 45, 48). Thus, in this study, the 15-day estrogen treatment and the 10-day estrogen, 5-day progester-
one treatment experiments did not show great differences in epithelial differentiation, but small differences did exist. In the estrogen-plus-progesterone treated oviducts, the nuclei of both the secretory cells and the ciliated cells were very compact and darkly-staining, not oval. In some cases, the cytoplasm as well as the nuclei of the ciliated cells was compressed between the secretory cells.

In tissue withdrawn from treatment, smaller oviducts with fewer tubular gland cells were seen, which supports the early indications of Strott (54) and of Fitzgerald (55), and the more recent investigations of Eroschenko and Wilson (56) on regressing quail oviducts. Those reported results indicated that cells are lost during regression, a time when estrogen is diminished in the animal due to decreased gonadotropin release (67, 68, 69). The epithelial cells appear to remain differentiated since they are reported by Eroschenko and Wilson (56) to retain cilia for up to 3 weeks after oviducal regression is induced in adult animals by reduction of daylight.

In chicks, withdrawal from hormone treatment was initially reported to result in no loss of cells, but only a reduction of cell size (45, 48). However, Yu et al (51, 52) have reported that DNA content is greatly reduced during regression in chick oviduct, and more recently, Palmiter (77) has reported that tubular gland cells make up 80% of the cells of a treated oviduct, but only 20% of the cells of an oviduct withdrawn from treatment. Therefore it appears that the chick oviduct also loses cells upon hormone withdrawal.

II. Assays of Total Growth: Weight, Protein, RNA and DNA Content

Estrogen caused a dramatic increase in the growth of the oviducts, resulting in a 700-fold increase in wet weight (Figure 3) from 2.5 mg on the
first day of treatment to an average value of 1.7 gm after 15 days of estrogen treatment. In birds receiving progesterone rather than estrogen for the last 5 days of treatment, the average oviducal weight reached 2.0 gm. The greatest rate of increase was between days 5 and 10 of treatment; the response slowed somewhat between days 10 and 15 of treatment.

Ten days after withdrawal from estrogen treatment, the wet weight had decreased only slightly. This difference is not significant (p = 0.04). However, oviducts which had received 10 days of estrogen treatment followed by 5 days of progesterone treatment, lost about 85% of their wet weight in the same 10-day withdrawal period, thus demonstrating a far greater decrease than the oviducts from birds withdrawn from estrogen treatment.

Similar changes were seen in the protein (Figure 4) and RNA (Figure 5) content of the oviducts. The greatest increase was always between 5 and 10 days of treatment. The increase observed when progesterone replaced estrogen from 10 to 15 days of treatment may be significant for the change in RNA content (p = 0.01), but is not significant for the change in protein content (p = 0.04). The concentration of protein and RNA per unit weight did not increase as greatly as the total amount of each component, due to the concomitant increase in wet weight (see Table I). The concentration of RNA remained fairly constant throughout treatment, whereas the protein concentration showed less variance and a greater increase. The changes in RNA and protein concentration throughout treatment were not significant. However, total protein and total RNA content increased approximately 1000-fold and 500-fold respectively over control values in the 15 days of estrogen treatment, indicating again the very rapid growth of the oviduct.
Figure 3. The Variation in Wet Weight as a Function of Treatment

Legend: o , treatment with estradiol.

● , treatment with progesterone.

----------, withdrawn from treatment.

..........., control oviducts.

Bars represent standard error.

Figure 4. The Variation in Protein Content as a Function of Treatment.

Legend as above.
Fig. 3: Time vs. Wet Weight

Fig. 4: Time vs. Protein Content
Figure 5. The Variation in Oviduct RNA Content as a Function of Treatment.
Legend as in Figure 3.

Figure 6. The Variation in Oviduct DNA Content as a Function of Treatment.
Legend as in Figure 3.
Fig. 5: Time vs RNA Content

Fig. 6: Time vs DNA Content

mg RNA/Oviduct

Day of Treatment

mg DNA/Oviduct

Day of Treatment

Treatment → Withdrawal

Treatment → Withdrawal
After 10 days of withdrawal from estrogen treatment, approximately one-third of the protein content, and one-half of the RNA content of the oviduct were lost. In contrast to this, in the oviducts that had received estrogen plus progesterone treatment, approximately 90% of both the RNA and the protein content was lost after 10 days of withdrawal. This greater rate of decrease after withdrawal from estrogen plus progesterone corresponds to the greater decrease in the wet weight of the organ when withdrawn from estrogen plus progesterone treatment rather than from treatment with estrogen only.

The changes in the DNA content (Figure 6) as a result of treatment varied from the other tissue components in 2 ways: 1) This is the only tissue component not increased by estrogen plus progesterone treatment relative to continued estrogen treatment. The DNA content of estrogen-plus-progesterone treated oviducts varies only slightly from the 10-day estrogen value, indicating that progesterone does not stimulate DNA synthesis, at least not at this point in the differentiation of the tissue. The concentration of DNA actually decreased significantly with progesterone treatment (see Table I). 2) Upon withdrawal from estrogen treatment, a loss of approximately 50% of the DNA content was noted, as in RNA content, but a loss of only two-thirds of the DNA content was noted in the oviducts withdrawn from estrogen plus progesterone treatment. This is in contrast to the loss of 90% of the RNA and protein content, and 85% of the wet weight in these same oviducts, indicating that less DNA was lost than other components. The concentration of DNA actually increased after withdrawal from progesterone, whereas it decreased after withdrawal from estrogen (see Table I). This concentration increase may be an indication of the greater loss of other components, such as wet weight and protein.
Table I. Summary of the Variations in the Content and Concentrations of Tissue Constituents in the Oviduct at Different Stages of Treatment.

<table>
<thead>
<tr>
<th>No. in sample</th>
<th>Treatment</th>
<th>Protein Mg/oviduct</th>
<th>Protein/wt Mg/gm</th>
<th>RNA Mg/oviduct</th>
<th>RNA/wt Mg/gm</th>
<th>DNA Mg/oviduct</th>
<th>DNA/wt Mg/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>5dE</td>
<td>41.5±1.3</td>
<td>98.52±3.24</td>
<td>4.96±0.38</td>
<td>11.86±0.52</td>
<td>2.49±0.09</td>
<td>6.01±0.19</td>
</tr>
<tr>
<td>9</td>
<td>10dE</td>
<td>185.08±9.48</td>
<td>137.26±3.13</td>
<td>15.72±0.86</td>
<td>11.73±0.56</td>
<td>8.18±0.27</td>
<td>7.86±0.18</td>
</tr>
<tr>
<td>9</td>
<td>15dE</td>
<td>309.33±17.77</td>
<td>188.82±15.18</td>
<td>20.43±0.95</td>
<td>12.40±0.68</td>
<td>11.81±0.52</td>
<td>11.82±0.56</td>
</tr>
<tr>
<td>4</td>
<td>10dE5dP</td>
<td>402.00±60.76</td>
<td>201.51±35.96</td>
<td>25.06±1.51</td>
<td>12.24±0.34</td>
<td>9.70±0.46</td>
<td>4.75±0.19</td>
</tr>
<tr>
<td>5</td>
<td>15dE10d0</td>
<td>204.80±16.92</td>
<td>147.99±3.16</td>
<td>12.73±1.59</td>
<td>9.08±0.32</td>
<td>5.37±0.36</td>
<td>3.97±0.37</td>
</tr>
<tr>
<td>5</td>
<td>10dE5dP10dO</td>
<td>48.48±9.13</td>
<td>155.23±8.15</td>
<td>2.82±0.37</td>
<td>9/30±0.52</td>
<td>2.93±0.33</td>
<td>9.74±0.40</td>
</tr>
</tbody>
</table>

Controls:

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0dC</td>
<td>0.0229</td>
<td>91.6</td>
<td>0.0222</td>
<td>8.9</td>
<td>0.0255</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>5dC</td>
<td>0.342</td>
<td>83.2</td>
<td>0.0443</td>
<td>10.8</td>
<td>0.0408</td>
<td>9.95</td>
<td></td>
</tr>
<tr>
<td>10dC</td>
<td>0.715</td>
<td>110.0</td>
<td>0.0485</td>
<td>7.46</td>
<td>0.067</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>15dC</td>
<td>0.757</td>
<td>85.2</td>
<td>0.0690</td>
<td>7.75</td>
<td>0.94</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>25dC</td>
<td>2.00</td>
<td>88.8</td>
<td>0.214</td>
<td>9.56</td>
<td>0.258</td>
<td>11.5</td>
<td></td>
</tr>
</tbody>
</table>
Control oviducts also increased in these components during the treatment period, although to a very slight extent. This may be due to very slight, initial estrogen-induced changes, or it may be the result of the general growth of the animal.

These results are comparable to those reported for the effect of estrogen on chick oviduct (41, 45, 46, 48, 51, 52). Estrogen treatment for 10 days caused an increase in all parameters in chick oviduct also, but there is a slight difference between the chick oviduct work and the data presented here for the quail, in the variance observed in the response. There is somewhat less variance in the work of Oka and Schimke (45), who used only the magnum portion of the chick oviduct for total growth assays, and in the work of Yu et al, who used whole oviducts. Yu et al (46, 51, 52) reported the value and standard deviation for DNA and RNA concentrations (as percent of dry matter) for pooled samples of whole oviducts to be 1.8±0.1% for DNA and 2.3±0.1% for RNA. These represent a smaller variance than that reported here, but the results are not strictly comparable, since representation in this thesis is percent of wet weight. No variance is indicated in their paper for total DNA content per oviduct. This slight difference in variance can probably be attributed to differences in experimental procedures, such as time schedule of injections, dose, and species differences.

Progesterone appeared to exert no antagonistic effect in this system, as has been reported for chicks. However, as mentioned earlier, the effect of progesterone on the estrogenic response depends on the stage of differentiation at which it is administered (45, 48, 43). The means by which progesterone inhibits the estrogenic growth response is by abolishing tubular gland cell differentiation (43), since these cells make up the vast bulk of the magnum.
However, if progesterone is given after these cells are already present, it does not inhibit, but actually enhances their functioning (45, 48). Thus if progesterone is given after estrogen induction, one would expect to see an increase in such tissue components as weight, protein, and RNA content, but not DNA content, as progesterone inhibits formation of new tubular gland cells. The results presented here support that prediction. The differences between oviducts treated for 15 days with estrogen, and those treated for 10 days with estrogen, 5 days progesterone are small. In the amount of total tissue constituents, the differences between these two groups are not significant (weight, p = 0.014; RNA, p = 0.022; DNA, p = 0.03; protein, p = 0.239). In the concentrations of tissue constituents, oviducts from these two groups are not different in protein concentration (p = 0.703), or RNA concentration (p = 0.84), but are significantly different in DNA concentration (p = 0.002). This would be due not only to the relatively greater synthesis of DNA in estrogen treated oviducts, but also to the relatively greater synthesis of protein and RNA in the estrogen plus progesterone treated oviducts. This is in keeping with the known effects of progesterone in chick oviduct, in inhibiting new tubular gland cell synthesis and in enhancing the functioning of those cells already present.

Withdrawal of hormone leads to a loss of all components, but DNA was lost to a lesser extent than any other tissue component. A greater loss occurred in all components after withdrawal from estrogen plus progesterone treatment than after withdrawal from estrogen. This may be due to 1) after receiving progesterone instead of estrogen for the last 5 days of treatment, these oviducts have actually been withdrawn from estrogen for 5 extra days; or 2) progesterone may be acting in such a manner as to make the tissue less
stable to a loss of hormone. If oviducts withdrawn from estrogen plus pro-
gesterone treatment are actually regressed to a greater extent because they
have been withdrawn from estrogen treatment 5 extra days, then this implies
that progesterone is less efficient as a long-acting stimulator than is
estrogen. This may be due to 1) Estrogen could precipitate out at the site
of injection, whereas progesterone, being more soluble, would not. This
would create a "depot" of estrogen, but not of progesterone, which would be
lost in the 5 days of progesterone treatment before withdrawal. 2) Progester-
one may be metabolized more efficiently than estrogen. This is known to be
the case in nearly all animals (78). 3) Estrogen could be associating with
lipids in the serum, which would create another type of "depot" (165).
Progesterone, being more polar, may not. This has been reported in rats (79).
In this consideration, it is relevant that estrogen induces serum lipemia in
chicks (80, 73, 170). The effects of progesterone on serum lipemia are not
known.

On the other hand, if progesterone is acting to make the tissue less
stable to a loss of hormone, this could be either by inducing greater meta-
bolism of estrogen and of itself, or by inducing a specific serum binder,
which would reduce the amount of active steroid.

The results of this study do not allow a choice between these possibili-
ties. All of them are likely. These results do indicate however, that those
oviducts from birds withdrawn from estrogen treatment are at an earlier
stage of withdrawal than those from birds withdrawn from estrogen plus
progesterone treatment.

The results reported here support those of Yu et al (51) for the chick
oviduct, and Eroschenko and Wilson for the quail oviduct (56). The study of
Eroschenko and Wilson reported only histological changes, but indicated that tubular gland cells were lost during regression of adult oviducts. The report of Yu et al (51) established that the DNA content of the chick oviduct varies from an average of 11,456 μg in a developing oviduct to 83,087 μg in a laying hen, to 17,486 μg in a regressing oviduct. These are contrary to the early reports of Oka and Schimke (45, 48), that the cells remained during hormone withdrawal, but ceased functioning. More recently, Palmiter has also indicated that, contrary to early reports, the percentage of tubular gland cells in oviducts withdrawn from treatment is only 20% as compared to 80% after 6 days of readministration of estrogen (77). Thus it appears that in the absence of hormone, not only cell functioning, but eventually the cells themselves are lost.

The pattern of loss of the various tissue components after withdrawal was not investigated in detail in this work, but in the study of Oka and Schimke (48), for chick oviduct, two different patterns were seen for the loss of components on hormone withdrawal. When hormone was withdrawn prior to day 10 of treatment, each component continued to increase until what would have been day 10 of treatment, then declined. If withdrawal began on day 10 or later, this continued increase did not occur, but an immediate decrease was seen in each component. The authors attributed the continued increase to a precipitation of estrogen at the site of injection, but did not explain why it did not occur after day 10, which presumably would have allowed for even more estrogen to precipitate. It may be that the treatment was inducing a serum binding protein after day 10, which would more effectively remove free estrogen from the circulation, and make the loss of hormone to the tissue more immediate. Alternatively, a new type of metabolism may have been induced. Thus, the pattern of loss of tissue components on withdrawal may depend on the
length of treatment prior to withdrawal, as well as the steroid used during treatment.

Withdrawal from estrogen plus progesterone treatment has not been reported in other studies. However, the results presented here concerning the withdrawal from estrogen treatment are at variance with those reported by Oka and Schimke (48). Those authors reported that 12 days of withdrawal after 10 days of estrogen treatment results in a far greater loss of wet weight (76% decrease) and RNA content (82% decrease) than DNA content (24% decrease). In this study, approximately the same amount of DNA, RNA and protein were lost during withdrawal from estrogen treatment.

The greater loss of weight and RNA in their study compared to this one may be due to the shorter treatment and the longer withdrawal period. However, that does not explain why, in their study, less DNA was lost than other components, whereas in this study, DNA was lost to the same extent as the other tissue components. It may be possible to explain this discrepancy by observing the pattern of withdrawal more closely. Oka and Schimke (48) demonstrated that initially, DNA is lost at a rate similar to lysozyme and wet weight, but that within 5 days after the beginning of withdrawal, the rate of loss of DNA had slowed greatly. In this study, estrogen was administered for 15 days, and it is probable that stimulation continues beyond that time as a result of the "depot" effect discussed earlier. These oviducts would therefore be in an earlier stage of withdrawal than those of Oka and Schimke. Initially, the rate of loss of DNA is similar to that of weight or lysozyme, but the rate slows greatly after about 5 days of withdrawal (48). If it is assumed that the oviducts from birds withdrawn from estrogen treatment in this study are in an earlier stage of withdrawal, due to the lengthier treatment,
than those of Oka and Schimke, then these results are compatible with those.

This same assumption would explain why oviducts from birds withdrawn from estrogen treatment lost about the same amount of DNA as RNA or protein, whereas those oviducts from birds withdrawn from estrogen plus progesterone treatment lost less DNA than RNA or protein.

III. Specific Proteins

Ovalbumin

Ovalbumin purified as described under Materials and Methods was demonstrated to be impure by Sephadex G-200 gel filtration (see Figure 7). It was therefore not useful in preparing a standard for the quantitation of antigen-antibody precipitates. Although it is approximately the same molecular weight as chick ovalbumin, as judged by the elution profile on Sephadex G-200, these two proteins are not completely immunologically identical, as can be seen from the Ouchterlony plate (refer to Plate 10a). This precludes the use of chick ovalbumin to construct a standard curve. In addition, as seen in Plate 10a and b, the antibody preparation is impure; there are two precipitating species, one of which is partially identical with chick ovalbumin. The second precipitating species has not been identified. That this second species is not simply an artifact of concentration is evident by the fact that it can be diluted out by serial dilution, to a point where the first species is still evident, but not the second (see Plate 10b).

Therefore, by demonstration of partial identity with chick ovalbumin, and identity with quail ovalbumin, ovalbumin has been detected in all treated oviduct magna, in all of the magna from animals withdrawn from estrogen treatment, and in one-half of the magna withdrawn from estrogen plus proges-
Figure 7. Elution Profile of Ovalbumin on Sephadex G-200.

1.5 ml of chick or quail ovalbumin (4 mg/ml) was applied to a column of height 60 cm, and diameter, 1.5 cm, and eluted with Tris buffer at a flow rate of 0.1 mg/min. Fractions of 2.1 ml were collected and absorbance at 280 nm was determined for each fraction.
Fig. 7  Elution Profile of Ovalbumen on Sephadex G-200
Plate 10a. Ouchterlony plate demonstrating the reaction of anti-chick ovalbumin antibodies with chick ovalbumin (CO), quail ovalbumin (QO), and magnum extract (ME) from birds withdrawn from estrogen treatment. 5 μl of Antibody preparation (1 μg antibody protein/ml) was applied to the center well, and 5 μl each of chick ovalbumin (100 μg/ml) quail ovalbumin (500 μg/ml) or oviduct extract (1%) were applied to the appropriate outer wells.

Plate 10b. Ouchterlony plate demonstrating the reaction of anti-chick ovalbumin with chick ovalbumin, quail ovalbumin, and magnum extract from birds treated for 10 days with estrogen. Legend and protocol as above. Serial dilutions: 1) 2% extract; 2) 1% extract; 3) 0.2% extract; 4) 0.1% extract.

Plate 10c. Ouchterlony plate demonstrating the reaction of anti-chick ovalbumin with chick ovalbumin, quail ovalbumin, and a magnum extract from a bird withdrawn from estrogen plus progesterone treatment. Legend and protocol as above. This is an example of a negative reaction.
terone treatment (refer to Plate 10c). No ovalbumin was detected in any of the control oviducts. The lower limit of the assay was 25 μg ovalbumin/ml.

Lysozyme

The lower limit of sensitivity of the assay for lysozyme is 2 μg/ml (see standard curve in appendix). The readings at the lower limit of the standard curve are therefore subject to a great deal of error. Concentration of the homogenate would have resulted in higher readings subject to less error, but this was not possible, due to the very small size of the control oviduct magna.

In recognition of this problem, it is concluded only that a trend is seen in lysozyme concentrations (see Figure 8) appearing (given the limitations mentioned), first on day 10 of estrogen treatment, and perhaps increasing when progesterone is substituted for estrogen between days 10 and 15 of treatment.

Lysozyme was present in one-half of the oviducts withdrawn from estrogen treatment, but in none of the oviducts withdrawn from progesterone treatment.

Estrogen therefore induced the synthesis of the egg-white proteins, ovalbumin and lysozyme, in quail oviduct. This has been reported to be the case in chicks as well (45, 48), and the synthesis of these proteins has been localized in the tubular gland cells (71). In chicks, only estrogen can induce the synthesis of these proteins during primary stimulation, whereas progesterone is also able to induce their synthesis during secondary stimulation (53). In this study, estrogen followed by progesterone treatment appears to cause a relatively greater lysozyme synthesis than continued estrogen treatment. However, due to the problems inherent in the lysozyme assay,
Figure 8. Variations in Lysozyme Concentration of Oviduct Magna as a Function of Treatment. Legend as in Figure 3.
Fig. 8: Time vs. Lysozyme Concentration

Treatment → Withdrawal

μg/mL Lysozyme / g.m. Magnum

Day of Treatment

0 5 10 15 20 25
which would cause a smaller than actual measurement at the lower end of the scale, this interpretation of the data presented here would probably not be valid.

An earlier genetic study of avian proteins indicated that proteins electropheretically similar to ovalbumin and lysozyme could be obtained from quail egg-white (85), and a similar, more recent study (141) has indicated that estrogen injection can induce the presence of ovalbumin in the oviducts of resting birds. These results support those studies.

The results reported here as the effects of withdrawal of treatment are at variance with those reported for chick oviduct. It has been shown that withdrawal of hormone results in rapid cessation of synthesis of cell-specific proteins, and that after 10 days of estrogen treatment and 10 days of withdrawal, the content of ovalbumin and lysozyme in the chick oviduct has diminished (45, 48). Yu et al have also demonstrated that during regression, hen oviduct magna do not make these proteins (52). It was therefore surprising to find that of the quail oviduct magna withdrawn from estrogen treatment, all contained ovalbumin, and one-half contained lysozyme. Of the magna withdrawn from estrogen plus progesterone treatment, one-half contained ovalbumin, and none contained lysozyme. This information is presented in Table II.

There are two possible explanations for this result: either some birds were regressing faster than others, or some of the birds had begun estrogen secretion naturally. The latter would normally be the case in birds of this age, as evidenced both by the stromal changes noted in control birds, and the report of Fertuck and Newstead (66) that tubular gland cells are present at 30 days of life in untreated quail. If this was the case, then as soon as the estrogen level fell to a point low enough to release the hypothalamus from
Table II

Summary of Weights, and the Presence or Absence of Specific Proteins in the Oviduct Magna of Birds Withdrawn from Treatment.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.57</td>
<td>+</td>
<td>+</td>
<td>0.25</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>0.77</td>
<td>+</td>
<td>+</td>
<td>0.20</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.67</td>
<td>+</td>
<td>+</td>
<td>0.24</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.33</td>
<td>+</td>
<td>-</td>
<td>0.21</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.17</td>
<td>+</td>
<td>-</td>
<td>0.10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.26</td>
<td>+</td>
<td>-</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
inhibition in the experimental quail, ovarian growth and estrogen secretion should have begun. Although the level of estrogen produced by the animal would have been extremely low as compared to the amount injected, it is a well known fact that the secondary stimulation of an oviduct requires far less hormone, and gives a faster and greater response than the primary stimulation (53). This suggested small difference in estrogen secretion in half of the oviducts withdrawn from estrogen treatment would therefore have created a marked difference in both size and tissue components, including cell-specific proteins, as compared to those oviducts withdrawn from treatment which had not begun estrogen secretion. There is no way of knowing from these studies whether the ovalbumin present in the oviducts withdrawn from estrogen treatment and the lysozyme found only in the larger half of these oviducts was newly made or simply not yet degraded. However, the fact that the birds with larger oviducts also had obviously larger ovaries indicates that they may have begun estrogen secretion, rather than there being two different rates of regression to explain this dichotomy.

Although the oviduct magna withdrawn from estrogen plus progesterone treatment also fell into two groups, as mentioned, it should be noted that both of these two groups were much smaller in terms of wet weight, than those withdrawn from estrogen treatment. This result is not surprising, if one recalls that the withdrawal from estrogen plus progesterone treatment resulted in a far greater loss of all components of organ growth than did the withdrawal from estrogen treatment. None of the oviduct magna withdrawn from estrogen plus progesterone treatment contained lysozyme, and only the larger half of them contained ovalbumin.
Avidin

Avidin was present only in those magna which had received progesterone treatment, not in the oviduct magna from birds receiving estrogen treatment only, or the tissue from birds withdrawn from treatment. The average concentration of avidin was 60.9±19.5 μg/gm wet weight.

The possibility of the existence of an inhibitor of avidin-biotin binding was investigated by adding a known amount of avidin and an excess of biotin to the homogenates of estrogen-treated oviducts. An enhancement of binding (approximately 20% increase) was found under these circumstances, as compared to when the avidin and biotin were incubated in simple aqueous solution.

Avidin has long been known to be under the control of estrogen plus some other steroid in chicks (32), although the nature of the in vivo second agent remains in question (24). Avidin has been demonstrated to be present in the goblet cells of the magnum epithelium (50) only after progesterone or anti-estrogen administration to estrogen-treated chicks (50). In this respect, the results reported here support the data for the chick: avidin is present only in oviduct magna of birds treated with estrogen plus progesterone; even prolonged estrogen treatment does not induce the synthesis of this protein in quail tissue.

IV. Summary of Results

The results reported here can be summarized as follows: Estrogen greatly enhances the growth and differentiation of the quail oviduct. Within 5 days of treatment, the mucosal folds have grown to fill the enlarged lumen, and there are abundant tubular gland cells in these. Vascularization is evident. By 10 days, tubular gland cells have greatly increased in number, and are
engorged with granules, and differentiation of epithelial cells is evident. By 15 days of treatment, the granules of the tubular gland cells have become so large and numerous as to obscure the nuclei of the cells, and epithelial cell differentiation is quite pronounced. Growth, as measured by increases in wet weight, RNA, DNA, and protein content, has increased greatly in this period. The egg-white proteins of ovalbumin and lysozyme are also induced by this estrogen treatment.

The effects of progesterone, when added after 10 days of estrogen treatment, are a small increase in epithelial cell differentiation, and a small relative increase in all tissue components except DNA. The difference between 15 days of estrogen treatment and 10 days estrogen, 5 days progesterone treatment is small; this is believed to be due to the prolonged estrogenic stimulation. However, the progesterone treatment displays a definite antagonism for the continued DNA synthesis seen between 10 and 15 days estrogen treatment. This could be accounted for by the anti-estrogenic effects of progesterone, since these affect cell proliferation, but not cell function. Ovalbumin and lysozyme were also present during continued progesterone treatment. In addition, progesterone induced the appearance of the protein avidin.

Withdrawal of hormone treatment led to a loss of all tissue components. This loss was greater after withdrawal from estrogen plus progesterone treatment than after withdrawal from estrogen treatment, indicating that hormone is necessary for maintenance of the differentiated state, as well as for the life of the cells. Oviducts withdrawn from both treatments fell into two groups, one group being about twice the weight of the other, and containing some of the specific proteins. The smaller oviducts in the group withdrawn from
estrogen plus progesterone contained no ovalbumin or lysozyme. It is believed that this dichotomy represents the result of resumed or initiated estrogen synthesis by the animals which had larger oviducts. It is at this time that differentiation of the oviduct normally occurs, and although this would be a very low amount of estrogen secretion, it is well-documented that the secondary response requires far less hormone than the primary response (53).

At all times during treatment, the ovaries or testis of the treated birds were notably small, probably due to a decreased gonadotrophin output. In birds withdrawn from hormone treatment, the gonads were of larger size in those birds with larger oviducts, thus indicating again that these may have begun estrogen secretion.

An analysis of the oviduct system is obviously complicated by the fact that more than one steroid is involved in the differentiation of this tissue. From these results, and those reported elsewhere, the effects of estrogen can be summed as follows:

1) stromal swelling and dispersion, vascularization, and blood cell invasion;
2) differentiation of progenitor cells to protodifferentiated cells and tubular gland cells, with concomitant synthesis of ovalbumin and lysozyme;
3) after prolonged treatment, the differentiation of ciliated cells and goblet cells;
4) continued presence of estrogen is necessary for support of the differentiated state; withdrawal leads to loss of differentiation and of cells.
Response #1: Stromal changes occur initially without the occurrence of increased vascularization; that appears to happen later, after the edema and dispersion of the cells. Thus cell-cell interaction which might arise from invasion of the stroma by blood cell, is not a factor in the early stromal changes, although it may be a factor in the later changes and induction of tubular gland cells.

Response #2: The differentiation of the progenitor cells to tubular gland cells occurs after vascularization. Therefore, cell-cell interactions may be involved (41, 45), either with cells from the blood, or with the stromal cells themselves. By this time the stromal cells are swollen and dispersed and have migrated out to the periphery. The fact that ovalbumin synthesis appears to be concomitant with and not subsequent to morphological differentiation (43) indicates that the genes for both of these are expressed simultaneously rather than sequentially. This indicates that morphologic differentiation is not a necessary prerequisite to biochemical differentiation.

Response #3: Very little is known about the epithelial differentiation to ciliated cells and goblet cells, except that it normally does not occur until after prolonged estrogen treatment (45, 48), that is, after tubular gland cells have stopped budding off. These processes may therefore be mutually exclusive. However, this is not indicated by the fact that, if estrogen and progesterone are given together, protodifferentiated cells can differentiate into other epithelial cell types containing both secretory granules and cilia (43).

Response #4: Withdrawal leads to loss of differentiation and of the cells. This may be due to the fact that estrogen supports the vascularization of the tissue. This would not, however, explain the more immediate loss of cell
functioning seen on withdrawal of the hormone in chick oviduct (48), nor would it explain the sequential nature of cell death, since tubular gland cells are lost while epithelial cells still remain differentiated.

The effects of progesterone can be summed as follows:

1) Antagonism of estrogen-induced cell proliferation.
2) Synergism with estrogen-induced cell functioning.
3) Enhancement of epithelial cell differentiation to ciliated cells and goblet cells.
4) Induction of avidin synthesis.

Response #1: The antagonism to the estrogenic response, occurs at the proto-differentiated tubular gland cell stage, where continued differentiation of these cells to tubular gland cells is abolished, and therefore the growth of the tissue is interfered with (43). It is evident in this study as an interference with the continued DNA synthesis noted between 10 and 15 days of estrogen treatment. Obviously, then, if progesterone is given after the tubular gland cells have differentiated, or nearly so, this response will not be so evident as when it is given at the beginning of treatment.

Response #2: The synergistic response with estrogen-induced cell functioning (45, 48), noted in chick oviduct, is seen in the quail as a slight increase in all tissue components except DNA. Both this response and response #4 are measures of the effect of progesterone on protein synthesis, but avidin synthesis is different in that it occurs in different cells from those which carry on the bulk of the protein synthesis, and it has an absolute requirement for progesterone induction (50), which the synthesis of the other proteins does not. The mechanism of progesterone synergism with estrogen-induced specific protein synthesis is believed to be via an increased message transcription, for
some of these proteins (97).

**Response #3:** The enhancement of epithelial differentiation to ciliated cells and goblet cells may be related to the abolition of the tubular gland cell differentiation, since these do not normally occur simultaneously. That is, the simple abolition of the one may be the enhancement of the other. This enhancement of epithelial cell differentiation can be seen even in oviducts not treated initially with estrogen (47); but other than this there is no information available on the mechanism of action of progesterone in enhancing epithelial differentiation.

**Response #4:** Avidin synthesis occurs in the epithelial goblet cells of the chick oviduct (50), and is known to require progesterone or an anti-estrogen for its induction. The amount of avidin synthesis is also known to be dependent on the length of estrogen treatment given before progesterone treatment is begun (49).

All of these responses lead towards increased growth and functioning of the tissue except the first response to progesterone, the antagonism of estrogen-induced cell proliferation. It may therefore be wise to use caution in attributing this latter response to the same mechanism of action as the others.
DISCUSSION: AN OUTLINE

A. Discussion of Results of this Experimentation

B. The Response as a Phenomenon of Induction
   1. The Nature of the Cellular Response to Estrogen: Palmiter's Model
      a. The Evidence
      b. Discussion of the Model
      c. Comparison to Other Steroid-Responsive Systems
   2. The Response to Progesterone: O'Malley's Model
      a. The Evidence
      b. Discussion of the Model
   3. Events a General Model Will Have to Explain

C. Comparison of this Induction Phenomenon to Embryonic Induction
   1. The Inducer
   2. The Competence of the Responding Cells

D. A Brief Assessment of the State of the Field
A. The Results of this Experimentation

The results reported here indicate that dramatic growth takes place, and that gene expression is radically altered, as a result of estrogen treatment. This is evident both in the appearance of new cell types and of new proteins. This gene expression appears to have a temporal or sequential characteristic as well, since the stromal changes occur first, then tubular gland cells appear, and epithelial differentiation occurs last. The temporal nature of the gene expression is also indicated by the fact that ovalbumin synthesis is induced before lysozyme synthesis.

It is also apparent from these results that the inducer does not simply trigger an already pre-programmed cell, which then proceeds on its own in accordance with its programming. This is evident from two observations: 1) the two steroids have different effects, indicating specificity in the inducer, and 2) withdrawal of hormone leads to a loss of gene expression. This latter is indicated by loss of cells in oviducts withdrawn from treatment, as well as loss of ovalbumin and lysozyme in some of these oviducts. In addition, although the epithelium remains ciliated, it clearly changes from its appearance at the time of treatment.

This gene expression is therefore dependent on the continued presence of the inducer. Such a very high amount of synthesis of cell-specific proteins (as judged by histology as well as biochemistry) over so sustained a length of time, would imply that the inducer activates or enhances the activity of the genes involved in this synthesis. Other possibilities, such as masked messenger (translation control), or activation of a pre-protein
molecule, seem implausible when the magnitude of the response is considered. It therefore seems probable that the new gene expression seen here as a result of estrogen treatment is the result of gene activation, or greatly enhanced transcription of genes already active.

These results would therefore support any model for the mechanism of estrogen action which includes these possibilities. Such a model would be that of Palmiter and co-workers, whose original idea it was to examine the induction of one protein (ovalbumin) by one steroid (estrogen), and to test the possibility of control at each of the steps in the induction process (89). That is, investigations were carried out to determine if control was exerted at the level of message transcription, message processing, transport to cytoplasm, translation of message or message degradation. The results of Palmiter's investigations supported a model in which estrogen acts via gene activation rather than by way of a masked messenger or the activation of a pre-protein. The results reported in this thesis are in keeping with that model.

The response that has been reported here to progesterone when it is given after estrogen treatment is three-fold in nature: the induction of avidin synthesis, the support of continued growth and functioning, and the inhibition of continued DNA synthesis. Thus it is clear that the response to progesterone encompasses not only unique action, but action synergistic to estrogen action, and action opposed to estrogen action.

Whether or not all three of these types of actions are mediated by the same mechanism is not known. Taken separately, each action is of small enough magnitude that it could be explained in a number of ways. The induction of avidin synthesis, for example, could be explained by new gene
activity, by a masked messenger hypothesis, or by an activation of a pre-protein. Work on the chick oviduct which uses avidin induction as a marker for progesterone action has led to a model of progesterone action involving new gene transcription (90). These results are compatible with that model, but can neither support nor contradict it.

The actions of progesterone that are synergistic with estrogen action (that is, the enhancement of tubular gland cell functioning) could be explained by enhanced transcription of estrogen-activated genes, or enhanced processing of messenger from these genes, or enhanced translation of messenger, or decreased degradation of messenger or of protein. Work on the chick oviduct which investigates the action of progesterone in inducing the synthesis of the tubular gland cell proteins indicates that progesterone, when given alone, on 2° stimulation, is able to initiate new gene activation (71), and when it is given with estrogen, to enhance transcription of some of the messenger RNAs involved (97). The increased synthesis of proteins and all tissue components except DNA reported here support that hypothesis. However, progesterone was not given together with estrogen, but following estrogen. Thus it is not possible to indicate whether the response to progesterone is one of initiating gene activity, or simply continuance of estrogen-activated transcription. Since estrogen presence is necessary for continued transcription, it seems likely that both of these possibilities are occurring.

The action of progesterone that is anti-estrogenic in nature is the inhibition of DNA synthesis. In the chick oviduct, this is the cause of the abolition of tubular gland cell differentiation and the growth response. There has been no elucidation of the mechanism of this action. It has been
proposed that estrogen stimulates cell proliferation in a different manner than cell functioning, such that progesterone can interfere with the first process, but not the second. The results reported here are compatible with such a hypothesis, but can neither support nor contradict it. There are a number of possible mechanisms by which this action could be mediated. Progesterone may interfere with one or many estrogen-activated genes, which code for a protein or proteins essential for this process; alternatively, progesterone or a progesterone-induced gene product may interfere with the functioning of such a protein. That is, this may be the result of a nuclear or a cytoplasmic action of progesterone, of the progesterone-receptor complex, or of a product induced by it.

B. The Response as a Phenomenon of Induction

The changes that are reported in this thesis closely parallel those known for the chick oviduct, and it is suggested that so great a response as observed in both birds must encompass the expression of many different genes. Most of the work on estrogen and progesterone-induced differentiation of oviduct tissue has been directed toward an explanation of this gene expression. It is therefore relevant to examine at this point the induction of oviduct differentiation, to consider the work done on similar systems, and to critically examine the models of steroid action that have been proposed to explain this effect.

The results reported here support or are compatible with both the model for estrogen action, and the model for progesterone action in chick oviduct. The first, based on Palmiter's work, hypothesizes that estrogen acts at the level of transcription, to independently induce activation of many genes (89).
The model for progesterone action, based on O'Malley's work, indicates that progesterone binds to its receptor, enters the nucleus, and there binds to acidic chromatin proteins, so activating new transcription (90, 91). Both models or hypotheses therefore have at base an activation of new transcription, although they are based on entirely different data.

Both Palmiter's hypothesis for estrogen action and O'Malley's for progesterone action on chick oviduct are extensions of the general model for steroid action (86-88). This general model is: that the steroid enters the cell, binds to a cytoplasmic receptor protein, enters the nucleus, and there acts in some as yet unknown way to alter transcription. Such receptor proteins have been demonstrated for estrogen, progesterone and testosterone in chick oviduct (139, 74, 75, 129-132), and for estrogen in quail oviduct (140). This mechanism of action is generally thought to be applicable to all cases of steroid induction in all organisms (86-88). The two specific cases mentioned here, of estrogen and progesterone in chick oviduct, are of relevance both because they represent leading work in the field, and because they relate directly to the problem presented here - the explanation of cellular response to a steroid inducer in the avian oviduct. These models will therefore be examined in terms of the evidence on which each is based, the controversial aspects of each, and how each compares to models for steroid action in mammalian systems.

The characteristics of this induction phenomenon will also be compared to those of embryologic induction phenomenon, in an effort to understand the common denominators of these processes.
1. The Nature of the Cellular Response to Estrogen: Palmiter's Model

When discussing the response of cells to the inducers, care must be taken to distinguish between the cell types and between the different stages of treatment as well. Two major classifications exist: the primary or initial response, and the secondary response, which is the response of a tissue that has been previously treated, withdrawn from hormone treatment, and then stimulated a second time. The primary response of the chick oviduct to estrogen has been outlined in the introduction, and outlined in detail in the results section of this thesis. The major work on the action of estrogen on the oviduct has made use of the secondary response, since that is somewhat more specific, because it omits a large part of the growth response, and involves primarily a return to functioning of cells already present.

The knowledge gained has been the work of Palmiter and co-workers. It was the original idea of this group to examine the induction of one protein (ovalbumin) by one steroid (estrogen), and to test the possibility of control at each of the steps in the induction process (89). That is, investigations were carried out to determine if control was exerted at the level of message transcription, message processing, transport of message to cytoplasm, translation of message, and message degradation.

This work will be examined first in terms of the evidence that has been reported, second in terms of the model that the author presents to account for the evidence, and then in terms of a comparison of this model and evidence to other steroid-responsive systems.

a. The Evidence

The results obtained by Palmiter and others concerning the effect of
estrogen and other steroids on oviducts withdrawn from hormone are summarized in the diagram on the following page. These results include the following:

The secondary response is faster than the primary one; following a lag period of 3 hours, ovalbumin is detected and begins increasing in amount (53). The beginning of this response, Phase I, is represented by an organization of polysomes from existing monosomes, rather than as a result of new rRNA synthesis. This phase occurs in the first 12 hours, and can be elicited by either estrogen or progesterone, or both together (53). The second phase (Phase II) is represented by continued increases in all constituents, with rRNA synthesis contributing new ribosomes to the pool of old and new ribosomes from which polysomes are drawn (53). Phase II can be elicited by estrogen only, not by progesterone, but progesterone with estrogen does not inhibit the response, rather, they act synergistically (53).

Ovalbumin is apparently synthesized and released at the same rate as other proteins, but its synthesis cannot be initiated in vitro (53). Ovalbumin message was isolated from ovalbumin-synthesizing polysomes by immunoadsorption to anti-ovalbumin antibodies, and was incubated with labelled precursors and viral reverse transcriptase, to make a labelled gene for ovalbumin (92, 93). Although consisting of only 200 nucleotides, it was nevertheless possible to use this "probe" to hybridize to cellular DNA, and demonstrate that in the induction of ovalbumin, gene amplification does not occur (93).

Ovalbumin message was later isolated from total average polysomal RNA (94), and reverse transcriptase from avian myeloblastosis virus was used in the same manner (95). The product of that reaction was the same small size, but was used to demonstrate, by hybridization, that 93% of hybridizable
Figure 9. Proposed Control of Ovalbumin Synthesis.

1) Gene amplification does not occur. One copy per haploid genome.
2) Message accumulation at a rate of 28 molecules/min/tubular gland cell, after a 3-hour lag, by de novo transcription, or by increased activity of genes already active.
3) Other messages synthesized at different rates.
4) If other steroids are present, their synergistic effect is mediated via increased messenger accumulation, not decreased degradation, by multiple control of a single rate-limiting step, or by additional rate-limiting steps.
5) Processing is extremely rapid, or absent; no stable pre-message exists; no stable pre-message is stored in withdrawal.
6) Transport to the cytoplasm is not a rate-limiting step; there is no nuclear pool of message, so reactivation of a transport to cytoplasm is not occurring.
7) Initiation of protein synthesis is not rate-limiting; there is no cytoplasmic pool of translatable or non-translatable message.
8) Translation is enhanced by increased rates of initiation and elongation of polypeptide chains; this is not specific for ovalbumin.
9) There is no evidence of a cytoplasmic repressor.
10) 93% of the message is in the polysomes.
11) The synergistic effects of progesterone or testosterone with estrogen are not exerted at translation.
12) Estrogen may stabilize the message, since it is degraded upon withdrawal; nuclei from tissue withdrawn from treatment contain one copy per nucleus.

E = Estrogen; $R_c$ = Cytoplasmic receptor.
material was in the polysomes, 5% in the nucleus, and 2% in the non-polysomal cytoplasm. This indicated that there was no pool of non-translated messenger in the cytoplasm, either in translatable or non-translatable form, thus supporting earlier studies which had assayed messenger by activity in a cell-free system (96) (see also ref. 137, 122, 138).

Two percent of the nuclear hybridization was a high molecular weight species with a sedimentation coefficient of 30S (the message is 16S-18S) (95). If this very small amount indicates a primary transcription product, then rapid conversion to a translatable form is indicated; not via a stable pre-message (95). The appearance of message in the polysomes without accumulation in the nucleus first, indicates that estrogen is not reactivating a transport to the cytoplasm (95).

The close correlation of the kinetics of ovalbumin synthesis and the appearance of hybridizable and translatable messenger, together with the lack of a significant pool of messenger in the non-polysomal cytoplasm, indicate that the initiation of protein synthesis is not a limiting factor (95).

The estimated level of message in the cells during periods of hormone withdrawal was one molecule per nucleus, so that it was not possible to demonstrate conclusively whether or not synthesis occurred during periods of hormone withdrawal; that is, whether re-administration caused enhancement of existing transcription, or de novo transcription (95). The loss of messenger activity on withdrawal of hormone indicated that continued estrogen treatment was necessary for continued presence of messenger; the authors considered it possible that the message was unstable in the absence of estrogen (96).

Other proteins - ovalbumin, conalbumin, ovomucoid, and lysozyme - are all made in the same tubular gland cells, following the secondary administration
of estrogen (71). Progesterone can also induce all four proteins, although in lower amounts, and with different time courses (71). Progesterone causes preferential synthesis of conalbumin relative to ovalbumin, and the ovalbumin synthesis begins more rapidly, but reaches a steady-state at a lower level (71). Conalbumin is preferentially synthesized at lower doses of estrogen, and conalbumin and ovomucoid are both selectively enhanced by progesterone and estrogen together, and by testosterone and estrogen together, relative to estrogen alone. This occurs in spite of the fact that testosterone is inactive alone (71). This selective increase is not due to messenger stabilization relative to estrogen treatment only, as judged by decay in the presence of actinomycin-D (71).

The increase in protein synthesis on secondary stimulation by estrogen was partially the result of an increase in the rate of elongation of polypeptide chains in tissue from treated animals, as compared to that in animals withdrawn from hormone, and partially the result of an increase in the rate of initiation of protein synthesis (97). Initiation is rate-limiting in tissue that has been withdrawn from hormone, whereas elongation is rate-limiting in the treated tissue.

The synergistic effects of progesterone with estrogen, and of testosterone with estrogen, relative to estrogen treatment alone, on ovomucoid and conalbumin synthesis, are not due to an increase in rates of initiation or elongation (97). They are therefore believed to reflect an increase in the concentration of translatable messenger for these proteins (97).

The "superinductive" effect of actinomycin-D was demonstrated to be due to enhanced translation, not to increased messenger, which ruled out the
postulated cytoplasmic repressor of Tomkins (99), at least in this system (99, 96, 98). Enhanced translation was tentatively attributed to the relatively long half-life of the message, which would therefore be increasingly able to compete for rate-limiting factors, as other messengers died out (98).

The synergistic effect of testosterone and estrogen together relative to estrogen treatment alone was shown to extend to rRNA synthesis, and to an increase in RNA polymerase activity, which was considered to be the result of more growing chains, rather than increased elongation rate (100). This supports the conclusion that testosterone and estrogen together cause an increase in messenger concentration relative to estrogen alone, and indicates that testosterone selectively enhances some estrogen-activated genes (100), and therefore does not act entirely by the same mechanism that estrogen or progesterone do, since these latter steroids also initiate gene activity.

The initial rate of ovalbumin message accumulation, and the steady-state rate of accumulation, as measured by estimation of its synthesis and half-life, were calculated to be 22 and 34 molecules per minute per tubular gland cell, which Palmiter believes to be close enough to represent a single value of 28 molecules per minute per cell, implying a simple, single-step model of constant rate increase (77). He also notes, though, that the time lag of 3 hours between steroid administration and message accumulation speaks against a simple one-step model (77). From comparison with other systems, it was calculated that a single gene can support this rate of transcription even if only 30-50% of the products of transcription reach the cytoplasm (77). The data indicate control at the level of synthesis or activation of messenger, and suggest vast differences in the rates of synthesis or activation of different messengers (77).
The ovalbumin messenger appears to be heterogeneous, by its partial binding to Millipore filters (92, 94), and by the broad band range produced on acrylamide gel electrophoresis, covering a range of molecular weight of 100,000 (94). The preparation from which this ovalbumin message is isolated is total average polysomal RNA, purified by sucrose sedimentation and gel electrophoresis, rather than by immunoadsorption. The immunoprecipitation of the product of translation in a cell-free system does not allow a demonstration that the preparation is pure, although calculations based on the percentage of messengers that are excluded by the procedure, and the percentage of messenger that is ovalbumin messenger, indicate that the preparation must be highly selected for ovalbumin message (94). The molecular weight of the message is 875,000, as judged by electrophoresis, and 550,000 as judged by sucrose sedimentation (94). This corresponds to 2640 and 1670 nucleotides respectively, of which 1161 will be translated to ovalbumin (94). At least part of the large untranslated portion is represented by polyA; of the remainder, it is thought that this is transcribed with the message (95). It has most recently been shown that the message does not undergo any significant change in molecular weight after its initial transcription, indicating that processing is insignificant or absent (95, 163). This points more directly to the process of transcription as the control point in the induction of this protein.

Palmiter feels that the induction of the four major proteins mentioned is not coordinated by a single regulatory factor (71) because: 1) conalbumin synthesis does not diminish faster than ovalbumin synthesis, which would be expected from the shorter half-life of its messenger (although he notes that this may be due to the gradual drop in estrogen to the level where it
preferentially induces conalbumin synthesis; 2) different steroids have selective effects on the induction of the proteins, which are not at the level of message degradation or translation; 3) there are differences in estrogen threshold level required for conalbumin induction relative to ovalbumin induction; and 4) other steroids elicit only part of the total response; that is, they do not elicit rRNA synthesis, DNA synthesis, etcetera (71). He therefore does not feel that the response to other steroids can be both coordinated and selective. Although he thinks it likely that each protein is independently regulated (71, 101), no mention is made of the possibility of a few intermediates, or one for each steroid.

Although the single rate of accumulation of message speaks for a simple, one-step induction of ovalbumin message, Palmiter feels that the 3-hour time lag involved would allow for any event in a cycle of transcription-translation-feedback events, and so speaks against a simple one-step model (77).

b. Discussion of the Model

A model of coordinated response due to a single intermediate factor is not favoured by Palmiter, due to the preferential synthesis of conalbumin at low doses, its longer-than-expected rate of degradation, and the selective effects of other steroids, including the lack of effect of these on peripheral events such as rRNA synthesis, DNA synthesis, etcetera (71). Palmiter notes that the preferential synthesis of conalbumin at low doses, and its longer-than-expected rate of decay on withdrawal of hormone, may be related in that the slowly falling levels of estrogen at withdrawal may preferentially enhance conalbumin synthesis (71). He suggests a study in the presence of an anti-estrogen (71), but to my knowledge, no further studies along that line have
been conducted. It is of interest here, that conalbumin has also been noted in the blood of immature, non-treated birds (52, 38, 102), and is therefore not thought to be under strict estrogenic control (102).

The three-hour time lag speaks against a simple one-step model of estrogen action (77), but cannot indicate in any way whether the responses are coordinated or independent. It would only allow time for intermediate actions; this could be in keeping with both possibilities. Such intermediate actions could include any imaginable sequence of events, including changes in nuclear ion concentrations, which are thought to be a controlling element in ecdysone-induced puffing patterns in *Chironomous* salivary gland chromosomes (103, 104). Such a possibility should be viewed in the light of the known effects of ion concentrations on steroid receptors (105).

The selective effects of other steroids, including the lack of effect on peripheral events, may speak for independent control, but these effects are nevertheless not at variance with a model of coordinated control, such as the Britten-Davidson model of gene activity in early development (106). In that case, the action of other steroids alone or in combination with estrogen, could be selective, but also coordinated. If the Britten-Davidson model were to be applied to steroid induction, then one would expect, using the Redundant Receptor Model, that all genes activated by estrogen have a receptor region to bind the activator RNA; that is, they would all have a common sequence associated with them. If the Multiple Integrator Model is used instead, this would not be the case; there would be different activator RNAs for different genes, or groups of genes. Thus it is possible that a single entity, or a few such entities, could mediate the pleiotropic responses, and still allow for the selective effects of other steroids. This model does not account for
the preferential synthesis of conalbumin at lower doses of estrogen. It does require that each steroid operate by a different intermediate.

A model requiring the independent induction of the cell-specific proteins would account for the available data regarding their induction at different doses and different steroid combinations, but such a model is intellectually displeasing when extended to the obviously coordinated responses of the primary stimulation, or even to the complete secondary response. It may be that more than one model should be considered; that is, it may be that there is more than one mechanism of action involved.

Whether these responses are independently or coordinately controlled bears directly on the nature of the model. Palmiter has pointed out (77) that if there is a single rate-limiting control step in the induction of each of the four proteins, then it must be subject to multiple regulation, otherwise one must postulate additional rate-limiting steps to account for the various effects of different steroids. He feels, however, that the genes for each of these proteins may be independently controlled by the steroid(s) (71, 101). If this model were extended to the primary response, one would imagine that a model of coordinated control would require measurably less estrogen bound in the nucleus than a model requiring that each gene be individually activated by estrogen. Such an investigation, however, would be hampered by the assumption that there is no intermediate(s), and by the difficulties involved in estimating nuclear binding.

The implied assumption to this point in the discussion has been that the process being controlled is transcription. Recent work demonstrating the absence of any precursor for ovalbumin message supports this assumption (163).
Again, intellectually, a model of estrogen action relying on the independent activation of each gene by estrogen or its intermediate is displeasing, since it does not explain the coordination evident in the primary response, when differentiation, division, and specific protein synthesis are all induced. One would expect these responses to occur in an orderly sequence in time, but such a sequence has not been established in detail, due possibly to the high doses of estrogen used, which preclude a resolution of the finer aspects of the response. The model of independent gene regulation, however, has only been suggested for the regulation of the egg-white proteins. If a general model for steroid action is assumed, then it would have to explain not only the primary and secondary actions of estrogen, but those of progesterone as well, which include the distinct anti-estrogenic effects of progesterone when it is given concomitantly with estrogen in the primary stimulation.

c. Comparison to Other Steroid-Responsive Systems

Because this secondary response of oviduct tissue is not directly comparable to either the full growth and differentiation response of the oviduct or of mammalian tissues, nor to the simple response of alteration of a cellular metabolic pattern seen with the corticoids, it is difficult to know what system to use for a comparison to the three-hour time lag. On primary stimulation, the time lag is 24 hours for synthesis of ovalbumin; on secondary stimulation, this occurs after only 3 hours, although rRNA synthesis does not occur until 12 hours after stimulation. The synthesis of specific protein before rRNA synthesis (53) has been reported in other systems as well (107).

It should also be noted that this avian system is useful for the same reason that it is anomalous: for the presence of biochemical markers for
steroid action. Mammalian tissues which grow and differentiate in response to estrogen and progesterone do not make luxury proteins for export. At least one specific protein, a peroxidase, has been reported to be induced by estrogen in mammalian systems; however, this is not a tissue-specific protein (108).

These two major differences, that ovalbumin is a luxury protein made for export, and that this secondary response is neither one of full growth and differentiation nor a simple metabolic response, make this phenomenon hard to classify, and difficult to compare. The very early responses to steroids measured in mammalian responsive tissues are definitely not leading to the synthesis of luxury proteins, and are likely to be functional in the continuation of the response. This difference in biological function is great enough to warrant caution in assuming a direct comparison between the two types of systems.

Palmiter states that the time lag allows for any in a cycle of transcription-translation-feedback events (77), and investigations of the early responses of other tissues to steroids are in agreement with this possibility. A similar situation occurs in chick liver, where the synthesis of the protein phosvitin is induced by estrogen treatment 12 hours after primary injection, or 6 hours after secondary injection (110). During the first 2 hours, there is an incorporation of label into nuclear proteins, and the accumulation of two specific proteins was noted (107). One of these was a basic, non-histone protein, and the other was an acidic protein, both of low molecular weight (26,000 and 20,000 respectively). The increase in these two proteins was thought to be due to transport from the cytoplasm, rather than to new synthesis (107). An alteration of nuclear non-histone constituents has also been noted in the effects of cortisol on rat liver (81), the effect of estrogen on
rat uterus (82), and in numerous other systems (133).

In mammalian systems, where the type of response measured is of a slightly different nature, there is still some evidence of a time lag in the response. In rat uterus, the early responses, which are not directly associated with growth, can be elicited by estradiol or estriol (109). The growth can be elicited only by estradiol, or, if repeated injections are given, by estriol as well (156). Anderson et al felt that growth could be correlated with the amount of receptor-estrogen complex in the nucleus at 6 hours, after the initial injection, but not at 1 or 3 hours. That is, the action of the complex in the nucleus at 6 hours is different from its action in the nucleus at 1 or 3 hours (109). It is highly unlikely that there is any difference in the complex itself between 1, 3 and 6 hours, and therefore, a difference in the nucleus would be indicated, either in the quality, quantity or arrangement of its constituents.

It therefore appears unlikely that the induction of ovalbumin message is the result of an initial direct activation of the genes by the estradiol-receptor complex. Regardless of whether the gene is activated independently of other genes or coordinately with them its activation is not likely the initial action of the receptor-estrogen complex in the nucleus.

In summary, the induction of ovalbumin synthesis occurs at the level of transcription of the messenger for ovalbumin, and this may be the control point for the induction of the other major proteins as well. If this is the only control point in the induction of specific protein synthesis, then it must be subject to multiple regulation, to account for the effects of other steroids. All of the effects of all of the steroids could not be mediated by a single intermediate factor, since this would not account for the selective
effects of various steroids, nor the different effects of one steroid at different doses. Therefore, two possibilities remain: either each gene is independently controlled by each steroid (whether by itself, or through an intermediate factor for each gene), or each steroid operates by one or a few intermediates (specific for that steroid), and the gene action is coordinated. There is as yet no evidence to distinguish between these possibilities. The data document quite nicely the events beginning with the induction of ovalbumin messenger transcription, but cannot indicate what happens prior to that event.

2. The Response to Progesterone: O'Malley's Model

The investigations into the response of oviduct tissue to progesterone have centered primarily around the induction of the synthesis of the protein avidin in response to a progesterone injection after estrogen treatment has been given. The work of O'Malley's group has centered around the induction of this protein, and the elucidation of a possible mechanism of action for progesterone. This work will also be reviewed first in terms of the evidence presented, then the model that has been proposed to account for it, and last, in terms of comparison to other systems.

a. The Evidence

The induction of avidin requires new protein synthesis (111, 112), and may or may not require new RNA synthesis (111, 112, 113); the studies using actinomycin-D give varying results. The phenomenon of "superinduction" occurs here (113), as well as with ovalbumin, but in this case it is possible to induce avidin synthesis in vitro (111-113), contrary to the situation with ovalbumin (99).
In its action on the chick oviduct, progesterone affects the synthesis of rapidly-labelled nuclear RNA, and the RNA polymerase activity which would cause it (113, 114), but the changes are small, and the use of whole nuclei precludes the distinction between amounts of enzyme, enzyme activity, and/or changes in template availability (114).

RNA-DNA hybridization studies (116, 117) indicate that oviducts treated with estrogen and progesterone contain all the RNA species present in estrogen-treated oviducts, plus some RNA species that are not present in the latter case (115, 116, 117). Other interpretations of this data include the possibility of the steroid stabilizing a rapidly turning-over minor population of RNA (116), which has been mentioned also as a possible explanation for the effect of estrogen on ovalbumin message (95, 97). Gene amplification, although unlikely, could also account for this data. There is no evidence that the proposed new RNA being produced is avidin message, and it is likely not, since the short incubation time used selects for redundant sequences, and since goblet cells of the magnum portion make up only a small percentage of the whole oviducts that were used. O'Malley and McGuire also point out that rapidly-labelled nuclear RNA is usually rRNA or precursor rRNA (111). However, this is not likely the case here, since progesterone is unable to induce Phase II of the secondary response; that is, it is unable to induce new rRNA synthesis (53).

An investigation of the effects of progesterone on the polysome profile indicates a negative effect of progesterone on polysome formation (118), but interpretation of these studies is hindered by the presence of an artifact in the technique used; i.e., the lack of use of detergent before centrifugation.
In the absence of detergent, only 10-20% of the polysomes will be recovered, due to a pelleting of polysomes bound to endoplasmic reticulum under these conditions (119).

Translatable messenger for avidin has been demonstrated to be present only after progesterone induction (120, 121). RNA from oviducts treated with estrogen and progesterone has the ability to induce avidin synthesis in an estrogen-treated oviduct, when it is injected into the lumen of the oviduct (157, 123). That this is a direct translation of that RNA is evident from cross-species transfers using immunologically non-cross-reactive avidin molecules (124).

The necessity for both estrogen and progesterone in the induction of avidin synthesis complicates the analysis of induction by adding the possibility that estrogen may induce the pre-message, which would then be activated by progesterone. Such a situation would be analogous to the masked messenger of amphibian oocytes (125). If totally untreated oviducts can synthesize avidin in response to progesterone, then this would not be the case. One such incident has been reported (111), but is at variance with most other studies (24, 49).

The matter is confused further by the report that avidin synthesis is under the control of estrogen in lizards, with progesterone and testosterone acting synergistically with estrogen in this regard (126). An evolutionary switch from estrogen to progesterone induction, would of course confer the advantage of arranging a supply of biotin in the yolk before the biotin-binding protein is made, but such a switch, again, could occur at the transcriptional or post-transcriptional level.
Receptor proteins for progesterone have been demonstrated in chick oviduct in cytoplasm and nuclei (127), which have sedimentation coefficients of 4S in the presence of salt, and 8S and 5S in its absence (127). Only testosterone and 5-α-pregnane-3,20-dione could compete with progesterone for binding to the receptor (127); these steroids also induce avidin synthesis (128, 24).

By fractionating and re-incubating the components of chromatin, and exchanging these components, the authors demonstrated a greater binding of the progesterone-receptor complex to the acidic chromatin proteins than to the histones (129). A more extensive fractionation of the acidic chromatin proteins, and re-incubation, was attempted in order to further identify the nature of the acidic proteins responsible for binding. A number of assays were employed to demonstrate the 3-dimensional structural fidelity of these hybrid chromatin (130). If fractionated chromatin components incubated in vitro actually return to their original 3-dimensional structure, then chromatin may be a self-assembly structure. However, it is not necessary to assume the 3-dimensional structural fidelity of the hybrid chromatin to accept that the complex binds to the acidic proteins.

A further investigation of the receptors indicated that there are two components (131), with the same steroid specificity and hormone binding kinetics (131), but that Component A binds to DNA, whereas Component B does not, and that Component B binds to chromatin, whereas Component A does not (132).

b. Discussion of the Model

O'Malley's model, then, is one in which the progesterone-receptor complex enters the nucleus and binds to the acidic chromatin proteins, thus acting at
the "nuclear or transcriptional level of protein synthesis, resulting in new gene transcriptions and eventuating in avidin synthesis" (116). This model, if it is to be a general model for progesterone action, would have to account for the early effects of progesterone as well.

Although the possibilities for coordination of the responses are obvious in this model, there is as yet no evidence to indicate that avidin synthesis results from new transcription, nor that such proposed new transcription is the result of the binding of the progesterone-receptor complex to acidic chromatin proteins. A possible causal relationship between the binding to acidic chromatin proteins and any of the other actions of progesterone remains to be demonstrated (133). Nevertheless, the following correlations are evident: 1) After 6 days of diethylstilbestrol (DES) treatment, chromatin of nuclei of tubular gland cells becomes condensed, and was described as "heterochromatin" by Kohler et al (50). At that dosage of DES, the peak of cell proliferation occurred at 6 days; thereafter, cell proliferation ceased and further growth was attributed to hypertrophy (50). 2) An injection of progesterone to treated chicks had greatest effect in inducing avidin synthesis at 6 days of treatment. Greater or lesser estrogen treatment inhibited this response to progesterone (49). 3) Acidic chromatin proteins of the nucleus are reported to change biphasically during treatment, increasing in amount and variety during the first 6 days of treatment, and decreasing thereafter (134). 4) The binding of the progesterone-receptor complex to the acidic chromatin protein varies in the same biphasic manner (135).

The correlation of changes in acidic chromatin proteins and chromatin condensation has been noted in other systems, and is discussed at length by LeSturgeon et al (136). However, any discussion of the effects of these
changes on avidin synthesis remains speculative. Again, it should be noted that chromatin from oviducts will probably not reflect to any appreciable extent the chromatin of the goblet cells in which avidin is made. Thus, there is a problem in using avidin synthesis as a marker for progesterone action: namely, it is specific to cells which represent a small percentage of the total tissue, and these cells generally differentiate after tubular gland cells, which are the major bulk of the tissue (50, 45, 58).

The difficulty with this model, as opposed to that of Palmiter, is that it indicates only the very initial events of steroid action, but does not correlate them with the later changes.

Spelsberg is of the opinion that receptor proteins for steroids are intracellular gene regulators (133), and that this control action is via the protein rather than the steroid; that is, he proposes that the steroid only acts to transport the proteins into the nucleus, where the proteins activate or derepress the responsive genes. He is also of the opinion that there are other proteins of a similar nature in cells. He points out that, for virtually every steroid-responsive system, an activation of transcription has been postulated, following a binding to a nuclear constituent, although the nature of the nuclear constituent implicated has varied widely. He indicates 1) that the reported binding to histones can be accounted for by contamination with acidic nuclear proteins; 2) that the reported binding to acidic chromatin proteins may be hindered by the use of proteases that simply digest the receptor proteins and release the labelled steroid, rather than by digesting the nuclear acceptor site, and releasing the complex; or 3) that extraction procedures may extract the receptor (an acidic protein), rather than the nuclear acceptor site. He states therefore, that identification of the
nuclear acceptor is not possible (using in vivo systems) by the present techniques, and he further points out that binding to DNA gives generally non-specific results, indeed that even digestion by nucleases give varying results. He has therefore pursued an in vitro analysis, but such in vitro work is hindered by the fact that it relies on fractionation under denaturing conditions, and reconstitution by re-incubation. Nevertheless, although the 3-dimensional structural fidelity of such chromatin is in doubt, it is not necessary to assume the 3-dimensional structural fidelity of the chromatin in order to accept that the complex may bind to the acidic chromatin proteins. This model is therefore quite feasible.

3. Events a General Model Will Have to Explain

Any model for steroid action on chick oviduct that is a general model will have to account for the following:

1) The sequential nature of the response. That is, why do the stromal cells respond first, and the progenitor tubular gland cells second? If this is a sensitivity to a lower estrogen threshold, how is it mediated? By higher concentration of the receptor, or by a nuclear constituent, or is it simply that the changes undergone by the stromal cells produce some condition necessary for the response of the progenitor tubular gland cells? Likewise, why do the ciliated cells and goblet cell differentiate last?

2) The 3-hour time lag in the secondary stimulation, and the 24-48 hour time lag in the primary stimulation. A simple binding of the receptor-steroid complex to any nuclear constituent and subsequent activation of transcription does not account for this time lag, if no intermediates exist.

3) The dose-effect of estrogen. The fact that a lower dose of estrogen
preferentially enhances conalbumin synthesis is hard to reconcile to a model of simple binding of receptor-steroid complex to a nuclear constituent and activation of transcription.

4) The selective effects of other steroids, both when they are given alone, and in combination with estrogen. This would include both the selective and synergistic effects of progesterone and testosterone with estrogen, and the anti-estrogenic effects of progesterone with estrogen when this is administered at the beginning of treatment.

C. Comparison of this Induction Phenomenon to Embryonic Induction

The type of induction discussed here exhibits differences from the normal embryonic induction, both in consideration of the inducer, and of the cell's response.

1. The Inducer

The specificity of the inducer in this case is very exact, and the variable parameters are known. This is not the case in embryonic induction, where in most cases the inducer is not only unknown, but may also be killed, heat-treated, or substituted by simple salt solutions and still induce some response.

2. The Competence of the Responding Cells

The differences extend also to the cell's competence to respond. In the steroid-induced differentiating systems discussed here, there appears to be no time limit to the competence of the tissue to respond. In other situations, as rat uterus, there is a time limit before which the cells do not respond fully, but there appears to be no subsequent time limit (148, 149, 150). In still other systems of steroid-induction, such as neonatal androgenization, there is a very definite, circumscribed time period before and after which the
steroid has no effect (158, 159). This latter situation is, of course, the normal case in embryonic induction.

From an embryological point of view, the difference between induction and competence is the difference between the control by the inducer over the cell's actions, and the control of the cell over its own response to the inducer. A great deal of work has centered around the control of the inducer over the cell's actions. The following question is also of interest: What control does the oviduct cell have over its own response? Such a control might possibly be mediated at any of the points investigated for the control of the cell-specific proteins.

Obviously, the cellular concentration of the receptor (139) could be a control point in the response of the cell to the steroid. However, much evidence indicates that the steroid determines the concentration of its own receptor, or the concentration of the receptor for other steroids (142, 89-91). In this manner, estrogen induces the production of progesterone receptor in guinea pig uterus, and progesterone may inactivate its own receptor (143-145). In chick oviduct, it has been reported that estrogen enhances the receptor for progesterone (146), although this is not a selective action (146, disc.). Progesterone has been shown to enhance the concentration of the estrogen receptor (147). These observations point to an important difference between the experimental and the natural situations: the presence of two or more hormones simultaneously, and the interactions of these.

The simple fact that the receptor is present, however, does not mean that the hormone will act. In rat uterus, the competence to respond to inducer is developed step-wise, over a period of 30 days, although the receptor is
present at birth, able to bind estradiol, and enter and bind in the nucleus (148, 149, 150). This would indicate that additional factors are necessary for each new step, and so indicate the possibility of additional control steps. This possibility is also evident in receptor-containing mutants of immunocytes that are resistant to glucocorticoid action, even though the receptors bind glucocorticoids and enter and bind in the nucleus (151).

In other systems development of competence is reflected in alterations of the type of binding observed in the target tissues. This has been reported for DES binding in rat brain (152, 160), androgen binding in rat epididymis (153, 161), and glucocorticoid binding in lung and other tissues (154).

Another difference between this type of induction and that seen commonly in embryonic systems is that the cells never become incapable of responding to the inducer when it is present, and never become capable of continued function in the absence of the inducer. This is partially related to the absence of a critical time period as discussed earlier. However, regardless of the fact that the cells do not stop responding, their response changes in the course of treatment. Kohler et al have reported that for the first 6 days of treatment, the growth response is primarily by hyperplasia (41); thereafter, it is primarily represented by cellular hypertrophy. There is, therefore, a point at which the response changes, at which the cells respond by functioning but no longer by dividing. This change in response may result from a change induced by the estradiol treatment, or, in the natural situation, it may be induced by other sources, such as other cells, or other steroids.

The absence of continued function of the oviduct tissue in the absence of inducer is also a notable exception to normal embryonic induction. The fact that continued stimulation by estrogen is necessary for continued transcription, and that other steroids do not have the same effect, although they have
similar effects, preclude the possibility that the inducer simply acts to trigger a gross control, with the cell then acting to regulate itself. In the oviduct system, the inducer obviously exerts a fine control as well.

The recent observation that the vaginal and uterine cervical epithelial cells of neonatally estrogenized female mice can proliferate and differentiate without estrogen, and are less sensitive to exogenous estrogen (155) is related to these characteristics of the necessity for continued estrogen presence and the inability to not respond in the presence of the inducer. Similar studies have been performed on neonatally androgenized rodents (83, 84). Although no equivalent of the neonatal critical period has been established in chicks, it is evident, in rodents at least, that the action of the steroid on its target tissue at different times in development can be quite different. In this respect it is reminiscent of Paul Weiss' statement, "Hormone action is probably not a one-shot operation; the agent involved more likely operates at different times, repeatedly, producing different results, depending on the time at which it works" (162). Obviously, the total action of the hormone on a tissue throughout the life of the tissue is a more complex matter than the simple induction to differentiation at the time of puberty.

D. A Brief Assessment of the State of the Field

The research that has been discussed here appears to have proceeded to the limit of the present technology. Minor advances may yet be made, such as the identification of the non-translated portion of the message. However, the major advances in this area will require a more complete knowledge of two areas: the structure of chromatin, and the process of transcription. Only when these areas are elucidated will it be possible to measure an alteration of these by the hormone treatment. Perhaps in recognition of this interdependency, the
1975 Conference of the Society for Developmental Biology will address itself to two areas: the mechanism of steroid action and the process of transcription. When these are understood, the phenomenon of cell differentiation may be on its way to being unravelled.
REFERENCES


19. __________ and __________ (1956) "Role of Sex Hormones in Albumin Secretion by Oviduct of Chickens". Poult. Sci. 35: 692.


25. Eakin, R. E., McKinley, W. A. and Williams, R. J. (1940) "Egg-White Injury in Chicks and Its Relationship to a Deficiency of Vitamin H (Biotin)". Science 92: 224.


Protein Assay

0.5 ml of sample or standard
+ 5.0 ml of Solution C
10 min at room temperature
+ 0.5 ml of Folin's reagent, diluted to 1 N
30 min at room temperature
Read absorbance at 660

Solution C = 50:1 of Solution A:Solution B (v/v)
Solution A = 2% Na₂CO₃ (w/v) in 0.1 N NaOH
Solution B = 0.5% CuSO₄·5H₂O (w/v) in 1% NaK Tartrate (w/v)

This procedure follows that of Lowry et al (60).
Extraction Procedure for Ribonucleic Acid Assay

1 ml of homogenate or standard
+ 2.5 ml cold 10% TCA (w/v)
centrifuge, keep pellet
resuspend in 2.5 ml 10% cold TCA
centrifuge, keep pellet
resuspend in 5 ml 95% ETOH
centrifuge, keep pellet
resuspend in 5 ml 95% ETOH
centrifuge, keep pellet
resuspend in 2 ml 0.1 N NaOH
heat 45 minutes at 80°C
cool on ice
+ 0.5 ml cold 10% TCA
centrifuge
save supernatant for ribonucleic acid assays

This procedure follows the Reference of Schneider (57), with the exception that the pellet was finally resuspended in 2 ml of 0.1 N NaOH, and heated for 45 minutes at 80°C, rather than being resuspended in 2 ml of 1.0 N NaOH, and let stand for 20 hours at 37°C, then neutralized with 0.4 ml 6 N HCl.
RNA Assay

\[ 2.5 \text{ ml FeCl}_3 (0.04\% \text{ (w/v)} \text{ in HCl}) \]
\[ + 0.5 \text{ ml orcinol reagent (50 mg orcinol/ml in 95\% ETOH)} \]
\[ + 1.0 \text{ ml of standard or sample} \]
\[ + \text{H}_2\text{O to 5.0 ml total} \]
\[ \text{heat 30 min in boiling water bath} \]
\[ \text{cool on ice} \]
\[ \text{read at 665} \]

This procedure follows the basic procedures of Mejbaum (59), Dische (169), and Schneider (57), with the following exceptions: 1) the FeCl\(_3\) is 0.04\% in con. HCl, rather than 0.1\% (Mejbaum, dissolved in orcinol reagent) or 0.5\% (Schneider, dissolved in orcinol reagent); 2) orcinol reagent is 50 mg/ml, rather than 100 mg/ml (all others); 3) the reaction was allowed to proceed for 30 min at 100\°C, rather than 20 min (Dische and Schneider), or 40 min (Mejbaum); sample volumes were also altered slightly.

<table>
<thead>
<tr>
<th>Mejbaum</th>
<th>Dische</th>
<th>Schneider</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% FeCl(_3) in 100 mg orcinol/ml 95% ETOH</td>
<td>0.5 ml of 0.1% FeCl(_3)·6H(_2)O added to 100 ml con. HCl</td>
<td>0.5 gm FeCl(_3) in 100 ml con. HCl; 1 gm orcinol/100 ml acid reagent</td>
</tr>
<tr>
<td>3 ml sample + 0.3 ml orcinol</td>
<td>1.5 ml sample + 3.0 ml acid reagent + 0.2 ml orcinol</td>
<td>0.2 ml sample + 1.3 ml H(_2)O + 1.5 ml reagent</td>
</tr>
<tr>
<td>40 min at 100\°C read at 670 nm</td>
<td>20 min 100\°C read at 665 nm</td>
<td>20 min at 100\°C read at 660 nm</td>
</tr>
</tbody>
</table>
DNA Assay: Extraction Procedure

1 ml homogenate or standard
↓
2.5 ml cold 10% TCA
↓
centrifuge
↓
resuspend in 2.5 ml cold 10% TCA (w/v)
↓
centrifuge, keep pellet
↓
+ 2.0 ml 5% PCA (v/v)
↓
heat at 70° for 20 min
↓
cool, use supernatant

This procedure follows that of Schneider, with the exception that it omits extraction with 95% ETOH, since this was determined to have no effect on the final values obtained.

DNA Assay: Diphenylamine Reaction

1.0 ml
↓
+ 2.0 ml reagent
↓
30°C 20 hours
↓
read at 600 nm

Reagent is 1.5 gm diphenylamine in 100 ml glacial acetic acid, with 1.5 ml con. H₂SO₄. At the time of use, 0.1 ml of acetaldehyde (16 mg/ml) is added to 20 ml of the above reagent, and this was used in the assay.

This assay follows the diphenylamine reaction according to the procedure of Burton (58).
Ovalbumin Ouchterlony Plates

Preparation of Agar Medium:

To 1 gm of agar, 5 ml of borate buffer, 95 ml of saline (8.5 gm/l) was added enough sodium azide to make 0.1% azide. The borate buffer consisted of: Boric acid (6.184 gm), borax (Na$_2$B$_4$O$_7$·10H$_2$O; 9.536 gm), NaCl (4.348 gm) and water, to make one liter. The pH was adjusted to 8.4-8.5.

The immunodiffusion was carried out on large microscope slides that had been cleaned, rinsed twice with alcohol, and twice with ether. 2 ml of agar was added to each slide and allowed to solidify. Holes were punched in the agar 1 cm apart, the samples were applied, and allowed to diffuse for 48 hours. The volume of sample applied was 5 microliters; the antibody protein was diluted to 1 mg of antibody protein/ml, and this was used in the diffusion plates. After 48 hours, the plates were washed in 0.9% saline for 2-3 days, then in distilled water for 3 hours, then dried in open air. They were stained in amido black for ½ hour, then washed in 2% acetic acid for 30 min.

This procedure follows that of Work and Work (62).
Lysozyme Assay

Preparation of Bacterial Cells:

1. inoculum of *Micrococcus lysodeikticus*
   - grow in liquid medium at 30° for 48-72 hours, in a shaker
   - collect by centrifugation
   - wash with 60 volumes of distilled water
   - collect by centrifugation
   - wash three times with cold acetone
   - collect by centrifugation
   - wash twice with cold ether
   - collect by centrifugation
   - dry by air and suction on a sintered glass funnel
   - store in a desiccator at 5°

The liquid medium used was: 0.5% Bacto-peptone, 0.5% NaCl, 0.3% beef extract, and 0.1% yeast extract.

This procedure follows the procedure of Litwack (63).
Lysozyme Assay

1 ml of bacterial cell suspension in cuvette

transmission adjusted to 50% at 645 nm

+ 0.1 ml extract or enzyme solution, cuvette inverted quickly twice, and timing started

read % transmission every 30 sec

Activity: change in % transmission between 30 and 60 seconds of reaction

Cell Suspension: 20 mg of prepared cells of *Micrococcus lysodeikticus* in 90 ml of 0.15 M phosphate buffer, pH 6.2, with 10 ml 1% NaCl.

This procedure follows that of Litwack (63), with the exception that 20 instead of 25 mg of cells/100 ml buffer were used. The reaction was read against a distilled water blank.

As can be seen from the standard curve on the next page, the lowest measurable amount was 2 \(\mu\)g/ml. This is believed to be due to the particulate nature of the substrate.
LYSOZYME STANDARD CURVE

% Transmittance Change (30 sec. to 60 sec.)

μgm. Lysozyme / ml.

0 2 4 6 8 10
Avidin Assay

Reagents and Standards are diluted in 0.2 M Ammonium Carbonate

0.5 ml of $^{14}$C Biotin

+ 0.1 ml of sample or standard

let stand 10 min at room temperature

+ 1.0 ml of ammonium carbonate with 10 mg bentonite

let stand 5 min

transfer to Millipore Filter, 0.45 μm pore size

rinse twice with ammonium carbonate

transfer filter to scintillation vial

dissolve with 10 ml of Bray's solution (65)

count

This procedure is essentially that of Korenmann and O'Malley (64). Background in counting was approximately 28-30 cpm; and efficiency was about 85%. Quench curve for correction to DPM is shown on following page, along with standard curve.