In Vitro Hormonal Induction of a Specific Protein (Avidin) in Chick Oviduct*

Bert W. O'Malley

ABSTRACT: Progesterone added *in vitro* to minced chick oviduct in tissue culture medium induced the synthesis of a specific oviduct protein, avidin. Avidin synthesis was apparent at 6 hr and reached a maximum at 48–72 hr; there was a 25-fold increase over preincubation levels of 0–0.2 μg/g of oviduct. The incubation product was indistinguishable from authentic avidin standard by disc gel electrophoresis, antibody precipitation studies, or assay of biological activity utilizing binding to [^{14}C]biotin. Using labeled amino acids, an increase in the rate of avidin synthesis over the course of the incubation occurred, indicating actual net synthesis of new avidin protein. Avidin synthesis was inhibited by cycloheximide (1–10 μg/ml) added at any time during the incubation. Avidin synthesis was prevented by actinomycin D added at zero time but not at 6 hr or later. The specificity of the induction was supported by the *absence* of an increase in the synthesis of oviduct total protein, ovalbumin, or lysozyme during the incubation. This study represents the first instance of *in vitro* steroid-induced synthesis of a specific protein (avidin) in minced tissue and should provide a simple, reliable system for elucidation of mechanism of steroid regulation of protein synthesis.

Elucidation of a mode of action of steroid hormones requires, in part, an experimental model in which specific biochemical events resulting from hormone action can be studied easily. Therefore, we have developed a system in which a hormone selectively induces synthesis of a measurable specific protein under chemically defined conditions.

Estrogenic substances are known to stimulate oviduct growth in newborn and older chicks (Brant and Nalbandov, 1956). We have found that estrogen markedly stimulates synthesis of nucleic acids and numerous proteins, similar to other model systems presently available. However, the administration of a single dose of progesterone *in vivo* to estrogen-stimulated chicks results in the induction of synthesis of a specific oviduct protein, avidin (Hertz *et al.*, 1943; S. G. Korenman and B. W. O'Malley, unpublished data). We now report induction of avidin synthesis in minced oviduct incubated *in vitro* with progesterone in tissue culture medium.

Materials and Methods

In vivo avidin studies were carried out on immature female Rhode Island red chicks. DES[1] (5 mg) was administered subcutaneously to 4-day-old chicks for 12–18 days. Progesterone (5 mg) was given as a single dose at zero time. The animals were sacrificed at the indicated times and the magnum portions of the oviducts were removed and homogenized at 4° with 16 strokes of a motor-driven Teflon pestle in six volumes of buffer A (0.07 M KCl–0.004 M $MgCl_2$–0.07 M NaCl–0.02 M phosphate buffer, pH 7.1). The homogenate was centrifuged at 5000*g* for 30 min and the supernatant was centrifuged at 105,000*g* for 2 hr. Avidin was measured in aliquots of the 105,000*g* supernatant.

In vitro studies were carried out on chicks treated with DES for 12–18 days as above. The oviduct from each chick was removed under sterile conditions, finely minced at 4°, and weighed. One-half of the tissue served as a control and the remainder was incubated with 5–10 μg of progesterone in 1 ml of biotin-free 199 medium (NIH) at 38° in a Dubnoff metabolic shaker under 95% O_2–5% CO_2 gas mixture. Crystalline penicillin G (35 μg/ml) was added initially. Experiments were terminated by freezing and the tissue and medium were homogenized in buffer A as above and analyzed for avidin. All *in vitro* studies were carried out under these identical conditions and all experiments were repeated on at least three separate occasions.

Unless otherwise indicated, avidin was determined in all subsequent studies by the method shown in Figure 1. The unknown soluble tissue fraction is reacted with an excess of *d*-biotin labeled with ^{14}C in the carboxyl group. Biotin is known to have a specific binding affinity for avidin. After a short incubation at room temperature, bentonite is added, binding the avidin nonspecifically. The mixture is pipetted onto a Millipore filter, washed with 0.2 M $(NH_4)_2CO_3$ buffer (pH 8.9), and the ^{14}C is counted in Bray's solution. No unbound [^{14}C]biotin remains on the filter. The blank of the method is zero and the sensitivity is 0.05 μg/g of oviduct tissue. Avidin present is calculated from a standard curve run under the same conditions using commercially purified avidin (Worthington). Details of this method have been pre-

* From the Endocrinology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received April 28, 1967.*

[1] Abbreviations used: DES, diethylstilbestrol; TCA, trichloroacetic acid.

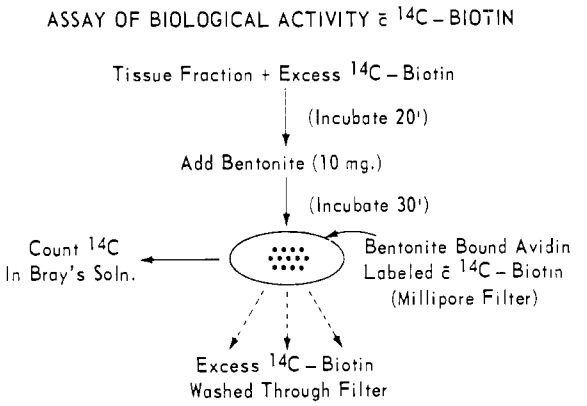

FIGURE 1: Method of avidin quantification utilizing biological capacity of avidin to bind [^{14}C]biotin. All additions are made in 0.2 M $(NH_4)_2CO_3$ buffer (pH 8.9).

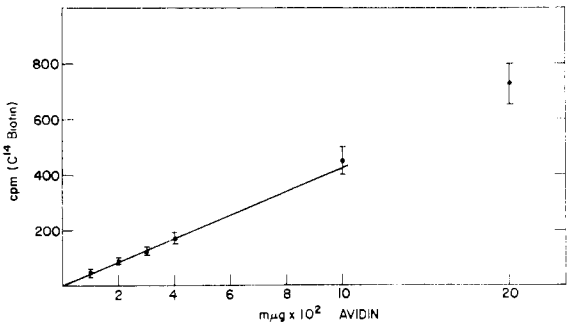

FIGURE 2: Capacity of avidin–antibody precipitate to bind [^{14}C]biotin as a function of amount of avidin in the precipitate. Assay conditions described in text. Addition of increasing amounts of avidin standard to incubations containing 105,000*g* supernatant and antiserum resulted in linear binding of [^{14}C]biotin relative to avidin present up to 1.0 μg. Each point represents mean plus and minus standard error of four determinations.

viously reported (Korenman and O'Malley, 1967). For convenience, avidin is expressed in the following studies as micrograms per gram of oviduct tissue because the results were identical when calibrated against oviduct-soluble protein determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

The incubation product was also assayed using a double-antibody precipitation method which allows the simultaneous analysis of avidin by both immunologic and biologic activity. Antiserum was obtained from rabbits immunized against commercially purified avidin. The avidin antiserum gave single precipitin lines of identity when tested against avidin standard and crude oviduct homogenate in agar gel double diffusion. An aliquot of the 105,000*g* supernatant obtained from the incubated oviduct after homogenization was reacted with the avidin antiserum, ^{14}C-labeled biotin, and an equal volume of 0.2 M $(NH_4)_2CO_3$ buffer (pH 8.9) for 2 hr at room temperature followed by the addition of an excess of sheep antirabbit globulin. The resulting precipitate was allowed to stand for 3 hr, washed three times with 0.01 M phosphate–0.9% NaCl buffer (pH 7.2), dissolved in NCS solubilizer (Nuclear-Chicago), and counted in toluene–phosphor solution. This assay was used to demonstrate incorporation of labeled amino acid into avidin during the incubation.

Analysis of 105,000*g* supernatants from oviduct tissue incubated with tritiated lysine showed an incorporation of tritium which was proportional to the amount of supernatant solution added. The precipitate also bound [^{14}C]biotin. This binding was shown to be proportional to the total amount of avidin present (Figure 2). When the avidin antiserum was omitted, the precipitate did not contain significant amounts of either ^{3}H or ^{14}C. Addition of ovalbumin–antiserum led to precipitation of counts presumably incorporated into ovalbumin but no [^{14}C]biotin was bound to the precipitate. Details of this method are to be presented elsewhere.

Ovalbumin was measured as described by Kabat and Mayer (1961) by quantitative immune precipitation. Agar gel diffusion studies using Ouchterlony plates showed that the bulk of precipitating protein was identical with ovalbumin. Lysozyme was assayed by the method of Litwack (1955).

Results

The normal *in vivo* induction curve for avidin synthesized by the estrogen-stimulated chick oviduct is seen in Figure 3. After a single 5-mg dose of progesterone given at zero time, the chicks are sacrificed at the indicated times and avidin is measured in the oviducts. Synthesis is first apparent at 10 hr and begins to plateau at 24 hr. No avidin appears in the oviduct either before or after estrogen treatment unless progesterone is administered. Details of this induction process are to be reported elsewhere (S. G. Korenman and B. W. O'Malley, 1966, unpublished observations).

A typical induction curve for avidin synthesis *in vitro* is also shown in Figure 3. Avidin synthesis was first noted at 6 hr and reached a maximum between 48 and 72 hr. Flasks occasionally became infected after 96-hr incubation (Figure 3), so experiments were generally terminated at 48–72 hr. The total avidin synthesized varied from 4 to 10 μg among different groups of animals. Age and amount of previous estrogen stimulation were variables carefully controlled in the subsequent studies. Homogenates of immature or estrogen-stimulated oviduct incubated with progesterone did not synthesize avidin.

In addition to measurement of newly synthesized avidin by the [^{14}C]biotin assay using Millipore filters (Figure 1), the incubation product was also analyzed by disc gel electrophoresis (Reisfeld *et al.*, 1962). A 7% monomer lower gel at pH 4.5 at 4° was used. An aliquot of the 105,000*g* supernatant from homogenized incu-

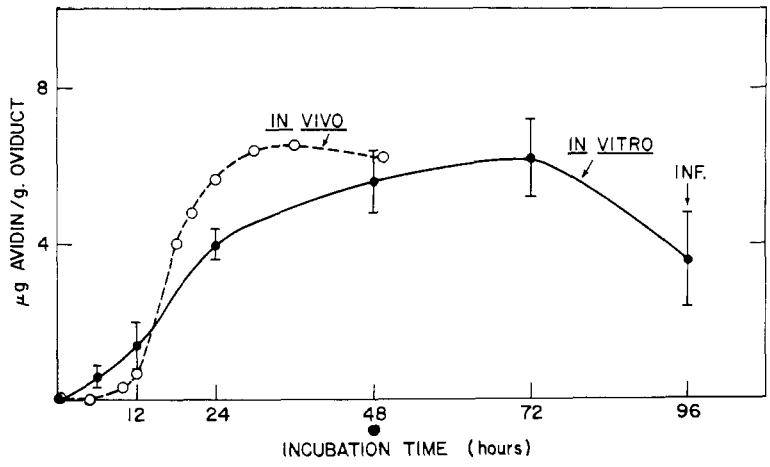

FIGURE 3: (- - - -) *In vivo* oviduct avidin induction curve after 5 mg of progesterone (subcutaneously) given to immature diethylstilbestrol-treated chicks. Each point represents mean plus and minus standard error of groups of four to six animals sacrificed at the indicated times. (———) *In vitro* avidin induction curve under conditions described in text. Each point represents the mean plus and minus standard error of four to six incubations.

bates was reacted with [^{3}H]biotin and layered on the disc gel. The labeled biotin was similarly reacted with commercially purified avidin standard (Worthington). Gels were run and then sliced into equal 1.3-mm slices and hydrolyzed with hydrogen peroxide or NCS solubilizer and counted. The biotin-binding protein synthesized during the incubation had an identical R_F (0.60) with that of authentic labeled avidin–biotin complex. Unbound biotin does not enter the separation gel in this system and the bulk of the tissue supernatant protein remains near the origin at this pH. Control tissue fractions showed no detectable avidin.

The decay curve for avidin under the conditions of the incubation is shown in Figure 4. Marked degradation occurs in the presence of liver tissue and less with muscle. Only 20% decay occurs over 48 hr in the presence of oviduct mince and less than 10% in the presence of a saline extract of chick oviduct. This study suggests that product decay is not a major determinant of avidin net synthesis in this *in vitro* system. However, since the amount of avidin added was in excess of that synthe-

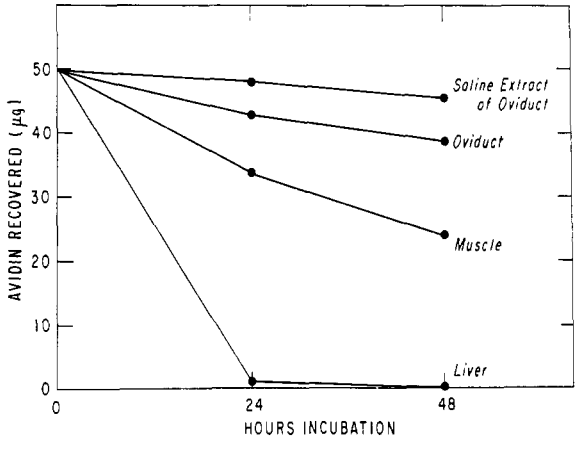

FIGURE 4: Determination of product decay under conditions of *in vitro* incubation at 38° and an atmosphere of 95% O_2–5% CO_2. Avidin standard (50 μg) was added at zero time to each flask containing 1 g of each of the above tissues and 1 ml of medium 199 (NIH). Incubations were terminated at the indicated times and analyzed for recovery of added avidin. Each point represents the mean of six flasks.

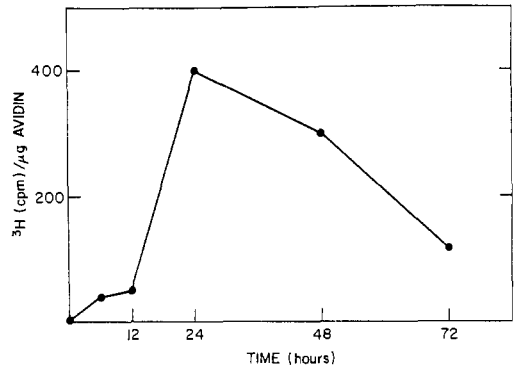

FIGURE 5: Rate of avidin synthesis over the course of a typical *in vitro* incubation. Progesterone (5 μg/ml) added at zero time. Each point represents the amount of ^{3}H incorporated into avidin–antiserum precipitate during a 60-min pulse of 10 μc of [^{3}H]lysine, divided by the total avidin mass (measured by assay described in Figure 1) present up to that point in the incubation. Treatment of precipitate is described in text. Each point represents the mean of triplicate determinations.

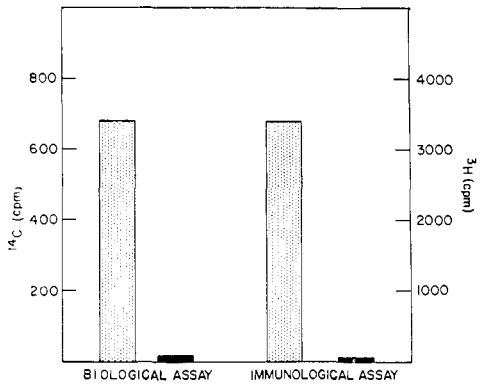

FIGURE 6: Effect on avidin synthesis of cycloheximide (10 μg/ml) added together with 5 μg of progesterone to oviduct minces at zero time. ^{14}C represents counts per minute of [^{14}C]biotin bound by 105,000*g* supernatant from 1 g of oviduct at termination of a 48-hr incubation. ^{3}H represents counts per minute of [^{3}H]lysine per gram of oviduct incorporated into avidin–antiserum precipitate during a 4-hr pulse at termination of the incubation. Assay procedures are described in text. Bars represent the mean of six incubates analyzed separately. The hatched area represents normal induction and the closed area + cycloheximide (20 μg/ml).

sized, the possibility still exists that degradation may be a factor in the incubation system where only 4–8 μg is synthesized. The rate of avidin synthesis was studied during the course of the incubation period (Figure 5). Each point represents the amount of tritiated amino acid incorporated into avidin during a 60-min pulse, divided by the total avidin mass present up to that point in the incubation. The marked change in tritiated amino acids incorporated into the avidin–antibody precipitate reflects an actual stimulation of the *rate* of new avidin synthesis. The peak rate of synthesis occurred between 12 and 24 hr. Synthesis was almost completed by 72 hr. These findings correlate well with the *in vitro* incubation curve shown in Figure 3.

Cycloheximide prevented the synthesis of avidin during a 48-hr incubation as measured by either biological or immunological methods (Figure 6). Addition of 10 μg/ml of the drug at any time during the incubation inhibited avidin synthesis. This concentration was sufficient to inhibit [^{3}H]lysine incorporation into TCA-precipitable material by 93%. The system was sensitive to cycloheximide at a dose of 1 μg/ml of medium. This supports the conclusion that the induction process is dependent on new protein synthesis. The effect of actinomycin D on avidin synthesis in this incubation system is shown in Figure 7. Addition of 1–20 μg/ml to the incubation system at zero time caused marked inhibition of avidin synthesis without inhibiting general protein synthesis as measured by incorporation of labeled amino acids into TCA-precipitable material. Addition of the antibiotic at 6 hr had no appreciable effect and additions at subsequent times led to increased avidin synthesis. Increases were consistantly noted with additions at 12, 24, or 48 hr and the stimulations were significant in all experiments, ranging from 25 to 60% above controls with no overlap in the values of the two groups. There was a tendency for greater superinduction with the higher doses (20 μg) of actinomycin D.

Figure 8 reflects the tissue:medium (T:M) ratio for avidin during the incubation. Avidin was rapidly released into the medium concomitant with its appearance in the tissue. At 6 hr the T:M ratio was only 0.6 and the ratio dropped to 0.3 at 24 hr. No major cell destruction, as observed by light microscopy, occurred during the first 18 hr of the incubation.

The specificity of the induction was confirmed by the absence of an increase in other major oviduct proteins

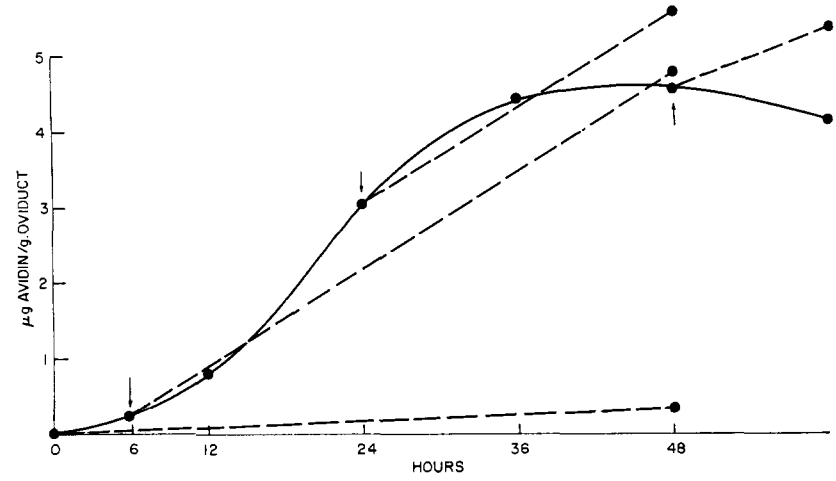

FIGURE 7: Effect of actinomycin D on avidin induction. (·———·) Avidin synthesis without actinomycin D under conditions described in Figure 3. (·– – – –·) Actinomycin D (10 μg/ml) added to replicate incubates at times indicated by arrows. Each point represents the mean of four separate incubation flasks.

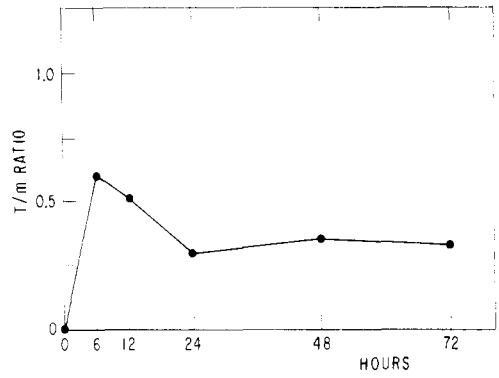

FIGURE 8: Tissue:medium (T:M) ratio during avidin induction under conditions described in text. Tissue and incubation medium were separately analyzed for avidin at the points indicated. Each point represents the mean of four separate incubation flasks.

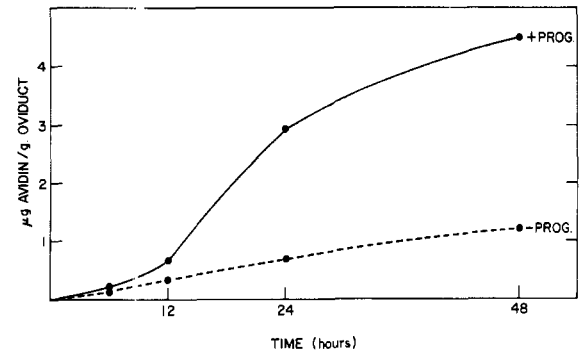

FIGURE 10: Avidin synthesis (·———·) in presence of 5 μg/ml of progesterone and (·- - - -·) without added progesterone during *in vitro* incubation under conditions described in text. Each point represents the mean of six separate flasks analyzed separately.

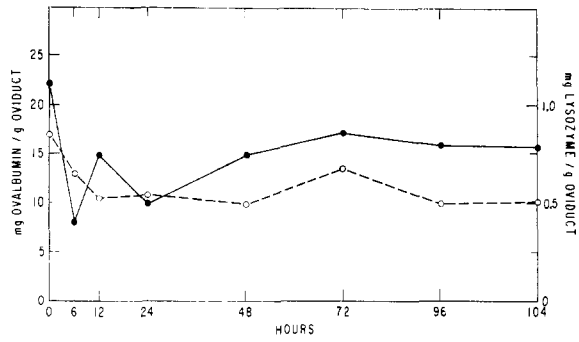

FIGURE 9: Levels of ovalbumin and lysozyme during course of normal avidin *in vitro* induction as described in Figure 3. Each point represents the mean of six flasks analyzed separately. (O–O) Lysozyme and (●—●) ovalbumin.

such as ovalbumin and lysozyme, during the incubation. It must be noted that these proteins are present at concentrations orders of magnitude greater than avidin. Therefore, if these proteins were being synthesized at a slightly faster rate it may not be detectable as a change in the total amount present (Figure 9). We cannot absolutely rule out induction of other unknown proteins. However, estimation of general protein synthesis measured by incorporation of successive pulses of tritiated amino acids into TCA-precipitable protein showed a progressive fall in the rate of synthesis of new protein over the course of the incubation. These events were in contrast to the increased rate of avidin synthesis occurring concomitantly.

In contrast to the results in *in vivo* studies there was avidin synthesis in oviducts incubated *in vitro* without progesterone (Figure 10), but progesterone-stimulated incubates generally synthesized 300–500% more avidin. The increase was linear with time and the material produced was avidin by all the previous criteria. Its synthesis was also inhibited by cycloheximide. Other studies lead us to suspect that the base-line induction of avidin synthesis is related to withdrawal of estrogen stimulation. Additions of DES to the incubation medium result in suppression of this spontaneous induction.

Discussion

The preceding studies have established a new *in vitro* system for study of synthesis and hormonal regulation of a specific protein. Avidin increased from an unmeasurable base line to 4–8 μg/g of oviduct. Induction of new enzyme protein has been recently reported using dexamethasone in hepatoma suspension culture (Thompson *et al.*, 1966). Griffin and Cox (1966a,b) have also reported increased enzyme activity using prednisolone in HeLa cell culture. To our knowledge, steroid-induced synthesis of a specific protein in normal tissue *in vitro* has not previously been reported.

The kinetics of avidin synthesis in minced chick oviduct differs from the kinetics of enzyme formation in bacteria after addition of inducer. During β-galactosidase induction in *Escherichia coli*, enzyme synthesis began within 3 min after adding inducer, and the rate of increase was linear (Pardee and Prestidge, 1961). In the oviduct tissue, there appeared to be two different rates of avidin synthesis occurring sequentially. Avidin was first measureable at 6 hr; then the slow accumulation of avidin was followed by a period of rapid synthesis at 12–24 hr. Similar hormone-mediated biphasic enzyme induction has been reported in other systems (Varner and Chandra, 1964; Griffin and Cox, 1966a). The small amount of base-line avidin induction which occurred in the absence of progesterone is not fully understood. No similar induction ever occurs without progesterone in the oviduct of the whole animal. The *in vitro* incubation conditions represent a dramatic change in environment for this tissue and other studies (B. W. O'Malley, un-

published data) point to estrogen withdrawal as a likely cause. The specificity of the system was confirmed by no increase in other major oviduct proteins such as ovalbumin or lysozyme.

Cycloheximide added at any time during the incubation prevented avidin synthesis. Studies utilizing labeled amino acid incorporation into avidin–antibody precipitates showed an actual stimulation in the rate of avidin synthesis. The findings suggest *de novo* synthesis of the protein. Actinomycin D added at zero time effected marked inhibition ($\gg$90%) of avidin synthesis. This would suggest that new RNA synthesis is specifically required for induction. However, numerous other effects have been noted with this antibiotic (Revel *et al.*, 1964; Korn *et al.*, 1965; Laszlo *et al.*, 1966) in addition to its well-known inhibition of DNA-directed RNA synthesis. Inhibition was no longer apparent at 6 hr and stimulation of synthesis occurred when the antibiotic was added at later time periods. This superinduction was more notable with higher doses of actinomycin D (20 μg/ml). A stimulatory action of actinomycin D has recently been reported in several other systems (McAuslan, 1963; Garren *et al.*, 1964; Sols *et al.*, 1965; Tomkins *et al.*, 1966). Studies on avidin degradation (10%/24 hr) in this system would rule out the possibility that the apparent actinomycin D stimulation (25–60%) is a result of product stabilization. The possibility exists that actinomycin D inhibits all mRNA synthesis and the avidin mRNA is relatively stable while the mRNA for a labile cytoplasmic repressor is rapidly turning over. Cytoplasmic (translational) derepression has been proposed as a mechanism of steroid regulation in other systems (Garren *et al.*, 1964; Tomkins *et al.*, 1966) but no other supportive evidence exists for a similar mechanism of action for progesterone in this *in vitro* system.

References

Brant, J. W. A., and Nalbandov, A. V. (1956), *Poultry Sci. 35*, 692.

Garren, L. D., Howell, R. R., Tomkins, G. T., and Crocco, R. M. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1121.

Griffin, M. J., and Cox, R. P. (1966a), *J. Cell Biol. 29*, 1.

Griffin, M. J., and Cox, R. P. (1966b), *Proc. Natl. Acad. Sci. U. S. 56*, 946.

Hertz, R., Fraps, R. M., and Sebrell, W. E. (1943), *Proc. Soc. Exptl. Biol. Med. 52*, 142.

Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, Springfield, Ill., C. C Thomas, p 361.

Korenman, S. G., and O'Malley, B. W. (1967), *Biochim. Biophys. Acta 140*, 174.

Korn, D., Protass, J. J., Leive, L. (1965), *Biochem. Biophys. Res. Commun. 19*, 473.

Laszlo, J., Miller, D. S., McCarthy, K. S., and Hochstein, P. (1966), *Science 151*, 1007.

Litwack, G. (1955), *Proc. Soc. Exptl. Biol. Med. 89*, 401.

Lowry, O. N., Rosebrough, N., Farr, A., and Randall, R. (1951), *J. Biol. Chem. 193*, 265.

McAuslan, B. R. (1963), *Virology 21*, 383.

Pardee, A. B., and Prestidge, L. S. (1961), *Biochim. Biophys. Acta 49*, 77.

Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature 195*, 283.

Revel, M., Hiatt, H. H., and Revel, J. (1964), *Science 146*, 1311.

Sols, A., Sillero, A., and Salas, J. (1965), *J. Cellular Comp. Physiol. 66*, 23.

Thompson, E. B., Tomkins, G. M., and Curran, J. F. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 296.

Tomkins, G., Thompson, E., Hayashi, S., Gelehrter, T., Granner, D., and Peterkofsky, B. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 349.

Varner, J. E., and Chandra, G. R. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 100.