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Purification and Properties of Dog Pancreas Ribosomes and Subunits*

Sherman R. Dickman and Eveline Bruenger

ABSTRACT: Dog pancreas ribosomes can be purified by chromatography over DEAE-cellulose. The particles are eluted by a gradient of KCl and are successively removed from the column as subunits: (1) 38 S, (2) 57 S, contaminated by some 38 S.

This distribution has been demonstrated by centrifugation of the peak fractions through a 10–30% sucrose gradient of similar composition as the solution in which they were eluted; *i.e.*, 0.01 M Tris, pH 7.3 (25°), 3 mM Mg^{2+}, 0.5 M KCl, and 15 mM mercaptoethanol. The optimum conditions for phenylalanine incorporation have been determined. On incubation in an amino acid incorporation system, the subunits reassemble to monomers. The 38S and the 57S particles have been further purified by preparative-scale sucrose gradient centrifugation. Their phenylalanine incorporation activity was tested. In the absence of polyuridylic acid, neither subunit separately, nor their mixture, demonstrated significant activity. Similarly, incorporation of isoleucine, valine, or lysine was essentially zero. Phenylalanine incorporation in the presence of polyuridylic acid was zero with the 38S particle, 40 $\mu\mu$moles/mg with the 57S particle, and 350 $\mu\mu$moles per mg with the mixture.

The relative ease of purification of bacterial ribosomes, their separation from endogenous mRNA by preincubation, and the stability exhibited by both monomers and subunits have all contributed to the rapid advances which utilize these nucleoprotein particles. In contrast, comparable studies with mammalian ribosomes have been less rapid, largely because of the difficulties in purifying active ribosomes. Since extraneous protein may decrease the functional activity as well as the stability of ribosomes, its removal is essential. Similarly, the presence of endogenous mRNA may compete or hybridize with that added, thus complicating the interpretation of the data. Our first objective in the study of ribosomes from a mammalian source has thus been to purify them according to these two criteria.

Most samples of mammalian ribosomes have consisted of RNA and protein in approximately equal amounts (Petermann, 1964). Beeley *et al.* (1968) obtained dog pancreas ribosomes which analyzed 60% RNA, but their polypeptide-synthesizing activity was not tested. We have found that ribosomes from dog pancreas can be purified by chromatography over DEAE-cellulose to yield biologically active particles of this composition. A KCl gradient is used for elution from the column. The ribosomes dissociate into subunits in the process and a preliminary separation can be made. The eluate is intermediate in composition between the conditions for dissociation established by Martin and Wool (1968) for muscle ribosomes (high ionic strength, pH, and Mg concentration) and that of T'so and Vinograd (1961) for reticulocyte ribosomes (moderate to low ionic strength and zero concentration of Mg). Crude subunits can be further purified by centrifugation through a sucrose gradient of high ionic strength. Under these conditions the endogenous mRNA is largely removed.

The work to be reported includes a description of the purification procedures, gross analyses, and sedimentation studies of the ribosomes and their subunits. Phenylalanine incorporation with and without poly U has been utilized as a measure of polypeptide synthesis capability and of endogenous mRNA content.

Experimental Procedure

Materials. The tissue for most of this work was obtained by excision of fresh pancreas from dogs. This was immediately chilled and stored at −20° or was purchased from Pel-Freez Biologicals, Inc., Rogers, Ark. PMSF[1] was obtained from

* From the Department of Biological Chemistry, University of Utah, Salt Lake City, Utah 84112. *Received November 8, 1968.* This investigation was supported by Grant No. AM 00803 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service. A portion of the work was presented at the 53rd Meeting of the Federation of American Societies for Experimental Biology, April 1969.

[1] The abbreviations used are: DOC, sodium deoxycholate; ME, mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; RNP, ribonucleoprotein; TES, tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

TABLE I: Yields, Composition, Spectral Ratios, and [^{14}C]Phenylalanine Incorporation Capabilities of Ribosomes.

Ribosomal Prepn	Yield (mg/g)	RNA Content (%)	RNA/ Protein	A_{260}/A_{235}	A_{260}/A_{230}	1% A_{260}	[^{14}C]Phe Incorp −Poly U ($\mu\mu$moles/mg of RNP)	[^{14}C]Phe Incorp +Poly U ($\mu\mu$moles/mg of RNP)
pH 8.5	25[b]		0.54	1.08	1.53	50	30–50	150–400
pH 8.5, DOC	9.0[b]	49	0.98	1.60	1.70	95	30	145–500
DEAE[a]	80[c]	60	1.5	1.96	1.72	130	10–30	80–500

[a] DEAE refers to ribosomes chromatographed over DEAE-cellulose and suspended in buffer which contains 3 mM Mg^{2+}. [b] Calculated as milligram of ribosomes/g of tissue pulp. [c] Calculated as the percentage of A_{260} units eluted from the column to those added.

Sigma Chemical Co. A 0.05 M stock solution was prepared in isopropyl alcohol. DL-[^{14}C]phenylalanine (21 or 44.2 mCi per mmole) was purchased from Nuclear-Chicago. Sodium desoxycholate (special enzyme grade) was bought from Mann Research Laboratories. Poly U was purchased from Miles Laboratories and DEAE-cellulose (0.90 mequiv/g) from Bio-Rad. Sucrose was Mallinckrodt, A.R. All solutions were prepared with glass-redistilled water. All other chemicals were reagent grade.

Preparation of Ribosomes. Frozen pancreas was thawed in a solution of 0.25 M sucrose which contained 1 mM PMSF.[2] All subsequent procedures were carried out at 0–2°. The tissue was trimmed of excess fat and passed through a stainless steel screen (1-mm holes) to remove connective tissue; 30 g of tissue pulp was homogenized in 10-g portions with 40 ml of the sucrose–PMSF solution in a Potter-Elvehjem-type glass homogenizer fitted with a Teflon pestle (eight strokes). The homogenate was centrifuged for 10 min at 13,000*g*.

The supernatant solution was carefully removed and adjusted to pH 8.5 by the addition of 1 N NH_4OH. It was then brought to concentrations of 0.15 M KCl and 3 mM Mg^{2+} and centrifuged at 1° for 90 min at 80,000*g*. The pellets (pH 8.5 ribosomes) were then suspended by gentle homogenization in 50 ml of medium 3 (0.01 M Tris, pH 7.4 at 25°, 0.15 M KCl, and 3 mM $MgCl_2$) which contained 0.1 mM PMSF. To this solution was added, with stirring, 5 ml of cold 5% DOC, prepared immediately before use. The solution was centrifuged for 90 min at 80,000*g*. The pellets (pH 8.5 DOC ribosomes) were gently homogenized in 10 ml of medium 3. Large aggregates were removed by centrifugation at 26,000*g* for 10 min.

Chromatography of Ribosomes. Washed DEAE-cellulose was equilibrated with buffer (0.01 M Tris, pH 7.4 at 25°, 0.025 M KCl, 15 mM ME, and 3 mM Mg^{2+}) at room temperature. The suspension was added to a column to form a bed 2.5 × 30 cm. The resin was thoroughly washed with buffer in the cold. The sample was applied and the column was washed with an additional 300–500 ml of cold buffer. The ribosomes were eluted by a linear gradient of KCl (0–1 M, 2-l. total volume) in this buffer. Washing and elution were carried out at approximately 75 ml/hr.

It is important to mention here that the temperature of the cold room can be decisive in affecting the phenylalanine incorporation activity of the ribosomes. Highest activity was obtained with ribosomes prepared at 0–2°, whereas slightly active or inactive preparations resulted with 4–6° preparations. This large effect of temperature was observed repeatedly.

The particles were generally eluted as a fairly sharp peak, as determined by A_{260} measurements, in the range 0.4–0.5 M KCl. When small fractions were collected (15 ml), the eluted material appeared as a double peak. Recovery of A_{260} material was usually in the range of 70–80% of that added to the column. The mixed eluates were centrifuged at 70,000*g* for 150 min. The pellets were rinsed with cold water, excess liquid was removed from the inside of the tubes, and the pellets were suspended in a total volume of 3 ml of solution by occasional agitation with a glass rod over a period of 2 hr. The influence of the composition of the solution on the degree of aggregation and incorporation activity of the particles is presented under Results.

Density Gradient Centrifugation. Linear sucrose gradients were prepared in the device described by Britten and Roberts (1960). They contained 10–30% sucrose and buffers as indicated. The samples to be analyzed were diluted with the same buffer as in the gradient such that 0.05 ml, which contained 2.5–5.0 A_{260} units, was routinely layered on the top of each polyallomer tube. The solutions were centrifuged at 1–3° in the SW39 rotor of a Spinco Model L centrifuge at 39,000 rpm and were removed from the tubes by displacement with 70% glycerol which was conducted to the bottom of the tube by a 20-gauge stainless steel needle attached to an aluminum cap. The effluent flowed through a flow cell (0.1-ml capacity, 0.2 cm thick) placed in a Gilford attachment of a Beckman DU spectrophotometer with the monochrometer set at 260 mμ. Absorbances were recorded automatically. Sedimentation constants were calculated by use of the equation of Martin and Ames (1961). For preparative purposes 2 ml (100–200 A_{260} units) of eluate was placed on 30 ml of gradient and centrifuged at 25,000 rpm for 14 hr in the SW25.1 rotor. The samples were displaced and monitored as described above.

Chemical Analyses. RNA was determined with the orcinol reaction (Schneider, 1957) and protein by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard. Ribosome concentration was often evaluated by A_{260} readings. Conversion factors are included in Table I.

Phenylalanine Incorporation into Polypeptide. Polypeptide

[2] Biologically active ribosomes can be prepared from fresh pancreas without the use of PMSF, but the presence of a trypsin inhibitor during thawing of frozen tissue is essential.

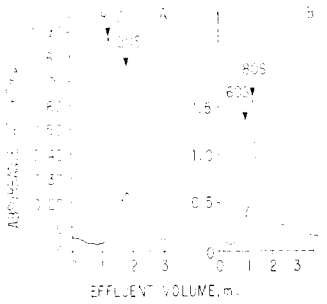

FIGURE 1: Sedimentation patterns of dog pancreas ribosomes. (A) pH 8.5 ribosomes. (B) pH 8.5 DOC ribosomes. An aliquot (0.05 ml) of each suspension was layered on top of a 10–30% sucrose gradient which contained medium 3. Experimental conditions are described in Methods.

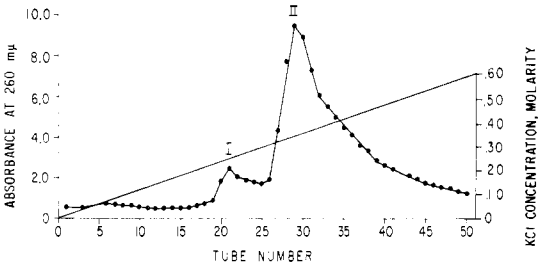

FIGURE 2: Chromatographic elution pattern of ribosomes on DEAE-cellulose column. pH 8.5 DOC ribosomes were suspended in 0.01 M Tris (pH 7.4, 25°)–3 mM Mg^2–0.025 M KCl–10 mM ME and placed on a 2.5 × 30 cm column of DEAE-cellulose at 0–2°. It was washed with 200–350 ml of this buffer and the ribosomes were eluted with a linear KCl gradient, 0–1 M in 2 l. of this buffer. (●—●) A_{260} values; (———) KCl molarity.

synthesis was measured by incubation of ribosomes in 0.5-ml total volume with the following materials: ATP, 1 mM; creatine phosphate, 5 mM; GTP, 0.2 mM; NH_4Cl, 0.1 M; $MgCl_2$, 7 mM; mercaptoethanol, 5 mM; dog or rat liver high-speed supernatant, 0.1 ml; TES or Tris buffers, 0.05 M; DL-[^{14}C]phenylalanine (21 or 44.2 mCi per mmole), 0.5 μCi; and poly U, 50 μg. In certain experiments the incorporation of 0.5-μCi quantities of [^{14}C]isoleucine, [^{14}C]valine, or [^{14}C]lysine was measured. The mixtures were incubated for 30 min at 37° and were extracted by a modification of the Siekevitz procedure (Gazzinelli and Dickman, 1962). The residues were dissolved in formic acid, evaporated on aluminum planchets, and counted in a Nuclear-Chicago automatic, windowless, gas-flow counter, with an efficiency of approximately 35%. This will be referred to as "standard incorporation conditions."

Results

Purification of Ribosomes. Isolation of organelles from pancreas is somewhat complicated by the presence of zymogen granules in this tissue. These dissolve at pH 7 or above with release of large amounts of deoxynucleases, proteases, and lipases (Hokin, 1955). Ribonucleases are absent in preparations from dog pancreas (Dickman and Bruenger, 1962; Sreebny and Johnson, 1967; Beeley *et al.*, 1968). On the other hand, aggregation of the microsomal fraction occurs below pH 6. To circumvent these complications as much as possible, pancreas pulp was homogenized in 0.25 M sucrose and 1 mM trypsin inhibitor (PMSF). The homogenate routinely measured in the range pH 6.3–6.7. Nuclei, zymogen granules, and mitochondria were removed by centrifugation, and the supernatant solution was then adjusted to 0.15 M KCl–3 mM $MgCl_2$ (pH 8.5) to detach ribosomes from the endoplasmic reticulum (Hamilton and Petermann, 1959). The particles (pH 8.5 ribosomes) were centrifuged and resuspended in medium 3 which contained 0.1 mM PMSF, and deoxycholate was added to a final concentration of 0.45%. These particles were likewise centrifuged and suspended in medium 3 (pH 8.5 DOC ribosomes). It was found that the yield of ribosomes was much higher if the deoxycholate was added to the isolated pH 8.5 ribosomes rather than directly to the low-speed supernatant. As shown in Table I, considerable protein is removed by the DOC treatment. This is also reflected in the spectral ratios. The phenylalanine incorporation activity of these two preparations was quite similar; 30 μμmoles phenylalanine/mg was incorporated due to endogenous mRNA and approximately 150 μμmoles/mg when poly U was present, although some preparations assayed as high as 500 μμmoles/mg.

The sedimentation patterns of both types of ribosomes centrifuged in a sucrose gradient are presented in Figure 1. pH 8.5 ribosome preparations are composed mostly of 80S material with small amounts of a dimer. pH 8.5 DOC ribosome preparations contain some of the larger subunit in addition to the 80S monomer.

Chromatography of *Escherichia coli* ribosomes over DEAE-cellulose has been utilized by Furano (1966) to isolate particles with an average content of 70% RNA. They retained about half of the incorporating activity of "washed" ribosomes. With pancreas ribosomes, however, a lower (3 mM) Mg^{2+} content in the buffer than was used by Furano was necessary to avoid aggregation. As with bacterial ribosomes, the particles are eluted from the column by a salt gradient.

Chromatographed ribosomes from dog pancreas are composed of 60% RNA and 40% protein (Table I). The A_{260}/A_{235} ratio of 1.96 indicates a very high RNA content by the criterion of Petermann (1964). The absorbancy of a 1% solution at 260 mμ is almost identical with that of Beeley *et al.* (1968). The over-all yield of purified ribosomes was 7–8 mg/g of tissue.

Ribosomes were also chromatographed in buffers which contained 1 or 0.5 mM Mg^{2+}. At these lower Mg^{2+} concentrations, the yield of particles was progressively reduced. Their phenylalanine incorporation activity was much less than those prepared at 3 mM Mg^{2+}. The yield of particles at 0.5 mM Mg^{2+} was too low to carry out incorporation studies.

Dissociation of Ribosomes on Chromatography. As shown in Figure 2, the particles were eluted from the column in two fractions. When peak 1 material was placed on a 10–30% sucrose gradient which contained 0.5 M KCl–3 mM Mg^{2+}–15 mM ME and centrifuged, the pattern of Figure 3A was obtained. The shape of this curve suggests a homogeneous population of small (38 S) subunits. Peak 2, on the other hand, contains a heterogeneous population as shown in Figure 3B. The small subunit is clearly separated from the large in the sucrose gradient. The salt gradient in the chromatography apparently is responsible for the dissociation of most but not all of the monomeric ribosomes into subunits.

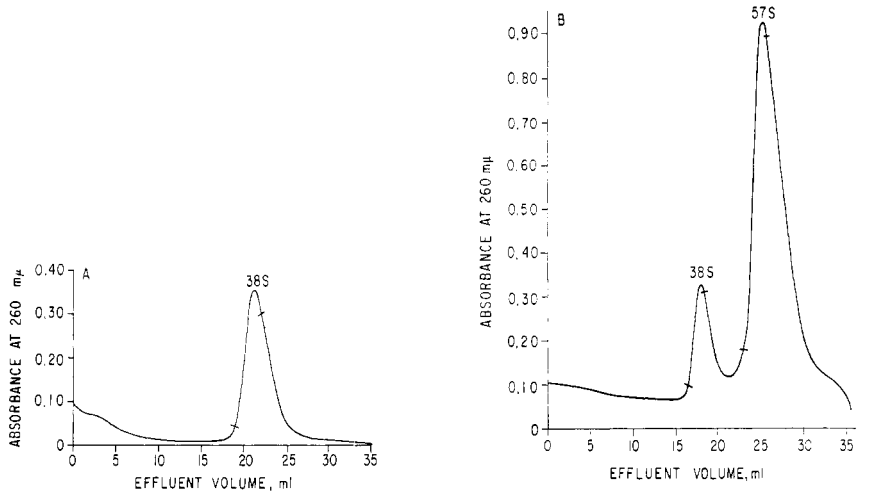

FIGURE 3: Sucrose gradient centrifugation of DEAE-cellulose peaks. (A) Peak 1 (2 ml) eluate was layered on a 10–30% sucrose gradient containing 0.01 M Tris (pH 7.4)–3 mM Mg–0.5 M KCl–10 mM ME. It was centrifuged overnight in the SW25.1 rotor at 25,000 rpm. (B) Peak 2 (2 ml) was treated similarly. Effluents were collected for further study as indicated.

TABLE II: Effect of Conditions of Storage on Phenylalanine Incorporation Capacity of Chromatographed Ribosomes.[a]

Ribosomal Suspending Solution	Incorporation Activity, Poly U[b]					
	Time of Storage in Days					
	0		1		2	
	−	+	−	+	−	+
H_2O	6	47	6	9	5	9
1 mM Mg^{2+}	20	75	22	56	22	50
3 mM Mg^{2+}	22	60	20	80	23	80

[a] Ribosomes were centrifuged and the pellets rinsed with H_2O and suspended by standing at 0–2° in: H_2O; 0.05 M Tris, pH 7.3 at 25°, 1 mM Mg^{2+}; or 0.05 M Tris–3 mM Mg^{2+}. Phenylalanine incorporation activity was measured with 1 mg of RNP/tube under standard conditions in the presence or absence of poly U. [b] In μμmoles/mg of RNP.

Ribosomes from Dog Liver. Fresh liver was processed by a very similar procedure to that used for frozen pancreas except that 0.1 mM PMSF was present in the sucrose used in homogenization. The chromatographed ribosomes from liver were as active in phenylalanine incorporation as those from pancreas but were obtained in a much lower yield.

Influence of Mg^{2+} on Chromatographed Particles. As shown in Figure 4, the solutions in which the chromatographed, pelleted ribosomes were suspended strongly influenced their sedimentation properties, even when centrifuged immediately. In the absence of added Mg^{2+} and at low cation concentration, the small subunit has associated to form larger particles as shown in Figure 4(0)a. On standing for 1 or 2 days, unfolding or breakdown occurred, and 18S and 38S particles were formed (Figure 4(1,2)a). We have not determined whether the 18S material is RNA or RNP. This suspension exhibited phenylalanine incorporation activity in response to poly U for a very short period (Table II). At 24 hr it was essentially inactive. When the pellet was suspended in Tris–1 mM Mg^{2+}, random association also occurred (Figure 4(0,1,2)b). These particles were fairly stable, however, as judged by their incorporation activity. In Tris–3 mM Mg^{2+}, monomers, dimers, and small aggregates rapidly formed and remained during 48 hr (Figure 4(0,1,2)c). With many preparations in this solution the phenylalanine incorporation with poly U increased on 48-hr storage (Table II). This suspension was the most stable as judged by either criterion and will be referred to as DEAE-ribosomes.

These experiments demonstrate quite clearly that the dissociation which occurred on chromatography can easily be masked or altered by the type of solution in which the pellets are suspended. Furthermore, the incorporation activity, stability, and degree of response to poly U are likewise affected by these factors. Much of the data presented in this paper may be considered to represent subsequent changes in the sedimentation properties and incorporation activity of these originally dissociated ribosomes.

Factors Which Affect Phenylalanine Incorporation Activity of DEAE-ribosomes

Mg^{2+} Concentration. DEAE-ribosomes show a sharp phenylalanine incorporation maximum at 7 mM Mg^{2+} in the

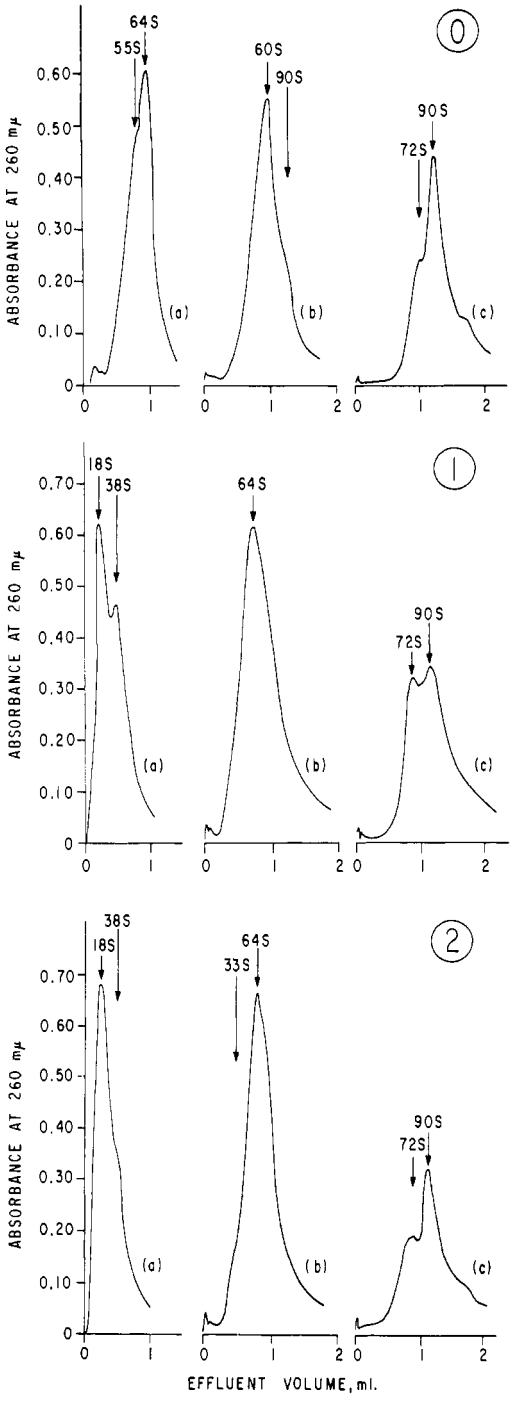

FIGURE 4: Influence of Mg^{2+} concentration on sedimentation patterns of DEAE-chromatographed ribosomes on storage at 0°. RNP was suspended in (a) H_2O, (b) 0.05 M Tris (pH 7.4 at 25°)–1 mM Mg^{2+}, (c) 0.05 M Tris (pH 7.4 at 25°)–3 mM Mg^{2+} by occasional agitation with a glass rod. Storage time in days, 0, freshly suspended; 1, after 24 hr; 2, after 48 hr. Each sample was layered on a sucrose gradient, which contained the corresponding buffer. It was centrifuged and the sedimentation pattern produced as described in Methods.

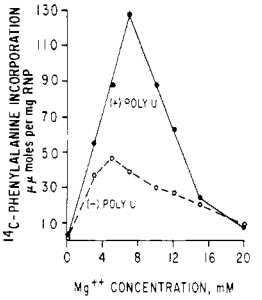

FIGURE 5: Influence of Mg^{2+} concentration on phenylalanine incorporation by DEAE-ribosomes in the presence (+50 μg) and absence of poly U. Standard incubation conditions, 1.3 mg of RNP/tube.

presence of poly U and a broader and lower optimum with a maximum at 5 mM Mg^{2+} in its absence (Figure 5). These concentrations are very similar to those obtained by Hultin and Pedersen (1963) with microsomes from ascites tumors and Campbell *et al.* (1964) with rat liver ribosomes.

pH. A comparison of incorporation activity in TES and Tris buffers is presented in Figure 6. The maximum activity in TES is exhibited at pH 7.5, the highest value tested, whereas in Tris maximum activity was found between pH 7.4 and 7.8. It is noteworthy that incorporation is consistently higher in Tris than in TES at comparable pH values (Good *et al.*, 1966).

Kinetics. The time course of phenylalanine incorporation is presented in Figure 7. The original rate decreased after 8 min but remained relatively constant thereafter up to 60 min. There was no indication of a lag period. This 52-min period of constant rate suggests that no single component becomes limiting nor that the ribosomes themselves are inactivated. It is of interest that the interval of active incorporation is longer than that found with liver ribosomes (Blobel and Potter, 1967) or microsomes (Weksler and Gelboin, 1967).

Concentration of Ribosomes. The amount of DEAE-ribosomes per tube was varied from 0.2 to 3.0 mg. Other components remained constant. Total phenylalanine incorporation (Figure 8) increased almost linearly up to 0.5 mg. At higher amounts, incorporation increased very little. When the data are plotted as micromicromoles of phenylalanine incorporated per milligram of RNP, a large decrease in unit activity with the larger amounts become strikingly evident. Less purified ribosomes (pH 8.5 DOC) reacted similarly. This situation might be caused by utilization of an essential com-

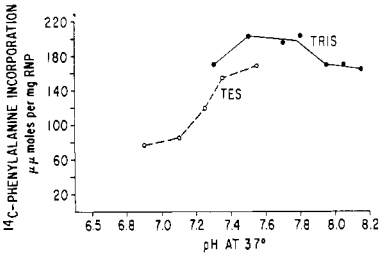

FIGURE 6: Influence of pH on phenylalanine incorporation by DEAE-ribosomes. The incubation mixtures were prepared with 0.05 M TES, sodium salt, or 0.05 M Tris–HCl of varying pH. The pH was measured at 25° before the addition of Mg^{2+} and again after 15 min at 37° during the incorporation run. Standard incubation conditions except for pH. All tubes contained 50 μg of poly U and 1.7 mg of RNP.

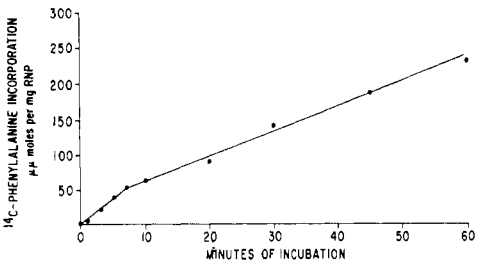

FIGURE 7: Time course of phenylalanine incorporation by DEAE-ribosomes. Standard incubation conditions except for time of incubation; 1.35 mg of RNP and 50 μg of poly U per tube.

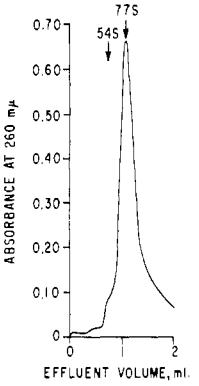

FIGURE 9: Monomer formation after preincubation of DEAE-ribosomes. Ribosomes were incubated under standard conditions but without [^{14}C]phenylalanine or poly U. They were then centrifuged at 88,000*g* for 90 min and the pellets were resuspended in H_2O. The solution was dialyzed at 2° overnight against medium 3 and the tenate was centrifuged on a sucrose gradient which contained medium 3. Conditions of centrifugation and A_{260} determination are given in the text.

ponent to the extent that it becomes limiting. Accordingly, the amounts of high-speed supernatant solution, ATP, and/or GTP were varied in the incorporation solutions. As shown in Table III, the extra quantities of ATP and GTP increased phenylalanine incorporation in the presence of poly U by approximately 25% over standard conditions. These relatively small effects suggest that shortage of one of these components is not responsible for the sharp drop-off in incorporation activity. The time study (Figure 7), in which total incorporation reached 320 μμmoles of phenylalanine, also supports the conclusion that some factor other than known components has become limiting.

Effects of Preincubation on DEAE-ribosomes. The technique of preincubation to remove endogenous mRNA from ribosomes has been widely employed since its introduction by Nirenberg and Matthaei (1961). It should be possible to isolate ribosomes which are free from mRNA in the absence of conditions which favor dissociation. DEAE-ribosomes were preincubated for 30 min at 37° in the usual incorporating medium minus [^{14}C]phenylalanine and poly U. The incubation mixture was chilled and centrifuged at 88,000*g* for 90 min, and the pellets were suspended in medium 3. A portion of the solution was dialyzed overnight against medium 3. The tenate was centrifuged through a sucrose gradient which contained medium 3 and produced the pattern presented in Figure 9. The particles had reassociated to monomers as evidenced by the major peak at 77 S with a small proportion of the large subunit. Similar patterns were obtained when the preincubated ribosomes were suspended in H_2O or in medium 3 and run directly on the gradient.

As shown in Table IV, these ribosomes exhibited some endogenous activity for phenylalanine incorporation which was not lowered significantly by preincubation. Preincubation increased the response factor primarily through much greater activity in the presence of poly U with the small samples tested. There was no significant difference in incorporation activity between the ribosomes that were suspended in medium 3 and those that were dialyzed overnight in this solution. These particles also demonstrated a much larger relative response to poly U when smaller amounts were tested.

Removal of mRNA from Ribosomal Subunits. Peak 1 and

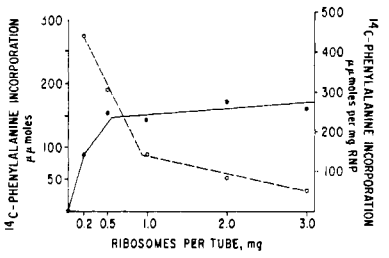

FIGURE 8: Influence of the amount of DEAE-ribosomes on phenylalanine incorporation. Standard incubation conditions. (●—●) Total μμmoles of phenylalanine incorporated; (○—○) micromicromoles of phenylalanine incorporated per milligram of RNP.

TABLE III: Effects of Variations in Concentration of Components on [^{14}C]Phenylalanine Incorporation.[a]

Component Added	Phe Incorp (μμmoles/mg of RNP)
1. High-speed supernatant (μl)	
50	34
75	50
100[b]	84
125	83
150	85
2. ATP (mM)	
1[b]	83
1.5	99
2.0	101
3. GTP (mM)	
0.2[b]	83
0.3	95
4. ATP and GTP (mM)	
1 and 0.2[b]	83
1.5 and 0.3	110

[a] Standard conditions of incubation except as noted; 1.5 mg of RNP and 50 μg of poly U per tube. [b] Amount added in standard incubation.

TABLE IV: Influence of Preincubation on Response of Chromatographed Ribosomes to Poly U.[a]

Ribosomal Preparation	Wt of Ribosomes/Tube (mg)	L-[^{14}C] Phenylalanine Incorp Poly U − ($\mu\mu$moles/mg of RNP)	Poly U + ($\mu\mu$moles/mg of RNP)	Response Factor
Nonpreincubated, suspended in medium 3	0.45	8	100	12.5
Preincubated, suspended in medium 3	0.40	9	150	17
Preincubated, suspended and dialyzed in medium 3	0.35	10	160	16
	0.50	15	165	11
	1.0	12	75	6.2

[a] Chromatographed ribosomes were suspended in 0.05 M Tris (pH 7.5 at 25°)–3 mM Mg^{2+} and stored at 0–2° overnight. An aliquot was preincubated under standard conditions except for omission of [^{14}C]phenylalanine and poly U. The solution was centrifuged at 80,000*g* for 90 min. The pellet was suspended in medium 3 and a portion was dialyzed *vs.* medium 3 at 0–2° overnight. All suspensions were incubated under standard conditions.

TABLE V: Removal of mRNA from Ribosomal Subunits by Gradient Centrifugation[a]

Preparation	[^{14}C]Phe Incorp Poly U ($\mu\mu$moles/mg of RNP) −	Poly U +
DEAE-cellulose column		
Peak 1	<3	<3
Peak 2	6	500
Peak 1 + 2	30	550
Sucrose gradient fractions		
38 S	<3	<3
57 S	<3	40
38 + 57 S	<3	350

[a] Aliquots (0.1 ml) from peak 1 (0.028 mg) and peak 2 (0.038 mg) eluates from a DEAE-cellulose column were assayed for phenylalanine incorporation activity under standard conditions in the presence and absence of poly U. Peak materials were purified by centrifugation through a 10–30% sucrose gradient as described in the text. Solutions which contained subunits were centrifuged at 230,000*g* for 4.5 hr. Each pellet was resuspended in 0.2 ml of medium 3–10 mM ME (38 S, 0.125 mg; 57 S, 0.27 mg) and were then assayed separately and together.

peak 2 material was tested for phenylalanine incorporation separately and together. In the absence of poly U, this activity can be considered as a measure of endogenous mRNA. In the presence of poly U, polyphenylalanine synthesis indicates the extent of cross contamination of subunits, the degree of monomer contamination of the large subunit, and the relative activity of the subunits when placed together. The data of Table V show that peak 1 RNP was inactive whether or not poly U was added. Peak 2 RNP, on the other hand, exhibited low activity without poly U and high (500 $\mu\mu$moles/mg) activity with poly U. These results suggest low endogenous mRNA content but considerable contamination with small subunit and/or monomers. When the two peak materials were combined, phenylalanine incorporation increased to 30 $\mu\mu$moles/mg without poly U and to 550 $\mu\mu$moles/mg with this polynucleotide. These increases suggest some mRNA in the peak 1 RNP, on the one hand, and the formation of active monomers from subunits in the mixture.

Peak 2 RNP was centrifuged through a 10–30% sucrose gradient which contained 10 mM Tris (pH 7.4)–0.5 M KCl–3 mM Mg^{2+}–15 mM ME, and cuts were made as shown in Figure 3B. The small and large subunits were tested separately and together as mentioned above. No activity with or without poly U was found with the small subunit or with the large subunit minus poly U. A low incorporation was observed with the 57S RNP with poly U.

When combined, activity without poly U was still at the background level but rose to 350 $\mu\mu$moles/mg when poly U was present. These results demonstrate the almost complete removal of endogenous mRNA from both subunit preparations as well as the removal of most of the 38S from the 57S subunit by centrifugation in this high-salt buffer.

To further evaluate the endogenous mRNA content of the purified subunits compared to that of DEAE-ribosomes, they were combined and tested by phenylalanine incorporation with and without the presence of poly U. In addition, the incorporation of isoleucine, valine, and lysine was measured. The data are set out in Table VI and demonstrate the practically complete lack of incorporation in all but the poly U directed incorporation of phenylalanine by the purified fractions. As judged by this operational test, endogenous mRNA can be considered to be removed by the centrifugation step.

Discussion

In the chromatographic purification procedure described in this paper, the RNA content of ribosomes was increased from approximately 40 to 60%. Yet there was very little change in the sedimentation constant of the monomer at various stages of purification. This suggests that the particle may be less compact in the purified state.

The marked decrease in unit incorporation activity with the larger amounts of ribosomes (Figure 8) is quite striking. From a practical standpoint, the data emphasize the need for a uniform amount of ribosomes in any study in which their specific incorporation activity is compared. This phenomenon hss been observed with a wide variety of ribosomes (Lamfrom and Knopf, 1965; Furano, 1966; Bloemendal *et al.*, 1966; Williamson *et al.*, 1967). Williamson *et al.* (1967) suggested that poly U becomes limiting at higher ribosome concentrations. Lamfrom and Knopf (1965) postulated that hemoglo-

TABLE VI: Incorporation of [^{14}C]Amino Acids by Ribosomes.[a]

Ribosomal Prepn	Amino Acid Incorp (cpm/mg of RNP)				
	Phe				
	Poly U				
	+	−	Ile	Val	Lys
DEAE	5460 (220)	400 (16)	355	40	95
Sucrose gradient combined subunits	5100 (205)	25 (1)	0	3	0

[a] A_{260} peak eluates from a DEAE-column were combined and centrifuged at 100,000*g* for 2.5 hr. The pellets were resuspended in 10 mM Tris–3 mM Mg^{2+}–15 mM ME and were tested after storage overnight at 4°. Purified subunits were prepared by centrifuging them through sucrose gradients as described in the text. The fractions were centrifuged at 230,000 *g* for 4.5 hr and the pellets were resuspended as above. Each type of ribosome was incubated under standard conditions with 0.5 μCi of the [^{14}C]amino acid. Numbers in parentheses refer to phenylalanine incorporation in units of micromicromoles per milligram of RNP.

bin chain initiation is limited by factors in the high-speed supernatant solution. Measurements of the ratio rate of chain initiation/rate of chain elongation with pancreas ribosomes will aid in evaluating the possibilities.

The attempt to determine the amount of endogenous mRNA in these ribosomes was found to be complicated by a number of factors. (1) A direct measurement of amino acid incorporation may be misleading if the preparation has become inactive. We have used the "response factor" to poly U as an indicator, but since this is a ratio of a large over a small number, it can vary widely with small differences in the endogenous mRNA activity. Thus, it is subject to a large error. (2) As mentioned above, the weight of particles taken per assay can affect incorporation activity, especially in the presence of poly U. (3) It is also evident that preincubation has not been as effective a procedure for removal (degradation?) of endogenous mRNA as it has been in the bacterial or liver systems. Lack of nuclease activity of dog pancreas ribosomes may be responsible for this difference.

Subunits of ribosomes have been prepared under a wide variety of conditions. Subunit formation is thought to be dependent upon the withdrawal of Mg^{2+} from the monomer. There have been two general procedures for accomplishing dissociation: (1) exposure of ribosomes at 0–2° to solutions which contain little or no Mg^{2+}, or contain Mg^{2+} binding agents such as EDTA, pyrophosphate, phosphate, or citrate (Chao, 1957; T'so *et al.*, 1958; Lamfrom and Knopf, 1965; Tashiro and Morimoto, 1966; Hamada *et al.*, 1968); (2) exposure of ribosomes at 37° to solutions of high ionic strength at pH 8 which contain appreciable (12.5 mM) amounts of Mg^{2+} (Martin and Wool, 1968).

The conditions of elution of pancreatic ribosomal subunits from DEAE-cellulose columns fall between these extremes and may be considered as a third procedure to obtain dissociation. It is of interest that dissociation at low ionic strength with high recovery of biological activity has been demonstrated solely with bacterial ribosomes. With both reticulocyte and muscle ribosomes, dissociation with recovery of polypeptide synthesis capability has been reported only at high ionic strength (0.5–1 M). Pancreatic ribosomal subunits can be randomly reassociated at 3 mM Mg^{2+} concentration by a drastic lowering of the ionic strength. This procedure leads to a preparation which contains monomers and larger aggregates (Figure 2). When this suspension is incubated under conditions of polypeptide synthesis (7 mM Mg^{2+} and 0.1 M NH_4Cl) a quite clean suspension of monomer (77 S) can be isolated (Figure 9). Besides these two procedures for the reassociation of pancreatic ribosomal subunits, we have dialyzed them against 0.01 M phosphate and subsequently against 0.01 M phosphate–3 mM Mg^{2+} (S. R. Dickman and E. Bruenger, 1968, unpublished data). The reactivation obtained, while positive, was not as striking as with the other treatments. Pancreatic ribosomes are less stable under conditions of low ionic strength than those from bacteria. It appears that a truly efficient reversible dissociation of pancreatic ribosomes is dependent on constant exposure to a certain minimum concentration of Mg^{2+}. The extent of dissociation at a given constant Mg^{2+} concentration is then determined by the cation concentration.

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Mode of Action of Berninamycin. An Inhibitor of Protein Biosynthesis*

Fritz Reusser

ABSTRACT: Berninamycin acts as a specific inhibitor of protein synthesis in *Bacillus subtilis* cells. Experiments with bacterial cell-free macromolecular biosynthetic systems showed that berninamycin interferes with amino acid incorporation into peptides directed by natural messenger ribonucleic acid and synthetic polyribonucleotides. Deoxyribonucleic acid and ribonucleic acid synthesis remained unaffected in cell-free systems. Individual reaction sequences occurring during polypeptide synthesis including aminoacyl transfer ribonucleic acid formation, aggregation to the ternary aminoacyl transfer ribonucleic acid–messenger–ribosome complex, messenger attachment to ribosomes, and peptide-bond formation remained insensitive to the antibiotic. No misreading of the code was apparent in a test system sensitive to streptomycin. These data suggest that the most likely site of berninamycin interaction during polypeptide biosynthesis occurs after formation of the peptide bond and might involve interference with transfer ribonucleic acid release, movement of the nascent peptide chain on the ribosomes, or movement of the messenger.

Berninamycin, an antibacterial agent, was isolated from the culture broth of *Streptomyces bernensis* sp. nova. The compound crystallizes in the form of light beige needles which have very limited solubility in aqueous solutions (<100 μg/ml). Preparation, isolation, characterization, and biological properties will be described elsewhere (O. O. Bergy and F. Reusser, in preparation). Upon acid hydrolysis of the antibiotic, a new sulfur-containing acid, two unknown amino acids, L-threonine, glycine, and a trace of alanine were isolated from the hydrolysate. Structural studies are continuing. The agent is highly inhibitory to gram-positive bacteria *in vitro*, but has been ineffective in the systemic treatment of experimental infections caused by gram-positive organisms in mice.

Materials and Methods

Bacillus subtilis UC-564 cells were grown in a medium containing the following ingredients per liter of deionized water: KH_2PO_4, 6 g; K_2HPO_4, 14 g; $MgSO_4$, 0.1 g; $(NH_4)_2SO_4$, 2 g; sodium glutamate, 2 g; glucose monohydrate, 4 g; and tryptone (Difco), 0.2 g. Shaken flasks containing 100 ml of medium were inoculated with 5 ml of seed derived from an overnight culture. The flasks were incubated on a rotary shaker at 37°. Antibiotic was added at the early logarithmic phase of cell growth.

*From the Department of Microbiology, The Upjohn Company, Kalamazoo, Michigan. *Received February 20, 1969.*

The cellular protein and nucleic acid fractions of *B. subtilis* were isolated and assayed as described previously (Reusser, 1967).

DNA and RNA polymerases from *Escherichia coli* were prepared and assayed as described by Reusser (1967).

The cell-free polyribonucleotide directed amino acid incorporation systems were prepared as described by Nirenberg (1963). The 30S fraction was used as an enzyme source. Phage f_2 RNA was obtained from Dr. Nomura, University of Wisconsin, Madison, Wis.

Poly C attachment to ribosomes was assessed by the filtration technique of Moore (1966). Poly U attachment to ribosomes could not be measured by this technique due to a high degree of self-adsorption of poly U to the filters. Poly U binding to ribosomes was thus measured by separating the ribosome–poly U complex by sucrose density gradient centrifugation.

E. coli soluble RNA (tRNA), stripped, was purchased from General Biochemicals. The enzyme source used to catalyze acylation of tRNA with one amino-^{14}C acid and 19 unlabeled amino acids was a dialyzed ribosomal supernatant solution (100 S) prepared as described by Nirenberg (1963). The exact composition of the reaction mixtures is shown in Table V.

The samples were incubated at 37° for 15 min. Separate studies had indicated that the reaction was complete after this time. The reaction was stopped by the addition of 0.5 ml of cold 10% trichloroacetic acid containing 0.5 mg of Celite/ml. The acid-insoluble product was collected on Millipore filters