Regulation of Polyribosome Formation and Protein Synthesis in the Uterus

ISOLATION OF CYTOPLASMIC RIBONUCLEOPROTEIN PARTICLES AND THE PRINCIPAL PROPERTIES OF THE CELL-FREE PROTEIN-SYNTHESIZING SYSTEM

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1. Three procedures for isolating ribonucleoprotein particles from the cytoplasmic fraction of rat-uterus homogenates are described. By procedure 1, ribonucleoprotein particles were isolated in the presence of 5 mM-Mg²⁺ and 25 mM-K⁺, and the postmitochondrial supernatant fraction was made to 1.3% (w/v) in potassium deoxycholate. About 50% of the RNA and protein of the microsomal fraction was recovered in the monomeric ribosomes isolated. By procedure 2, ribonucleoprotein particles were isolated in the presence of 10 mM-Mg²⁺ and 0.1 M-K⁺, and in the absence of detergent. The ribosomes obtained were primarily polymeric, but recovery of microsomal RNA and protein was only 32%. By procedure 3, ribonucleoprotein particles were isolated according to procedure 1 but without the use of detergent. A mixture of polymeric and monomeric ribosomes was obtained, and the recovery of microsomal RNA and protein was about 60%. 2. Uterine polymeric and monomeric ribosomes, isolated by procedure 3 and designated 'polyribosomal preparation', were examined for protein-synthesizing capabilities. The principal properties of the cell-free protein-synthesizing system containing the polyribosomal preparation are described. The efficiency of amino acid incorporation in the complete system incubated for 30 min. and containing the polyribosomal preparation was found to be either 2.5 molecules of [¹⁴C]leucine or 2.2 molecules of [¹⁴C]-valine incorporated/ribosome. Assay of the preparation in the complete cell-free system containing 10 mM-sodium fluoride indicated that 40% of the incorporation activity is a result of initiation of new polypeptide chains and 60% is due to completion of previously existing chains. Monomeric ribosomes obtained by various treatments of the polyribosomal preparation with sodium fluoride, ribonuclease and potassium deoxycholate had decreased incorporation activity in the cell-free system. However, monomeric ribosomes obtained by treatment with sodium fluoride only had an incorporation activity 50% greater than that of monomers obtained by treatment with ribonuclease only. 3. The results indicate that uterine polymeric and monomeric ribosomes are sites of amino acid incorporation *in vivo* and *in vitro*. It is concluded that most polymeric and monomeric ribosomes occurring in the cytoplasmic fraction of the uterus are free and unattached to membranes, and that the polyribosomes are relatively unstable.

Nuclear RNA synthesis is stimulated before enhancement of cytoplasmic protein synthesis during early oestrogen action in the uterus of the ovariectomized rat (Hamilton, Widnell & Tata, 1965; Means & Hamilton, 1966b). The rates of nuclear RNA synthesis and turnover to the cytoplasm also rise and fall in association with corresponding fluctuations in the concentrations of cytoplasmic RNA and protein and in size of the uterus during the oestrous cycle (Hamilton,

Widnell & Tata, 1967). These and other observations suggest that regulation of cytoplasmic protein synthesis in the uterus results in part at least from hormonal control of genetic transcription (Tata, 1966; Segal & Scher, 1967; Hamilton, 1968).

A more comprehensive view of the hormonal regulation of protein synthesis in the uterus would be possible if there were available a cell-free amino acid-incorporation system for characterized preparations of polymeric and monomeric ribosomes

isolated from the cytoplasm of uterine cells. The response of this system could then be described for the effects *in vitro* as well as *in vivo* of oestrogenic and other steroidal hormones known to have uterotrophic functions.

We have thus investigated the general mechanism of cytoplasmic protein synthesis in the uterus of the normal rat in dioestrus. We find that the uterus, like other mammalian organs, contains polymeric and monomeric ribosomes that are active sites of protein synthesis *in vivo* and *in vitro*. In the present paper we describe and evaluate several procedures for isolating RNP* particles from the cytoplasmic fraction of the uterus. The majority of the particles are unattached to membranes, and the polymeric ribosomes appear to be relatively unstable. The principal properties of the cell-free protein-synthesizing system containing a mixture of polymeric and monomeric ribosomes are described.

METHODS AND MATERIALS

Animals and their management. Normal and ovariectomized female Sprague-Dawley rats, each weighing 160–180 g., were obtained from Hormone Assay Co., Chicago, Ill., U.S.A. No restrictions on intake of water or food (oestrogen-free Purina Laboratory Chow) were placed on animals before they were killed. All animals used to obtain uterine RNP particles were in the dioestrous phase of the oestrous cycle. The phase of the oestrous cycle was determined for individual animals by the fresh weight of the uterus taken immediately after the rat was killed, and by microscopic examination of vaginal cell types (Hamilton *et al.* 1967). In one experiment, animals were 'pulse-labelled' by the administration intraperitoneally of 20 μ C of 14 C-labelled acid protein hydrolysate 10 min. before they were killed. Ovariectomized rats used for the preparation of the AS₇₀-enzyme fraction described below were killed not less than 3 weeks after surgery.

Isolation of cytoplasmic RNP particles. Normal animals were killed by cervical dislocation and the unruptured uteri were removed immediately. These were stripped of adhering fat and mesenteries, blotted on a moist cloth, weighed on a Mettler B balance, and quickly frozen at -70° . Before homogenization, pooled uteri (usually six per group) were thawed at $0-2^{\circ}$, minced finely with curved iris scissors, and suspended in 6 vol. of a standard homogenizing medium (final concns. 50 mM-tris, 0.25 M-sucrose, 5 mM-MgCl₂, 25 mM-KCl, 5 mM-2-mercaptoethanol and 0.2 mg. of polyvinyl sulphate/ml., pH 7.6). All subsequent procedures were performed at $0-2^{\circ}$ unless otherwise stated. Cytoplasmic RNP particles were then isolated from the minced uterine tissue by one of the following procedures.

Procedure 1. Minced tissue suspended in the standard homogenizing medium was homogenized for 2 min. at -10° by an Uita-Turrax TP 18/2 tissue disintegrator (Janke und Kunkel K.G., Staufen i. Br., Germany) run at 60 v. The homogenate was filtered through two layers of nylon bolting cloth (110 mesh), and the whole cells and nuclei were removed by centrifugation at 900 g for 5 min. The

supernatant fraction was collected and centrifuged at 27000 g for 15 min. to sediment mitochondria. The supernatant fraction was collected and made to 1.3% (w/v) in potassium deoxycholate. Then 20 min. later 3 ml. samples of this fraction were each layered over 3.1 ml. of 0.5 M-sucrose layered over 2.3 ml. of 2.0 M-sucrose (Wettstein, Noll & Penman, 1964). The 0.5 M- and 2.0 M-sucrose solutions used here were prepared in medium A (final concns. 50 mM-tris, 5 mM-MgCl₂, 25 mM-KCl, pH 7.6). RNP particles were then pelleted by centrifugation in the 40 rotor of a Spinco model L ultracentrifuge run at 105000 g for 9 hr.

Procedure 2. RNP particles were isolated according to procedure 1, with the exceptions that the homogenizing medium contained 10 mM-MgCl₂ and 0.1 M-KCl, the homogenizing period was 1 min., and potassium deoxycholate was not used.

Procedure 3. RNP particles were isolated according to procedure 1, with the exception that potassium deoxycholate was not used.

Throughout the text and in the relevant Figures and Tables, the expression 'polyribosomal preparation' refers to a mixture of polymeric and monomeric ribosomes isolated by procedure 3. The expression 'ribosomal preparation' refers to one of only monomeric ribosomes obtained from the polyribosomal preparation, as described in the following paragraph.

Preparation of monomeric ribosomes. Uterine polyribosomal preparation was suspended in medium A, adjusted to 10 mM-NaF, and incubated at 37° for 15 min. The preparation was then quickly chilled at $0-2^{\circ}$ and centrifuged at 105000 g for 2.5 hr. The pelleted ribosomes were smeared and suspended in medium A. The suspension was made to 0.6% in potassium deoxycholate and adjusted to pH 7.6 with 1.0 N-KOH. This suspension was then incubated for 10 min. at 37° , and quickly chilled to $0-2^{\circ}$. To 1 ml. samples, 10 ml. of medium B (final concns. 1 mM-tris, 1 mM-MgCl₂, 50 mM-KCl, pH 7.6) were added, and the preparations were centrifuged at 105000 g for 2 hr. The pelleted ribosomes were washed twice by resuspension and recentrifugation in medium B. Monomeric ribosomes were also prepared by incubation of the polysomal preparation in medium B containing 5 μ g. of ribonuclease/ml. for 5 min. at 37° , or by incubation of the preparation in medium A made to 0.6% in potassium deoxycholate for 10 min. at 37° . The ribosomes were then collected and washed twice.

Distribution and characterization of polymeric and monomeric ribosomes. Isolated RNP particles were suspended in medium A containing 0.12 M-sucrose. Samples (1 ml.) were separately layered over 10–34% (w/v) continuous sucrose density gradients. The sucrose used had been dissolved in medium A, and the gradients were prepared at room temperature and maintained overnight at 4° . The layered gradients were centrifuged in the SW25 rotor at 50000 g for 2 hr., and 28 fractions were collected after the bottoms of the rotor tubes had been punctured (Britten & Roberts, 1960). E_{260} and E_{280} values were determined for each fraction. Fractions containing 14 C-labelled RNP particles were assayed for acid-insoluble radioactivity as described by Moore & Hamilton (1964). For the characterization of uterine monomeric ribosomes distributed on the sucrose density gradient, purified 80S ribosomes, isolated from rabbit reticulocyte cells previously incubated in the presence of 10 mM-NaF (obtained from Dr Boyd Hardesty,

* Abbreviation: RNP, ribonucleoprotein.

Department of Chemistry, University of Texas, Austin), were used as a marker. Sedimentation coefficients of 78s and 110s for uterine monomeric and dimeric ribosomes were determined by analyses in the Spinco model E analytical ultracentrifuge.

Preparation of AS₇₀-enzyme fraction. An enzyme fraction containing activating enzymes, transfer factors and soluble RNA was isolated from the cytoplasmic fraction of liver taken from ovariectomized rats. Liver fragments (12g.) were minced finely and suspended in 4 vol. of 0.1M-tris, pH 7.5. The minced tissue was homogenized in a glass homogenizer equipped with a tight-fitting Teflon pestle. The homogenate was filtered and centrifuged at 105000g for 4 hr., and the supernatant fraction was collected and diluted to 100 ml. by addition of the tris buffer. By the use of powdered ammonium sulphate, the 40–70% saturation precipitate was isolated and dialysed according to the procedures of Allen & Schweet (1962). The dialysed solution, designated AS₇₀-enzyme fraction, was adjusted to 25 mM-GSH and 2.5 mM-EDTA and stored in separate tubes at –20°.

The cell-free amino acid-incorporation system. The complete incubation medium used was essentially that described by Maxwell (1962) and by Talal (1966). The medium contained (final concns.) in a final vol. of 0.5 ml.: 50 mM-tris (pH 7.5 at 37°; pH 7.9 at 0°), 7 mM-MgCl₂, 70 mM-KCl, 6 mM-2-mercaptoethanol, 1 mM-ATP, 0.1 mM-GTP, 5 mM-creatine phosphate, 20 μg. of creatine phosphokinase, 0.25 mM-¹⁴C-labelled amino acid (0.05 μC with specific radioactivity of 10), 0.25 mM each of the other 19 unlabelled amino acids, about 0.5 mg. of protein of isolated RNP particles suspended in 0.25 M-sucrose in medium A, and 1.5 mg. of protein of AS₇₀-enzyme fraction. After incubation for 30 min. at 37°, incorporation was terminated by precipitation with 0.5 ml. of 20% (w/v) trichloroacetic acid. After 30 min. at 0–2°, the precipitate was collected by centrifugation and washed three times with 10% (w/v) trichloroacetic acid. The precipitate was then resuspended in 0.5 ml. of 10% trichloroacetic acid, heated at 90° for 15 min., and centrifuged again. The pellet was smeared and suspended in aq. 95% (v/v) ethanol. The precipitate was again collected, smeared in Bray's (1960) solution and suspended in 15 ml. of the solution for assay of radioactivity in a Packard Tri-Carb scintillation spectrometer. The counting efficiency was 50%.

Chemical and other determinations. Protein, RNA and DNA were determined respectively by the procedures of Lowry, Rosebrough, Farr & Randall (1951), Ceriotti (1955) and Burton (1956).

Specific activity of polyribosomal or ribosomal preparation assayed in the cell-free system was determined as counts of ¹⁴C-labelled amino acid incorporated/mg. of protein of the preparation added to the system. The amount of protein in the preparation added was determined by the procedure of Lowry *et al.* (1951).

The efficiency of amino acid incorporation by the polyribosomal preparation was determined as molecules of ¹⁴C-labelled amino acid incorporated/ribosome during 30 min. of incubation at 37°. The method of calculation was that of Warner & Rich (1964), and the concentration of ribosomes in the preparation was determined as described by Lin, Mosteller & Hardesty (1966).

Materials. Tris (Trizma base, reagent grade) and potassium deoxycholate were obtained from Sigma Chemical

Co., St Louis, Mo., U.S.A. L-[¹⁴C]Leucine (200 mc/mole), L-[¹⁴C]valine (200 mc/mole), ATP (low sodium content) and GTP were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Acid protein hydrolysate labelled with ¹⁴C was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Creatine phosphate (disodium salt tetrahydrate, A grade), creatine phosphokinase (A grade prepared from rabbit skeletal muscle), 2-mercaptoethanol and GSH were from Calbiochem, Los Angeles, Calif., U.S.A. Powdered ammonium sulphate (enzyme grade) and NaF were from Mann Research Laboratories, New York, N.Y., U.S.A., and polyvinyl sulphate was from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Bentonite was obtained from Amend Drug and Chemical Co., New York, N.Y., U.S.A., and purified by the procedure of Fraenkel-Conrat, Singer & Tsugita (1961). Ribonuclease inhibitor from the rat-liver supernatant fraction was prepared by the procedure of Lawford, Langford & Schachter (1966).

RESULTS

Isolation and distribution of polymeric and monomeric ribosomes. The sedimentation profile of uterine cytoplasmic RNP particles isolated according to procedure 1 and distributed on the sucrose density gradient demonstrated only a 78s peak

Table 1. Recovery of microsomal RNA or protein in preparations of RNP particles isolated from the cytoplasmic fraction of the uterus

Uteri from 12 normal rats were pooled, minced finely and divided into three equal portions by weight. From these portions, RNP particles were isolated according to procedures 1, 2 and 3 respectively. For each procedure, the filtered homogenate was divided into two equal volumes. From one volume, the RNP particles were isolated as described in the text. From the other volume, the microsomal fraction was isolated from the postmitochondrial supernatant fraction (Tata *et al.* 1963). Samples of the portions of minced tissue or of the paired homogenate volumes were determined for DNA. The DNA content of the portions or of the paired volumes varied less than 1%. DNA, RNA and protein were determined chemically as described in the text.

Isolation procedure	RNA or protein/mg./g. of tissue obtained		Percentage of microsomal RNA or protein recovered in RNP particles
	Microsomal fraction	RNP particles	
Amount of RNA			
1*	0.44	0.22	50
2	0.43	0.14	32
3	0.39	0.24	62
Amount of protein			
1*	1.69	0.83	49
2	1.85	0.60	32
3	1.85	1.10	60

* Postmitochondrial supernatant fraction made to 1.3% in potassium deoxycholate.

Fig. 1. Sucrose-density-gradient analyses of uterine polymeric and monomeric ribosomes. Polyribosomal preparation was isolated from the cytoplasmic fraction of pooled uteri of normal rats by procedure 3. The preparation was incubated in medium A for 30 min., in medium A containing 5 μ g. of ribonuclease/ml. for 5 min. or in medium A containing 10 mM-NaF for 30 min. After incubation at 37°, the preparation was rapidly chilled, pelleted, resuspended in 1.0 ml. of medium A containing 0.12 M-sucrose and layered over a 10–34% (w/v) continuous sucrose density gradient for centrifugation and determination of E_{260} as described in the text. About 1.0 mg. of protein of RNP preparation was layered over each gradient. Determination of sedimentation coefficients and the other experimental details were as described in the text. ●—●, Polyribosomal preparation; ●—---●, ribonuclease-treated ribosomes; ---, NaF-treated ribosomes; —, 80s reticuloocyte ribosomes.

for monomeric ribosomes. In this procedure for isolating ribosomes, the homogenizing medium contained 5 mM-Mg²⁺ and 25 mM-K⁺ (Wettstein, Staehelin & Noll, 1963), the homogenizing period was 2 min., and the postmitochondrial supernatant fraction was made to 1.3% in potassium deoxycholate (Breillatt & Dickman, 1966) to release the ribosomes bound to membranes. Approximately 50% of the RNP of the homogenate microsomal fraction was recovered in the ribosomes isolated (Table 1). The sedimentation profile of RNP particles isolated according to procedure 2 indicated the presence of abundant polymeric ribosomes in relation to the concentration of dimeric and monomeric ribosomes. In this procedure, the homogenizing medium contained 10 mM-Mg²⁺ and 0.1 M-K⁺ (Munro, Jackson & Korner, 1964), the homogenizing period was 1 min., and potassium deoxycholate was not used. Though this procedure permitted isolation

of RNP particles having a more classical polyribosomal distribution, the recovery of microsomal RNP was only 32% (Table 1).

Fig. 1 shows the typical sedimentation profile of RNP particles isolated by procedure 3. Two peaks of increased extinction are discernible: one of 78s monomeric ribosomes and one of 110s dimeric ribosomes in greater concentration, with larger polymers decreasing in concentration in the denser region of the gradient. The procedure employed for isolating the RNP particles was the same as in procedure 1, with the exception that the potassium deoxycholate was not added to the postmitochondrial supernatant fraction (Bloemendal, Bont & Benedetti, 1964). The recovery of microsomal RNP was 60%, and the yield of ribosomes was approximately 1.1 mg. of protein/g. of uterine tissue (Table 1). Incubation of the RNP particles in the presence of 5 μ g. of ribonuclease/ml. or of 10 mM-sodium fluoride before they were layered over the sucrose density gradient resulted in a disappearance of the 110s peak of dimeric ribosomes and an increase in the concentration of monomers (Fig. 1). Sodium fluoride was more effective than ribonuclease in dissociating polymeric ribosomes into monomers. That incubation in the absence of ribonuclease or of sodium fluoride for 30 min. at 37° did not result in a dissociation of the polymeric ribosomes was shown by their distribution on the sucrose density gradient.

We recognize the possibility that the relatively large number of dimeric and monomeric ribosomes obtained by procedure 3 and shown in Fig. 1 might be a result of endogenous ribonuclease activity during isolation (Shortman, 1961; Blobel & Potter, 1966). However, the use of homogenization medium and of medium A containing the ribonuclease inhibitor of the rat-liver supernatant fraction, or containing different concentrations of bentonite (0.5–2.0 mg./ml.), and phosphate rather than tris buffer, resulted in the isolation of RNP particles having the same distribution as described for the particles isolated by procedure 3.

Amino acid incorporation by polymeric and monomeric ribosomes in vivo. Animals were 'pulse-labelled' with 20 μ C of ¹⁴C-labelled acid protein hydrolysate 10 min. before they were killed, and the uterine polymeric and monomeric ribosomes were isolated according to procedure 3. Fig. 2(a) shows that the polyribosomal preparation distributed on a sucrose density gradient exhibited several peaks of radioactivity. These peaks correspond to the known positions of 78s monomers and 110s dimers, and the probable positions of uncharacterized tetramers or greater aggregates. If the polyribosomal preparation was incubated in the presence of 5 μ g. of ribonuclease/ml. before fractionation, most of the radioactivity in the denser

Fig. 2. Amino acid incorporation by uterine polymeric and monomeric ribosomes *in vivo*. Each animal was 'pulse-labelled' by the administration of $20\mu\text{C}$ of ^{14}C -labelled acid protein hydrolysate; 10min. later it was killed. Uteri were then pooled, and polyribosomal preparation was isolated according to procedure 3, and incubated in medium A in the absence or presence of $5\mu\text{g.}$ of ribonuclease/ml. for 5 min. at 37° . The RNP particles were pelleted, washed twice and resuspended in medium A containing 0.12M -sucrose for layering over the sucrose density gradient. Approximately 1.0mg. of protein of RNP preparation was layered over each gradient. The other experimental details were as described in Fig. 1 and in the text. (a) Profile of RNP preparation incubated in the absence of ribonuclease; (b) profile of RNP preparation incubated in the presence of ribonuclease. ●—●, E_{280} ; ○---○, radioactivity.

region of the gradient disappeared, and the radioactivity in the monomer peak and in the less dense region increased (Fig. 2b). It is not known for this system if the radioactivity associated with ribosomes was first initiated on polymeric ribosomes, and remained attached to monomeric ribosomes when the polymeric ones dissociated. Such a temporal sequence for polypeptide synthesis has been demonstrated for other amino acid-incorporation systems (Gilbert, 1963; Hardesty, Miller & Schweet, 1963). The time selected for 'pulse-labelling' *in vivo* (10min.) has been shown for other mammalian systems to be sufficiently brief for the avoidance of labelling of structural ribosomal protein (Talal, 1966; Wettstein & Noll, 1965). Our experiment indicates only that cytoplasmic polymeric and monomeric ribosomes of the uterus are sites of amino acid incorporation *in vivo*.

Amino acid incorporation by polymeric and monomeric ribosomes in vitro. Cytoplasmic polyribosomal preparation containing mixed polymeric and monomeric ribosomes was isolated according to procedure 3 from pooled uteri taken from normal rats. The principal characteristics of the cell-free protein-synthesizing system containing the polyribosomal preparation and ^{14}C leucine are given in Table 2. Amino acid-incorporation activity was quite reproducible, and decreased with the omission of individual constituents of the complete system. Incorporation of ^{14}C leucine was decreased 90% or more by the omission of the polyribosomal preparation, the ATP or the AS_{70} -enzyme fraction, or by the addition of ribonuclease or puromycin. Cycloheximide was less effective as an inhibitor of incorporation, and a concentration of $100\mu\text{g./ml.}$

Table 2. *Principal characteristics of the uterine cell-free system for protein synthesis*

Polyribosomal preparation was isolated from pooled uteri of normal rats by procedure 3. The complete system contained about 0.5mg. of protein of the polyribosomal preparation, $0.5\mu\text{C}$ of ^{14}C leucine and 1.5mg. of protein of the AS_{70} -enzyme fraction. The other constituents of the complete system and the remaining experimental details were as described in the text.

Incubation medium	^{14}C Leucine-incorporation activity (counts/min./mg. of protein)
Complete system	406
Complete system, minus polyribosomal preparation	17
Complete system, minus 19 unlabelled amino acids	196
Complete system, minus ATP	62
Complete system, minus GTP	250
Complete system, minus AS_{70} -enzyme fraction	28
Complete system, plus puromycin ($4 \times 10^{-4}\text{M}$)	48
Complete system, plus cycloheximide ($50\mu\text{g.}$)	244
Complete system, plus cycloheximide ($100\mu\text{g.}$)	164
Complete system, plus ribonuclease ($5\mu\text{g.}$)	58
Complete system, plus polyvinyl sulphate ($2.5\mu\text{g.}$)	375

was necessary to obtain more than 50% inhibition. Significant decreases in incorporation (40–50%) also occurred if either GTP or the mixture of the other

Fig. 3. Total incorporation of [^{14}C]leucine as a function of the concentration in mg. of protein of polyribosomal preparation in the incubation medium. The polyribosomal preparation was isolated from pooled uteri by procedure 3. Incorporation values are given for each assay tube. The other experimental details were as described in the text.

19 unlabelled amino acids was omitted. Only a small decrease (6–8%) occurred if polyvinyl sulphate was added to the complete system to a final concentration of $2.5\text{ }\mu\text{g./ml.}$ The characteristics of the uterine cell-free system described in Table 2 were also observed when ^{14}C -labelled acid protein hydrolysate rather than [^{14}C]leucine and the mixture of the other 19 unlabelled amino acids was used.

Duplicate experiments indicated that the efficiency of amino acid incorporation by the uterine polyribosomal preparation in the complete cell-free system was approximately 2.5 molecules of [^{14}C]leucine/ribosome of polyribosomal preparation. For [^{14}C]valine the corresponding value was 2.2. For the rabbit reticulocyte cell-free system, the corresponding values for leucine and valine are 6.2 and 4.9 respectively (Lin *et al.* 1966).

In determining the optimum conditions for amino acid incorporation into protein in the complete cell-free system described above, the following analyses of kinetics and of constituent requirements were performed. Fig. 3 shows the total incorporation of [^{14}C]leucine as a function of different amounts of polyribosomal preparation added to the cell-free system. Incorporation was linearly dependent on concentration from 0.25 mg. to 1.0 mg. of protein of the preparation. The time-course for the incorporation of [^{14}C]leucine was approximately linear for the first 30 min., and levelled off thereafter (Fig. 4a). Fig. 4(b) shows the incorporation of [^{14}C]leucine as a function of the concentration of the AS $_{70}$ -enzyme fraction. The effect of the concentration of magnesium chloride

Fig. 4. (a) Time-course of the incorporation of [^{14}C]leucine into the complete cell-free system. (b) Incorporation of [^{14}C]leucine as a function of the concentration in mg. of protein of the AS $_{70}$ -enzyme fraction in the complete cell-free system. In both (a) and (b), each assay tube contained about 0.5 mg. of protein of uterine polyribosomal preparation. The other experimental details were as already described.

or potassium chloride on incorporation is shown in Fig. 5.

Lin *et al.* (1966) have demonstrated that sodium fluoride added to the reticulocyte cell-free system to a final concentration of 10 mM inhibits almost completely the initiation of new polypeptide chains, but not the completion of old ones. Fig. 6 shows the effect of the concentration of sodium fluoride in the cell-free system on the [^{14}C]leucine-incorporation activity of the uterine polyribosomal preparation. Incorporation decreased with increased concentration of sodium fluoride, but at a concentration of 10 mM or greater the salt caused no further decrease in incorporation. These results indicate that approximately 40% of the amino acid-incorporation activity of the uterine polyribosomal prepara-

Fig. 5. Effect of concentration of MgCl_2 (a) or KCl (b) on the incorporation of $[^{14}\text{C}]$ leucine into the complete cell-free system. Each assay tube contained about 0.5 mg. of protein of uterine polyribosomal preparation.

tion is due to the initiation of new polypeptide chains, and 60% is due to the completion of previously existing chains.

Amino acid incorporation by monomeric ribosomes in vitro. Monomeric ribosomes were obtained from the polyribosomal preparation by incubation in the presence of 5 $\mu\text{g.}$ of ribonuclease/ml., 10 mM-sodium fluoride or 0.6% potassium deoxycholate, or by sequential incubations in the presence of 10 mM-sodium fluoride and 0.6% potassium deoxycholate (Lin *et al.* 1966). Monomeric ribosomes thus prepared were washed and distributed on sucrose density gradients. The sedimentation profile indicated in each instance the presence of only a single peak of 78s monomeric ribosomes. Table 3 shows that each preparation of monomeric ribosomes had lowered amino acid-incorporation activity in the cell-free system, compared with that

Fig. 6. Effect of concentration of NaF on the incorporation of $[^{14}\text{C}]$ leucine into the complete cell-free system. Each assay tube contained about 0.5 mg. of protein of uterine polyribosomal preparation.

Table 3. $[^{14}\text{C}]$ Leucine-incorporation activity of polyribosomal preparation or of monomeric ribosomes in the uterine cell-free system

Polyribosomal preparation containing mixed polymeric and monomeric ribosomes was isolated from pooled uteri of normal rats by procedure 3. Monomeric ribosomes were prepared as described in the text. About 0.5 mg. of protein of either polyribosomal or ribosomal preparation was added to each assay tube. The assay of $[^{14}\text{C}]$ leucine-incorporation activity was as described in Table 2 and in the text. The results are representative of replicate experiments.

Incubation medium	$[^{14}\text{C}]$ Leucine-incorporation activity (counts/min./mg. of protein)
Complete system containing polyribosomal preparation	410
Complete system containing ribonuclease-treated ribosomes	197
Complete system containing NaF -treated ribosomes	302
Complete system containing ribosomes treated with potassium deoxycholate	209
Complete system containing ribosomes treated with NaF and then with potassium deoxycholate	94

of the polyribosomal preparation, which contained mixed polymeric and monomeric ribosomes. Ribosomal preparation obtained by sequential treatments with sodium fluoride and potassium deoxycholate had 15–20% of the activity exhibited by

the polyribosomal preparation. Ribosomal preparations obtained by treatment with either ribonuclease, sodium fluoride or potassium deoxycholate were more active, having 50–75% of the activity of the polyribosomal preparation. It was demonstrated in Fig. 1 that sodium fluoride was more effective than ribonuclease in dissociating polymeric ribosomes. Assayed in the cell-free system, however, sodium fluoride-treated ribosomes exhibited a higher amino acid-incorporation activity than ribonuclease-treated ribosomes (Table 3). Sodium fluoride-treated ribosomes in the reticulocyte cell-free system also actively incorporate amino acids (Lin *et al.* 1966). Treatment of polyribosomes with sodium fluoride may result in their dissociation to monomeric ribosomes, with messenger RNA remaining attached to some of the monomers. This problem remains to be investigated.

DISCUSSION

The programme of research in our Laboratory (Means & Hamilton, 1966a,b; Hamilton, 1968) has recently centred on the rate of microsomal protein synthesis *in vivo* and its hormonal regulation in the uterus. We therefore accepted a lower percentage of polymeric ribosomes isolated for the sake of increased recovery of microsomal RNP (Table 1), and used procedure 3 rather than procedure 2 to obtain a mixture of polymeric and monomeric ribosomes for analyses in the cell-free amino acid-incorporation system. The principal characteristics of this system (Table 2) are in general agreement with those described by Kalman & Opsahl (1961) and Wilson (1962) for chick oviduct tissue, and by Greenman & Kenny (1964) for the rat uterus. In the latter investigation RNP particles were isolated from a whole-tissue homogenate treated with sodium deoxycholate, and the sedimentation characteristics of the particles were not reported.

The results reported in the present paper indicate that uterine polyribosomes are relatively unstable. In procedure 3, the homogenizing medium contained 5 mM-Mg²⁺ and 25 mM-K⁺, and 60% of the microsomal RNP was recovered in mixed polymeric and monomeric ribosomes. In procedure 2, the medium contained 10 mM-Mg²⁺ and 0.1 M-K⁺, and, although the ribosomes isolated were primarily polymeric, only 32% of the microsomal RNP was recovered (Table 1). Comparable observations have been reported for pancreatic and cerebral RNP particles isolated at the low or higher concentrations of Mg²⁺ and K⁺ (Breillatt & Dickman, 1966; Zomzely, Roberts, Brown & Provost, 1966). Zomzely *et al.* (1966) concluded from such observations that cerebral polyribosomes are relatively unstable.

Although treatment of the postmitochondrial

supernatant fraction with potassium deoxycholate dissociated uterine polymeric ribosomes into monomeric ones, the recovery of microsomal RNP was only 10% less than in the absence of the detergent (Table 1). This observation indicates that most of the microsomal RNP particles in the uterus are not attached to membranes. The same observation has been made for spleen polyribosomes (Talal, 1966), whereas hepatic and pancreatic polyribosomes have been found to be mostly membrane-bound (Siekevitz & Palade, 1960; Henshaw, Bojarski & Hiatt, 1963; Breillatt & Dickman, 1966).

We cite three lines of evidence suggesting that the polymeric ribosomes isolated by procedure 3 are held together by messenger RNA and not by Mg²⁺ ions: (a) treatment with ribonuclease or sodium fluoride resulted in a dissociation of the polymeric ribosomes into monomeric ones (Fig. 1); (b) treatment with ribonuclease, sodium fluoride, or potassium deoxycholate before assay in the cell-free system reduced the amino acid-incorporation activity (cf. Table 2 and Table 3); and (c) investigation by electron microscopy of tissue slices and of the RNP particles isolated revealed aggregates of 3–6 ribosomes linearly distributed on longitudinal units presumed to be messenger RNA (G. Maul, C.-S. Teng & T. H. Hamilton, unpublished work). The evidence of (a) does not, however, rule out entirely the possibility that the prominent dimer peak observed in Fig. 1 is a result of binding by Mg²⁺ ions. Treatment with either ribonuclease or sodium fluoride might have resulted in a dissociation of dimeric ribosomes previously united by ionic forces. We emphasize that in the experiments of (b) we intentionally dissociated polymeric ribosomes into monomeric ones. The activity in the cell-free system of these monomers previously treated with enzyme, salt or detergent (Table 3) may be different from that of those occurring naturally in uterine cells. We further emphasize that, by procedure 3, RNP particles are obtained from a whole-tissue homogenate not treated with detergent (Bloemendal *et al.* 1964). This procedure results in the isolation of microsomal RNP particles, and thus results in a selection of only part of the total uterine population of polymeric and monomeric ribosomes.

A general feature of uterine RNP particles appears to be their relatively unstable nature. Greenman & Kenny (1964) demonstrated that 4 days after ovariectomy the uterine cytoplasmic concentration of RNP had decreased by 100%. Moore & Hamilton (1964) demonstrated that both the rate of synthesis and the cytoplasmic concentration of uterine ribosomes increased by 300% between 4 hr. and 24 hr. after the administration of oestradiol-17 β to the ovariectomized rat. The effect of the hormone on ribosomal synthesis was blocked by pretreatment of the animals with actino-

mycin D, an inhibitor of DNA-dependent synthesis of ribosomal RNA as well as of messenger RNA (Reich & Goldberg, 1964; Trakatellis, Axelrod & Montjar, 1964). Means & Hamilton (1966a) demonstrated that, between 2hr. and 8hr. after hormone administration, the specific activity of microsomal protein rapidly labelled *in vivo* increased by 250%. These observations considered together raise the possibility that regulation of cytoplasmic protein synthesis in the uterus by oestrogen occurs by variations in the number and in the activity of polyribosomes. Further, there is the possibility that these variations are regulated by genetic transcription regulated in turn by the hormone. These possibilities are explored in the following paper (Teng & Hamilton, 1967).

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