Ribonucleoprotein Particles of Bovine Anterior Pituitary Gland. Physicochemical and Biosynthetic Characteristics*

P. Radhakantha Adiga,† Robert O. Hussa,‡ and Theodore Winnick

ABSTRACT: Bovine adenohypophyseal ribonucleoprotein (RNP) preparations have been analyzed in terms of sedimentation properties, capacity to incorporate labeled amino acids into protein and hormone fractions, and ability to dissociate into subunits and reassociate back into ribosomes. The following results were obtained. (1) Ultracentrifugal analysis revealed components with sedimentation coefficients of 55, 72, 108, 137, and 160. Removal of Mg^{2+} caused a disappearance of the heavier species, with a concomitant increase in the 55S and 72S peaks. (2) Amino acid incorporating ability of fractionated pituitary RNP preparations was confined to the polysome region; very little incorporation occurred with monoribosomes. (3) Polyuridylic acid (poly U) could bind to pituitary ribosomes and stimulated [^{3}H]phenylalanine incorporation by the latter. This effect increased following preincubation of the ribosomes. On the other hand, polysomes showed poor ability to bind poly U and to stimulate poly U directed [^{3}H]phenylalanine incorporation. (4) Chelating agents dissociated ribosomes into 44S and 27S subunits, containing 28S and 18S ribosomal ribonucleic acid (rRNA), respectively. Dissociation of prelabeled ribosomes resulted in the release of a large proportion of nascent protein and hormones from the ribosomes, while a small amount of radioactivity remained bound to both subunits. (5) Reconstitution of ribosomes from subunits could be accomplished by the careful addition of Mg^{2+}. Such reconstituted structures had the capacity to bind poly U, but were not able to stimulate poly U directed [^{3}H]phenylalanine incorporation. However, addition of spermine, together with poly U, resulted in a marked stimulation of [^{3}H]phenylalanine incorporation by the reconstituted ribosomes.

Extensive experimental evidence has accumulated during the past few years that ribonucleoprotein[1] particles serve as the primary site of translation of the genetic message in the protein synthetic machinery of a variety of living cells. Until recently, two main types of basic ribosomal units had been recognized: a particle with a $s^0_{w,20}$ of approximately 80S, apparently characteristic of animal and yeast cells, and containing 28S and 18S rRNA; and a 70S species, found mostly in bacterial cells, with 23S and 16S RNA (Taylor and Storck, 1964). However, a third variety with an s value of 80S, but containing 25S and 16S RNA, has recently been encountered in the cytoplasm of plant cells (Stutz and Noll, 1967; Click and Tint, 1967). In addition, a spectrum of sizes (20–300 S) of RNP particles has been observed in extracts of several cell types, in proportions governed by various factors (Petermann, 1964). The smaller entities (20–62 S) may represent either dissociation products, or precursors of the basic 70–80S unit (Mangiarotti and Schlessinger, 1966; McConkey and Hopkins, 1965). The heavier particles, often associated with the major biosynthetic activity, are commonly termed polyribosomes or polysomes. These appear to be orderly ribosomal aggregates, held together by a strand of mRNA (Warner *et al.*, 1963; Wettstein *et al.*, 1963).

A ribosome-dependent cell-free system from bovine anterior pituitary glands, capable of incorporating labeled amino acids into mixed tissue protein and an ACTH fraction, has been recently reported from this laboratory (Adiga *et al.*, 1966). Subsequently, the same system has been found to incorporate radioactive amino acids into growth hormone and prolactin (Rao *et al.*, 1967a). The apparent stability and high biosynthetic potency of bovine pituitary ribosomes over prolonged periods of incubation invited the present detailed investigation of these particles.

Experimental Procedures

Materials. L-[3,4-^{3}H]Proline (5 Ci/mmole), L-[^{3}H]leucine (5 Ci/mmole), and L-[^{3}H]phenylalanine (3 Ci/mmole) were purchased from New England Nuclear Corp. [^{3}H]Poly U (ammonium salt, 24.7 mCi/mmole) and unlabeled poly U were products of Miles Chemical Co. and Sigma Chemical Co., respectively. EDTA was obtained from California Corp. for Biochemical Research. The sources of the other biochemicals used have been described in a previous paper (Adiga *et al.*, 1966). Growth hormone and prolactin standards were provided by the Endocrine Study Section of the National Institutes of Health.

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† Present address: Thyroid Laboratory, Royal Victoria Hospital, Montreal 2, Can.

‡ Predoctoral fellow of the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: RNP, ribonucleoprotein; DOC, deoxycholate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

Preparation of the Constituents of the Anterior Pituitary Cell-Free System. The methods of preparing mixed ribosomes, pH 5 enzyme, and the "cocktail" (containing the energy sources, [^{12}C]amino acid mixture, and cofactors) are those previously described (Adiga *et al.*, 1966), with the following modifications. In the preparation of the ribosome fraction, residue II was discarded. The microsome pellet (residue IV) was suspended in one part of supernatant IV and five parts of medium S, prior to DOC treatment. The presence of the supernatant phase in this step significantly enhanced the labeled amino acid incorporating ability of ribosomes, presumably due to a stabilization effect (Lawford *et al.*, 1966). Addition of a fraction of the pH 5 supernatant phase, derived by precipitation at 65% saturation with ammonium sulfate, followed by dialysis for 2 hr at 4° against medium M, improved the biosynthetic activity of the complete system. This fraction, hereafter termed pH 5 supernatant factor, was routinely added to the cell-free incubation system at a level of 1 mg of protein/ml.

When the polysome-enriched fraction was desired, the procedure of Wettstein *et al.* (1963) was followed. A discontinuous sucrose density gradient was prepared by layering 2.5 ml of 0.5 M sucrose in medium M on 3.0 ml of 2 M sucrose (also in medium M). Then 5.5 ml of the DOC-treated microsomal suspension was layered on this gradient. Centrifugation at 140,000*g* was carried out for 4 hr at 0–3° in a Spinco Model L ultracentrifuge. The supernatant fluid was discarded. The pellet was first rinsed with medium M, and then gently suspended in medium S.

The RNP preparation, when required for either sedimentation or sucrose gradient analysis, was clarified by low-speed centrifugation (3000*g* for 5 min) at 0–4°, to remove large-particle aggregates. The resultant preparation is hereafter referred to as "native" ribosomes.

"Derived" ribosomes were prepared by first preincubating the mixed ribosomes with the complete cell-free system at 37° for a specified time interval, and then reisolating the ribosomes by centrifugation for 1 hr at 140,000*g*. After dialysis against medium M at 4° for 12 hr, the ribosomes were clarified by the above low-speed centrifugation step.

Dissociation of Ribosomes. This process was usually accomplished by the addition of 2 μmoles of EDTA/mg of ribosomes. After 2 min, ultracentrifugal or sucrose density gradient analysis was begun. A second method of dissociation involved dialysis of the ribosomes for 15 hr at 4° against 0.01 M Tris–0.05 M KCl (pH 7.6) containing 1×10^{-4} M EDTA. The dialysis tubing was pretreated with a boiling solution of 0.1 M EDTA (pH 7.6) for 10 min and washed thoroughly with water before use.

Sedimentation Analysis in the Analytical Ultracentrifuge. A Spinco Model E instrument, equipped with phase-plate schlieren optics, was used for analysis of the ribosomal preparations. An AN-D rotor, containing a cell of 1.2-cm light path, was employed in all cases. Timing was begun when a speed of 29,500 rpm was attained, except when dissociation of ribosomes was being followed. In the latter case, the speed used was 50,740 rpm. All runs were made at 24.5° in specified media, at a ribosome concentration of 1–6 mg/ml. Sedimentation rates were calculated from the photographic plates with the aid of a Gaertner microcomparator. Observed sedimentation coefficients were corrected for differences in viscosities and densities of the suspending media and water, and are expressed as either $s^0_{w,20}$ or $s_{w,20}$. Viscosity was measured with an Ubbelohde-type viscometer (Cannon Instrument Co.), and density was determined with a pycnometer. In calculations, a partial specific volume of 0.65 ml/g was assumed for all classes of RNP particles (Petermann and Hamilton, 1961). Relative proportions of the separated components were calculated by planimetry.

Sucrose Density Gradient Analysis. A 5–20% (w/v) linear sucrose gradient was employed. A mixing device similar to that described by Britten and Roberts (1960) (Buchler Instrument Co.) was used, and the gradient was prepared in 1.3 × 5 cm cellulose tubes. The sucrose solutions were generally prepared in either medium M minus mercaptoethanol or in 0.01 M Tris–0.05 M KCl buffer (pH 7.6). However, when rRNA was to be analyzed, the sucrose solutions were made up in 0.1 M NaCl–0.01 M sodium acetate–0.1% SDS (Henshaw, 1964). Ribosomal suspension (0.3 ml) (1–3 mg of rRNA/ml) was carefully layered on the gradient phase. Centrifugation was performed at 0–3° in a Spinco Model L ultracentrifuge for specified time intervals at required speed, using an SW 39 rotor. For gradient analysis of rRNA preparations, centrifugation was carried out at 15° for 12 hr at 20,000 rpm.

After centrifugation, the bottom of the tube was carefully punctured, and 2-drop fractions (4 drops in the case of SDS gradients) were collected. Absorbance of each fraction at 260 mμ was measured, after suitable dilution with water, in a Beckman DU spectrophotometer. When biosynthetic activity of freshly prepared ribosomes was to be measured following sucrose gradient centrifugation, alternate fractions were analyzed for absorbance. The remaining samples were supplemented with the various components of the cell-free system plus a labeled amino acid (usually 5 μCi of [^{3}H]-proline), in a total volume of 0.25 ml. These mixtures were then incubated for 1 hr at 37°.

For some experiments, "derived" ribosomes were prelabeled by incubation in the cell-free system containing an isotopic amino acid. After gradient centrifugation, individual fractions were diluted with 1 ml of water, and absorbance at 260 mμ was measured. Then 2 mg of crystalline bovine serum albumin was added (as carrier) to each fraction, and the radioactivity of protein insoluble in hot TCA was determined. In certain instances, the labeled growth hormone and prolactin content of ribosomes and ribosomal subunits was determined, following fractionations on the sucrose gradient. In these cases, the individual fractions corresponding to each absorbance peak were pooled. The desired hormone was then isolated and purified from this pooled material by methods involving the use of authentic carrier (Rao *et al.*, 1967b; R. O. Hussa, P. R. Adiga, and T. Winnick, unpublished data). The radioactivity associated with the carrier hormone was then measured.

TABLE I: Some Physicochemical Properties of Bovine Anterior Pituitary RNP Preparations.

Preparation	RNA (%)	Protein (%)	RNA:Protein	$A_{260\ m\mu}:A_{235\ m\mu}$	Extinction Coef at 260 mμ[a]
Mixed ribosomes	47.8	52.0	0.92	1.46	132
Polysome-enriched fraction	53.2	46.5	1.15	1.52	138

[a] Per centimeter of 1% solution in medium M.

Chemical Procedures. rRNA was measured by the procedure of Scott *et al.* (1956), as modified by Fleck and Munro (1962), with crystalline bovine serum albumin as added carrier. Protein was determined by the method of Lowry *et al.* (1951).

The rRNA from unfractionated pituitary and liver ribosomes was prepared and purified by the procedure of Hiatt (1962). When the RNA components of ribosomal subunits were required, the latter were isolated by density gradient fractionation. The pooled fractions from each peak were made 1% with respect to SDS, and 0.1 M in NaCl. The RNA was then precipitated by the addition of 2.5 volumes of 95% ethanol. After standing overnight at −20°, the precipitate was collected and washed with two volumes of 66% ethanol. Lastly, the RNA precipitate was suspended in a medium containing 0.1 M NaCl and 0.01 M sodium acetate, prior to layering on the sucrose gradient.

Radioactivity Determination. Tritium associated with each fraction from sucrose gradient experiments was measured either directly, as in [^{3}H]poly U binding experiments, or after isolation of the protein fraction precipitated by hot TCA (Adiga *et al.*, 1966, 1968). All radioactivity measurements were made in an automatic Packard liquid scintillation spectrometer (Model 3042). Corrections for zero time values and background were applied when required.

Results

Some Physicochemical Properties of Pituitary RNP Preparations. In Table I are presented data summarizing some of the physicochemical characteristics of the mixed ribosomes and polysome-enriched fraction. The higher RNA content and the higher RNA : protein ratio of the latter preparation are noteworthy. In this connection, the preparative procedure for polysomes involved sedimentation through a dense sucrose solution which favored collection of the heavier aggregates. The two preparations were found to have very similar ultraviolet absorption spectra, with maxima and minima at 260 and 235 mμ, respectively (data not shown). The curves were in fact quite similar to those for rat liver ribosomes (Petermann, 1964). It was concluded that the purity of the two pituitary RNP preparations was comparable with that of similar preparations from other mammalian tissues (Munro *et al.*, 1964; Tashiro and Siekevitz, 1965, Zomzely *et al.*, 1966).

The mixed ribosomes, suspended in medium M, exhibited several discrete peaks in the analytical ultracentrifuge (Plate IA). The $s_{w,20}$ of these components were 55, 72 (major component), 108, 137, and 160 S. In addition, one or two still heavier species were evident. Extrapolation of the 72S value to zero concentration resulted in an $s^0_{w,20}$ of 75 S. This latter figure will be used for the monomer peak throughout this paper. Polysome-enriched preparations showed similar schlieren patterns, except that the proportion of larger particles was relatively more abundant than in mixed ribosomes.

From the large volume of information on ribosomes from different biological sources (Petermann, 1964), it was anticipated that the stability of pituitary ribosomal aggregates would be dependent on magnesium ion concentration. In practice, it was found that the removal of Mg^{2+} by dialysis at 4° against Tris–KCl buffer led to a gradual disappearance of the heavier species, accompanied by an increase in the monomer peak. At 12 hr, the larger aggregates had disappeared almost completely, leaving only the 55S and 75S units (Plate IB).

Mixed ribosomes maintained at 0° in medium M were stable for 7–8 days, even though amino acid incorporating ability was completely abolished. A gradual increase in the 55S species was noted, presumably due to autodegradation of ribosomes (Tashiro and Siekevitz, 1965). Both types of RNP preparations, when frozen in medium M and then thawed, yielded random heavy aggregates. However, freeze–thawing in medium S, or in more concentrated sucrose solutions, prevented this phenomenon.

The ribosomes could be completely dissociated into subunits by the addition of chelating agents in appropriate amounts. Ribosomal suspensions in Tris–KCl buffer, when treated with EDTA at a concentration of 20 μmoles/ml, and then analyzed in the ultracentrifuge, showed two components with $s_{w,20}$ values of 37 and 21 S, and in proportions of about 2:1, respectively (Plate IC). The sedimentation values for the two particles, corrected to zero concentration ($s^0_{w,20}$), were 44 and 27 S. A further decrease in s values was observed when the EDTA concentration was increased, presumably due to changes in shape and degree of hydration of the RNP particles. Sodium pyrophosphate (0.02 M) (Henshaw, 1964) was found to cause similar dissociation of the pituitary ribosomes. The 44S and 27S ribosomal subunits are probably counterparts of the 47S and 32S subunits which were shown by Tashiro and Siekevitz (1965)

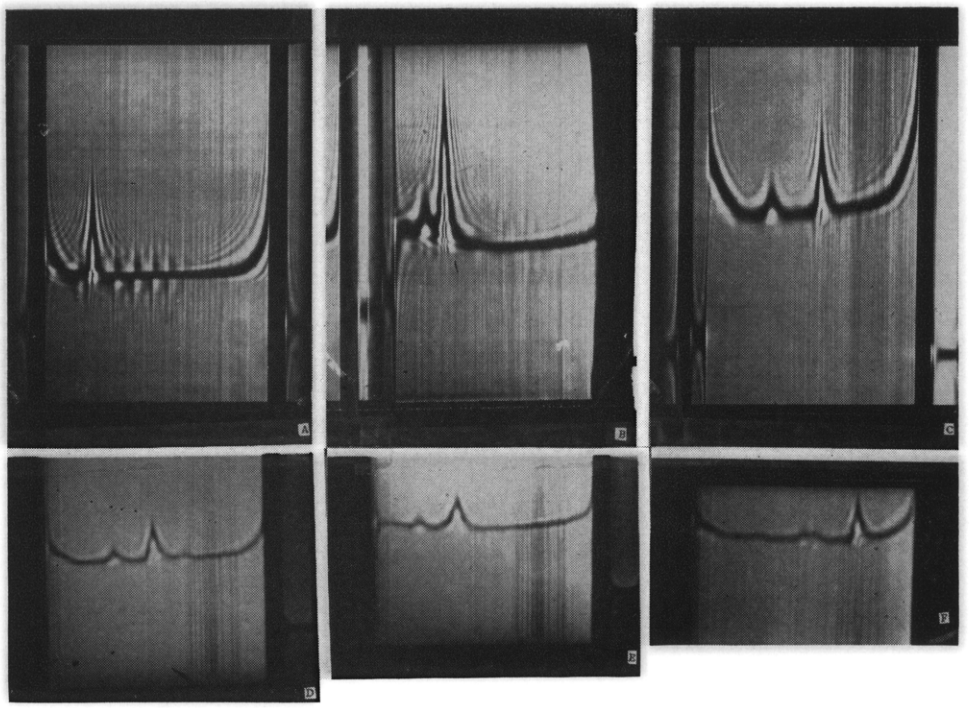

PLATE 1: Sedimentation patterns of anterior pituitary ribosomal preparations. The direction of sedimentation is from left to right. (A) Ribosomes (6 mg/ml) stored frozen in medium S and dialyzed overnight at 4° against medium M. The $s_{w,20}$ values were: 55, 72 (large peak), 108, 137, and 160 S. (B) Mixed ribosomes (4 mg/ml) dialyzed 12 hr at 4° against 0.01 M Tris–0.05 M KCl (pH 7.6). $s_{w,20}$ values were 55 and 75 S. (C) Ribosomes (1.2 mg) in Tris–KCl buffer, mixed with 2 μmoles of EDTA. $s_{w,20}$ values are 21 and 37 S. Concentration ratio of 21 S/37 S = 1:2. (D) Ribosomes (2 mg) in medium M treated with 4 μmoles of EDTA, followed by 4 μmoles of magnesium acetate. $s_{w,20}$ values are 32, 50 (large peak), and 66 S. (E) Ribosomes (2 mg) dialyzed 16 hr at 4° against Tris–KCl containing 1×10^{-4} M EDTA. $s_{w,20}$ values are 24 and 44 S. Concentration ratio of 24 S/44 S = 1:10. (F) Ribosomes (1 mg) in medium M mixed with 7.5 μmoles of EDTA/ml prior to centrifugation. $s_{w,20}$ values: 53, 58, 72, and 112 S (not shown).

to result from the action of chelating agents on liver ribosomes. These investigators found that the *s* coefficients of the subribosomes were quite dependent on the experimental conditions employed. Plate ID shows that the pituitary subunits could be at least partially reconstituted.

When a pituitary ribosomal suspension was dialyzed against Tris–KCl buffer containing 1×10^{-4} M EDTA, a similar dissociation phenomenon was observed (Plate IE). However, the relative proportions of the subunits were altered in favor of the larger particle, presumably due to greater instability of the smaller component.

Some evidence for the occurrence of an intermediate stage of dissociation was obtained. Treatment of the ribosomes in medium M with EDTA (7.5 μmoles/ml) revealed a particle with an $s_{w,20}$ of 58 S, in addition to 53S, 72S, and 112S species (Plate IF). This 58S component is probably analogous to the "60S" liver ribosome intermediate of Tashiro and Siekevitz (1965).

Sucrose Gradient Separation of Ribosomal Subunits and RNA Analysis. The undissociated (75S) pituitary ribosomes were found to give a single peak, typical of such particles (Tashiro and Siekevitz, 1965). Following the addition of EDTA, two well-resolved subribosomal components were observed, as expected (data not included). The heavier particles most probably corresponded to the 44S species encountered in schlieren patterns under similar conditions, and the lighter component to the 27S unit.

RNA from undissociated pituitary ribosomes gave a pattern indistinguishable from that of a control bovine liver rRNA preparation (data not shown). Since the latter has been found to consist of 28S and 18S components (Hall and Doty, 1959), it was assumed that these same values applied to pituitary rRNA. The rRNA profiles of pituitary subunits are shown in Figure 1. The larger particle appeared to consist predominantly of 28S RNA (Figure 1A), while the smaller species seemed to contain almost exclusively 18S RNA (Figure 1B). A small proportion of the 18S RNA encountered with the 44S subunit could be due to a slight contamination of the latter with either the 27S subunit or with the native 55S particle. Both types of rRNA have been reported in native 60S ribosomes of other mammalian species (Ristow and Köhler, 1967).

Ribosome Dissociation and Release of Nascent Protein and Hormones. In preliminary experiments it was consistently found that the polysome-enriched fraction was at least twice as active as the mixed ribosomes (per milligram of rRNA) in stimulating incorporation of labeled amino acids into protein in the cell-free system. The ribosomes isolated following the incubations were found to retain sizable amounts of radioactive protein. It has been suggested (Gilbert, 1963) that such labeled, bound

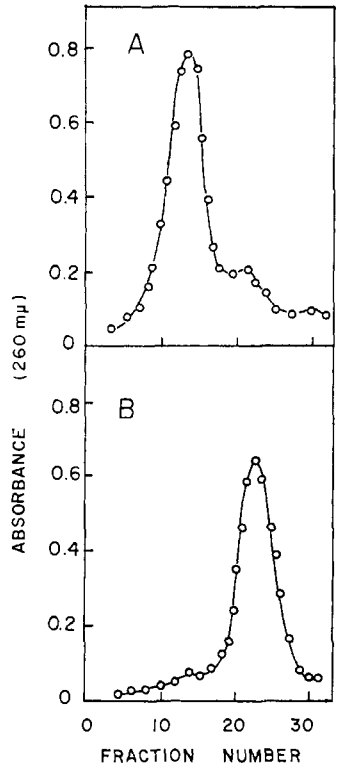

FIGURE 1: Sucrose gradient analysis of RNA from: (A) 44S and (B) 27S pituitary ribosomal subunits. In this and in all subsequent figures, the direction of sedimentation was from right to left.

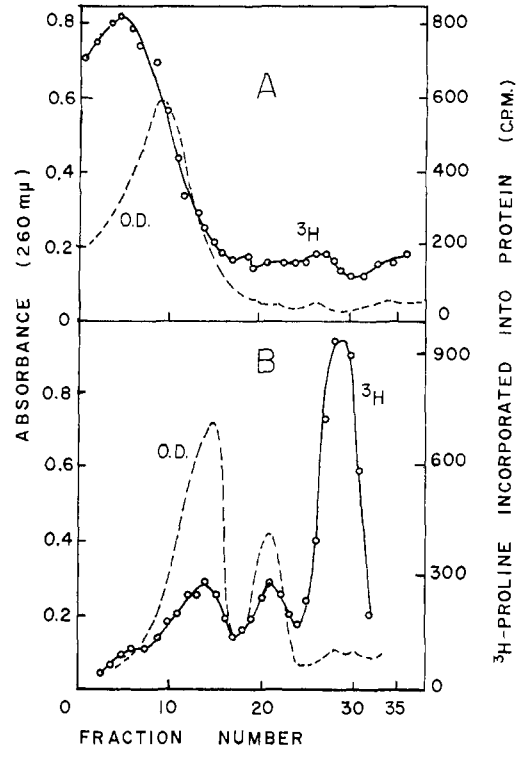

FIGURE 2: Release of labeled protein upon ribosomal dissociation. (A) Prelabeled 75S ribosomes, subjected to sucrose gradient centrifugation (5 hr at 28,000 rpm). (B) Same as A, but with EDTA treatment prior to fractionation.

material represents polypeptide chains with tRNA at the growing end.

It was of interest to study in more detail the radioactive proteins associated with pituitary ribosomes. Figure 2A gives the pattern of labeling following sucrose density gradient centrifugation of such a preparation. It may be seen that a considerable quantity of isotope was found in the 75S region. Figure 2B shows that most of the radioactivity was released upon dissociation of the ribosomes and appeared at the top of the gradient. However, significant amounts of ^{3}H-labeled protein were retained by both of the subunits.

It was next desired to determine whether any of the ribosomal-bound or solubilized radioactive protein could be assigned to specific hormones. In order to enhance the degree of labeling, three isotopic amino acids were simultaneously employed in a cell-free incubation experiment with polysome-enriched fraction. Sucrose density gradient fractions corresponding to undissociated ribosomes, ribosomal subunits, and the released material at the top of the gradient were used for the isolation of labeled prolactin and growth hormone with the aid of carriers. The data in Table II suggest that the two hormones were bound to ribosomes and released in the same manner as the labeled protein fraction.

Protein Synthesis by Ribosomes Fractionated on the Sucrose Gradient. In view of the above presumptive evidence that polysomes were active in the pituitary cell-free system, it was of interest to determine whether the monomeric ribosomal component (the major sedimenting species) was also functional. For this purpose, polysome-enriched fraction was centrifuged on a sucrose gradient, and individual fractions were incubated with labeled amino acid in the cell-free system. The results, presented in Figure 3A, show that most of the amino acid incorporating ability was confined to heavier fractions in the polysome region. Very little, if any, radioactive labeling was observed in the monosome region. Similar results were obtained with mixed ribosomes, except that quantitatively less radioactivity was recovered in the heavier aggregate region (R. O. Hussa, unpublished data).

Figure 3B represents an experiment in which the polysome-enriched fraction was labeled by preincubation with [^{3}H]proline in the complete cell-free system and subsequently analyzed on a sucrose gradient. In this case, a somewhat different distribution of radioactivity was noticed. A shift was observed in the labeling profile, from the heavier toward the lighter region. Considerable radioactivity was found associated with single ribosomes near the top of the gradient. However, much isotopic labeling persisted in regions heavier than monomers.

When the ribosomes were prelabeled as before, but reisolated prior to gradient centrifugation, a major displacement of the radioactivity pattern occurred, such that virtually all the labeling was within the monomer peak (Figure 3C). In the context of the conclusion that polysomes are functional in the pituitary system, it is suggested that the observed shift in radioactivity distribution from heavier to lighter aggregates was due to

TABLE II: Association of Labeled Hormones with Ribosomal Components.[a]

Labeled Starting Material	Radioactivity Recovered (cpm)	
	Growth Hormone	Prolactin
75S ribosomes	600	6100
Released protein	110	1600
44S subunit	30	750
27S subunit	110	670

[a] Polysome-enriched fraction (approximately 10 mg of rRNA) was incubated at 37° in 6 ml of the cell-free system plus 60-μCi quantities of [^{3}H]leucine, [^{3}H]-proline, and [^{3}H]phenylalanine. After 90 min, the total ribosomes were sedimented, suspended in medium M, and dialyzed overnight (to remove free labeled amino acids). Half the ribosomal material was subjected directly to sucrose gradient centrifugation (using Tris–KCl buffer); the other half was treated with 20 μmoles of EDTA and then similarly fractionated on a gradient.

an orderly breakdown of polysomes during amino acid incorporation.

[^{3}H]Poly U Binding and Poly U Directed Phenylalanine Incorporation by Native, Derived, and Reconstituted Pituitary Ribosomes. It was pertinent to compare the ability of reconstituted and nondissociated pituitary ribosomes to bind [^{3}H]poly U and to stimulate poly U directed phenylalanine incorporation. It was first found, as expected, that "native" ribosomes bound [^{3}H]poly U to a very limited extent (Figure 4A). A somewhat greater degree of association was observed when ribosomes were preincubated for 0.5 hr (Figure 4B). Upon increasing this time interval to 1 hr, a further enhancement of the binding capacity occurred (Figure 4C).

In order to study the effect of reversible dissociation on subsequent ability of ribosomes to bind poly U, it was important to employ optimal experimental conditions for the reconstitution process. Schlieren patterns in the analytical ultracentrifuge had indicated a reconstitution of EDTA-dissociated ribosomes at restored Mg^{2+} concentration. However, random aggregation was observed when such preparations were analyzed on a sucrose gradient in medium M. This effect was presumably due to alterations in the intrinsic stability of reconstituted ribosomes upon reexposure to a high ionic environment. It was found that aggregation could be avoided when fractionation was performed on a sucrose gradient in solution D of Tashiro and Siekevitz (1965) (0.001 M Tris–0.05 M KCl–0.002 M $MgAc_2$, pH 7.6). Under these conditions almost quantitative recovery of reconstituted ribosomes was obtained. Such restored preparations were analyzed on the sucrose gradient and found to possess significant capacity to bind [^{3}H]poly U (Figure 4D).

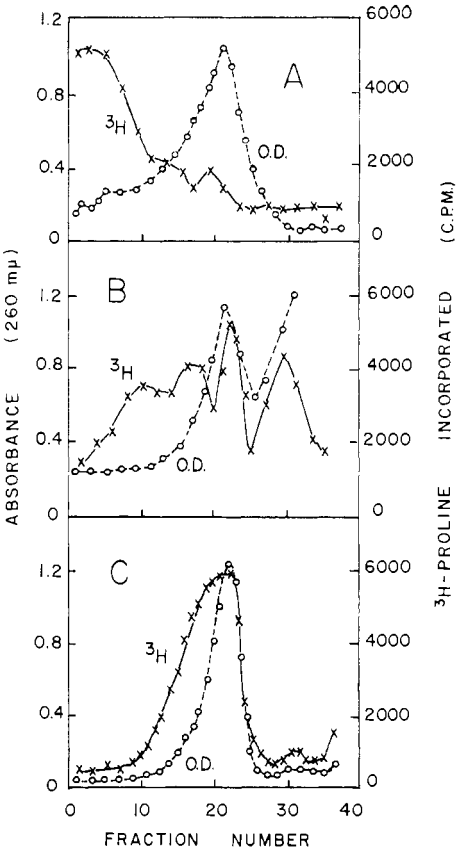

FIGURE 3: Protein synthesis by polysome-enriched fraction, treated in different ways. (A) Centrifuged on a sucrose gradient in medium M for 2 hr at 28,000 rpm. The individual fractions were then assayed in the cell-free system, plus [^{3}H]proline. (B) Prelabeled in the cell-free system and then subjected to gradient fractionation as in A. (C) Same as in B, except that the ribosomes were reisolated after incubation and then run on a 0.01 M Tris–0.05 M KCl gradient.

To rule out the possibility that [^{3}H]poly U association with ribosomes under different conditions was nonspecific, *i.e.*, did not necessarily reflect the messenger capacity of the synthetic polynucleotide, it was considered pertinent to study the ability of the latter to promote isotopic phenylalanine incorporation into polypeptide combination. Figure 5 gives the results obtained with "native" pituitary ribosomes. While labeling in polyphenylalanine was not demonstrated, it is a reasonable assumption that it accounted for much of the radioactivity found in the hot TCA-insoluble protein fraction. A definite response to the addition of poly U prior to gradient fractionation is apparent. A still greater degree of stimulation of [^{3}H]phenylalanine incorporation was observed when the poly U was added subsequent to fractionation on the sucrose gradient. These results clearly demonstrate that the pituitary "native" 75S ribosome had significant ability to support labeled phenylalanine uptake in the presence of the synthetic polynucleotide.

It was desired to compare "derived" ribosomes with the "native" particles tested in the above experiment, since preincubated preparations might be relatively free of endogenous mRNA. Figure 6 shows the low basal

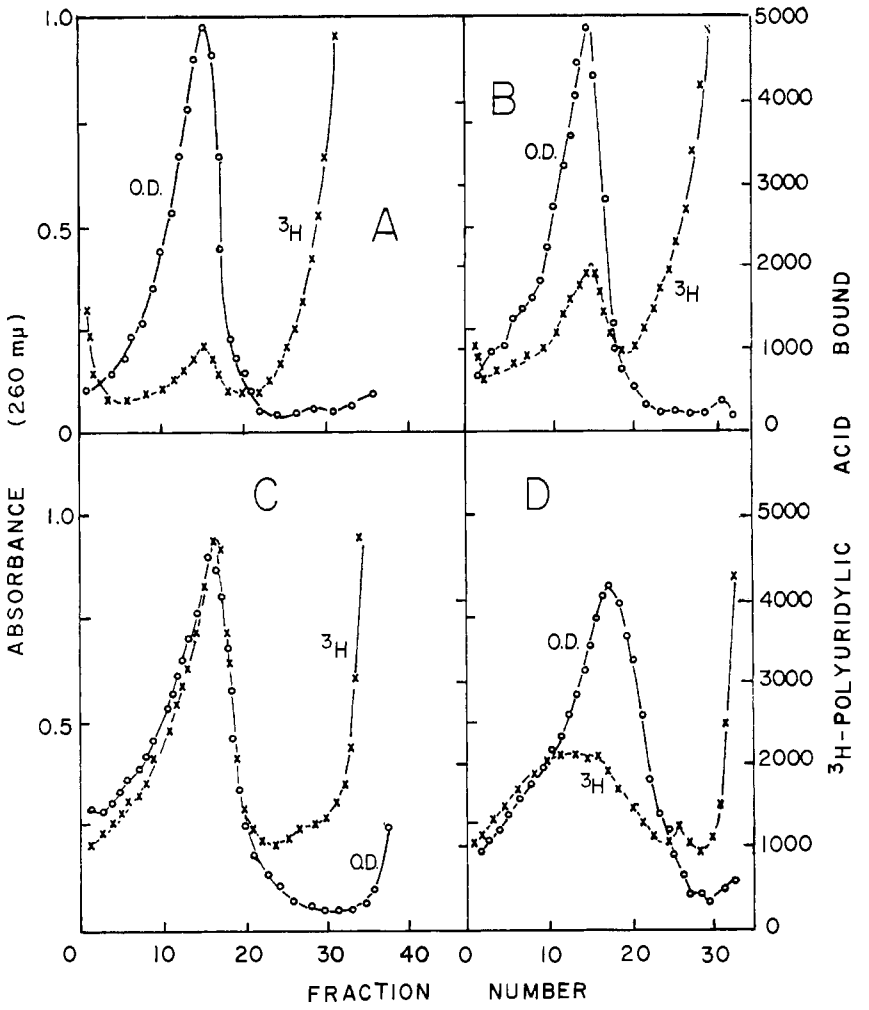

FIGURE 4: Binding of labeled poly U by ribosomes under different conditions. (A) Direct treatment of ribosomes with 0.5 μ Ci of [^{3}H]poly U, followed by sucrose gradient centrifugation. (B) Ribosomes were preincubated for 30 min at 37°, then treated with [^{3}H]poly U, prior to fractionation. (C) Same as B, but with preincubation time increased to 60 min. (D) Ribosomes were first dissociated to subunits by the addition of 2 μmoles of EDTA/mg of ribosomes, then reconstituted by adding an equimolar quantity of Mg^{2+}, treated with [^{3}H]poly U, and centrifuged on a sucrose gradient. In A–C, gradients were prepared in medium M minus mercaptoethanol; while in D, solution D of Tashiro and Siekevitz was used. Conditions of centrifugation in this, and in all subsequent experiments, were as in Figure 3.

level of [^{3}H]phenylalanine uptake when assays were performed in the absence of poly U. When the labeled polynucleotide was added to the mixed ribosomes prior to gradient centrifugation, the utilization of labeled amino acid was markedly stimulated. A further three- to fourfold increase in [^{3}H]phenylalanine incorporation was obtained when the poly U was added to individual fractions from the sucrose gradient, prior to assay of the samples in the cell-free system.

Similar experiments were performed with ribosomes which had been dissociated into subunits with EDTA and then recombined. In Figure 7A it is seen that such reconstituted particles had a relatively low capacity to incorporate radioactive phenylalanine into protein in the presence of poly U. When spermine, as well as poly U, was added to the gradient fractions before assay (Figure 7B), a pronounced stimulation occurred. Figure 7C indicates that spermine alone was ineffective when the reconstituted ribosomes were tested with [^{3}H]phenylalanine in the absence of poly U.

Discussion

The present work has been centered about the study of two RNP preparations from bovine anterior pituitary glands: "mixed ribosomes" and "polysome-enriched fraction." Both materials were quite comparable, on the basis of chemical composition and ultraviolet spectra, with corresponding preparations from other mammalian tissues (Tashiro and Siekevitz, 1965; Munro *et al.*, 1964). Furthermore, both RNP preparations showed the expected heterogeneity of particle size in the analytical ultracentrifuge (Plate I), except that heavier aggregates were more abundant in the polysome-enriched fraction. There was the usual dependency on magnesium ion concentration for stability of aggregates and monomeric ribosomes.

The incomplete reconstitution of the subunits at 24° may have reflected the relative instability of re-formed ribosomes to temperature and ionic strength. A similar instability of reconstituted ribosomes was found in

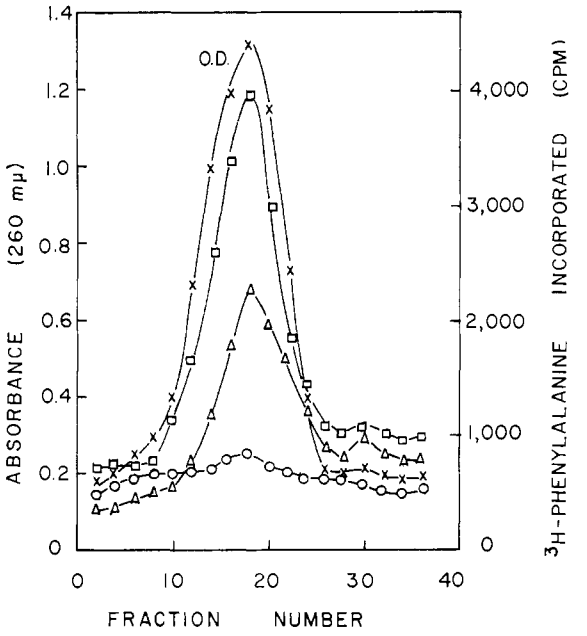

FIGURE 5: Poly U directed incorporation of labeled phenylalanine into protein fraction, using native ribosomes and the cell-free system. [^{3}H]Amino acid (2.5 μCi) was employed per assay. (○) Activity of individual sucrose density gradient fractions in the absence of poly U (control). (△) Poly U (100 μg) added per 3 mg of ribosomes, before fractionation on the sucrose gradient. (□) Poly U (25 μg) added to individual fractions after gradient centrifugation, before incorporative assay.

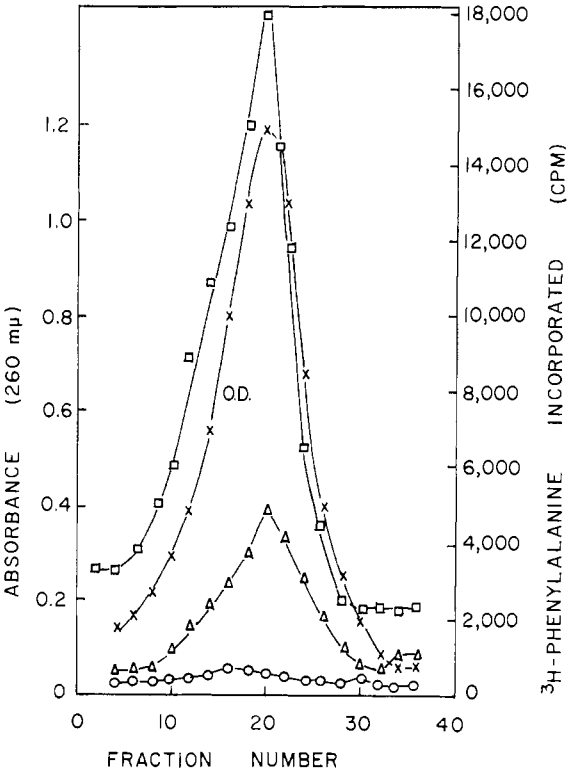

FIGURE 6: Poly U directed incorporation of [^{3}H]phenylalanine into protein fraction, with derived ribosomes and the cell-free system. The experiments and their corresponding symbols are the same as in Figure 5.

Escherichia coli (Pestka, 1966). The apparent preferential destruction of the smaller 27S subunit upon dialysis against Tris–KCl buffer containing EDTA seems in accord with similar observations for preparations from bacteria (J. D. Watson, personal communication) and liver (Tashiro and Siekevitz, 1965). The 58S particles observed at certain EDTA concentrations may correspond to the 60S ribosomes found by Tashiro and Siekevitz, and thought by them to represent an intermediate stage of dissociation.

The good resolution of pituitary subribosomes obtained by the sucrose density gradient method was utilized in prelabeling experiments, which demonstrated the presence of tightly bound radioactive substances on ribosomes reisolated from the incubation system. While most of the labeled material was released (apparently as soluble protein), the presence of residual radioactivity on both the 27S and 44S subunits (Figure 2) agrees with the finding of Philipps (1965) for rabbit reticulocyte ribosomes. By contrast, Gilbert (1963) found polyphenylalanyl-tRNA to be associated with only the 50S subparticle of *E. coli*. This distinction may be due to such factors as different stabilities of mammalian *vs.* bacterial ribosome–mRNA complex, and to different behavior of synthetic polynucleotides, as compared with natural mRNA.

The qualitative demonstration (Table II) that some of the nascent radioactive protein, both solubilized and attached to dissociated ribosomes, was inseparable from two authentic hormones (added as carriers) is of considerable interest. One implication is that some of these newly synthesized polypeptide chains, still attached to ribosome subunits, were comparable in size with growth hormone and prolactin (mol wt 20,000–26,000) (Andrews, 1966). These experiments are exploratory in nature and the reason for the observed distribution pattern of the labeled hormones with respect to the ribosomes, subunits, and soluble protein is not presently understood.

The technique of assaying the incorporative ability of consecutive samples taken from sucrose gradient centrifugation of the polysome-enriched fraction clearly showed that activity was concentrated in the heavier region of particle size (Figure 3A). When prelabeled, polysome-enriched fraction was analyzed by gradient centrifugation (Figure 3B), the persistence of labeling in the heavy aggregate region suggested that the polysomes had resisted destruction to a considerable extent. When labeled ribosomes were reisolated from the cell-free system prior to gradient centrifugation (Figure 3C), the radioactivity shifted completely to the monosome region, presumably due to polysome instability in the absence of Mg^{2+}.

Although there are several examples of functionally active monosomes, the relatively low protein biosynthetic activity of the pituitary monosomes is not without precedent for other mammalian systems (Wettstein *et al.*, 1963; Lamfrom and Knopf, 1965; Munro *et al.*, 1964). de Groot *et al.* (1967) have listed the following possible causes for inactivity of monosomes: (a) an absence of attached mRNA, (b) presence of nonfunctional

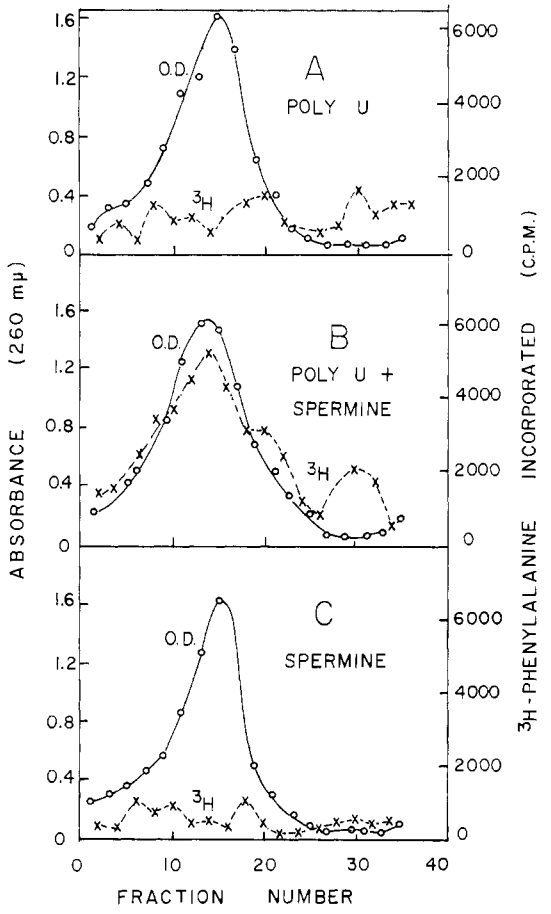

FIGURE 7: Poly U directed incorporation of [^{3}H]phenylalanine into protein fraction with reconstituted ribosomes plus the cell-free system. (A) Poly U (25 μg) added to each fraction collected in sucrose gradient centrifugation. (B) Same as A, but with the addition of 2 μmoles of spermine. (C) Same as A, but with 2 μmoles of spermine substituted for the poly U.

fragments of mRNA, (c) attachment of inhibitory substances, and (d) lack of initiation factors.

In considering the role of endogenous mRNA in relation to the pituitary monosomes, it was helpful to study the interaction of these particles with the synthetic messenger, poly U. No systematic attempt was made to develop optimum conditions for such interactions. However, the finding was significant that native ribosomes showed a greatly increased capacity to bind labeled poly U, following prolonged preincubation at 37° (Figure 4B,C). Presumably such pretreatment facilitated removal of natural mRNA from ribosomal sites, to which poly U could subsequently be attached.

The demonstration (Figure 5) that the 75S pituitary ribosomes promoted the incorporation of [^{3}H]phenylalanine (presumably into the polyamino acid) under the influence of poly U supports the view that the latter substance functioned in a manner analogous to mRNA. The higher activity with derived ribosomes (Figure 6), as compared with native particles (Figure 5), in this process, is in accord with the poly U binding experiments and is probably due to a greater proportion of poly U responsive particles (resulting from preincubation). Attention is called also to the further elevation in labeled phenylalanine incorporation when the poly U was added to individual ribosome fractions taken *after* sucrose gradient centrifugation as compared with the experiment in which the polynucleotide was added to the original mixed ribosomes *before* gradient fractionation. This contrast may be due to a relatively higher available poly U concentration in the former case.

To the best of our knowledge, restoration of biosynthetic activity upon reconstitution of subribosomal particles from mammalian sources has been demonstrated only with reticulocyte preparations (Miller *et al.*, 1967). In the pituitary experiments, reconstitution by restoration of magnesium ions, followed by sucrose density gradient centrifugation in medium D of Tashiro and Siekevitz (1965), gave 75S particles capable of binding labeled poly U (Figure 4D). However, these reassembled ribosomes showed poor ability to promote utilization of [^{3}H]phenylalanine in the presence of poly U (Figure 7A). This again suggests the lack of an essential factor in these particles.

In the light of the finding of Pestka (1966), that spermidine exerts a stabilizing effect on *E. coli* ribosomes, it was encouraging that the addition of spermine markedly stimulated the incorporation process with reconstituted pituitary ribosomes in the presence of poly U. The failure to observe true protein synthesis even in the presence of spermine (Figure 7C) suggests that a required substance may have been lost during dissociation of the original ribosomes. This could be a chain initiation factor (Miller *et al.*, 1967). The further investigation of anterior pituitary polysomes from the standpoints of their physical properties and biosynthetic activity is the subject of the second paper in this series (Adiga *et al.*, 1968).

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Studies Concerning the Behavior of Actinomycin in Solution*

Donald M. Crothers,† Steven L. Sabol,‡ David I. Ratner,‡ and Werner Müller

ABSTRACT: Dimerization and conformational change of actinomycin are investigated by equilibrium centrifugation and optical rotatory dispersion. A specific dimer forms in aqueous solutions of actinomycin, with a dimerization constant of roughly 10^3 M^{-1}. The heat and entropy of dimerization are relatively large and negative. The optical rotatory dispersion spectrum of actinomycin depends on the nature of the solvent, presumably reflecting differences in molecular conformation. Changes in the optical rotatory dispersion spectrum seem to be correlated with the surface tension of the solvent. Results are discussed in terms of some qualitative ideas about the nature of hydrophobic interactions.

Actinomycin, an oligopeptidic substance, has a structure intermediate in complexity between small molecules and proteins. In our studies of the complex formation between actinomycin and DNA (Müller and Crothers, 1968), we have found it useful to regard this system as a very simple model for the interaction of protein with nucleic acid. This analogy would require some similarity between the solution properties of actinomycin and more complicated protein molecules. Many proteins undergo conformation changes as a result of changes in external conditions, and also are able to form specific aggregates at appropriate concentrations. In this paper we show that actinomycin exhibits both of these characteristics. A further purpose of the work reported here is to aid the interpretation of experiments on the actinomycin–DNA complex by elucidating the properties of one of the components.

Recent work (Müller and Emme, 1965) has disclosed that actinomycin aggregates in aqueous solutions but not in most organic solvents. These studies, employing the ultracentrifuge for molecular weight determination, concluded that actinomycin C_3 (see Brockmann, 1960,

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