STUDIES ON STEROID REGULATION OF SYNTHESIS OF A SPECIFIC OVIDUCT PROTEIN IN A NEW MONOLAYER CULTURE SYSTEM

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Steroid hormones are known to have marked effects on protein synthesis,[1, 2] and there is convincing evidence that they play a major role in regulation of tissue growth and functional differentiation of responsive tissues. Since the mechanisms of hormone action are complex, it would be desirable to study regulation in a simple system. Cell cultures may represent such systems since they are composed of relatively homogeneous cell populations and have fewer extracellular variables. Unfortunately, cell cultures frequently do not maintain the differentiated functions of the tissue of origin for any practical length of time. This has been true for most cultures of endocrine and hormone-responsive tissue, although reports of functioning endocrine tumor cell cultures exist.[3]

We have recently demonstrated changes from fibroblastlike to epithelioid cell patterns in chick oviduct monolayer cultures when estrogen was added to the medium.[4] We have also reported the *in vitro* induction of avidin, the biotin-binding egg-white protein, using minced chick oviduct incubated in the presence of progesterone.[5] The present study demonstrates the induction of synthesis of the specific oviduct protein, avidin, by progesterone in monolayer cultures of chick oviduct tissue.

Materials and Methods.—Cell cultures: Four-day-old female Rhode Island Red chicks were injected subcutaneously with 5 mg diethylstilbestrol (DES) daily for 18 days. The chicks were then sacrificed and 1-mm diameter explants of epithelial tissue from the mucosal surface of the oviducts were placed in 30-ml plastic flasks with a surface area of 25 cm^2. The cells were grown at 37° in Parker's 199 medium with 20% fetal bovine serum and 50 units of penicillin and 50 μg streptomycin/ml for a period of 6–10 weeks prior to testing. At that time, a mixed monolayer culture of epithelioid cells and fibroblasts had completely covered the surface of the flasks. Complete changes of media were made every 1 or 2 days throughout the study. After a control period, 5 μg diethylstilbestrol/ml was added to the media for 14 days, followed by incubation with 5 μg progesterone/ml of medium. Avidin, ovalbumin, and lysozyme were assayed on aliquots of the medium.

Assay of avidin: Avidin was quantified by an assay[6] involving specific binding of the avidin to D-biotin-carboxyl-C^{14}, adsorption of the complex onto bentonite, and trapping the complex on a cellulose acetate filter.

To demonstrate that the biotin-binding protein was also immunologically identical to avidin, studies were carried out utilizing a double-antibody immunoassay.[7] New Zealand rabbits were injected with commercially purified avidin and avidin antiserum was obtained. Aliquots of the medium were incubated with the avidin antiserum and C^{14}-labeled biotin. It has been previously shown that biotin does not interfere with an avidin-antiserum reaction, nor is the binding capacity of avidin for biotin altered by a prior attachment of antibody to the avidin molecule.[8] Sheep antirabbit globulin was then added and the resulting precipitate was washed, dissolved in NCS solubilizer, and counted in a toluene-phosphor solution.

Disc gel electrophoresis was also employed to examine the avidin incubation product. Gels were run at pH 4.5 (7% gel) where the bulk of the culture media protein remained in the upper third of the gel but the avidin-biotin-C^{14} complex ran in the bottom third. Gels were sliced into equal 1.3-mm slices and hydrolyzed with hydrogen peroxide or NCS solubilizer and counted.

Ovalbumin and lysozyme assay: Ovalbumin was precipitated by specific antibody and quantified by protein estimation of the precipitate using the Folin-phenol procedure.[9] Agar gel diffusion studies on Ouchterlony plates demonstrated that the large bulk of precipitating protein was identical with ovalbumin. Lysozyme was assayed by the method of Litwack.[10]

Labeled amino acid incorporation into protein: The cells and media were removed and the protein was precipitated with 10 vol of 10% cold trichloroacetic acid (TCA). After at least 1 hr at 4°, the suspension was centrifuged and washed twice with 10 vol 5% TCA. The precipitate was suspended in 3–5 ml 5% TCA and heated for 30 min at 90° in a water bath to hydrolyze RNA and free acyl-bound amino acids. The solution was centrifuged and the supernatant discarded. The pellet was washed twice with 95% ethanol, ethanol:chloroform:ether (1:2:1), and ether. The precipitate was mixed with 0.05 ml H_2O and 1 ml NCS solubilizer, and, after standing overnight at room temperature, was counted in toluene-phosphor scintillation liquid. Similar aliquots of culture hydrolysates were analyzed for total protein by the method of Lowry.[11]

Estimation of rate of DNA synthesis: The rate of DNA synthesis was determined utilizing 12-hr pulses of H^3-thymidine. The H^3-thymidine was added to medium 199 without carrier thymidine to a concentration of 2 μc/ml. Two ml of this medium containing either 5.0 μg of DES/ml or 0.5 μl of ethanol/ml as a control were added to the monolayer cultures. The cells were harvested after 12 hr by cooling to 4° followed by trypsinization. The cells were covered with 6 vol of acetone and allowed to stand at 4° for 8 hr. Carrier DNA (Herring sperm, 200 μg) was added and the mixture was precipitated with 10% TCA. The pellet was washed three times using 5% TCA containing thymidine (2 mg/ml). The precipitate was then washed three times with ether: ethanol (1:3), and the pellet was dissolved in NCS solubilizer and counted in a toluene-phosphor solution. Corrections were made for quenching.

Estimation of RNA: Cells were harvested from culture flasks by cooling to 4°, followed by scraping. The cells were mixed with 3 ml of 10% TCA, and, after 2 hr at 4°, the precipitate was washed twice with 10% TCA and centrifuged. The precipitate was washed with cold 95% ethanol, warm 95% ethanol, and then with ether. The pellet was suspended in 1 ml 5% TCA and heated at 90° for 30 min with occasional stirring. The suspension was centrifuged and the soluble material saved; the procedure was repeated and the soluble extracts were combined. Aliquots were taken for counting and the remainder was analyzed for RNA by an orcinol method.[12]

Materials and radioactive compounds: Culture flasks were obtained from B-D Laboratories, Inc., Baltimore, Md. Purified avidin and ovalbumin were purchased from Worthington and NCS solubilizer from Nuclear-Chicago. Herring sperm DNA was obtained from Calbiochem. H^3-thymidine (6.7 c/mM) and H^3-uridine (10 c/mM) were purchased from New England Nuclear.

Results.—The morphologic appearance of a typical 6-week-old chick oviduct monolayer culture treated with DES is shown in Figure 1. The cellular pattern is mixed but epithelioid cells predominate.

Induction of avidin synthesis: The rate of induction of avidin synthesis in monolayer cell cultures of estrogen-stimulated oviduct tissue is shown in Figure 2. Progesterone was added at zero time and avidin was first detectable at 12 hours. The maximum rate of induction occurred between 12 and 24 hours and synthesis reached a plateau by 48 hours.

The reason for the decreased rate of avidin synthesis between 24 and 48 hours is not clear. The inducing steroid is not completely metabolized at this time and studies involving addition of purified avidin to the cultures show no definite product inhibition. Depletion of some critical medium ingredient or build-up of toxic metabolites are possibilities since addition of new medium to the cultures leads to an increased rate of avidin synthesis.

The possibility that previously synthesized avidin was only released and not synthesized *de novo* during progesterone administration was ruled out by analyses of subcellular fractions of the cells. No intracellular accumulation of avidin occurred until progesterone was introduced. Experiments involving induction of

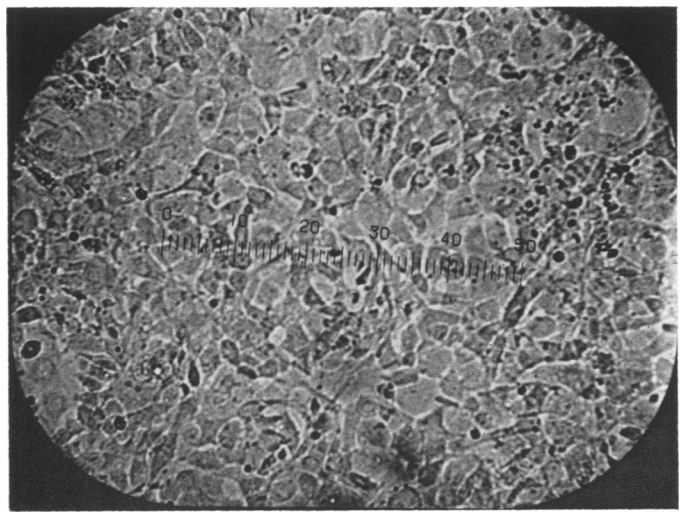

FIG. 1.—Light-microscopic photograph of a typical 5-week-old chick oviduct monolayer culture (magnification ×960).

avidin synthesis have been repeated on over 300 different monolayer cultures. Approximately 70 per cent of the total groups of cultures prepared have functioned to produce avidin. The response within any given group was uniform. Avidin synthesis did not occur in cultures of oviduct sub-epithelial stroma.

Spontaneous initiation of avidin synthesis by oviduct tissue maintained in culture longer than three weeks has occasionally occurred. This problem may be related to the steroid content of the particular batch of fetal bovine serum. Progesterone has been measured in certain lots of this material used in experiments where premature initiation of avidin synthesis has been noted. Careful control of this variable by sampling all cultures prior to testing is an absolute necessity.

Immunological and electrophoretic identification of incubation product: To demonstrate that the biotin-binding protein was immunologically identical to avidin, studies were carried out utilizing a double-antibody immunoassay with rabbit antiserum to avidin (Fig. 3). Aliquots of the medium were incubated with the avidin antiserum, followed by addition of C^{14}-biotin. The amount bound to the

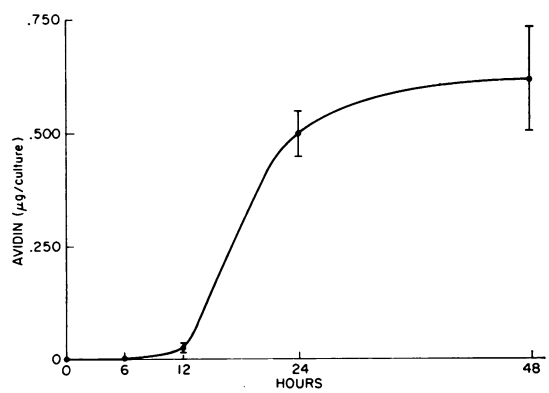

FIG. 2.—A 48-hr time curve for induction of avidin synthesis in cultured chick oviduct. Progesterone (5 μg/ml) was added to the incubation medium at zero time. Each value represents the mean ±SE of 10 separately analyzed cultures.

avidin-antibody precipitate is a measure of the amount of avidin present which is immunologically identical to purified avidin antigen. No immunologically identifiable avidin was noted in either untreated cultures (*I*) or in those incubated with DES (*II*). When progesterone was added to the culture, avidin was present as defined by the precipitation of the C^{14}-biotin by avidin antibody (*IV*). The dose-response curve was linear with respect to both increasing amounts of cell medium and added avidin standard (*V*). No biotin-binding material was present in the precipitate when avidin-antiserum was replaced by normal rabbit serum (*III*).

Disc gel electrophoresis was employed as a third criterion for avidin identity. Gels were run at pH 4.5 (7% gel) where the bulk of the culture media protein remained in the upper third of the gel but the avidin-biotin-C^{14} complex ran in the bottom third. The progesterone-treated cell cultures secreted a material with an

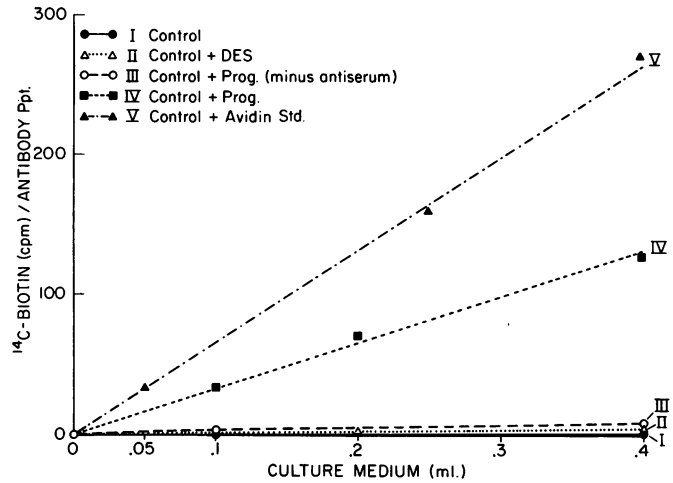

FIG. 3.—Analysis of amount of C^{14}-biotin bound to precipitates of aliquots of media incubated under various conditions and reacted with avidin-antiserum. All cultures incubated at 37° for 48 hr with: *I* = no hormone; *II* = 5 μg/ml diethylstilbestrol; *III* = 5 μg/ml progesterone but no subsequent addition of avidin antiserum; *IV* = 5 μg/ml progesterone; *V* = no hormone, but purified avidin standard added to medium prior to analysis. Each value represents the mean of four determinations.

identical electrophoretic mobility (*Rf* = 0.87) to avidin standard, whereas no detectable avidinlike material was present in control or diethylstilbestrol-treated cultures. Thus, the newly synthesized incubation product was immunologically, electrophoretically, and biologically identical to authentic avidin.

Product degradation: To examine product decay under the conditions of the incubation, 33 μg of avidin was added to 20 cultures and the cultures were assayed at 0, 24, and 48 hours (Table 1). No significant metabolism of avidin occurred during a 48-hour sterile incubation. Therefore, inhibition of degradation cannot be a mechanism of avidin induction in this system.

Specificity of induction: The specificity of response of this cell culture induction system is shown in Figure 4. During a 48-hour incubation with progesterone, no significant increases in ovalbumin or lysozyme occurred. This is in contrast to the

TABLE 1

ESTIMATION OF PRODUCT (AVIDIN) DECAY

Hours incubation	Recovery
0	29.8 ± 0.22
24	31.0 ± 0.14
48	30.8 ± 0.27

Thirty-three μg of purified avidin standard were added to 20 oviduct monolayer cultures and incubated at 37° for 48 hr. Cultures were sampled at the times indicated and avidin was measured by the biotin-binding assay. Each value represents the mean ± SE of 20 separately analyzed cultures.

TABLE 2

ESTIMATION OF TOTAL OVIDUCT CULTURE RNA

Additions	Total RNA (μg/culture)	SA of RNA (cpm/μg RNA)
No hormone	440 ± 17	111 ± 14
Diethylstilbestrol	490 ± 31	116 ± 13
Progesterone	440 ± 41	119 ± 18

Replicate cultures were exposed to 5 μg/ml DES or 5 μg/ml progesterone for 24 hr. Two μc of H^3-uridine (SA = 11.9 c/mM) were incubated with each monolayer culture for 6 hr. RNA was extracted as in text. Each value represents the mean ±SE of 6 separately analyzed cultures.

increased rate of avidin synthesis which occurred concomitantly. We cannot absolutely rule out induction of other unknown oviduct proteins.

Effect of the steroids on general protein synthesis: The effect of DES and progesterone on general protein synthesis of monolayer cells was determined by incorporation of H^3-amino acids into TCA-precipitable cell and media protein (Fig. 5). When DES was introduced into the culture, a significant stimulation of labeled amino acid incorporation into protein occurred. Progesterone, however, did not significantly affect general protein synthesis of the cultured oviduct tissue.

Effect of the steroids on DNA and RNA synthesis: The effect of DES and progesterone on the rate of cell culture DNA synthesis is shown in Figure 6. Diethylstilbestrol stimulated H^3-thymidine incorporation into DNA but progesterone did not. No changes in total cell RNA were noted when DES or progesterone was added to the incubation medium (Table 2). Additional experiments using H^3-uridine (2 μc/ml) pulses for six hours showed no alteration of RNA specific activity.

Effect of inhibitors on avidin induction: The effect of inhibitors was tested during a 48-hour incubation with progesterone (Fig. 7). The induction process was dependent on new protein synthesis since cycloheximide prevented induction completely. When actinomycin D and progesterone were added together, there was a 90 per cent inhibition of avidin synthesis, suggesting, but not proving, that new RNA synthesis is required. At the concentration of actinomycin D used (0.1 μg/ml), there was less than 7 per cent inhibition of general protein synthesis as measured by labeled amino acid incorporation into TCA-precipitable protein.

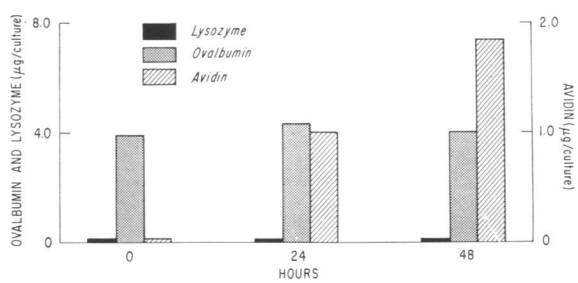

FIG. 4.—Estimation of ovalbumin and lysozyme concomitant with avidin induction in chick oviduct monolayer cultures. All cultures contained progesterone (5 μg/ml) added at zero time and were sampled at 0, 24, and 48 hr. Bars represent the mean of 6 separately analyzed cultures.

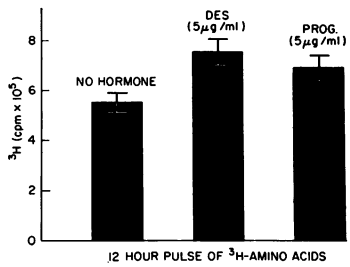

FIG. 5.—Incorporation of H^3-amino acids into oviduct monolayer protein. Cultures were incubated at 37° for 48 hr with diethylstilbesterol (5 μg/ml), progesterone (5 μg/ml), or no hormone. During the last 12 hr of the incubation H^3-leucine (2 μc/ml) and H^3-lysine (2 μc/ml) were added to the cultures. Experiments were terminated by freezing and analyzed for labeled protein as described in the text. Bars represent the mean ±SE of 6 separately analyzed cultures.

To determine whether continued DNA synthesis was a prerequisite for avidin induction, hydroxyurea, a specific inhibitor of DNA synthesis, was added to the culture medium to a final concentration of 0.001 *M*. Avidin synthesis was not inhibited at a dose which inhibited cell culture DNA synthesis 75 per cent (Fig. 8).

Discussion.—The present studies demonstrated that normal chick oviduct tissue could be maintained in a functional differentiated state in monolayer culture for at least three months. Introduction of progesterone into the oviduct monolayer cultures induced the synthesis of the specific oviduct egg-white protein, avidin. The newly synthesized incubation product was identical to authentic avidin standard by immunological, electrophoretic, and biological (biotin-binding) assay. Essentially no degradation of avidin occurred during a 48-hour sterile incubation. Therefore, inhibition of degradation could not be a mechanism for avidin induction. This concept is further supported by the studies utilizing avidin antiserum to demonstrate an increase in antibody-precipitable protein in cultures incubated with progesterone. The induction appeared to be dependent on new protein synthesis, since cycloheximide inhibited the process. Actinomycin D inhibition of progesterone-induced avidin synthesis suggested that new RNA synthesis was necessary. However, the numerous other actions of this antibiotic[13, 14] in addition to its effect on DNA-directed RNA synthesis would make the evidence only suggestive. New DNA synthesis was not necessary for avidin induction since hydroxyurea, an inhibitor of

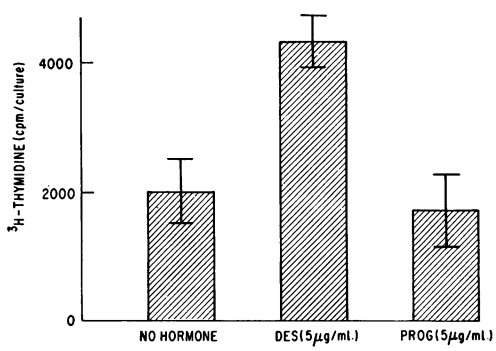

FIG. 6.—Incorporation of H^3-thymidine into oviduct monolayer DNA. Cultures were incubated at 37° for 48 hr with H^3-thymidine (2 μc/ml) and diethylstilbestrol (5 μg/ml), progesterone (5 μg/ml), or no hormone. Experiments were terminated by freezing and analyzed for labeled DNA as in text. Bars represent the mean ±SE of 8 separately analyzed cultures.

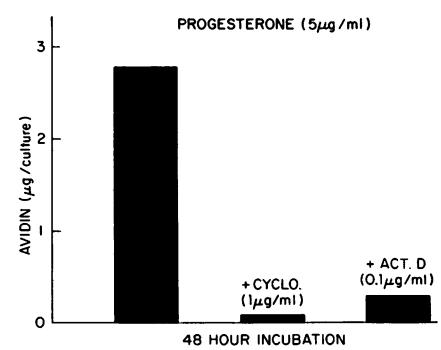

FIG. 7.—Effect of cycloheximide and actinomycin D on avidin synthesis by oviduct monolayers. Progesterone (5 μg/ml) was added to all cultures at zero time and incubated at 37° for 48 hr alone or with cycloheximide (1 μg/ml) or actinomycin D (0.1 μg/ml). Bars represent the mean of 12 separately analyzed cultures.

DNA synthesis thought to act on ribonucleotide reductase, did not prevent new avidin synthesis. Progesterone and DES did not stimulate total cell RNA in oviduct monolayer cultures which were actively making induced proteins. The cell response in this system is specific enough so that major RNA changes might not be expected. Tomkins *et al.*[15] have also noted that glucocorticoid induction of tyrosine α-ketoglutarate transaminase in hepatoma suspension culture is accompanied by no changes in total cell RNA.

No significant stimulation of oviduct general protein synthesis is noted with progesterone. The specificity of the induction is further supported by the lack of a measurable increase in synthesis of other major oviduct proteins, such as ovalbumins or lysozyme. None of the steroids in the concentrations used produced toxic effects

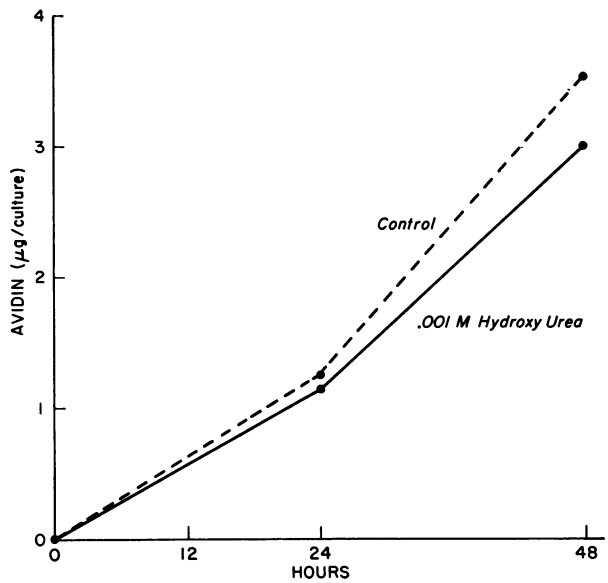

FIG. 8.—Effect of hydroxyurea on avidin synthesis in oviduct monolayer. Progesterone (5 μg/ml) was added to all cultures at zero time and incubated alone or with hydroxyurea (added to 0.001 *M*) for 48 hr. at 37°. Each point on curve represents the mean of 5 separately analyzed cultures.

in the monolayer cultures as evaluated by RNA, DNA, and general protein synthesis.

Only a few culture systems now exist which are appropriate for study of steroid regulation of specific protein synthesis. Thompson *et al.*[16] have recently reported that glucocorticoids would induce synthesis of tyrosine α-ketoglutarate transaminase in cells of the ascites form of Morris hepatoma. Similarly, Griffin and Cox[17] have reported increases in alkaline phosphatase activity of HeLa cells, cultured in monolayer, after exposure to prednisolone. Prednisolone was later reported to cause an actual increase in "activity" of the existing individual enzyme molecules rather than an increase in new enzyme protein.[18]

Oviduct monolayer culture is a system composed of normal cells in which induction of a specific protein can occur. The use of normal rather than tumor tissue,

however, has the disadvantage that an identical continuing cell line cannot be established and propagated. Tissue must be replanted frequently and variation in the biology and preparation must be circumvented with appropriate controls. Trypsinized oviduct monolayer cultures have retained the capacity to synthesize avidin. However, trypsinization and subculturing frequently caused temporary or permanent loss of oviduct cell function in our system similar to that reported by others.[3]

The induction of avidin synthesis by progesterone in oviduct monolayer culture reproduces the observations reported from this laboratory in the oviduct of the whole animal treated with progesterone[19] or in minced chick oviduct tissue incubated with progesterone *in vitro*.[5] Only progestational or anti-estrogenic steroids acted as inducers of avidin synthesis.[20] The exact mechanism of the induction is unknown but we have demonstrated recently that progesterone stimulated oviduct rapidly labeled nuclear RNA and RNA polymerase prior to induction of new avidin synthesis.[21] This would support the concept that this steroid acts initially at the nuclear or transcription level of protein synthesis. The oviduct monolayer culture system promises to be a simplified, efficient, and specific tool for further study of hormonal control of protein synthesis.

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