Protein Biosynthesis on Chick Oviduct Polyribosomes. I. Changes during Estrogen-Mediated Tissue Differentiation*

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ABSTRACT: The effect of diethylstilbestrol (DES) upon oviduct translation during hormone-mediated differentiation was investigated by studying the changes which occur in synthesis and activity of polyribosomes. DES stimulation results in an increase in the amount of polyribosomes recoverable by centrifugation of a postmitochondrial supernatant over 2 M sucrose, as well as the presence of larger species of polyribosomes, as detected by sucrose gradient centrifugation within 24 hr following a single injection to immature chicks. Concomitant with these DES-mediated changes is an increased capacity of the newly assembled polyribosomes to synthesize protein as assayed in a cell-free system. Moreover, it was shown that the peptide products synthesized *in vitro* by polyribosomes isolated from unstimulated and DES-treated oviduct exhibited marked qualitative differences upon analysis by polyacrylamide gel electrophoresis. Furthermore, DES was demonstrated to cause striking qualitative changes in the protein populations present in a 105,000*g* supernatant fluid of oviduct. Finally chick oviduct nuclear RNA was isolated and purified at various stages of DES-mediated differentiation. This purified nuclear RNA was demonstrated to possess "messenger" activity as evidenced by its ability to direct peptide synthesis in a heterologous cell-free system. Furthermore, DES administration caused a striking increase in messenger activity in concert with the qualitatively different peptide populations synthesized by oviduct polyribosomes *in vitro*. It is concluded that DES promotes the polyribosomal synthesis of qualitatively different peptides which may reflect earlier hormone-mediated changes in oviduct messenger RNA populations. Maximal stimulation of these biosynthetic events was shown to occur at 3–4 days of hormone-treatment and, thus, prior to completion of tissue cytodifferentiation. These results are compatible with the possibility that DES alters nuclear gene expression as a primary event in its mechanism of action. This alteration could result in the coordinated synthesis of ribosomal and new species of messenger RNA which are subsequently transported into the cytoplasm. Here new polyribosomes are formed from which the cell-specific proteins necessary for oviduct growth and differentiation are translated.

Administration of estrogen to the immature chick results in differentiation of 3 distinct types of epithelial cells from the homogeneous population of primitive mucosal cells present in the undifferentiated oviduct (Brant and Nalbandov, 1956; Ljungkvist, 1967). At least one of these new cell types, the tubular gland cells, synthesizes cell-specific proteins in response to continuing exposure to estrogen (diethylstilbestrol, DES) (Kohler *et al.*, 1968; O'Malley and McGuire, 1968a). The major oviduct protein synthesized by the gland cells is the egg-white protein ovalbumin the induction of which is first apparent at about 4–6 days of DES (O'Malley *et al.*, 1967, 1969). Previous results from these laboratories have demonstrated that DES-induced changes in nuclear transcription such as nuclear RNA synthesis (McGuire and O'Malley, 1968; Dingman *et al.*, 1969), chromatin template activity (O'Malley *et al.*, 1969), and new species of hybridizable RNA (O'Malley and McGuire, 1968b; O'Malley *et al.*, 1968, 1969) precede the appearance of cell-specific proteins. However, the events which couple nuclear changes in gene expression with induction of ovalbumin have not been deliniated.

Total protein biosynthesis has been demonstrated to increase during DES action on the oviduct (O'Malley and McGuire, 1968a; O'Malley *et al.*, 1970; Means and O'Malley, 1970). Since the appearance of new cell types must be accompanied by major changes in the proteins synthesized by the oviduct and since all proteins are synthesized upon cell polyribosomes, we decided to investigate changes in these particles during DES-mediated oviduct differentiation. Polyribosomal protein biosynthesis can be regarded as a means by which to assess the state of oviduct messenger RNA populations during DES-mediated differentiation both quantitatively and qualitatively. Quantitative changes are reflected by total mass of polyribosomes and total capacity of these particles for protein biosynthesis. Again qualitative differences are reflected by analysis of the peptide products synthesized *in vitro* by polyacrylamide gel electrophoresis.

Thus it will be demonstrated that DES stimulates the formation of oviduct polyribosomes. Concomitant with the increase in polyribosomes is a stimulation of their biosynthetic activity assayed in a cell-free system. Furthermore, marked qualitative differences are revealed in the peptides synthesized *in vitro* from polyribosomes isolated at various stages of DES-mediated oviduct differentiation upon analysis by polyacrylamide gel electrophoresis. Finally, these changes in oviduct protein biosynthesis during estrogen action are suggested as reflecting earlier changes in total "messenger" activity of oviduct nuclear RNA.

Materials and Methods

Chemicals. Diethylstilbestrol was purchased from Merck and Co., whereas sucrose (ribonuclease-free), Tris (Ultra

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Pure), and [^{12}C]amino acids were obtained from Mann. Calbiochem was the source for ATP, GTP, phosphoenolpyruvate, and pyruvate kinase. Thioglycerol was obtained from Aldrich, pancreatic ribonuclease (5 × recrystallized) from Worthington, and poly(uridylic acid) from Miles. Sodium dextran sulfate was purchased from Pharmacia (mol wt 2 × 10^6 composed of 17% sulfur). Uniformly labeled L-[^{14}C]valine (209 mCi/mmole) and L-[^{3}H]valine (8.0 Ci/mmole) were from New England Nuclear. The purity of valine preparations was tested in two systems of chromatography as previously described (Means and Hall, 1967).

Preparation of Polyribosomes. Immature Rhode Island Red chicks were used for all studies. Each chick, beginning at 6 days of age, was given a daily subcutaneous injection of diethylstilbestrol (5 mg in sesame oil) for 10–12 days. Chicks were killed by cervical dislocation and the magnum portions of the oviducts were removed and placed in a beaker at 4°. All subsequent procedures were carried out at 0–4°. Oviducts were cut into small pieces and homogenized in 2.5 ml of medium H (0.035 M Tris-HCl, 0.025 M KCl, 0.01 M $MgCl_2$, and 0.25 M sucrose, pH 7.6) per g of tissue by means of a Polytron Pt-10 tissue disintegrator. Homogenization was continued for 30–40 sec at a setting of 3.3. Following centrifugation at 14,000*g* for 15 min the supernatant fluid (10-ml aliquots) was layered on 4 ml of a 2.0 M sucrose solution containing the same concentration of salts as in medium H. Samples were then centrifuged for 3.5 hr at 150,000*g* (R_{av}). The pellet was resuspended in medium A (0.05 M Tris-HCl, 0.025 M KCl, 0.001 M $MgCl_2$, and 0.005 M thioglycerol, pH 7.6, at 4°) to a final concentration of 4 mg of protein/ml. Material isolated in this manner is designated as a "polyribosomal preparation" following the nomenclature established by Wettstein *et al.* (1963). However, as will become evident from the sucrose gradient profiles, single ribosomes are included. The polyribosomes isolated in the manner described above had an RNA to protein ratio of 0.86 to 1.0 and the ratio of absorbency at 260 and 280 was always found to be in the range of 1.55–1.65.

Preparation of the pH 5 Enzyme Fraction. A crude pH 5 enzyme fraction was obtained from 198,000*g* (R_{av}) supernatant fluid of oviduct by the procedure of Campagnoni and Mahler (1967). The pH 5 precipitate was resuspended in medium A and dialyzed for 24 hr against 3 changes of the same medium. The protein concentration was then adjusted to 2.5 mg/ml with medium A and frozen at −196° in 2-ml aliquots. No detectable loss in activity was demonstrable for at least 3 months.

Sucrose Gradient Centrifugation. Linear sucrose gradients (27 ml, 0.3–1.0 M) were prepared as previously described (Means *et al.*, 1969). Polyribosomal material equivalent to 10.0 A_{254} was applied to each gradient using band-forming caps for the Spinco SW 25.1 rotor. Centrifugation was continued for 2 hr at 63,100*g* (R_{av}). Gradients were fractionated using an ISCO Model D density gradient fractionator which monitors continuously the A_{254}.

Protein-Synthesizing System. The complete system for amino acid incorporation by polyribosomes *in vitro* is modified from the procedure described for rat testis (Means *et al.*, 1969). Components included in a final volume of 1.0 ml were: Tris-HCl (pH 7.6), 30 mM; $MgCl_2$, 7.5 mM; ATP, 1 mM; GTP, 0.4 mM; phosphoenolpyruvate, 10 mM; pyruvate kinase, 20 eu; thioglycerol, 2 mM; 19 [^{12}C]amino acids (excluding valine), 0.05 mM each; L-[^{14}C]valine, 0.5 μCi; pH 5 enzyme fraction, 125 μg of protein; and polyribosomes, 400 μg (as protein).

Incubations were carried out at 37° for various periods of time. Following incubation tubes were placed in ice and the reaction was stopped by the addition of 10 μmoles of [^{12}C]valine. Protein was then precipitated with trichloracetic acid added to a final concentration of 10%. After 30 min at 0°, the samples were centrifuged at 1000*g* for 10 min. Pellets were resuspended in 5% trichloroacetic acid, covered, and heated in a 90° water bath for 30 min. Tubes were then cooled, and the contents were collected on glass filter disks and washed ten times with large volumes of 5% trichloroacetic acid. Zero-time controls were those samples carried throughout the procedure but not incubated at 37°.

Determination of Radioactivity. Each filter disk was transferred to a glass scintillation vial. Protein was dissolved by addition of 50 μl of water and 1.0 ml of Nuclear-Chicago solubilizer (NCS) and allowing the samples to remain at room temperature overnight. Ten milliliters of a scintillation fluid prepared by diluting 42 ml of Liquiflor (New England Nuclear) to 1 l. with toluene (Baker) was added to each vial. Radioactivity was determined using a Beckman LS-250 liquid scintillation system. In all cases the counts per minute of zero controls ranged from 75 to 90 and counting efficiency for ^{14}C varied from 85 to 92%.

Determination of Protein. Protein was determined by the method of Lowry *et al.* (1951). Results are expressed as pmoles of [^{14}C]valine incorporated per mg of polyribosomal protein; one pmole of [^{14}C]valine corresponds to 438 dpm in the acid-precipitable material.

Polyacrylamide Gel Electrophoresis. Following incubation in the complete system nascent peptides were released from polyribosomes by the addition of NaF to a final concentration of 10 mM and continuing incubation for an additional 10 min. Samples were then centrifuged in the SW 65 rotor for 2 hr at 150,000*g* (R_{av}). Supernatant fluid was then dialyzed overnight against 3 changes of 0.01 M $NaHCO_3$ containing 5 mM thioglycerol. Finally, the dialysate was concentrated to 0.5–0.75 ml by further dialysis against 75% sucrose. Samples so prepared were analyzed by polyacrylamide gel electrophoresis.

Electrophoresis was performed at 0° in a Büchler Polyanalyst using a resolving gel composed of 10% acrylamide and 0.1% methylene bis(acrylamide) (Bis) buffered at pH 8.83 with Tris-HCl. Potassium persulfate (60 μg/100 ml) and riboflavin (2 mg/100 ml) were present as initiators of the polymerization reaction and 10 μl/100 ml of tetramethylethylenediamine (TEMED) was added as an accelerator. The stacking gel was 1.25% acrylamide and 0.31% Bis buffered to pH 6.67 with Tris-phosphate. Concentrations of initiators were identical with those described above and TEMED was added at 25 μl/100 ml. Each gel was polymerized photometrically for 30 min. Upper buffer was Tris-glycine (pH 8.90) and lower buffer was Tris-HCl (pH 7.51). The resulting running pH was 10.2. Electrophoresis was continued for approximately 2.3 hr at 0° from cathode to anode. Gels were then removed from the glass tubes and fractionated using a Savant Auto-gel Divider. Radioactivity was determined in a Beckman Model LS-250 scintillation spectrometer following addition of a scintillation fluid composed of 100 ml of Beckman BBS-3 and 50 g of Cab-O-Sil thixotropic gel per liter of Liquifluor–toluene. In all cases, sufficient radioactivity was allowed to accumulate to reach a preset error of 0.2%.

Counting efficiency for single-label experiments was 65% for ^{14}C and 38% for ^{3}H. For double-label experiments efficiencies were 42% for ^{14}C and 29% for ^{3}H with a spillover of ^{14}C into the ^{3}H channel of 26%. Data were appropriately corrected before presentation in the figures.

Oviduct Nuclear RNA Extraction. Nuclei were isolated from oviduct as described in detail by O'Malley and McGuire (1968a,b). Nuclei were disrupted in 1 M Tris-HCl (pH 8.3) containing 1.5% (w/v) sodium dodecyl sulfate for 2 min at 23°, an equal vol of hot (60°) phenol solution (phenol–*m*-cresol–8-hydroxyquinoline, 7.9:1:0.1, v/v) was added, and the mixture was vigorously shaken for 10 min at 23° followed by 5 min at 60°. The emulsion was cooled rapidly to 4° and centrifuged at 17,000*g* for 10 min. Aqueous supernatant fluid and interphase material were reextracted in 0.5 M NaCl containing 0.6% sodium dodecyl sulfate for 1 min at 23°, hot phenol solution was again added, followed by vigorous shaking for 4 min at 23° then for 3 min at 60°, and the mixture was recentrifuged at 17,000*g* for 10 min. Resulting aqueous phase was removed and nucleic acids were precipitated by addition of 2 vol of ethanol–*m*-cresol. DNA was removed on a spindle and the remaining solution was placed at −20° overnight. After centrifugation at 800*g* for 10 min the RNA pellet was washed twice with 3 M potassium acetate and finally dissolved in distilled water to a final concentration of 5 μg/μl.

"Messenger" RNA Assay. Ribosomes and soluble factors (the S-30 cell-free extract) were prepared from *Escherichia coli* MRE 600 by the method of Nirenberg (1963). Messenger activity of synthetic and natural nucleic acids was then determined (Nirenberg, 1963). In this assay when utilizing polyuridylic acid, incorporation of [^{14}C]phenylalanine was linear up to 15 μg of poly(U) and the lower level of sensitivity was 0.15 μg. Assays were performed in duplicate and contained the following components in a final volume of 300 μl: 24.5 μmoles of Tris-HCl (pH 7.8); 2.1 μmoles of magnesium acetate; 12 μmoles of NH_4Cl; 246 nmoles of ATP; 7.2 nmoles of GTP; 0.1 μl of mercaptoethanol; 1.86 μmoles of phosphoenolpyruvate; 5 eu of pyruvate kinase; 49.5 μmoles each of 19 unlabeled amino acids (excluding phenylalanine); 0.5 μCi of [^{14}C]Phe (350 mCi/mmole); 200 μl of S-30 extract; and nucleic acid as indicated in the tables. Incubation was continued for 20 min at 37°. Incorporation of [^{14}C]amino acid into peptide was determined by the filter paper technique of Mans and Novelli (1961). Activity of complete assay mixture without added nucleic acid is subtracted from each determination. This value was approximately 0.07 pmole per mg of ribosomal protein. One pmole of [^{14}C]Phe in the acid-insoluble material is equivalent to 730 dpm.

Results

Characterization studies were carried out on immature chicks which had been treated for 10 days with DES. With such chicks the recovery of polyribosomes was 1.2–1.7 μg of polyribosomal protein per mg of oviduct wet weight. The distribution of such a preparation on sucrose gradient is shown in Figure 1A. Seven peaks of absorbance at 254 nm are demonstrable in the figure in which the size of polyribosomal aggregates increases from left to right (*i.e.*, top to bottom of the gradient). The first peak always represents the monomers. Figure 1B shows that addition of deoxycholate (0.5% final concentration) to the postmitochondrial fraction prior to sedimentation through 2 M sucrose resulted in no detectable change in the profile. On the other hand, incubation of the isolated polyribosomes with 1 μg/ml of ribonuclease for 5 min at 37° before gradient centrifugation caused a disaggregation of the polyribosomes into monosomes and disomes (Figure 1C).

Requirements for Cell-Free Protein Synthesis. Requirements

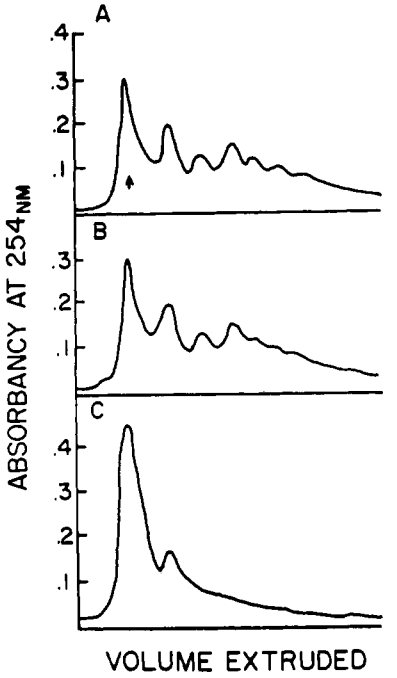

FIGURE 1: Sucrose gradient profile of polyribosomes isolated from oviducts of immature chicks treated daily for 10 days with 5 mg of DES. Polyribosomes were isolated as described in Materials and Methods. The polyribosomal suspension (10 A_{254} units) was applied to each gradient of 27 ml (0.3–1.0 M). Centrifugation was performed for 2 hr at 25,000 rpm in the SW 25.1 rotor of the Beckman L265B at a temperature of 2°. Direction of sedimentation is from left to right (top to bottom) and the first peak (arrow in A) in this and subsequent figures represents single ribosomes: (A) polyribosomes isolated from 15,000*g* oviduct supernatant fluid; (B) polyribosomes isolated from 15,000*g* oviduct supernatant fluid previously treated with sodium deoxycholate to a final concentration of 0.5%; (C) isolated polyribosomes preincubated with RNase (1 μg/ml) for 5 min at 37°.

for protein biosynthesis by the cell-free system from chick oviduct are illustrated in Table I. This system exhibits an absolute requirement for polyribosomes, Mg^{2+}, ATP, and an ATP-generating system. Omission of any one of these components resulted in a 95–100% decrease in activity. Maximal activity of the oviduct system is also highly dependent upon GTP and a pH 5 enzyme fraction. Finally, omission of the 19 unlabeled amino acids or thioglycerol resulted in depressions of 38 and 24%, respectively. Under these optimal conditions the incorporation of [^{14}C]valine into protein increased at a near linear rate throughout 1 hr of incubation.

Metal Ion Requirements. Figure 2A illustrates the effect of Mg^{2+} concentration on the incorporation of [^{14}C]valine into peptides by oviduct polyribosomes *in vitro*. Maximal activity was demonstrated between 6 and 10 mM and raising or lowering the concentration of Mg^{2+} beyond this range resulted in a sharp reduction of activity. On the other hand, the oviductal system did not exhibit a demonstrable requirement for K^+. As can be seen from Figure 2B increasing the concentration of monovalent cation above that already present in the medium (approximately 2 mM) caused a linear decrease in activity. At 100 mM K^+ the incorporation activity of oviduct polyribosomes was only 20% of that observed in the absence of this metal (*i.e.*, the complete system).

Effects of Inhibitors. The effects of various metabolic inhibitors upon activity of the polyribosomal system are shown in Table II. Both puromycin and cycloheximide

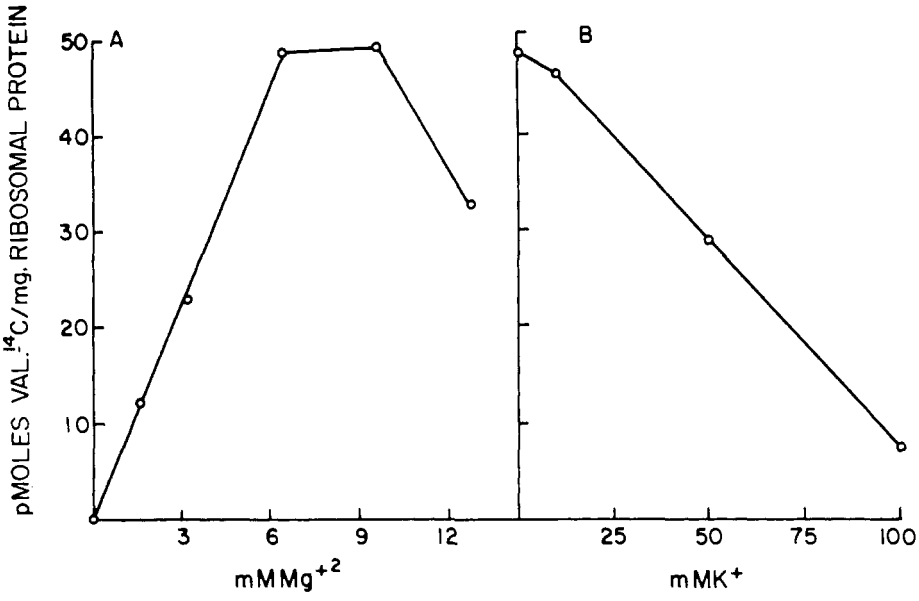

FIGURE 2: Effect of concentration of metal ions upon incorporation of [^{14}C]valine by the cell-free system from oviduct. Incubation was carried out for 30 min at 37° and polyribosomes were from 10 day DES-treated chicks: (A) Mg^{2+} concentration; (B) K^{+} concentration.

caused significant reductions in protein biosynthesis. Deoxyribonuclease did not inhibit incorporation whereas ribonuclease completely abolished activity.

Effect of Ribonuclease Inhibitors on Protein Synthesis by Oviduct Polyribosomes in Vitro. Polyribosome preparations were demonstrated to exhibit ribonuclease activity (1.6×10^{-7} μg per mg of ribosomal protein) when assayed utilizing the thionine dye release assay of Schrader and Greenman (1971). Therefore, various inhibitors of this enzyme were added during the homogenization procedure in order to determine whether the presence of these compounds would (a) affect the level of RNase demonstrable in the polysomal pellet, and (b) affect the protein-synthesizing ability of the isolated oviduct polyribosomes. Table III provides data which illustrate that the protein-synthesizing activity of isolated polyribosomes is unaltered by addition of seven different inhibitors of RNase to the homogenizing medium. On the other hand all of these inhibitors resulted in undetectable levels of ribonuclease in polyribosomal preparations (<0.005 μg/ml). Therefore in all subsequent studies heparin was added to the homogenizing medium (medium H plus 100 μg/ml of sodium heparin).

Polyribosome Accumulation during DES-Mediated Oviduct Differentiation. Table IV shows the effect of DES upon the content and accumulation of polyribosomal protein of

TABLE I: Requirements for Amino Acid Incorporation by Polysomes Isolated for Chick Oviduct.[a]

Component	Specific Activity (pmoles of [^{14}C]Val/mg of Ribosomal Protein)	% Complete
Complete	35.4	(100)
Deletion		
ATP	0.7	2
GTP	12.4	35
ATP-generating system	1.8	5
Mg^{2+}	0.7	2
Thioglycerol	26.9	76
19 Amino acids	21.9	62
pH 5 enzyme fraction	5.7	16
Polysomes	0	0

[a] Incubation with the complete system was performed for 30 min at 37° as described under Methods. The remaining tubes were incubated similarly except for omission of the component shown.

TABLE II: Effect of Inhibitors on Protein Synthesis by Chick Oviduct Polysomes *in Vitro*.[a]

Inhibitor	Concentration (μg/ml)	Specific Activity (pmoles of [^{14}C]Val/mg of Ribosomal Protein)	Inhibn (%)
None		36.7	
Puromycin	50	5.5	85
Cycloheximide	50	11.8	68
DNase	5	36.5	0
RNase	1	0.4	99

[a] Conditions of incubation are described under Methods. Each compound was present from the beginning of the incubation period which was for 30 min at 37°. Polyribosomes (400 μg/ml) were isolated from oviducts treated with DES for 10 days.

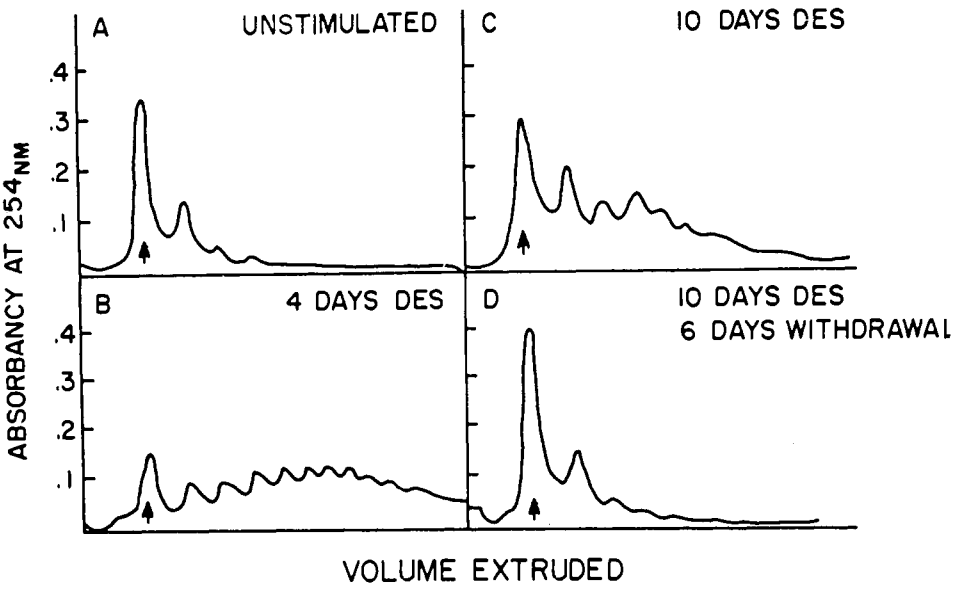

FIGURE 3: Sucrose gradient profiles of polyribosomes isolated from oviduct at various stages of DES-mediated differentiation. Ten A_{254} units was applied to each 27-ml gradient (0.3–1.0 M sucrose). Centrifugation was continued in the SW 25.1 rotor for 2 hr at 25,000 rpm. Gradients were fractionated using an ISCO Model D density gradient fractionator. Direction of sedimentation was from left to right and the arrrow represents location of single ribosomes: (A) unstimulated; (B) 4-day DES; (C) 10-day DES; (D) 10 days of DES then hormone discontinued for an additional 6 days.

chick oviduct. An increase in polyribosomal protein can be seen within 1 day of DES treatment whether data are expressed as micrograms per milligram of oviduct or as microgram per oviduct. Oviductal content of polyribosomes continues to increase for at least 7 days of hormone administration but has begun to decline by 10 days. If estrogen treatment is discontinued after the 10th day and polyribosomes are isolated 16 days hence (10 + 16 w) a precipitous decrease is seen in amount of oviduct polyribosomes. The concentration of polyribosomes (in micrograms per milligram of oviduct) is considerably less 16 days after estrogen withdrawal than the amount isolated from unstimulated chicks.

Effect of DES on Distribution of Ribosomes. A typical sucrose gradient profile of polyribosomes from unstimulated immature chicks is shown in Figure 3A. Sedimentation is from left to right and the arrow signifies the single ribosome peak. Monomers and dimers comprise the bulk of the ribonucleoprotein particles present in the undifferentiated oviduct. Administration of DES for 4 days results in a marked change in the polyribosome pattern (Figure 3B). Ten major peaks can now be observed and a large proportion of the polyribosomes exist as aggregates of two or more monomers. Further stimulation with estrogen (10 days) causes still another pattern change. Some of the heavy polyribosomes

TABLE III: Effect of Presence of Ribonuclease Inhibitors during Isolation of Oviduct Polyribosomes Upon Incorporation Activity *in Vitro*.[a]

Inhibitor	Concentration	Peptide Synthesis (pmoles of [^{14}C]Val/mg of Ribosomal Protein)
None		35.4
Heparin	100 μg/ml	38.1
Polyvinyl sulfate	200 μg/ml	34.8
Polyvinyl pyrrolidone	200 μg/ml	32.0
Diethyl oxidiformate	25 mg/ml	33.2
Bentonite	0.05%	34.2
Sodium dodecyl sulfate	0.05%	35.2

[a] Various inhibitors of ribonuclease activity were added to the homogenization medium in the concentrations indicated below. Polyribosomes were then isolated from oviduct homogenates as described in Methods. Particles were assayed for ability to synthesize protein in a cell-free system (Means *et al.*, 1969). All chicks had received DES for 10 days prior to experimentation.

TABLE IV: Effect of DES-Treatment upon Polyribosomal Protein Isolated from Chick Oviduct.[a]

DES (days)	Ribosomal Protein	
	μg/mg of Oviduct	μg/Oviduct
0	0.6	12.3
1	1.1	39.5
4	1.7	370.5
7	2.1	1062.4
10	1.5	842.5
10 + 16 w[b]	0.1	37.4

[a] DES (5 mg) was administered subcutaneously each day for the times indicated. Polyribosomes were isolated from oviduct as described in Materials and Methods. [b] For this group estrogen was injected daily for 10 days then discontinued for the subsequent 16 days.

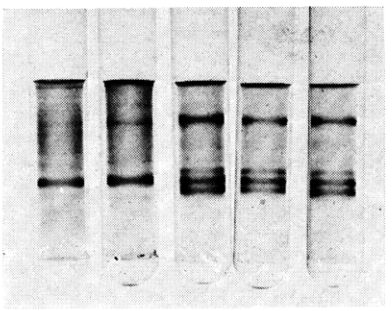

FIGURE 4: Polyacrylamide gel patterns of proteins present in a 105,000*g* supernatant fluid of oviducts at various stages of estrogen-induced differentiation. Approximately 100 μg of protein was applied to each gel. Conditions of gel electrophoresis are described in Materials and Methods and migration was from cathode (top) to anode (bottom). Following electrophoresis for 2.3 hr at a running pH of 10.2, gels were fixed in 12.5% trichloroacetic acid and stained with coomassie blue. From left to right gels represent unstimulated and 1, 4, 7, and 10 days of DES.

present at 4 days are now absent (Figure 3C) and there is an increase in the proportion of monosomes and disomes. When DES-treatment is discontinued for 16 days (Figure 3D), the distribution of polyribosomes now closely resembles the pattern seen in unstimulated oviduct. There is now a predominance of monomers and absence of large polymers.

Incorporation Activity of Polyribosomes. The effect of DES upon protein biosynthesis by isolated chick oviduct polyribosomes is demonstrated in Table V. A twofold increase in incorporation activity is observed within 24 hr following a single injection of DES to immature unstimulated chicks.

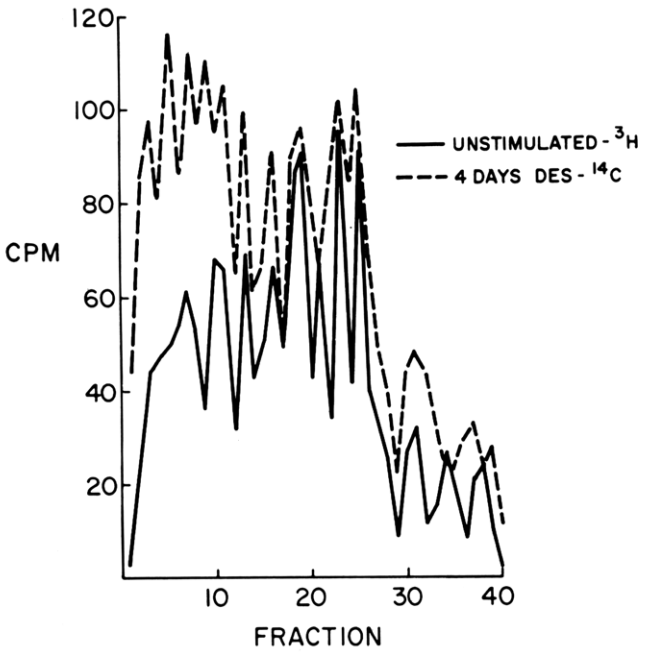

FIGURE 5: Electrophoretogram of peptides synthesized by oviduct polyribosomes *in vitro* at 0 and 4 days of DES treatment. Peptides were double labeled as follows: polyribosomes from unstimulated chicks were incubated with [^{3}H]valine and those from 4 days of DES with [^{14}C]valine. Following incubation for 45 min, samples were pooled and carried through remaining procedures together (see Materials and Methods). Electrophoresis was from cathode (left) to anode (bottom). Gels were fractionated and radioactivity of each fraction determined under optimal conditions for double labeling: ———, ^{3}H (unstimulated); – – –, ^{14}C (4-day DES).

TABLE V: Effect of DES on Protein Synthesis by Isolated Chick Oviduct Polysomes *in Vitro*.[a]

DES (days)	Specific Activity (pmoles of [^{14}C]Val/mg of Ribosomal Protein)
0	19.1
1	39.9
4	75.8
7	60.0
10	36.7
10 + 16 w[b]	18.8

[a] Polyribosomes were isolated from oviducts of chicks which had received estrogen for the times indicated. Polyribosomes (400 μg/tube) were incubated in a cell-free system for 30 min at 37° (Means and Hall, 1969, and Materials and Methods). All assays were performed in triplicate. One pmole of [^{14}C]valine is equivalent to 438 dpm in the acid-precipitated material. Counting efficiency was 88%. [b] For this group estrogen was injected daily for 10 days then discontinued for the subsequent 16 days.

By 4 days of hormone treatment the rate of protein biosynthesis has increased nearly 4 times over that of control polyribosomes and activity is maximal at this time. Decreases are noted at 7 and 10 days of DES, at which point incorporation activity declines to a plateau of approximately double the unstimulated value (unpublished observations). Withdrawal from DES for 16 days results in an incorporation activity very similar to the unstimulated value.

Gel Analysis of Oviduct Soluble Protein. Polyacrylamide gel patterns of protein present in a 105,000*g* supernatant fluid of oviduct at various stages of estrogen-mediated tissue differentiation are shown in Figure 4. Gels represent from left to right the unstimulated pattern and the protein present after 1, 4, 7, and 10 days of DES treatment. It can be seen that large differences occur between 0 and 1 and between 1 and 4 days of hormone administration. On the other hand, the pattern shown for 7 and 10 days exhibit few differences from the 4-day gel. The changes which occur in the soluble protein during the first few days of the differentiation process are of such magnitude that gels at 0 and 4 days do not even appear to represent the same tissue.

Polyacrylamide Gel Analysis of Peptide Products. Since the soluble protein population showed such large changes we examined the peptide products synthesized *in vitro* in order to determine whether the changes in soluble proteins demonstrated *in vivo* (Figure 4) during DES-mediated differentiation were reflected in the cell-free system. Oviduct polyribosomes from unstimulated and 4-day DES-treated chicks were incubated in the cell-free system. Peptide products were isolated and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. Figure 5 illustrates an electrophoretogram of a typical double-label experiment where peptides synthesized from unstimulated polyribosomes are labeled with [^{3}H]valine and those from 4-day treated particles with [^{14}C]valine. Radioactive peaks can be seen throughout the gel length indicating synthesis of a variety of peptides of different sizes (or charge density) by both groups of polyribosomes. However, differences do appear

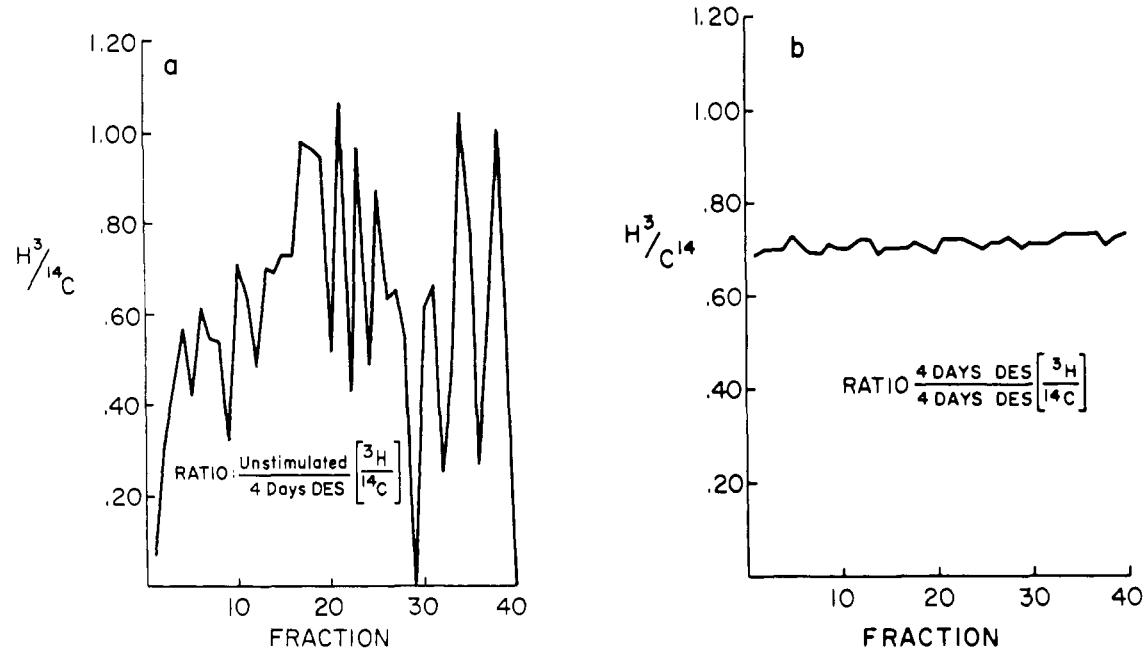

FIGURE 6: Ratio of $^{14}C/^{3}H$ cpm of double-labeled peptides synthesized by oviduct polyribosomes *in vitro*. (a) Ratio of corrected cpm of ^{3}H (unstimulated) to ^{14}C (4-day DES) calculated from the electrophoretogram shown in Figure 3. (b) Ratio of $^{3}H/^{14}C$ of an experiment performed exactly as described in Figure 5 except that both groups of polyribosomes (labeled with ^{14}C or ^{3}H) were from oviduct of 4-day DES-treated chicks.

to be present in the labeling patterns. In order to clarify the differences the ratio of corrected ^{3}H cpm to ^{14}C cpm were plotted separately. The results are exhibited in Figure 6a. Ratio of $^{3}H/^{14}C$ varies throughout the gel and ranges from 0 to 1.06. Moreover marked changes were still apparent if the labels were reversed, that is, if polyribosomes from unstimulated chicks were incubated with [^{14}C]valine and those from 4-day DES chicks with [^{3}H]valine. As a comparison a similar double-label experiment was performed utilizing only polyribosomes from 4-day treated chicks. The ratio of $^{3}H/^{14}C$ is shown in Figure 6b. It can be seen that the ratio is nearly constant at every point along the gel (0.69–0.73). These data demonstrate that major differences do exist in the peptides synthesized *in vitro* by polyribosomes from unstimulated chicks and those treated for 4 days with DES.

Messenger Activity of Various Nucleic Acids. Messenger activity of several nucleic acids, both synthetic and natural, is shown in Table VI. All assays were performed by the procedures described by Nirenberg (1963) which utilize an S-30 extract of *E. coli* MRE 600 as the source of ribosomes and soluble factors. Using polyuridylic acid as message, linear incorporation of poly(U)-directed incorporation of [^{14}C]Phe was demonstrated up to a maximum of 15 g of poly(U) (Table VI). Under these conditions 260 pmoles of [^{14}C]Phe was utilized per mg of ribosomal protein. *E. coli* RNA was also shown to possess messenger activity in this assay system and this activity was destroyed by preheating the RNA. Transfer RNA and DNA isolated from *E. coli* were similarly inactive.

This bacterial system for assessing messenger activity was demonstrated to be a valid assay for RNA from heterologous mammalian sources since nuclear RNA from chick liver was shown to be able to direct the incorporation of [^{14}C]Phe into peptide (Table VI). Moreover this incorporation was linear with respect to increasing amounts of RNA up to a maximum of 400 μg. Finally oviduct nuclear RNA demonstrated messenger activity and this activity was abolished by ribonuclease whereas ribosomal and transfer RNAs isolated from the same tissue were inactive.

The effect of estrogen upon the messenger activity of oviduct nuclear RNA is illustrated in Figure 7. Estrogen produces a threefold stimulation in total messenger activity within 24 hr following a single injection to immature chicks. Daily

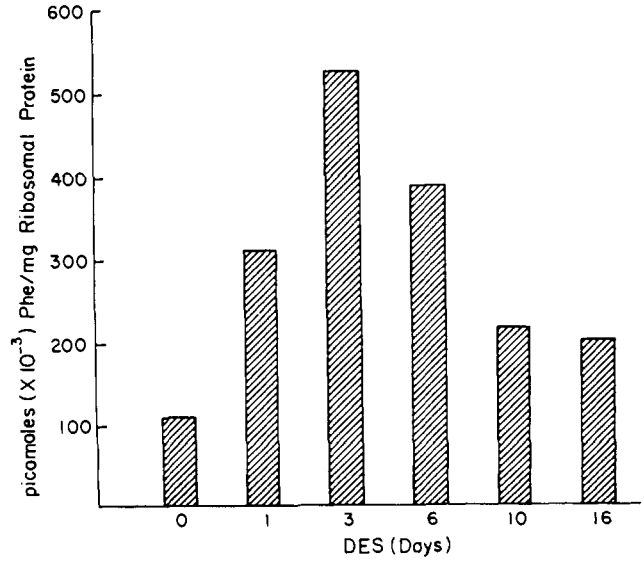

FIGURE 7: Messenger activity of oviduct nuclear RNA at various stages of DES-stimulated development. Nuclear RNA (200 μg) was used for each determination and assays were performed by the method of Nirenberg (1963). Assays were performed in triplicate and activity of assay mixture without added RNA (0.07 pmoles of [^{14}C]-Phe mg of ribosomal protein) was substracted from each value.

TABLE VI: Messenger Activity of Synthetic and Natural Nucleic Acids.[a]

Nucleic Acid		Product Specific Activity (pmoles of [^{14}C]Phe/mg of Ribosomal Protein)
Type	Amount (μg)	
Poly(U)	5	87.8
	10	196.4
	15	257.5
	20	263.7
E. coli RNA	200	3.9
Heated *E. coli* RNA	200	0.3
E. coli tRNA	200	0.3
E. coli DNA	200	
Chick liver RNA	100	0.3
	200	0.8
	300	0.9
	400	1.4
	500	1.4
Oviduct nuclear RNA	200	1.9
Oviduct nuclear RNA	200	
plus RNase	20	
Oviduct rRNA	200	0.05
Oviduct tRNA	200	

[a] Messenger activity was determined by the method described by Nirenberg (1963). Details of this procedure and for extraction of natural nucleic acids are found in Methods. Values represent the mean of triplicate determinations and have been corrected for activity of complete assay mixture without added nucleic acid. One pmole of [^{14}C]Phe is equivalent to 730 dpm in the acid-insoluble material and activity of assay mixture without added nucleic acid (0.07 pmole of [^{14}C]Phe/mg of ribosomal protein) was substracted from each value. Counting efficiency for ^{14}C was 65%.

injections of estrogen resulted in a maximal effect at 3 days and by 6 days messenger activity has begun to decline. Moreover gel analysis of the peptide products synthesized *in vitro* reveal differences when unstimulated and 3-day DES-treated nuclear RNAs are added to the system (I. B. Abrass, A. R. Means, and B. W. O'Malley, unpublished data). These data demonstrate that estrogen-induced changes in nuclear messenger activity occur in concert with major alterations in translational events and offer further evidence that these major biochemical alterations necessary for oviduct cytodifferentiation are nearing completion after only a few days of estrogen treatment.

Discussion

The magnitude of the protein synthetic response of the immature undifferentiated oviduct to DES is considerable and this model system provides one of the few instances where morphologic differentiation of animal cells is not restricted to the period of embryogenesis. Biochemical specialization, *i.e.*, induction of synthesis of cell-free proteins, has been shown to be preceded by and dependent upon hormone-mediated effects at the nuclear level of gene transcription (O'Malley and McGuire, 1968b; O'Malley *et al.*, 1968, 1969, 1970; McGuire and O'Malley, 1968; Dingman *et al.*, 1969). Thus, DES-treatment stimulates within 1 hr rapidly labeled nuclear RNA synthesis (McGuire and O'Malley, 1968), and the RNA produced has been demonstrated to have a different nucleotide composition than nuclear RNA from unstimulated oviduct (O'Malley *et al.*, 1969). Furthermore, the populations of DNA-hybridizable RNA from unstimulated and DES-treated chicks have been demonstrated to show marked qualitative differences (O'Malley and McGuire, 1968b; O'Malley *et al.*, 1968, 1969). Again these changes can be demonstrated within the first 48 hr of DES-action. Thus nuclear synthetic events are apparent within hours of DES-administration whereas cell-specific protein synthesis does not occur until 4–6 days (O'Malley *et al.*, 1967, 1969).

Data presented herein provide evidence as to the nature of biosynthetic events which occur subsequent to nuclear interaction but prior to induction of cell-specific protein synthesis. In this regard DES was shown to result in an increase of oviduct ribosomes recoverable by centrifugation through 2 M sucrose within 24 hr following a single injection to unstimulated chicks (Table IV). Moreover, a striking change occurs in the distribution of ribosomes on sucrose gradients which favors larger aggregates within 4 days of DES-treatment (Figure 3). Furthermore these polyribosomes synthesize at least fourfold more peptide *in vitro* than did the preparation isolated from unstimulated chicks (Table V). These data would be compatible with an increased synthesis of ribosomes or a conversion of monomers to polyribosomes or to a combination of both events. Although many hormones have been demonstrated to increase polyribosomal protein biosynthesis (Korner, 1959; Liao and Williams-Ashman, 1962; Tata, 1967; Teng and Hamilton, 1967; Means and Hall, 1969), few have been reported to concomitantly influence the conversion of monosomes into polyribosomes. Notable exceptions in this regard include the actions of insulin on skeletal muscle (Stirewalt *et al.*, 1967) and epidermal growth factor on chick epidermis (Cohen and Stastny, 1968). Diabetes reduces the proportion of large polyribosomes which can be isolated from rat skeletal muscle. Insulin has been demonstrated to cause a reassembly of preexisting ribosomes resulting in a complete restoration of polyribosomes (Stirewalt *et al.*, 1967). Similarly epidermal growth factor causes a conversion of ribosomes into functional polyribosomal structures in epidermis from chick embryo cultured *in vitro* (Cohen and Stastny, 1968). It should be emphasized, however, that in both chick epidermis and rat skeletal muscle the formation of polyribosomes occurs primarily from preexisting ribosomes. On the other hand, during DES-mediated differentiation of the chick oviduct the increased numbers of large polyribosomes and their increased biosynthetic activity is apparently accompanied by an increased synthesis of ribosomes (Table IV).

We (O'Malley *et al.*, 1967) and Oka and Schimke (1969) have previously demonstrated that if DES-stimulated chicks are withdrawn from hormone ovalbumin synthesis decreases. Palmiter *et al.* (1970) have recently suggested that cessation of ovalbumin synthesis is a consequence of polyribosome disaggregation. The data presented herein would support this suggestion. Readministration of DES to withdrawn chicks causes a resumption of ovalbumin synthesis which is preceded by a reassembly of polyribosomes from preexisting

monomers (Palmiter *et al.*, 1970). These data suggest, therefore, that polyribosome population may be controlled by the availability of messenger RNA. Further support for this suggestion arises from the finding that the "messenger" activity of oviduct nuclear RNA is stimulated very early following estrogen (Figure 7). Collectively these data point out dissimilarities in the action of one hormone on a single target tissue. On the one hand, protein biosynthesis appears to be limited by availability of messenger RNA and polyribosomes necessary for synthesis of developmental and secretory proteins during DES-mediated differentiation. On the other hand, oviduct protein biosynthesis following readministration of DES to previously differentiated chicks undergoing hormone withdrawal may be limited only by the availability of messenger RNA.

Acrylamide gel electrophoresis of soluble protein (105,000*g* supernatant fluid) obtained from oviduct during DES-mediated differentiation reveal marked changes in patterns with the most striking changes occurring between 4 and 7 days of hormone treatment (Figure 4) (Means and O'Malley, 1970). Acrylamide gel electrophoretograms of peptides synthesized by oviduct polyribosomes *in vitro* (Figures 5, 6) also reveal major differences. Again greatest changes are observed at approximately 4 days of DES-treatment. These differences seen in the peptides synthesized *in vitro* may reflect synthesis of new peptides in response to DES or may only reflect differential rates of synthesis of similar peptides. At any rate the polyribosomes isolated from the chick oviduct are capable of synthesizing a variety of peptides *in vitro*. Moreover, although no conclusion can be made from such data, it is interesting that the R_F's of the major bands of ovalbumin and lysozyme correspond to gel fractions 16, 20, 22, and 25 (*i.e.*, R_F's of 0.371, 0.453, 0.509, and 0.562) and that these four fractions all represent "peak" fractions with respect to radioactivity content. This observation raised the possibility of demonstrating the cell-free synthesis of a major oviduct protein such as ovalbumin. In this regard preliminary studies from our laboratory have shown that approximately 25% of the newly synthesized peptide released from the polyribosomes after a 30-min incubation period is precipitable by a specific antibody to ovalbumin. Similar results have been reported for the synthesis of thyroglobulin by thyroid polyribosomes (Salabe *et al.*, 1969) and synthesis of myosin by polyribosomes isolated from chick skeletal muscle (Heywood and Nwagnu, 1968).

Protein biosynthesis as assessed by all criteria described in the present study was observed to decrease by 10 days of DES-administration. Morphologic differentiation of the oviduct is nearly complete by this time and by 15 days of DES treatment ovalbumin comprises 60% of the total oviduct protein (O'Malley *et al.*, 1967). Therefore, the decrease in recoverable ribosomes, change in polyribosomal pattern and decreased rate of protein biosynthesis observed at 10 days may represent the cessation of synthesis of proteins required for oviduct differentiation. Continuous synthesis of relatively fewer proteins would then be required for maintenance of the differentiated state.

The appearance of new proteins synthesized by oviduct polyribosomes during estrogen-mediated differentiation may reflect earlier changes in the population of messenger RNA. Indeed oviduct messenger RNA activity (assayed by the procedure of Nirenberg (1963)) is increased by DES-treatment and the peak in activity occurs prior to the maximal level of protein biosynthesis (Figure 7). The only real measure of messenger RNA, is, of course, the ability of an isolated RNA fraction to direct the synthesis of a specific protein in a cell-free system. Such a claim has recently been reported for the cell-free synthesis of hemoglobin on reticulocyte ribosomes (Lockard and Lingrel, 1969, 1970).

We conclude that estrogen stimulates both morphologic and biochemical differentiation of the chick oviduct. DES was demonstrated to increase the number and activity of polyribosomes isolated from a postmitochondrial supernate of chick oviduct. Furthermore, DES promoted the polyribosomal synthesis of qualitatively different peptides *in vitro* which may reflect earlier estrogen-mediated changes in messenger RNA populations. Finally, these translational events are coupled with primary effects of estrogen upon nuclear transcription resulting in the expression of new RNA species (O'Malley and McGuire, 1968b; O'Malley *et al.*, 1968, 1969). This model system now permits the sequential investigation of coordinated nuclear and cytoplasmic events during steroid-induced target tissue differentiation.

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Protein Biosynthesis on Chick Oviduct Polyribosomes. II. Regulation by Progesterone*

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ABSTRACT: Progesterone neither initiates oviduct cytodifferentiation nor elicits an overall increase in cellular hyperplasia. However, this hormone does specifically induce synthesis of the egg white protein avidin. The effect of progesterone on regulation of oviduct protein biosynthesis was investigated by examining synthesis and activity of polyribosomes following a single injection of this hormone to either unstimulated or estrogen-treated immature chicks. Progesterone stimulates polyribosomal protein synthesis within 24 hr following an injection to immature chicks. Moreover, analysis of the peptide products synthesized *in vitro* reveal differing patterns suggesting qualitative changes in protein populations produced by progestins.

These observations indicate that overall protein biosynthesis can be regulated by progesterone in the undifferentiated oviduct. This regulation may be mediated by earlier changes at the level of gene transcription resulting in synthesis of new messenger RNAs. On the other hand, progesterone administration to chicks previously treated with estrogen results in a disaggregation of polyribosomes and a decreased rate of ribosome synthesis. Twenty-four hours after injection, the ability of isolated polyribosomes to synthesize peptide *in vitro* is reduced by 40% and electrophoretic analysis of these newly synthesized peptides reveal qualitative differences. Furthermore, the radioactivity ratios of double-labeled gels suggest that progesterone may repress some nuclear genes normally active in the estrogen-treated oviduct as well as selectively activate a few previously restricted genes. These conclusions are compatible with the overall decrease in protein biosynthesis caused by progesterone in the differentiated oviduct coupled with the concomitant induction by this hormone of specific proteins such as avidin. Progesterone then appears to regulate protein biosynthesis in unstimulated or estrogen-differentiated oviducts in different manners. However, the primary effects must occur at the level of genetic transcription in both cases with at least one similar result—induction of avidin synthesis.

Progesterone specifically induces synthesis of the egg-white protein avidin within several hours following a single injection to DES-stimulated or unstimulated chicks (Korenman and O'Malley, 1968; O'Malley and McGuire, 1968a). Indeed the versatility of this response is remarkable since progesterone has also been shown to induce avidin synthesis *in vitro* in minces of oviduct tissue (O'Malley, 1967) and in monolayer cell culture (O'Malley and Kohler, 1967). In each case the rate of induction reaches a maximum between 12 and 24 hr after hormone administration.

Unlike the oviductal response to estrogen (Brant and Nalbandov, 1956; Kohler *et al.*, 1969; O'Malley *et al.*, 1969), progesterone neither initiates tissue differentiation and growth nor causes an overall increase in protein biosynthesis (O'Malley and McGuire, 1968a; O'Malley *et al.*, 1969, 1971). In fact, the effect of progesterone on protein biosynthesis is complex since administration of this steroid to chicks previously treated with DES results in a diminished rate of total protein biosynthesis (O'Malley *et al.*, 1971) whereas injection into unstimulated (O'Malley and McGuire, 1968a, O'Malley *et al.*, 1971) or estrogen-withdrawn (Palmiter *et al.*, 1970) animals brings about an increase in this parameter.

We have recently reported the interaction of progesterone with a specific cytoplasmic protein which may represent the initial biochemical response of the oviduct to this steroid (O'Malley *et al.*, 1970, 1971). Subsequent to this initial interaction the bulk of the evidence presently available points towards early primary effects upon nuclear transcription (O'Malley *et al.*, 1969, 1971; O'Malley and McGuire, 1968b, 1969). In order to provide evidence concerning the cyto-

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