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Stimulation of Cell-Free Polypeptide Synthesis by a Protein Fraction Extracted from Chick Oviduct Polyribosomes*

John P. Comstock, Bert W. O'Malley, and Anthony R. Means

ABSTRACT: Estrogen induces synthesis of the specific protein ovalbumin in chick oviduct. We have previously demonstrated completion of ovalbumin chains *in vitro* on oviduct polyribosomes isolated from estrogen-treated chicks. The present experiments were undertaken in an attempt to establish a cell-free ribosomal system capable of *de novo* synthesis of ovalbumin. Extraction of oviduct polyribosomes with sucrose, EDTA, dithiothrietol, and KCl yields a soluble fraction (AvF) which is capable of stimulating both rate and extent of protein synthesis on washed ribosomes by 6- to 10-fold. This stimulation occurs in the presence of saturating amounts of aminoacyl synthetases and elongation factors and is observed whether protein synthesis is directed by endogenous (natural) or exogenous synthetic (polyuridylic acid) mRNA. The AvF-enhanced synthesis of protein is dependent upon time, number of ribosomes, and amount of AvF fraction. A protein nature is suggested for AvF factors since they are inactivated by heat (60°), Pronase, or *N*-ethylmaleimide but not by RNase. Protein synthesis is stimulated by AvF subsequent to aminoacylation of tRNA since stimulation occurs using both [^{14}C]-Val and [^{14}C]Leu-tRNA as labeled substrate. Furthermore, addition of AvF results in a distinct lowering of the Mg^{2+} concentration required for optimal protein synthesis. Again this "Mg^{2+} shift" occurs both with natural message using [^{14}C]Val or [^{14}C]Leu-tRNA and with polyuridylic acid using [^{14}C]Phe-tRNA. Stimulation of polyphenylalanine synthesis and lowering of Mg^{2+} optimum occur in the presence of saturating amounts of partially purified T_1 and T_2 indicating that AvF is distinct from elongation factors. Finally, as a method for identifying and separating a specific protein product of the AvF-stimulated reaction, peptides synthesized and released *in vitro* were subjected to antiovalbumin affinity chromatography. This procedure demonstrated that in the presence of AvF, 14% of the released peptides was immunologically similar to ovalbumin.

Administration of estrogen to the immature chick results in both morphologic differentiation and biochemical specialization of the oviduct (O'Malley *et al.*, 1969). Following cellular differentiation one of the new cell types (the tubular gland cell) produces the specific protein ovalbumin (O'Malley *et al.*, 1969; Kohler *et al.*, 1968). Moreover, once differentiation has occurred, production of this protein is regulated by estrogen and this regulation appears to occur at the level of nuclear transcription (O'Malley *et al.*, 1969; O'Malley and McGuire, 1968a,b).

We have recently demonstrated that estrogen administration results in the assembly of oviduct polyribosomes as well as an increase in their biosynthetic activity assayed in a cell-free protein-synthesizing system (Means *et al.*, 1971). This polyribosomal system is capable of synthesizing immunologically identifiable ovalbumin *in vitro* (Means and O'Malley, 1971). However, it is possible that the immunologically competent protein product represents only completion and release of previously existing nascent chains. Consequently we have begun to further define the cell-free system in order to prepare cellular materials capable of initiating and completing ovalbumin molecules entirely *in vitro*. It is anticipated that such a system would be capable of translating exogenous mRNA and, therefore, might be useful in assaying the unique products of estrogen directed transcription.

Miller *et al.* (1967) were the first to report a cell-free system derived from rabbit reticulocytes which was capable of *de novo* globin synthesis. This system has subsequently been shown to contain initiation factors and to be able to translate exogenous mRNA (Prichard *et al.*, 1970; Nienhuis *et al.*, 1971). Therefore, we undertook to develop a similar system from the oviduct. This report documents the isolation of an avian protein fraction (AvF) from oviduct polyribosomes which stimulate both total protein synthesis and ovalbumin synthesis *in vitro*. The data are consistent with the AvF-dependent stimulation of chain initiation.

Experimental Section

Preparation of Oviduct Ribosomes and AvF Factors. Female Rhode Island Red chicks (7 days old) were given daily sub-

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cutaneous injections of diethylstilbestrol (5 mg) for 10–12 days. Polyribosomes were prepared from the magnum portion of the oviduct as previously described (Means *et al.*, 1971). Following centrifugation through 1.85 M sucrose, the polyribosomes were resuspended in SED solution (0.25 M sucrose, 0.1 mM Na_2EDTA (pH 7.0 with NaOH), and 1 mM dithiothreitol; the concentration was adjusted to 120 A_{260}/ml with SED. Polyribosomes were then washed as first described by Miller *et al.* (1967). The suspension was immersed in an ice bath and 4.0 M KCl was added slowly to a final concentration of 0.5 M while constantly spinning the tube. After standing at 2° for 15 min, the suspension was centrifuged at 105,000*g* (R_{av}) for 60 min. The upper three-fourths of the supernatant fluid was then collected and stored at −70° in 200-μl aliquots. Protein concentration of this AvF fraction was determined by the method of Lowry *et al.* (1951) and was always within the range of 3–4 mg/ml. The ribosome pellet was resuspended in SED to a final concentration of 120 A_{260}/ml and 200-μl aliquots were stored at −70°. Sucrose gradient analysis of the pellet revealed that more than 95% of the material consisted of monomeric ribosomes and ribosomal subunits. Protein content of the ribosome preparation was approximately 7–10 mg/ml and the RNA concentration, determined by the Ceriotti method (Ceriotti, 1955), was 7–8 mg/ml.

Preparation of Crude Enzyme Factors from Oviduct Supernatant Fluid. The clear portion of the 150,000*g* (R_{av}) supernatant fraction (*i.e.*, crude cytosol) obtained during the preparative ultracentrifugation step necessary for preparation of polyribosomes (Means *et al.*, 1971) was collected and stored at −70°. Subsequently this fraction was used for the isolation of fractions containing aminoacyl synthetases and polypeptide chain elongation factors. Three different procedures were tried in order to obtain a preparation with optimal activity when it was found that the crude cytosol inhibited protein synthesis.

pH 5 ENZYME FRACTION. Crude cytosol was adjusted to pH 5.1 by the addition of 1.0 N acetic acid. After standing at 4° for 30 min the suspension was centrifuged at 10,000*g* for 15 min. The resulting supernatant fraction was collected and dialyzed for 18 hr against medium I (0.05 M Tris-HCl (pH 7.5)–0.1 mM EDTA (pH 7.0)–1.0 mM dithiothreitol). Protein concentration of the dialysate was 5.0 mg/ml.

AS_{70} ENZYME FRACTION. A protein fraction precipitating between 35 and 70% ammonium sulfate saturation (at 4°) was prepared from crude cytosol by the method of Arlinghaus *et al.* (1968). This AS_{70} fraction (26.8 mg/ml) was used following dialysis against medium I.

G-25 CYTOSOL. Crude cytosol (5 ml) was passed through a 1.5 × 20 cm column of Sephadex G-25 (medium). The supernatant fluid was eluted with medium I and the eluate was continuously monitored at A_{280}. The peak absorbancy fractions were combined and concentrated by Diaflo pressure dialysis. Final protein concentration was 15.1 mg/ml.

All of these preparations were stored in small aliquots at −70°.

Preparation of [^{14}C]Aminoacyl-tRNAs. Purified chick oviduct tRNA was prepared by the procedure described by Anderson (1969). Nucleic acids were separated by phenol extraction of a 16,000*g* supernatant fluid followed by ethanol precipitation. The bulk of large molecular weight RNAs were removed from the total RNA fraction by precipitation with 1 M NaCl. The tRNA was then separated from the remaining large molecular weight materials by Sephadex G-200 chromatography as described by Delihas and Staehelin (1966). The second A_{260} peak contained the tRNA. This fraction was concentrated, dialyzed, and lyophilized. The yield of tRNA was 4 A_{260} units/g of oviduct. A fraction containing aminoacyl synthetases was made from crude cytosol by the method of Muench and Berg (1966). Finally, [^{14}C]Leu-tRNA (5.0% charged with a specific activity of 684 dpm/pmole) containing 19 [^{12}C]aminoacyl-tRNAs and [^{14}C]Phe-tRNA (0.6% charged with a specific activity of 1001 dpm/pmole) were prepared as previously described (Anderson, 1969). The preparations were lyophilized and stored at −70°.

Cell-Free Protein Synthesis. The incubation mixture contained the following components in a final volume of 100 μl: Tris-HCl (pH 7.2 at 23°), 30 mM; ATP (neutralized to pH 7.0), 1.0 mM; GTP (neutralized to pH 7.0); 0.5 mM; phosphoenolpyruvate, 7.5 mM (neutralized to pH 7.0); pyruvic kinase, 1.5 EU, $MgCl_2$, 5.0 mM; dithiothreitol, 1.0 mM; [^{14}C]valine, 1.0 μM (0.1 μCi); and 19 [^{12}C]amino acids, 20.0 μM each. In addition, reaction mixtures usually contained ribosomes (1.4 A_{260}; 110 μg of RNA), AvF fraction (70 μg of protein) and AS_{70} enzyme fraction (180 μg of protein). Samples were incubated for 20 min at 37° and reactions were terminated by addition of 1.0 ml of 10% trichloroacetic acid. Precipitates were then prepared for liquid scintillation counting as previously described (Means *et al.*, 1969). Counting efficiency for ^{14}C was 85–88%.

Assay conditions were modified when [^{14}C]Leu-tRNA was used as the radioactive tracer. For these experiments the incubation mixture contained the following components in a volume of 100 μl: Tris-HCl (pH 7.2), 30 mM; GTP (pH 7.0), 0.5 mM; phosphoenolpyruvate (pH 7.0); 7.5 mM; pyruvate kinase, 1.5 EU; dithiothreitol, 1.0 mM; $MgCl_2$, 5.0 mM; KCl, 100 mM; ribosomes, 35 μg of RNA; AvF fraction, 60 μg of protein; AS_{70} enzyme fraction, 180 μg of protein, and 8.0 pmoles of (5470 dpm) of [^{14}C]Leu-tRNA and 19 [^{12}C]aminoacyl-tRNAs. Incubation was carried out at 37° for 3 min; 1.0 ml of 10% trichloroacetic acid was then added to stop the reaction. Samples were processed as described previously (Means *et al.*, 1969, 1971).

Affinity Chromatography of Ovalbumin. An ω-aminoalkyl derivative of Sepharose-4B was prepared as described by Cuatrecasas (1970). To this was coupled a highly specific antibody to ovalbumin. The resulting antibody–Sepharose gel had a capacity for ovalbumin of 200 μg/ml of packed gel volume. The egg proteins lysozyme and avidin or bovine serum albumin were not bound to the gel. Furthermore, radioactive ovalbumin, isolated and purified by the method of Rhodes *et al.* (1958) from an egg labeled *in vivo* with [^{3}H]Val was bound and released quantitatively from the gel as assayed by counts. Ovalbumin was released from the column with 6 M guanidine-HCl and a 1.0-ml fraction was collected. Following dialysis against medium I samples were counted by liquid scintillation spectrometry. Purified ovalbumin following elution with 6 M guanidine-HCl was found to be no longer immunologically reactive with the antibody. We were therefore unable to specifically identify the retained material as ovalbumin.

Materials and Methods Chemicals. Diethylstilbestrol was purchased from Merck and Co. Sucrose (ribonuclease free), Tris (Ultra Pure), ammonium sulfate (Ultra Pure), guanidine-HCl (Ultra Pure), and [^{12}C]amino acids were obtained from Schwarz-Mann Research Laboratories. Calbiochem supplied ATP, GTP, phosphoenolpyruvate, pyruvate kinase, dithiothreitol, Pronase (B grade), and *N*-ethylmaleimide. Polyuridylic acid (ammonium salt) was purchased from Miles Laboratories. Ribonuclease (code R) was obtained from Worthington Biochemical Corp. Uniformly labeled L-[^{14}C]-

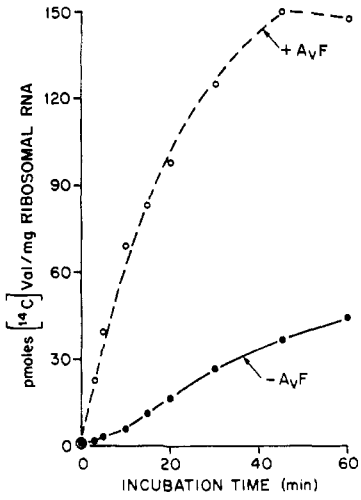

FIGURE 1: Time course for the incorporation of [^{14}C]valine into protein. A 100-μl aliquot was removed at each time point from a 1000-μl reaction mixture which contained 1.10 mg of RNA (14.0 A_{260} units), 200 μl of AvF (700 μg of protein), 1.8 mg of AS_{70} protein, and the other components as described in the Experimental Section. A zero-time blank of 1.1 pmoles was subtracted from each point. [^{14}C]Valine (1 pmole) is equivalent to 572 dpm.

TABLE I: Dependence of Protein Synthesis on Various Components of the Reaction Mixture.[a]

Component	pmoles of [^{14}C]Val Incorpd/mg of rRNA
Complete	141.1
Deletion	
ATP	24.4
GTP	109.4
ATP-generating System	32.0
Mg^{2+}	1.1
Dithiothreitol	136.9
19 amino acids	59.8
Tris	90.7
Ribosomes	0
AS_{70} enzyme fraction	85.1
AvF fraction	19.2

[a] The concentration of each component of the reaction mixture is noted in the Experimental Section. Each reaction mixture contained, unless deleted, 110 μg of rRNA (1.4 A_{260} units), 70 μg of AvF protein, and 180 μg of AS_{70} protein. Incubation was for 20 min at 37°.

valine (260 mCi/mmole) and L-[^{14}C]phenylalanine (450 mCi/mmole) were from Schwarz BioResearch, Inc., and uniformly labeled L-[^{14}C]leucine (312 mCi/mmole) was from Amersham–Searle.

Results

Rate of protein synthesis over a 60-min time period is shown in Figure 1 for oviduct ribosomes incubated in the presence or absence of the AvF fraction. Under the incubation conditions utilized AvF resulted in a marked stimulation in the rate of protein synthesis and linear kinetics are observed for 20–30 min.

The effect of increasing amounts of AvF on oviduct protein synthesis by a rate-limiting amount of ribosomes is shown in Figure 2A. It can be seen that a linear increase is noted up to 30 μg of AvF protein and saturation is achieved at approximately 60 μg. High concentrations of protein (120 μg) begin to inhibit the incorporation of [^{14}C]valine into protein by oviduct ribosomes. The inhibition may result from the high KCl concentrations which are reached with larger amounts of AvF since the KCl optimum for the system is 100 mM (present in 60 μg of AvF). However, attempts to lower the KCl concentration of AvF by dialysis results in a loss of activity. AvF, however, can be made 0.15 M KCl by dilution with SED, absorbed to DEAE-cellulose and batched off with 0.4 M KCl without a significant loss in activity (Gilbert and Anderson, 1970). Figure 2B demonstrates that protein synthesis is linear up to 140 μg of ribosomes (expressed as RNA) when incubated in the presence of a saturating amount of AvF. Between 140 and 350 μg of ribosomes, amino acid polymerization is not limited by the amount of ribosomes in the reaction mixture.

The requirements for the cell-free system in order to obtain maximal protein synthesis are shown in Table I. The system is highly dependent on Mg^{2+}, ATP, and an ATP-generating system as well as upon AvF fraction and ribosomes. Presence of GTP, 19 [^{12}C]amino acids, and Tris-HCl buffer are also necessary for optimal protein synthetic activity. On the other hand, no requirement could be demonstrated for additional dithiothreitol. It should be pointed out, however, that this compound is also present in the AvF fraction and ribosomes and, indeed, is required for the stability of the stimulatory activity of AvF.

Table II illustrates that essentially no activity is observed in the cell-free system in the absence of ribosomes. This suggests that the AvF fraction is not grossly contaminated with ribosomes. Moreover, only minimal activity occurs when ribosomes and AS_{70} enzyme fractions are incubated in the absence of AvF whereas significant protein synthesis results with ribosomes and AvF alone. Addition of a crude cytosol (cell sap) as a source of transfer factors and aminoacyl synthetases inhibit protein synthesis. This inhibition could be removed by passing the cytosol over a Sephadex G-25 column. Finally AS_{70} enzyme fraction, G-25 cell sap or a pH 5 enzyme fraction appear to be equally effective in promoting maximal protein synthesis in the presence of AvF.

The capacity for stimulating protein synthesis is destroyed by heating the AvF fraction for 5 min at 60° prior to assay. Furthermore, pretreatment with *N*-ethylmaleimide or pronase inactivates the fraction whereas full activity is maintained upon pretreatment with RNase. These experiments with inhibitors were performed as described by Miller and Schweet (1968). Appropriate control tubes were included on each experiment so that the effective concentration of inhibitor (incubated directly with AvF) did not alter the rate of polymerization if added to the entire reaction mixture. These data suggest that the stimulatory factors of AvF are proteins.

All data presented in the preceding figures were obtained using a free amino acid as the tracer substrate. Figure 3A shows data using [^{14}C]Leu-tRNA as the labeled material. The time course for protein synthesis obtained in the presence or absence of the AvF fraction can be seen in Figure 3A. AvF results in significant stimulation of the rate of protein synthesis but since the synthetase reaction is by-passed in this study, the time of a linear rate of protein synthesis was shortened considerably (compare to Figure 1). Figure 3B shows that in

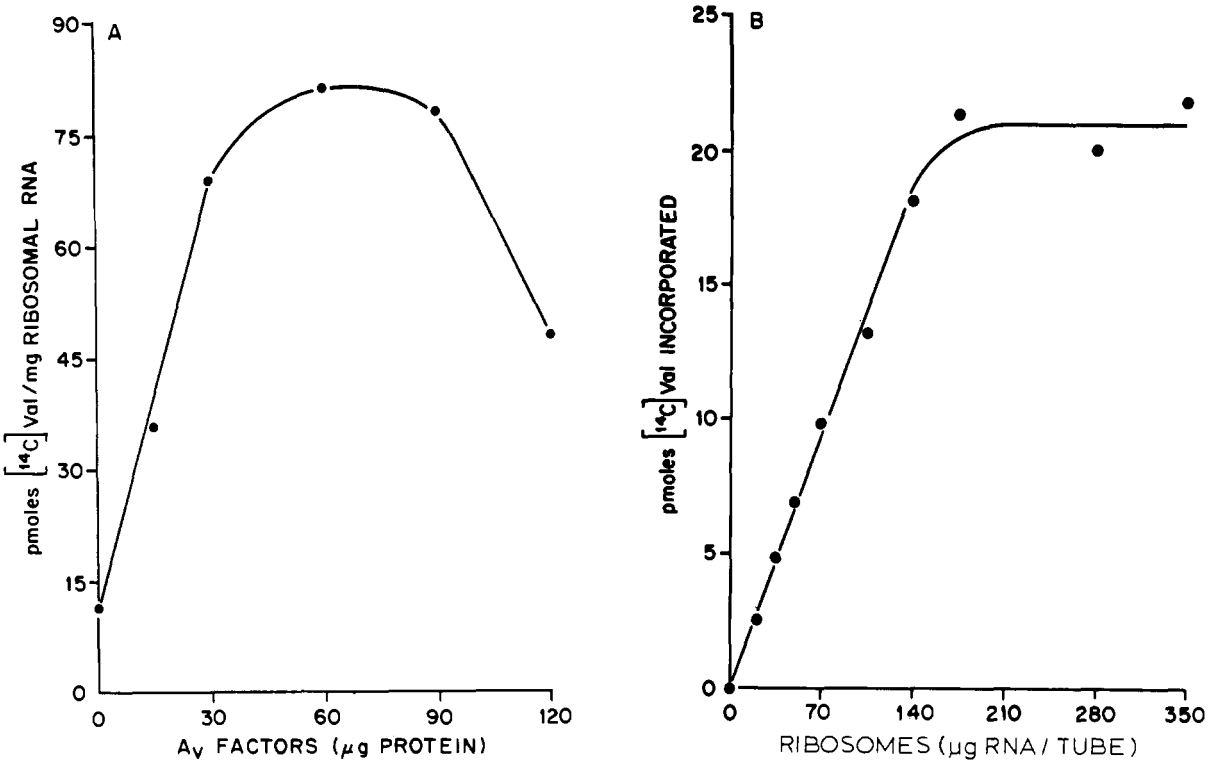

FIGURE 2: [^{14}C]Valine incorporation into protein. (A) As a function of the amount of AvF fraction. Each 100-μl reaction mixture contained 110 μg of RNA (1.4 A_{260} units), 180 μg of AS_{70} protein, and the other components as described in the Experimental Section. Incubation was for 20 min at 37°. (B) As a function of the amount of ribosomes. Each 100-μl reaction mixture contained 15 μl of the AvF fraction (70 μg of protein), 5 μl (180 μg) of AS_{70} protein, and the other components as described in the Experimental Section. Incubation was for 20 min at 37°. A blank of 0.3 pmole was obtained in the absence of ribosomes and was subtracted from each point.

the presence of AvF protein synthesis increased linearly over a wide range of ribosome concentrations.

The Mg^{2+} concentration required for maximal protein synthesis is shown in Figure 4. In the absence of AvF the Mg^{2+} optimum is 7 mM. However, when protein synthesis is measured in the presence of AvF there is a distinct lowering of the Mg^{2+} requirement to 4 mM. This lower optimum occurs when either [^{14}C]valine or [^{14}C]Leu-tRNA is used as substrate, indicating that the acylation of tRNA which may be stimulated in the presence of AvF since the salt wash contains aminoacyl synthetases (Gilbert and Anderson, 1970) is not responsible for lowering the Mg^{2+} optimum.

Table III illustrates the ability of washed oviduct ribosomes to polymerize [^{14}C]Phe in the presence of poly(U). It can be seen that addition of poly(U) to the complete system (*i.e.*, containing AvF) results in an eightfold stimulation in [^{14}C]Phe incorporation. Thus, whereas oviduct polyribosomes will not accept poly(U) (Means and O'Malley, 1971), the KCl-washed oviduct ribosomes used in the present experiments respond very well to the addition of exogenous mRNA.

Figure 5 shows the Mg^{2+} concentration required for the poly(U)-directed polymerization of [^{14}C]Phe-tRNA. The conditions were set so that a rate-limiting amount of rabbit reticulocyte ribosomes (washed twice in high salt) were incubated for 2 min (rate point) in the presence of saturating amounts of the partially purified rabbit reticulocyte elongation factors, T_1 and T_2. In this system AvF both stimulates the rate of phenylalanine polymerization and lowers the Mg^{2+} optimum from 10 to 6 mM.

Figure 6 offers some evidence as to the product synthesized in the AvF-dependent natural message system. A large reaction mixture was incubated for 60 min at 37° and then centri-

TABLE II: Dependence of Protein Synthesis on Various Components of the Reaction Mixture.[a]

Ribosomes	Ribosomal Wash Fraction	Supernatant Enzyme Fraction: Source	Supernatant Enzyme Fraction: Amt (μg)/Tube	pmoles of [^{14}C]Val Incorpd
R	AvF	AS_{70}	180	15.8
R				0.1
	AvF			0.1
		AS_{70}	180	0.1
	AvF	AS_{70}	180	0.1
R		AS_{70}	180	2.4
R	AvF			9.5
R	AvF	pH 5	25	14.2
R	AvF	Cell sap	70	5.7
R	AvF	Cell sap	140	4.5
R	AvF	G-25 Cell sap	300	16.7

[a] The reaction mixture contained 110 μg of rRNA (1.4 A_{260} units), 70 μg of AvF protein, AS_{70} enzyme fraction, and other components described in the Experimental Section. Supernatant enzyme fractions (containing transfer factors and aminoacyl synthetases) were prepared as described in the Experimental Section and added in saturating amounts as determined in separate experiments. The crude cell sap, 150,000*g* (R_{av}), routinely inhibited incorporation in all amounts tested. Incubation was 20 min at 37°.

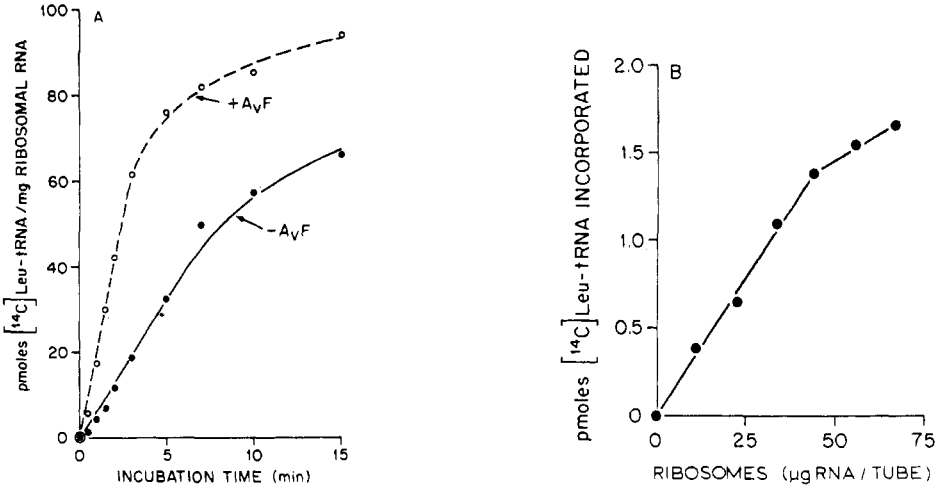

FIGURE 3: (A) Time course for the incorporation of [^{14}C]leucyl-tRNA into protein. Each 100-μl reaction mixture contained 39 μg of rRNA (0.84 A_{260} unit), 20 μl of AvF (60 μg of protein), 180 μg of AS_{70} protein, 8 pmoles of chick oviduct [^{14}C]leucyl-tRNA (580 cpm/pmole), 19 [^{12}C]-aminoacyl-tRNAs, and other components as described in the Experimental Section. Incubation was for 3 min at 37°. A zero-time blank of 1.5 pmoles has been subtracted from each point. (B) Incorporation of [^{14}C]leucyl-tRNA into protein as a function of the amount of ribosomes. Incubations were performed under the conditions as described in the legend to Figure 5. A blank of 0.13 pmole obtained in the absence of ribosomes was subtracted from each point.

fuged at 105,000g (R_{av}) for 60 min. An aliquot of the supernatant and a portion of the ribosomal pellet were prepared for determination of acid-insoluble radioactivity as previously described (Means *et al.*, 1971). Under these conditions 73% of the incorporated radioactivity remained associated with the ribosomes whereas 27% was released into the supernatant fluid. The supernatant fluid was passed through a Sepharose column to which antiovalbumin was coupled. The column was then washed thoroughly with buffer. The bound counts were eluted with 6 M guanidine-HCl and the appropriate fractions containing radioactivity were pooled and dialyzed. It can be seen that 14% of the radioactive peptides which had been released into the supernate during incubation bound to the column. These data indicate that a significant fraction of the released peptides are immunologically similar to ovalbumin. This column is being used in an initial step to separate a specific product of the cell-free system from the reaction mixture in order that further chemical analysis of such a product can be performed.

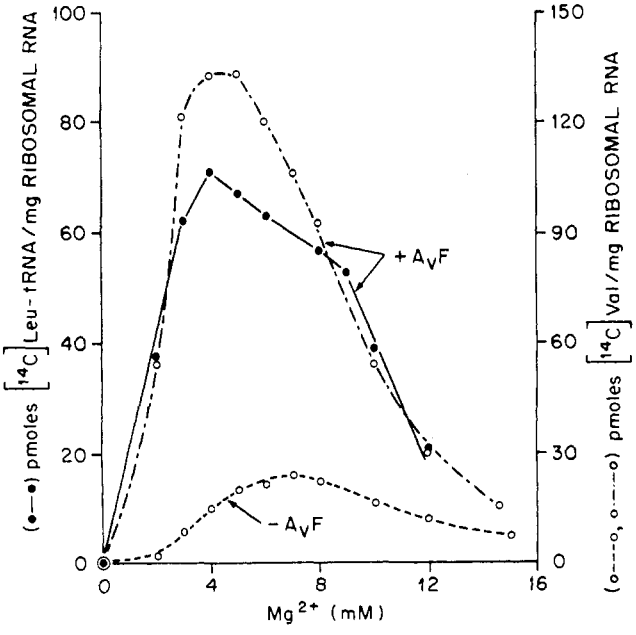

FIGURE 4: [^{14}C]Valine and [^{14}C]leucyl-tRNA incorporation into protein as a function of Mg^{2+} concentration. Each reaction mixture contained the appropriate components for the different assay conditions as described in the legends to Figures 3 and 6. Blanks of 1.3 pmoles of [^{14}C]valine and 1.8 pmoles of [^{14}C]leucyl-tRNA obtained for each assay condition in the absence of Mg^{2+} were subtracted from the appropriate points.

Discussion

We have described significant refinements of a cell-free protein synthesizing system from a hormone-dependent nucleated tissue. This sytem is now highly dependent upon protein substances removed from polyribosomes by washing with a high concentration of KCl in the absence of Mg^{2+}. The crude wash (AvF fraction) stimulates both rate and extent of amino

TABLE III: Poly(U)-Directed Phenylalanine Polymerization on Washed Oviduct Ribosomes.[a]

System	pmoles of [^{14}C]Phe Incorpd
Complete + poly(U)	12.5
Complete − poly(U)	1.6

[a] The complete system contained 100 μg of rRNA (1.2 A_{260} units), 60 μg of AvF protein, 180 μg of AS_{70} protein, and 0.70 A_{260} unit of poly(U). Other components were ATP, 1.0 mM; GTP, 0.5 mM; phosphoenolpyruvate, 3.0 mM; pyruvate kinase 1.5 EU; dithiothreitol, 1.0 mM; Tris-HCl (pH 7.2), 30 mM; [^{14}C]phenylalanine, 20 μM (0.1 μCi); KCl, 100 mM (supplied partially in the AvF fraction); and Mg^{2+}, 6 mM. Incubation was for 15 min at 37°. A blank of ribosome plus poly(U) was 0.1 pmole. [^{14}C]Phe (1 pmole) is equivalent to 1001 dpm.

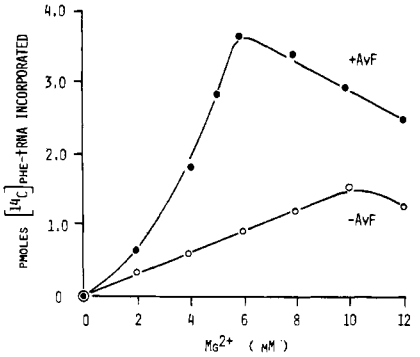

FIGURE 5: [^{14}C]Phe-tRNA polymerization as a function of Mg^{2+} concentration. Each 50-μl reaction mixture contained 0.2A_{260} of twice-washed (0.5 M KCl) rabbit reticulocyte ribosomes (incorporation was linear up to 0.35 A_{260} unit, 0.75 A_{260} unit of poly(U), 5.5 pmoles of chick oviduct [^{14}C]Phe-tRNA (840 cpm/pmole), and 30 μg of AvF protein. Saturating amounts of partially purified rabbit reticulocyte T_1 protein (20 μg) and T_2 protein (3.0 μg) were used in all tubes. The reaction mixture contained Tris-HCl (pH 7.2), 30 mM; GTP (pH 7.0), 0.5 mM; phosphoenolpyruvate (pH 7.0), 3.0 mM; pyruvate kinase, 1.5 EU; dithiothreitol, 1.0 mM; and KCl, 100 mM (partially supplied by AvF fraction). Incubation was for 2 min at 37°. A blank of 0.24 pmole obtained in the absence of Mg^{2+} was subtracted from each point. Incorporation without the addition of poly(U) was 0.10 pmole in the presence of AvF and 6 mM Mg^{2+}.

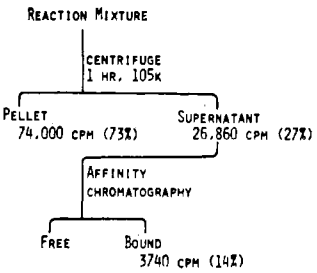

FIGURE 6: AvF-dependent synthesis of immunologically reactive ovalbumin on oviduct ribosomes *in vitro*. The reaction mixture (2000 μl) on which the analysis was carried out contained 2.0 mg of RNA (20.0 A_{260} units), 400 μl of AvF (1200 μg of protein), 3.6 mg of AS_{70} protein, and other components as described in the Experimental Section. Following incubation for 60 min at 37° the reaction mixture was centrifuged at 105,000*g* (R_{av}) for 60 min. The supernatant was carefully removed, duplicate 100-μl aliquots were prepared for counting (5), and the remainder applied to an antiovalbumin affinity column (5 ml). The bound counts were collected in 2 ml following elution with 6 M guanidine-HCl, dialyzed against medium 1, and 1 ml was counted in Spectroflor (Amersham–Searle)–toluene–Triton X-100 (42:958:500). The ribosomal pellet was resuspended in water and prepared for counting as previously described (Means *et al.*, 1971).

acid polymerization on washed ribosomes and under optimal conditions the system demonstrates linear incorporation kinetics for 30 min. Moreover the cell-free system exhibits linear dependence upon both the number of ribosomes and the amount of AvF fraction.

Saturating amounts of transfer factors and aminoacyl synthetases are provided by an AS_{70} fraction of oviduct cytosol. Addition of this material results in 40% increase in amino acid polymerization over the stimulation observed in the presence of AvF alone. This suggests that the AvF fraction does contain T factors and synthetases but probably not in saturating amounts. On the other hand, in the presence of saturating amounts of these cytosol factors alone the protein-synthesizing system is capable of polymerizing only 15% of the complete system, that is a system containing both cytosol enzymes and AvF.

High salt-washed ribosomes contain saturating amounts of tRNA (Gilbert and Anderson, 1970). It is unlikely, therefore, that AvF which also contains tRNA (Gilbert and Anderson, 1970) is stimulating protein synthesis by the addition of tRNA to the reaction. This is indicated by the data which show AvF stimulates polymerization in the presence of aminoacyl-tRNAs at a 2-min rate point and the stimulatory activity remains following elution of AvF, but not tRNA from DEAE-cellulose at 0.4 M KCl (J. P. Comstock, B. W. O'Malley, and A. R. Means, unpublished results).

A distinctly low Mg^{2+} optimum of 4 mM exists for the AvF-dependent system as contrasted to the higher optimum of 7 mM required in the absence of the factors. AvF, therefore, appears to shift the Mg^{2+} optimum to a lower concentration. Moreover the same low Mg^{2+} optimum of 4 mM is observed when acylated tRNA is used as the tracer in the polymerization assay. These data suggest that charging of tRNA in the presence of AvF is not producing the decrease in Mg^{2+} optimum.

The requirement for a low Mg^{2+} concentration in polypeptide synthesis is well documented for bacterial and reticulocyte cell-free systems (Prichard *et al.*, 1970; Miller and Schweet, 1968; Nakamoto and Kolakofsky, 1966; Revel and Hiatt, 1965). Moreover a shift to this lower optimum is associated with chain initiation and fidelity of translation (Miller and Schweet, 1968; Salas *et al.*, 1967; Lucas-Lenard and Lipmann, 1967). Miller and Schweet (1968) first reported that *de novo* hemoglobin synthesis was achieved in the rabbit reticulocyte system at low Mg^{2+} concentrations using a KCl ribosomal wash fraction. More recently Shafritz *et al.* (1970), again employing the reticulocyte system, were able to demonstrate a low Mg^{2+} optimum in a poly(U)-dependent phenylalanine polymerization assay. This Mg shift required two protein factors isolated from the crude salt wash of reticulocyte ribosomes. Furthermore these same investigators have shown that these protein factors are distinct from chain elongation factors T_1 and T_2 (Shafritz and Anderson, 1970a). Thus, by utilizing Met-$tRNA_F$ binding (Shafritz and Anderson, 1970b), the Phe-tRNA polymerization at low Mg^{2+} and Met-puromycin release (Shafritz *et al.*, 1971), these proteins were demonstrated to be initiation factors and were designated M_1, M_2.

Similarly the stimulatory AvF fraction appears to be distinct from T_1 and T_2 and our data suggest that AvF contains proteins necessary for chain initiation. The following observations support these suppositions: (1) AvF stimulates protein synthesis in the presence of saturating amounts of fractions known to contain T_1 and T_2 (*i.e.*, AS_{70} enzyme fraction); (2) there is a shift in the Mg^{2+} optimum to a lower concentration associated with AvF stimulation of the natural message system; (3) AvF stimulates polymerization of the Phe in the poly(U)-directed system in the presence of saturating amounts of purified T_1 and T_2; and finally (4) in the presence of AvF, the synthetic message system shows maximal activity at low Mg^{2+} concentrations.

Investigations were begun in order to identify a specific product of the oviduct AvF-dependent system. This approach was necessary since definitive proof of *de novo* polypeptide synthesis requires the demonstration of uniform labeling throughout a particular protein molecule. Ovalbumin comprises nearly 60% of the protein mass of estrogen-stimulated oviduct and a specific immunologic assay is available for this protein (O'Malley *et al.*, 1969; O'Malley and McGuire, 1968a;

O'Malley, 1967). Moreover we have previously demonstrated immunologically competent ovalbumin to be a product of the polysomal cell-free system (Means *et al.*, 1971; Means and O'Malley, 1971). For these reasons we have directed our efforts toward the identification of ovalbumin. When oviduct ribosomes are incubated for 60 min in the presence of AvF, approximately 27% of the acid-precipitable radioactivity is released into the supernatant fluid. Of the released peptides 14% are immunoreactive as ovalbumin when tested using a column assay comprised of antiovalbumin coupled to Sepharose gel. These data demonstrate that the AvF stimulated system is capable of the synthesis of ovalbumin on washed oviduct ribosomes. However, definitive experiments are yet required to prove the initiation of ovalbumin chains *in vitro*. Studies of this nature are now in progress.

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