Protein Biosynthesis in the Testis. IV. Isolation and Properties of Polyribosomes*

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ABSTRACT: A procedure is described for the isolation of a polyribosomal preparation from rat testis. Sucrose gradient centrifugation, analytical ultracentrifugation, and electron microscopy reveal that this testicular preparation contains some single ribosomes characterized by a sedimentation coefficient of 77 S, and a population of polyribosomes ranging from dimers to hexamers. Larger polymers containing 10–50 ribosomes were also observed. The yield of polyribosomes is shown to depend upon the procedure adopted to homogenize the testis (four strokes only with a pestle clearance of 0.9 mm) and upon the concentration of deoxycholate (0.25%). Conditions are described for optimal incorporation of [^{14}C]valine into peptide by a cell-free system. The incorporation system shows an absolute requirement for polyribosomes, adenosine triphosphate, and Mg^{2+}, guanosine triphosphate, a pH 5 enzyme fraction, and an adenosine triphosphate generating system; the 19 unlabeled amino acids are also necessary for maximal protein biosynthesis. The optimal Mg^{2+} concentration is 6.4 mM and the activity of the cell-free system is not dependent upon added K^+. Incorporation is inhibited by addition of ribonuclease or NaF. Inhibition is also caused by puromycin and to a lesser extent by similar concentrations of cycloheximide. The extent of inhibition of protein biosynthesis produced by omitting guanosine triphosphate or by addition of NaF suggests that incorporation of amino acids into peptides under the conditions used represents both chain initiation and addition of single amino acids to existing incomplete peptides.

Protein biosynthesis in testis from immature rats is stimulated within 30 min of a single injection of FSH;[1] other hormones, which are known to possess FSH-like activity (namely, pregnant mare's serum and human chorionic gonadotrophin), exert the same effect (Means and Hall, 1967, 1968a). On the other hand, glucose stimulates protein synthesis in testes from mature rats (Means and Hall, 1968a; Davis and Morris, 1963). Further investigation of the actions of these two agents upon the testis revealed that FSH and glucose stimulate different cell types (Means and Hall, 1968a). In order to explore the mechanisms by which FSH and glucose stimulate testicular protein biosynthesis, it was decided to characterize testicular polyribosomes and to determine optimal conditions for the incorporation of amino acids into peptide bonds by such polyribosomes.

Methods

Preparation of Polyribosomes. Testes were obtained from mature male rats (200–250 g) of the Sprague–Dawley strain and all procedures thereafter were performed at 0–4°. Following removal of the tunica albuginea, testicular tubules were homogenized in 2.5 ml of medium H (0.035 M Tris, 0.025 M KCl, 0.01 M $MgCl_2$, and 0.25 M sucrose, pH 7.6, at 4°) per g of tissue by four up-and-down passes in a mechanically driven glass homogenizer fitted with a Teflon pestle. Preliminary experiments showed that pestle clearance is critical if high yields of polyribosomes are to be obtained. The optimum clearance was found to be 0.9 mm; therefore, this clearance was used in all subsequent experiments. Following homogenization the solution was filtered through two layers of mira cloth and the filtrate was centrifuged for 10 min at 1000*g*. The supernatant was decanted and centrifuged at 15,000*g* (R_{av}) for 20 min. The supernatant from this second centrifugation was removed to within 2 cm of the pellet and 0.05 ml of freshly prepared 5% deoxycholic acid (in 0.03 M Tris, pH 8.0, at 4°) was added per ml of supernatant. Polyribosomes were then isolated by the method of Wettstein *et al.* (1963). The protein concentration of the polyribosomal preparation was adjusted to approximately 1 mg/ml with medium B (0.05 Tris, 0.025 M KCl, 0.001 $MgCl_2$, and 0.25 M sucrose, pH 7.6, at 4°) for subsequent studies. The ratio of absorbancy at 260 and 280 was found to be in the range of 1.5–1.6 for all preparations (Petermann, 1964).

Preparation of the pH 5 Enzyme Fraction. The pH 5 enzyme fraction was prepared by the procedure described by Campagnoni and Mahler (1967).

Sucrose Gradient Centrifugation. Sucrose gradients (0.3–1.0 M) were prepared at 4° using a Lucite mixing chamber. Sucrose solutions were prepared in medium

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[1] Abbreviation used are: FSH, follicle-stimulating hormone.

A (medium B minus sucrose) and all sucrose used in these experiments was ribonuclease free (Mann Research Laboratories). Polyribosomes equivalent to 200–250 μg of protein were applied to a 27-ml gradient using the Spinco band-forming caps for the SW25 rotor. Centrifugation at 0° was continued for 2 hr at 53,500g (R_{av}). The A_{254} was monitored continuously using an ISCO Model D density gradient fractionator (Instrumentation Specialities Co.), and when fractions were collected an ISCO fraction collector was used (Model 270).

Analysis of Polyribosomes by Ultracentrifugation. Sedimentation velocity experiments were performed in a Spinco E ultracentrifuge at 31,410 rpm and 4°, employing schlieren optics. Resuspended polyribosomes (0.5–1.2 mg of protein/ml) were dialyzed for 12 hr against three changes of medium C (medium A plus 5 mM thioglycerol) at 4° prior to sedimentation. Sedimentation coefficients, s, were calculated from the rates of movement of the maximum ordinates of peaks, measured with the aid of a two-coordinate comparator (Nikon Model 6C), accurate to 2×10^{-4} cm. Values of s were corrected to 20° in water to give $s_{20,w}$ values (Svedberg and Pedersen, 1940); the viscosity and density of the solvent were assumed to be those of water and the partial specific volume of all polyribosomes was taken as 0.66 ml g^{-1} (Petermann, 1964). Plots of $s_{20,w}$ against protein concentration were constructed and extrapolated to zero concentration to give $s^0_{20,w}$ values.

For the analysis of a single polyribosomal species, the polysomal suspension (before centrifugation) was layered on sucrose gradients (1 mg of protein/gradient) and centrifuged as before. Absorbance at 254 mμ was monitored continuously by a Gilford recording spectrophotometer and fractions of 10 drops each were collected and kept at 0–2°. Fractions from eight sucrose gradients were pooled and the pooled fractions were dialyzed and examined in the ultracentrifuge as described above.

Electron Microscopy. Polyribosomes were fractionated on sucrose gradients as described above and 10 drop fractions were collected. The fractions corresponding to the A_{254} peaks were investigated by electron microscopy. Polyribosomal fractions were kept at 0–2° throughout the procedure and all samples were transferred to grids within 1 hr of the time of fractionation. A small amount of the suspension (<1 drop) was placed on a smooth-surfaced agar block where the moisture was absorbed. A thin layer of 0.25% formvar (polyvinylformal in ethylene dichloride, w/v; Delaware Scientific Laboratories, Inc.) was then layered over the polyribosomes. The thickness of this layer or "support" was kept as close as possible to 600–1000 Å by observing the interference colors (silver to gold). The film of polyribosomes and formvar was floated off the agar block and picked up on 400 mesh copper grids. The grids were washed with decreasing concentrations of sucrose in medium A (5% changes) ending with a wash in medium A. The washed grids were then shadowed with platinum and coated with carbon. Pictures were taken with a Hitachi HU-11C Electron Microscope operated at an accelerating voltage of 75 kV and using a high-resolution specimen holder.

Protein-Synthesizing System. The complete system for amino acid incorporation is a modification of one described for rat liver by Wettstein *et al.* (1963) and contained the following components in the concentrations indicated: Tris, 30 mM; $MgCl_2$, 6.4 mM; thioglycerol (Aldrich Chemical Co.), 2 mM; ATP (P. L. Biochemicals), 1 mM; GTP (P. L. Biochemicals), 0.4 mM; phosphoenolpyruvic acid (Sigma), 10 mM; pyruvic kinase (Calbiochem), 20 EU/ml; 19 amino acids (Mann), 0.05 mM each; [$^{14}C_5$]valine (New England Nuclear, 209 mM/mmole), 1.0 μCi; sucrose (Mann), 0.24 M; polyribosomes, 400 μg/ml; pH 5 enzyme fraction, 250 μg/ml; and water to a final volume of 1.0 ml.

For zero-time controls, the reaction was stopped by addition of 10 μmoles of valine (unlabeled) followed by trichloroacetic acid to a final concentration of 10%. Incubations were performed for 30 min at 37° in a Dubnoff metabolic shaker set at 144 oscillations/min with air as the gas phase. Incubations were terminated as described above for zero-time controls. After 30 min at 0°, the samples were centrifuged at 1000g for 5 min. Pellets were broken with a Vortex mixer after discarding the supernatant and 1.5 ml of 5% trichloroacetic acid (w/v) was added to each tube. Samples were then mixed, covered, and heated in a 90° water bath for 30 min. Tubes were then cooled to 0° before collecting the precipitate on fiber glass filter disks (Reeve Angel, 2.4 cm). Each disk was washed six times with large volumes of 5% trichloroacetic acid, dried briefly on a hot plate, and transferred to a glass scintillation vial. NCS reagent (1 ml; Nuclear-Chicago) was added to each vial and vials were kept at 40° for 2–4 hr in order to dissolve the protein for measurement of radioactivity.

Measurement of Radioactivity. The ^{14}C content of the protein solution was measured by adding 15 ml of scintillation fluid prepared by dissolving 4 g of 2,5-diphenyloxazole (Packard) and 50 mg of 1,4-bis[2-(5-phenyloxazole)]benzene (Packard) per l. of scintillation grade toluene (Mallinckrodt) was added to each vial. Radioactivity was then measured by liquid scintillation spectrometry using a Packard Tri-Carb Model 3315. In all cases sufficient radioactivity was allowed to accumulate to give a probable error of less than 5%. Disintegrations per minute were determined by automatic external standardization. Counting efficiency for ^{14}C varied from 79 to 85%.

Determination of Protein. Protein was determined by the method of Lowry *et al.* (1951) and results are expressed at micromicromoles of [^{14}C]valine incorporated per milligram of polyribosomal protein; 1 $\mu\mu$mole of [^{14}C]valine corresponds to 420 dpm in the acid-precipitable material.

Results

Physical Characteristics of Polyribosomes. A typical sucrose gradient of testicular polyribosomes is shown in Figure 1. Eight peaks of absorbance at 254 mμ are

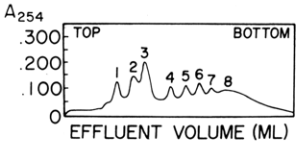

FIGURE 1: Sucrose gradient profile of polyribosomes from rat testis. Approximately 200 μg of polyribosomal protein was applied to each gradient of 27 ml. Centrifugation was performed for 2 hr at 25,000 rpm in the SW25 rotor of the Spinco Model L-2 at a temperature of 0°. Numbers 1–8 indicate cuts of the gradient taken for electron microscopy.

PLATE 1: Electron micrograph of material corresponding to the fifth peak of Figure 1. The pentagonal groups shown are representative of the material in this peak. Platinum shadowed, magnification 108,000 ×.

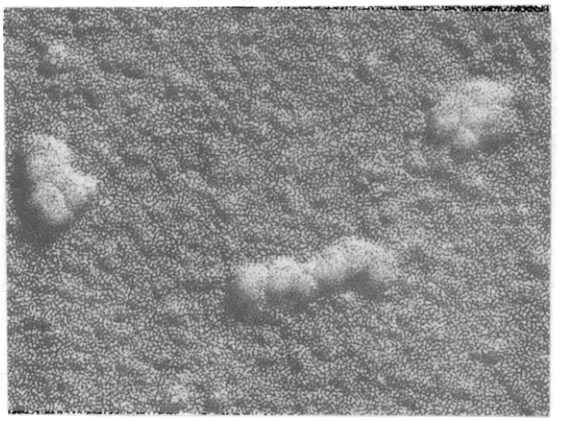

PLATE 2: Electron micrograph of sixth peak. This peak contains large numbers of hexasomes. Platinum shadowed, magnification 108,000 ×.

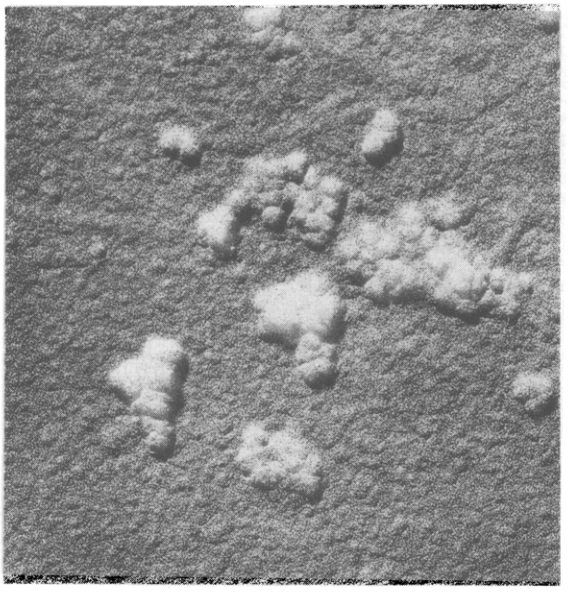

PLATE 3: Electron micrograph of the seventh peak. Large aggregates of polyribosomes can be seen together with smaller clusters. Platinum shadowed, magnification 72,000 ×.

shown in the figure in which the size of polyribosomal aggregates increases from left to right. Fractions representing each of the eight peaks were investigated by electron microscopy. Peaks 1–4 could always be identified as monomers through tetramers, respectively, and since these particles were confirmed by analytical ultracentrifugation the micrographs are not shown. Peaks 5 and 6 were composed primarily of pentamers and hexamers, respectively, although each of these fractions was contaminated with other species of polyribosomes (Plates 1 and 2). No distinct size range could be assigned to the particles in peaks 7 and 8 although both of these fractions contained heterogeneous clusters of ribosomes each consisting of more than six ribosomes (Plates 3 and 4).

In order to establish that the particles represented in Plates 1–4 were polyribosomes and not simply aggregates of monomeric ribosomes resulting from the fixing procedure, an aliquot of each fraction was treated with ribonuclease (1 μg/ml), then applied to grids, and shadowed. The results of such treatment (using fractions 5 and 8) are shown in Plates 5 and 6. It can be seen that in each case the electron micrographs are composed predominantly of single ribosomes. Treatment of material from the remaining five peaks (*i.e.*, other than the monosome peak) gave the same results.

In order to confirm the assignment of polyribosomal size, preparations were investigated by means of the analytical ultracentrifuge.

A series of schlieren patterns was obtained with a sample of testicular polysomes (1.2 mg of protein/ml) and photographs were taken at 4-min intervals. Figure 2 represents an enlargement of a frame taken after 12-min centrifugation in which six peaks are numbered (I–VI). The direction of sedimentation is left to right. The preparation was analyzed at four concentrations prepared by diluting the material used for the study shown in Figure 2. Figure 3 shows sedimentation

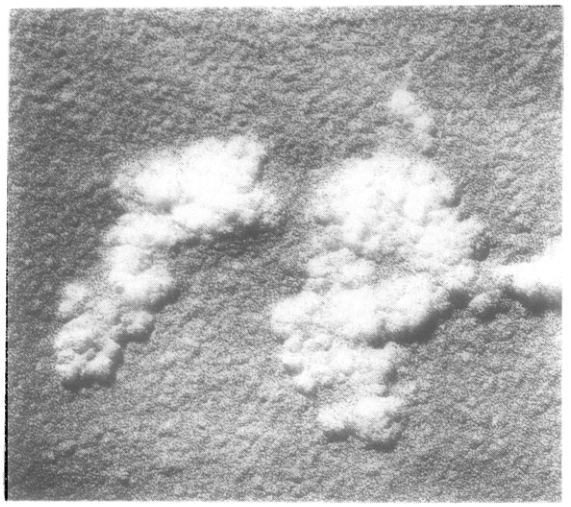

PLATE 4: Electron micrograph of the material at the bottom of the gradient (peak 8). Note predominance of large clusters. Platinum shadowed, magnification 72,000 ×.

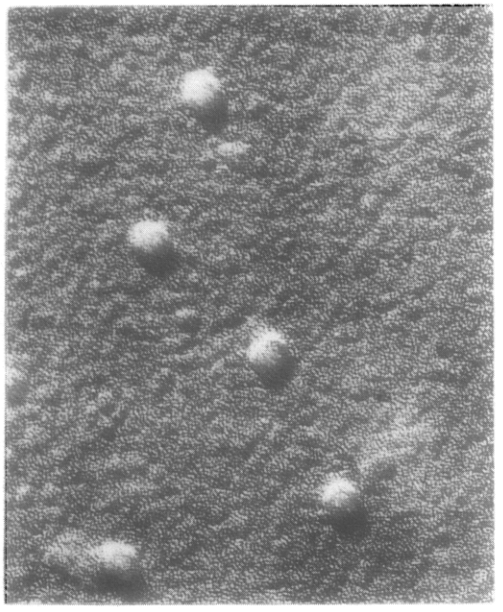

PLATE 5: Electron micrograph of material from fifth peak treated with 1 μg/ml of ribonuclease for 10 min at 37°. The monomer is the only species present in this picture. Platinum shadowed, magnification 108,000 ×.

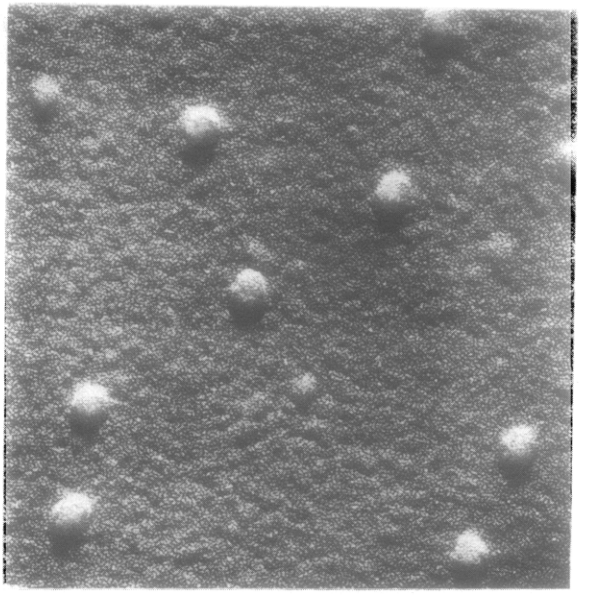

PLATE 6: Electron micrograph of material from peak 8 treated with 1 μg/ml of ribonuclease for 10 min at 37°. Again only monomers can be seen. Platinum shadowed, magnification 108,000 ×.

TABLE I: Properties of Ribosomal Aggregates from Rat Testes.[a]

Peak on Sucrose Gradient	Electron Micrograph	Analytical Ultracentrifuge $s^0_{20,w}$ (S)
First	Monomers	77 (III)
Second	Dimers	128 (IV)
Third	Trimers	172 (V)
Fourth	Tetramers	216 (VI)
Fifth	Pentamers	
Sixth	Hexamers	

[a] Roman numerals in the third column indicate the corresponding peak number in Model E photograph (Figure 2).

coefficients as a function of protein concentration and the corresponding values for $s^0_{20,w}$ calculated by extrapolation to zero concentration are given in Table I. When fractions corresponding to peak 1 of sucrose gradients (Figure 1) were pooled and examined in the ultracentrifuge, a single peak with a sedimentation coefficient $s_{20,w}$ of 76 S was observed at a protein concentration of 1.6 mg/ml. From Figure 3 it can be seen that such an $s_{20,w}$ value corresponds to values observed with peak III of the schlieren pattern (see Figure 2). Clearly peak 1 of the sucrose gradient corresponds to peak III of the schlieren pattern. This peak shows a value for $s^0_{20,w}$ of 77 S and is therefore composed of single ribosomes in keeping with the electron microscopic appearance of this fraction.

Peaks I and II in the schlieren patterns (Figure 3) presumably represent ribosomal subunits in various stages of breakdown. Peaks IV–VI in Figure 2 showed curvilinear plots for $s_{20,w}$ as a function of concentration clearly demonstrating that linear extrapolation to zero concentration is not justified. The divergence from linearity is more pronounced in the peaks corresponding to larger polyribosomal aggregates. As the result of curvilinear extrapolation our values for $s^0_{20,w}$ differ from those reported by other workers, *e.g.*, Campagnoni and Mahler (1967), and the difference

TABLE II: Requirements for the Polyribosomal Amino Acid Incorporation System from Rat Testis.[a]

Component	μμmoles of [^{14}C]Valine Incorpd/mg of Polysomal Protein	% Complete
Complete	11.7	(100)
Deletion		
Mg^{2+}	0.5	4
ATP	0.4	3
GTP	2.9	25
ATP-generating system	6.4	55
Thioglycerol	10.5	90
19 amino acids	5.6	45
pH 5 enzyme fraction	2.5	21
Polyribosomes	0.0	0

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

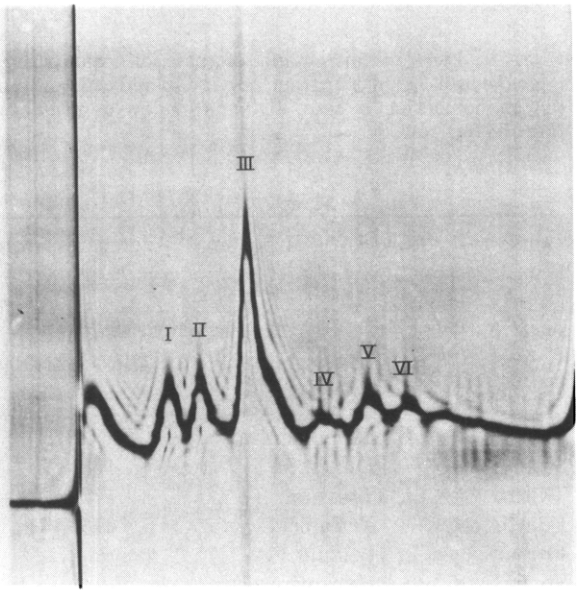

FIGURE 2: Schlieren pattern of polyribosomes from rat testes 12 min after reaching 31,410 rpm. Concentration of ribosomal protein was 1.2 mg/ml in medium C, pH 7.6, at 4°. Peaks are numbered in increasing order of sedimentation coefficient (I–VI).

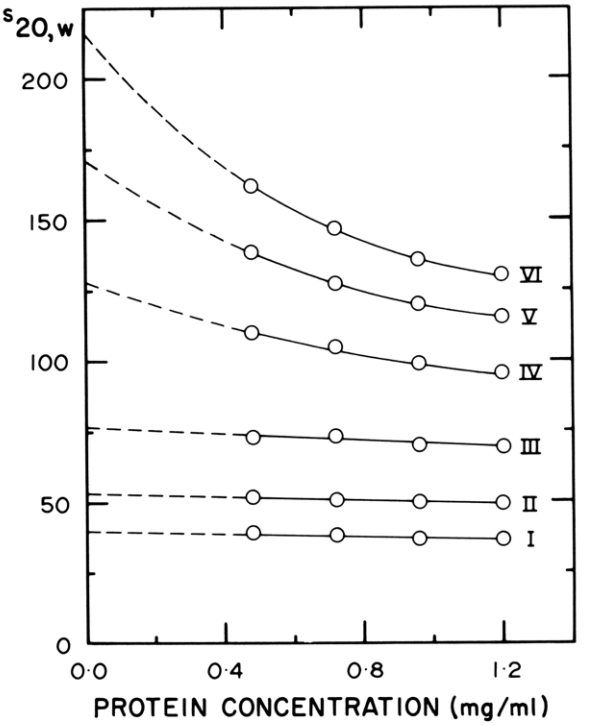

FIGURE 3: s_{20} as a function of protein content. A preparation of polyribosomes from 80 testes was suspended in medium C (1.2 mg of protein/ml). The sample was examined in the analytical ultracentrifuge (Figure 2) and was reexamined three times after dilution with medium C to provide the protein concentration shown. Values of $s_{20,w}$ at each concentration were calculated for each peak and $s^0_{20,w}$ was obtained by extrapolation to zero concentration.

is greater with larger aggregates (Table I). It is interesting that these authors reported that some values of $s_{20,w}$ were unexpectedly high (see legend to their Table I). It is clear from Figure 3 that linear extrapolation to zero concentration is unacceptable and that the values reported in Table I of the present paper are free from this source of error.

Table I also summarizes the microscopic appearance and sedimentation properties of the six peaks seen with sucrose gradients. It will be seen that pentamers and hexamers were not observed in the analytical ultracentrifuge. Careful examination of Figure 2 reveals small irregularities in the schlieren patterns beyond (to the right of) peak VI. These irregularities were consistently observed from run to run and represent heavy material corresponding to larger aggregates each of which is present in amounts too small to appear as a discrete peak. This is not surprising in view of the fact that the total concentration of protein in the sample was only

TABLE III: Effect of Inhibitors upon the Incorporation of [^{14}C]Valine by a Cell-Free System from Testis.[a]

Inhibitor	Concn (μmoles/ml)	$\mu\mu$moles of [^{14}C]Valine Incorpd/mg of Protein	% Inhibn
None		13.3	
Puromycin	10	9.6	28
	50	6.4	52
	100	5.7	57
Cycloheximide	10	13.2	1
	50	10.3	23
	100	8.3	28
Deoxyribonuclease	5	13.2	1
Ribonuclease	5	0.1	99
NaF	420	3.5	74

[a] Conditions of incubation are described under Methods.

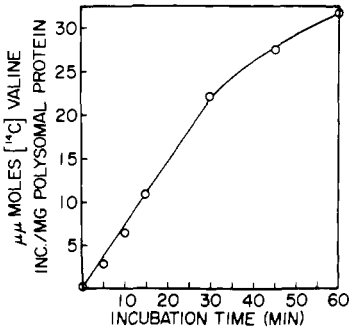

FIGURE 4: Time course for the incorporation of [^{14}C]valine by the complete polyribosomal system (see Methods).

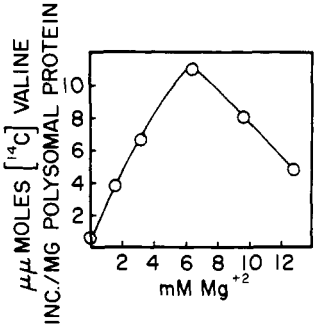

FIGURE 5: Effect of concentration of Mg^{2+} upon incorporation of [^{14}C]valine by the cell-free system. Incubation was for 30 min at 37° using conditions described under Methods.

1.4 mg/ml. This preparation required 80 testes (120 g of tissue). It is possible that larger amounts of tissue may have provided peaks corresponding to pentamers and hexamers but all attempts to demonstrate such larger aggregates in schlieren patterns failed to produce peaks sufficiently well resolved to enable *s* values to be measured.

Requirements for Cell-Free Protein Synthesis. The time course for the incorporation of [^{14}C]valine into protein by the polyribosomal system from rat testis is shown in Figure 4. Incorporation was linear during the first 30-min incubation and continued to increase between 30 min and 1 hr although the rate was less during the second 0.5 hr than during the first.

The requirements for protein biosynthesis by the cell-free system are shown in Table II. This system exhibits an absolute requirement for polyribosomes, Mg^{2+}, and ATP. Omission of any one of these components resulted in a 96–100% decrease in activity. The system is also highly dependent upon pH 5 enzyme fraction, GTP, the 19 amino acids, and an ATP-generating system, but exhibited 90% of the original activity in the absence of thioglycerol. Finally maximal incorporation of [^{14}C]amino acid into protein was found to occur at a [^{14}C]valine concentration of 4.8 mμmoles/ml (1.0 μCi/ml).

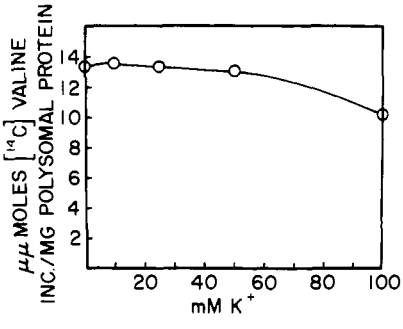

FIGURE 6: Effect of concentration of K^+ upon incorporation of [^{14}C]valine by the cell-free system.

Metal Ion Requirements. Protein biosynthesis with the polyribosomal system was found to be extremely sensitive to the Mg^{2+} concentration. Figure 5 shows that maximal activity occurred in the presence of 6.4 mM Mg^{2+} and raising or lowering the concentration of this cation caused a sharp reduction in incorporation. On the other hand, the testicular system did not exhibit a requirement for K^+. Increasing the K^+ concentration from 0 to 50 mM had no effect on the incorporation of [^{14}C]valine and 100 mM K^+ was slightly inhibitory (Figure 6).

Effects of Inhibitors. Table III shows the effects of various metabolic inhibitors upon activity of the polyribosomal system. It can be seen that both puromycin and cycloheximide caused significant reduction in incorporation at concentrations of 50 and 100 mM ($P < 0.01$). Cycloheximide, however, was not effective at 1 mM, whereas a similar concentration of puromycin caused 28% inhibition. At all concentrations tested puromycin was a more effective inhibitor of protein synthesis *in vitro* than cycloheximide. Deoxyribonuclease (5 mM) did not inhibit incorporation whereas ribonuclease (5 mM) completely abolished activity. Moreover the addition of 10 mM NaF to the incubation medium inhibited incorporation by 74%.

Discussion

These experiments demonstrate for the first time the presence of polyribosomes capable of protein biosynthesis in rat testis. That the particles isolated in these studies are polyribosomes (that is, aggregates of ribosomes joined by RNA; Warner *et al.*, 1963; Penman *et al.*, 1963) is supported by the following evidence: (i) analysis of ribonucleoprotein from rat testis by sucrose gradient centrifugation revealed a high proportion of A_{254} material with sedimentation characteristics typical of ribosomal aggregates (Figure 1 and references); (ii) ribonuclease and NaF were each capable of converting the heavy aggregates into material behaving on sucrose gradients like monomeric ribosomes; (iii) fractions from sucrose gradient were unequivocally identified by electron microscopy which revealed aggregates of particles showing the size and shape of ribosomes in groups of two to six or greater (Plates 1–4); (iv) such fractions when exposed to ribonuclease or NaF broke down to monomeric ribosomes as demonstrated by electron microscopy (Plates 5 and 6); and (v) the assignment of particle size indicated by sucrose gradients and electron microscopy was confirmed by analytical ultracentrifugation and subsequent calculation $s^0_{20,w}$ values for each peak (Table I).

A likely explanation of the varying relationship between $s_{20,w}$ values and protein concentration (Figure 3) is that frictional interactions between the various constituents of the sample increase as the environment in which the various species sediment becomes more complex. For example, $s_{20,w}$ of peak VI (Figure 2) refers to the sedimentation of tetramers in the presence of all other polyribosomes of smaller size, while that of peak IV refers to the sedimentation of dimers in the absence of tetramers. It is possible that increased frictional interactions may result from increasing asymmetry of larger polyribosomes. Such asymmetry is apparent in Plates 1–4 although the shapes observed in electron micrographs cannot be taken to represent the shapes assumed by aggregates in solution. The nonlinear concentration dependence seen in Figure 3 suggests that a Johnston–Ogston (1946) effect may have been encountered during centrifugation. For this reason no attempt has been made to analyze the system by means of peak areas.

It is clear that characterization of polyribosomes on the basis of $s_{20,w}$ values is without meaning unless values can be extrapolated to give $s^0_{20,w}$ values, and that linear extrapolation is not valid for aggregates of more than two ribosomes, at least in the testicular preparation herein described.

The polysomal preparation from rat testis is capable of protein biosynthesis and this biosynthetic activity shows the usual requirements seen with other systems *in vitro*, *i.e.*, amino acids, pH 5 enzyme, Mg^{2+}, ATP, GTP, etc. (Table II).

In order to achieve optimal yields of polyribosomes from rat testis a pestle clearance of 0.9 mm is important. This finding is in keeping with observations by Ganoza *et al.* (1965) with rat liver. Presumably smaller clearance causes disruption of lyosomes with the release of proteolytic and other enzymes (Bucher, 1953). A second factor of importance for good yields of polyribosomes is the concentration of detergent used and this again agrees with other systems. For example, Wettstein *et al.* (1963) reported that 1.3% deoxycholate was optimal for the preparation of hepatic polysomes while Campagnoni and Mahler (1967) and Talal (1966) obtained best results with rat cerebral cortex and rat spleen respectively by using no detergent. In our experiments the yield of polysomes was very low without detergent, suggesting that a large proportion of ribosomes are membrane bound in the testis. The same appears to be true for liver and pancreas (Siekevitz and Palade 1960; Breillatt and Dickman, 1966). However the *activity* (as opposed to yield) of testicular polyribosomes expressed as micromicromoles of [^{14}C]amino acid incorporated into peptides per milligram of polyribosomal protein was almost the same when polyribosomes isolated without detergent (free polyribosomes) were compared with those isolated with deoxycholate (membrane-attached polyribosomes). Similar findings have been reported for the incorporation activity of free and membrane-attached polyribosomes from rat liver (Manganiello and Phillips, 1965).

The characteristics of protein biosynthesis by polyribosomes from rat testis *in vitro* are similar to those described for other systems, *e.g.*, rat liver (Wettstein *et al.*, 1963), reticulocytes (Lin *et al.*, 1966), calf lens (Spector and Travis, 1966), and rat uterus (Teng and Hamilton, 1967). However, the requirements for metal ions appear to vary considerably among various systems. Although all systems so far reported require Mg^{2+} for protein synthetic activity, in some cases the concentration of this ion required for maximal activity is not sharply defined (*e.g.*, rat brain, Campagnoni and Mahler, 1967; Zomzely *et al.*, 1966). On the other hand, some systems show optimal activity over an extremely narrow range of Mg^{2+} concentrations (*e.g.*, rat uterus, Teng and Hamilton, 1967; rat spleen, Talal, 1966). Our studies show that testis belongs to the second group (Figure 5) since activity declines sharply on either side of the optimal concentration of 6.4 mM. Potassium requirements also vary from such systems as brain (Campagnoni and Mahler, 1967) and uterus

(Teng and Hamilton, 1967), on the one hand, which require K^+, to liver (Wettstein *et al.*, 1963) and calf lesns (Spector and Travis, 1966), on the other hand, which do not require added K^+. The testis again belongs to the second group since K^+ is without effect until concentrations of 100 mM are reached when inhibition of protein synthesis occurs (Figure 6).

The dependence of the testicular polyribosomal preparation upon GTP is of considerable interest since 25% of the incorporation seen with the complete system occurs in the absence of added GTP (Table II). All cell-free systems require GTP for protein biosynthesis but the extent of inhibition when GTP is not present varies considerably (Spector and Travis, 1966; Campagnoni and Mahler, 1967; Teng and Hamilton, 1967). A second important observation is that NaF (100 mM) also causes 75% inhibition of incorporation in the present system. A cell-free system in rat uterus was inhibited by NaF to the same extent as by omission of GTP (40% inhibition) (Teng and Hamilton, 1967).

GTP appears to discharge two functions in protein biosynthesis, firstly the nucleotide promotes binding of aminoacyl-tRNA to ribosomes (Ravel *et al.*, 1966), this action does not seem to require hydrolysis of GTP (Anderson *et al.*, 1967) and is less specific for this nucleotide than the second function in which hydrolysis of GTP allows translocation of peptidyl-tRNA from the donor to the acceptor site on the ribosome (Ohta and Thach, 1968). The persistence of some incorporation of amino acids in the absence of added GTP is likely to arise in our system from two sources, namely, (i) small amounts of GTP bound to ribosomes and not removed during preparation of the polyribosomal system, and (ii) addition of one amino acid to incomplete nascent chains. In the first case endogenous GTP would by hydrolyzed to GDP and this would be converted into GTP by the ATP-generating system present in our incubation mixture (pyruvic kinase). In the second case one aminoacyl-tRNA would bind to the ribosomal donor site (assisted by ATP) and translocation would fail in the absence of GTP.

Sodium fluoride appears to inhibit chain initiation but not to prevent addition of amino acids to established chains (Lin *et al.*, 1966). If these considerations apply to the present system of testicular polysomes it is likely that 25% of activity measured with the complete system results from addition of one amino acid only to nascent chains, and as much as 75% of activity may involve formation of new chains although we have no direct evidence that the activity of the testicular system involved chain initiation. It now appears that in reticulocytes chain initiation may not require *N*-formylmethionine (Culp *et al.*, 1968) but GTP is required for the binding of aminoacyl-tRNA to the donor site (Mukundan *et al.*, 1968; Ravel *et al.*, 1966). In so far as these considerations apply to the testis the above suggestion seems plausible.

The polyribosomal system described here appears to have been characterized in sufficient detail to enable the regulation of protein biosynthesis in the testis to be studied in a cell-free system. Experiments are now in progress in these laboratories to determine the influence of FSH upon protein synthesis by testicular polysomes.

Acknowledgment

The authors are grateful to Mr. Richard Mena for excellent technical assistance and to Dr. Boyd Hardesty for stimulating advice and discussions.

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