Dissociation of Rat Liver Ribosomes to Active Subunits by Urea*

Mary L. Petermann† and Amalia Pavlovec

ABSTRACT: Since ribosomal subunits are sensitive to degradative enzymes, adult male rats were treated with phenobarbital to decrease the liver RNase content. The ribosomes were isolated, dissociated, and reassociated in the presence of 1 mM dithiothreitol. When their bound magnesium was reduced to 0.3 equiv/mole of RNA phosphate, and they were then treated with urea, they dissociated to subunits. The urea was removed by dialysis against a low-magnesium buffer, and the subunits were fractionated in a zonal rotor. With 2 M urea about 50% of the small subunits was recovered in a 40S fraction, 5% was found in a 28S form, and the rest had dimerized to a 60S form that sedimented with the large subunit (59 S) fraction. A better large-subunit preparation was obtained when the ribosomes were dissociated in 2.7 M urea, which prevented dimerization but converted most of the small subunits into the 28S form. The large subunits contained the theoretical amount of 5S RNA. When the 40S and 59S fractions were mixed and the bound magnesium was increased to 0.6 equiv/mole of RNA phosphate, the subunits reassociated to 81S ribosomes. These ribosomes showed very little endogenous phenylalanine incorporation but were highly active in the presence of polyuridylic acid.

The ribosomes of *Escherichia coli* can readily be dissociated to 30S and 50S subunits by a moderate reduction in their bound magnesium. When the magnesium is restored to its initial level these subunits reassociate to 70S ribosomes that are active in protein synthesis (Staehelin and Meselson, 1966). Mammalian 80S ribosomes, on the other hand, do not usually dissociate until their bound magnesium has been reduced to a very low level, and this type of dissociation frequently results in unfolding of the subunits, detachment of the 5S RNA, and irreversible loss of activity (Petermann *et al.*, 1969). Active subunits can be obtained from animal ribosomes by a high-salt treatment (Martin and Wool, 1969), but since a high-salt wash may remove important factors (Heywood, 1970; Arnstein, 1970) a low-salt procedure may sometimes be preferable. Such a procedure is described in the present paper. Rat liver ribosomes were dissociated to active subunits by removing half their bound magnesium and adding urea. The 5S RNA was not detached, and the subunits remained in compact forms that could reassociate to 81S particles that were active in protein synthesis.

Methods

Ribosomes. In most of these experiments adult male rats were given 25 mg of phenobarbital/day, in the drinking water, for 7 days before sacrifice, to reduce the amount of RNase in the liver (Louis-Ferdinand and Fuller, 1970). Liver ribosomes were isolated as described by Petermann and Pavlovec (1963), with some modifications. Dithiothreitol (DTT),[1] 1 mM, was present throughout the procedure; 0.6% deoxycholate was used to disrupt the microsomal membranes; the first ribosome pellets were washed in 0.5 mM $MgCl_2$–20 mM $KHCO_3$ (pH 8.0), suspended in 0.5 mM $MgCl_2$–1 mM potassium phosphate (pH 6.8), and centrifuged at 20,000*g* for 15 min. To this supernatant 0.11 volume of 0.5 M $MgCl_2$ was added, to precipitate the ribosomes. The precipitate was washed with 0.05 M $MgCl_2$–1 mM potassium phosphate (pH 7.0), suspended in 1 mM phosphate (pH 7.3), and dialyzed against 0.2 mM $MgCl_2$–1 mM phosphate (pH 7.3) for 2 days on a rocking dialyzer. These preparations have been called P ribosomes. When the phenobarbital pretreatment was not used only 0.5% deoxycholate was used; such preparations have been called N ribosomes. All preparations were mixed with 0.05 volume of 40% sucrose, frozen rapidly in a Dry Ice–alcohol mixture, and stored under nitrogen at −20° (Petermann, 1971).

Isolation of Subunits. To reduce the bound magnesium 60–80 mg of ribosomes, in a volume of 6 ml, was mixed with 1 ml of 0.7 M KCl and dialyzed for 2.5 hr against buffer KD containing 0.2 mM $MgCl_2$ in a small Zeineh dialyzer (Biomed Instruments Inc., Chicago). Large and small subunits were prepared in separate experiments. For the large subunits one-half volume of 8 M urea, freshly dissolved in water and treated with charcoal (Hamilton and Ruth, 1967), was added slowly, with good stirring, at 5°. After standing for 30 min at 5° the material was dialyzed for 1 hr in the Zeineh dialyzer against buffer G. The solution was divided into three portions, adjusted to contain an inverted gradient of ribosomes (Britten and Roberts, 1960), and layered on a 10–20% sucrose gradient, in buffer G, in a Beckman B XIV zonal rotor. The rotor and all solutions were prechilled in ice, and the gradient-mixing chamber was set in a Dry Ice–alcohol bath at −18°. The subunits were centrifuged for 17 hr at 25,000 rpm at 5° (Petermann, 1971). The 59S fractions (2.7 M urea large subunits) were frozen and stored as described above. The small subunits, most of which had been converted into a 28S form, were discarded.

* From the Sloan-Kettering Institute for Cancer Research, New York, New York 10021. *Received March 9, 1971.* This research was supported by Grant CA 08748 from the National Cancer Institute, U. S. Public Health Service; National Science Foundation Grants GB 6308 and GB 8717; and Atomic Energy Commission Contract AT(30-1)-910. A preliminary report on this work was presented to The American Society of Biological Chemists, Atlantic City, N. J., April 17, 1969.

[1] Abbreviations used are: P ribosomes, material from rats pretreated with phenobarbital; N ribosomes, material from untreated rats; DTT, dithiothreitol; buffer KD, 30 mM KCl–1 mM DTT–1 mM potassium phosphate, pH 7.3; buffer G, 10 mM KCl–1 mM DTT–0.1 mM $MgCl_2$–1 mM potassium phosphate, pH 7.8; *r*, equivalents of magnesium bound per mole of RNA phosphate.

To obtain active (40 S) small subunits the procedure was similar except that only one-third volume of 8 M urea was added, and the subsequent dialysis was for 45 min. The 59S fraction (2 M urea large subunits) was stored at −20°, but the 40S fraction was used immediately.

Reassociation of Subunits. Freshly isolated 40S subunits (20–50 A_{260} units) were mixed with twice the amount of 2.7 M urea large subunits. One-fifth volume of 130 mM KCl–17.5 mM $MgCl_2$–1 mM DTT–1 mM potassium phosphate (pH 7.3) was added, with good stirring, over a period of about 45 min. The mixture was stirred for an additional 45 min, then diluted to twice its original volume with buffer KD containing 3 mM $MgCl_2$ and centrifuged for 19 hr at 78,000*g*. The pellets were suspended in a small volume of the immediately overlying supernatant solution, so that the total volume was 1–2 ml. Large or small subunits alone were treated similarly. A sample of unfractionated 2 M urea subunits in buffer G was kept for 1 day at 5°, then dialyzed for one day against the 3 mM $MgCl_2$ buffer, and a portion of the initial ribosome preparation, dialyzed briefly against the 3 mM $MgCl_2$ buffer, served as control. Since the initial ribosome solutions contained disomes, trisomes, and larger polysome fragments, some of the N ribosomes were pretreated to increase the proportion of monosomes by incubation for 20 min at 37° in 15% ethanol (Haga *et al.*, 1970). The solution was then chilled in ice, dialyzed against buffer KD containing 0.2 mM $MgCl_2$, and dissociated with 2 M urea as described above.

Analysis of Ribosomal Fractions. Samples containing 2 mg of ribosomes or subunits per ml were examined in the analytical ultracentrifuge in 30-mm double-sector cells at 5° and 44,000 rpm, with schlieren optics. Samples from the zonal fractionations were dialyzed free of sucrose, diluted to 60 μg/ml, and examined in 12-mm cells, with ultraviolet optics, at 5°. Sedimentation coefficients were measured in buffer G. To determine the relative amounts of small and large subunits in a fraction, NaEDTA (pH 7.0) was added to a final concentration of 2 mM. RNA was determined by the orcinol method (Petermann, 1964). Bound magnesium was measured as described by Petermann and Pavlovec (1967) and expressed as equivalents per mole of RNA phosphate (*r*).

To measure the amount of 5S RNA in the large subunit two 59S preparations, made by the ethanol–2 M urea procedure from N ribosomes, were combined and concentrated by centrifuging at 78,000*g* for 17 hr in buffer KD containing 0.5 mM $MgCl_2$. The RNA was isolated as described by Petermann and Pavlovec (1963), except that the ethanol precipitate was centrifuged at 78,000*g* for 30 min (Knight and Darnell, 1967). The precipitate was dissolved in 0.1 M NaCl–1 mM sodium phosphate (pH 7.0) and the 18S and 28S RNAs were precipitated with 1 M NaCl. The supernatant solution was analyzed on Sephadex G-100 in 1 M NaCl (Christman and Goldstein, 1969). A sample of whole N ribosomes was treated in the same way. The identities of the 5S and tRNA fractions were checked by electrophoresis on 10% polyacrylamide gels (Peacock and Dingman, 1967).

Amino Acid Incorporation. The ability of the various fractions to incorporate phenylalanine, endogenously or in the presence of 100 μg of poly(U), was measured as described by Weinstein *et al.* (1966), with 2 A_{260} units of ribosome/assay tube.

Results

The initial ribosome preparations had a magnesium to phosphate ratio, *r*, of 0.55, and their ultracentrifugal patterns showed chiefly monosomes (82 S) and faster sedimenting material, plus a small 60S boundary. Although the first dialysis reduced the bound magnesium to $r = 0.3$, no additional subunits appeared until urea was added; in 2 M urea, however, the ribosomes were 80% dissociated (Figure 1A). When the urea was removed the pattern (Figure 1B) showed little change. After overnight storage at 5° in this buffer the subunits gave an identical pattern, and in the high-magnesium buffer they recombined to give 81S and larger particles, with an ultracentrifugal pattern like that in Figure 1E. The N ribosomes (not shown) gave similar patterns. The zonal fractionations also showed 28S, 40S, and 59S peaks (Figure 2A,B); when P ribosomes were used the 28S peak was smaller, and only 2% of the ultraviolet-absorbing material remained at the top of the gradient. On analytical ultracentrifugation the pooled 40S fractions gave a sharp boundary, with a small 50S shoulder (Figure 3A), and the 59S pattern resembled Figure 3B. Addition of EDTA converted the large subunit into a 35S form and the small subunit to a 24S form that was clearly distinguishable. The 40S fraction contained about 10% of large subunits, and the 2 M urea 59 S showed a significant contamination with small subunits (Figures 3C and 2A).

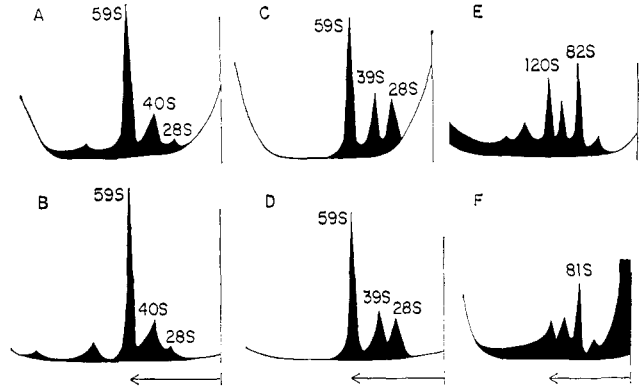

FIGURE 1: P ribosomes and subunits; analytical ultracentrifugation with schlieren optics, (A and B) 2 M urea subunits; (A) in 23 mM KCl–0.75 mM DTT–0.15 mM MgCl–0.75 mM potassium phosphate (pH 7.3)–2 M urea; after 22 min; (B) in buffer G, 18 min; (C and D) 2.7 M urea subunits; (C) in 20 mM KCl–0.67 mM DTT–0.13 mM $MgCl_2$–0.67 mM potassium phosphate (pH 7.3)–2.7 M urea, 22 min; (D) in buffer G, 18 min; (E) control ribosomes, in buffer KD containing 3 mM $MgCl_2$, 6 min; (F) 2 M urea 40 S + 2.7 M urea 59 S, in the same buffer plus 6% sucrose, 10 min.

To improve the yield of small subunits and the purity of the large-subunit fraction a number of techniques were tried. Layering the subunits in an inverse gradient improved the separation of the 40S and 59S particles, but the chief difficulty was the sedimentation of small subunits in the 60S region. When monosomes were prepared by the ethanol treatment or isolated in a sucrose gradient, then dissociated, the yield of 40 S was somewhat higher, but the 59S fraction still contained 10–12% of small subunits. When the 59S fraction was resedimented in buffer KD containing 2.5 mM $MgCl_2$, the small subunits were removed, by reassociation with large ones to 81S ribosomes, but since the large subunits also dimerized to 90S particles the yield of 59 S was very low.

The best procedure for obtaining large subunits was dissociation in 2.7 M urea (Figures 1C,D, 2C). Although most of the small subunits were converted into the 28S form, the large ones were still mainly 59 S. This 59S material gave a single boundary in buffer G (Figure 3B) and showed only a trace of small subunits when examined in the presence of EDTA (Fig-

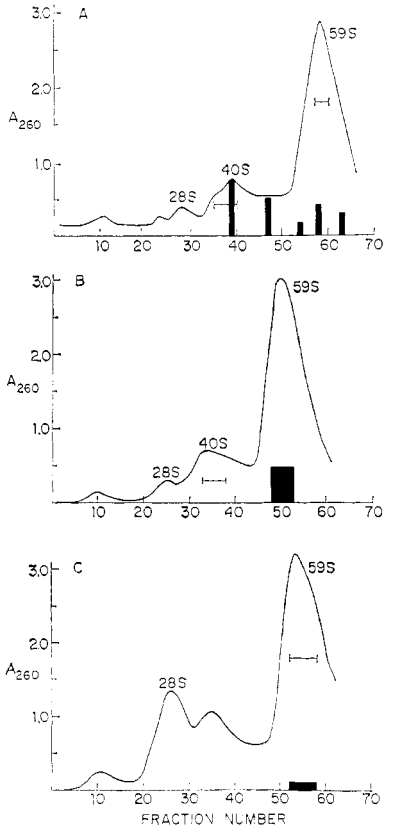

FIGURE 2: Fractionation of ribosomal subunits in the zonal rotor in buffer G. The sedimentation coefficients were measured in the analytical ultracentrifuge, after removing the sucrose. The vertical bars show the relative amounts of small subunits in the various fractions, as determined from the 24S component observed in the analytical ultracentrifuge in the presence of EDTA. The brackets show the fractions used to study phenylalanine incorporation. (A) N ribosomes dissociated in 2 M urea. The subunits were layered on the gradient in one sample, in 5% sucrose. (B) P ribosomes dissociated in 2 M urea; (C) P ribosomes dissociated in 2.7 M urea. B and C were layered in inverse gradients.

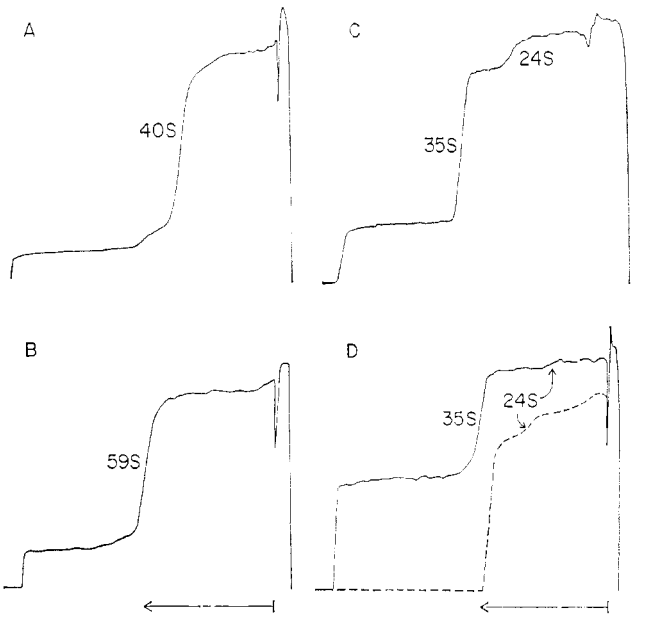

FIGURE 3: Analytical ultracentrifugation of isolated P subunits, with ultraviolet optics. (A) 2 M urea small subunits; (B) 2.7 M urea large subunits, in buffer G. (C) 2 M urea large-subunit fraction containing 17% of small subunits; (D) 2.7 M urea large subunits containing 8% of small ones, in buffer G plus EDTA. In D the dash line shows the pattern obtained at four times the usual subunit concentration.

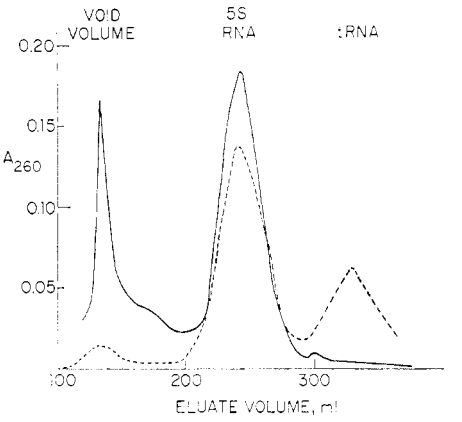

FIGURE 4: Gel filtration of the soluble fraction of ribosomal (– – – –) and large-subunit (———) RNAs on G-100 Sephadex in 1 M NaCl.

ure 3D). A more concentrated sample was run at the same time in a prism cell and photographed on the same film. After correction for the concentration difference the 24S boundary accounted for 4–8% of the total ultraviolet absorption. When r was increased to 0.6 these 59S subunits reassociated with the 2 M urea small subunits to 81S ribosomes and larger particles (Figure 1F); except for the large sucrose boundary at the meniscus the pattern was similar to that of the control ribosomes in the same buffer (1E). The N ribosomes (not shown) behaved similarly.

When total RNA was precipitated with 1 M NaCl, and the supernatant was fractionated on Sephadex (Figure 4) a small amount of material, probably 28S or 16S RNA, was excluded from the gel, and distinct 5S and tRNA peaks appeared. With the 2 M urea large subunit 5.6% of the RNA was soluble in 1 M NaCl. The gel filtration showed an excluded fraction and a 5S peak, but no peak in the tRNA region. The 5S peak contained 42% of the RNA in the 1 M NaCl supernatant, corresponding to 2.3% of the total RNA in the subunit. Correcting for the presence of 10% of small subunits in the 59S fraction increased the yield to 2.6%; the theoretical value is 2.2%. In an earlier experiment, where the large-subunit RNA was treated similarly, except that the ethanol precipitate was centrifuged at low speed, 85% of the theoretical amount of 5S RNA and no tRNA were recovered.

Phenylalanine Incorporation. In the presence of poly(U) the control P ribosomes were highly active in phenylalanine incorporation (Table I). The unfractionated 2 M urea subunits were 70–80% as active as the controls; in expt 3 and 4 this loss of activity occurred during the first dialysis. The activities of the various large-subunit fractions were roughly proportional to their content of small subunits. The small subunits were inactive alone (expt 2 and 4), but markedly stimulated incorporation when added to the large ones. A mixture containing 2.6 A_{260} units of large subunits and 1.0 A_{260} unit of small ones (expt 4) was less active than the usual 2:1 mixture.

The N ribosomes were about 30% less active than the P ribosomes, when tested with the same pH 5 fraction. The unfractionated N subunits were active (Table I), but the isolated, remixed subunits had lost most of their activity. The pH 5

TABLE I: The Incorporation of [^{14}C]Phenylalanine by Ribosomes and Subunits, in Counts per Minute per Unit of Optical Density at 260 mμ.[a]

Expt No.	Sample	s in L (%)	Incorpn with Poly(U)		Endogenous Incorpn	
			Total	% of Control	Total	% of Control
			P Ribosomes			
1	Control		6,000		2000	
	Unfractionated		4,890	82	408	20
	L	4	1,093	18	135	7
	L + S		4,610	77	154	8
2	Control		29,100		2725	
	Unfractionated		23,000	79		
	S		610	2		
	L	8	6,870	24		
	L + S		16,200	56		
3	Control		16,970			
	Dialyzed[b]		11,510	68		
	Unfractionated		12,820	76		
	L (2 M urea)	12	5,340	31		
	L	7	1,800	11		
	L + S		8,760	52		
4	Control		9,489		1420	
	Dialyzed[b]		5,433	57		
	Unfractionated		6,690	71		
	S		236	2		
	L	6	877	9		
	L + S		5,076	53		
	L + S[c]		3,524	37		
			N Ribosomes			
	Control		10,920		2260	
	Unfractionated		9,590	88	243	11
	S		(−335)[d]	0	177	8
	L	5	394	4	(−514)[e]	0
	L + S		2,162	20	(−295)[e]	0

[a] "Unfractionated" subunits represent material dissociated in 2 M urea (see Methods). Small subunits (S) were dissociated in 2 M urea; large subunits (L) were dissociated in 2.7 M urea, except where indicated. All incubated for 30 min at 37° with the pH 5 fraction. [b] After dialysis against buffer KD containing 0.2 mM $MgCl_2$. [c] Ratio of L to S was 2.6. [d] The pH 5 fraction alone incorporated 2380 cpm. [e] The pH 5 fraction alone incorporated 2000 cpm.

fraction used in this experiment apparently contained some ribosomes, since it was quite active when tested alone. The addition of small subunits decreased this activity. Since earlier experiments had shown that 2 M urea large subunits containing 15% of small ones were two-thirds as active as the controls, the unstable fraction appeared to be the isolated small subunits.

In the absence of poly(U) the P control ribosomes incorporated much less phenylalanine. Although the unfractionated subunits, which still contained 82 S (Figure 1B), retained some activity (expt 1), the isolated subunits were almost inactive. The N control ribosomes were active, but the subunits showed little or no activity.

Discussion

Animal ribosomes do not usually dissociate until their bound magnesium has been reduced to a very low level, and the resulting subunits are often unstable. Active subunits have, however, been obtained from a variety of animal ribosomes, including those of reticulocytes (Hamada *et al.*, 1968; Bonanou *et al.*, 1968; Arnstein, 1970) and rat liver (Stahl *et al.*, 1968; Barden and Korner, 1969; Martin and Wool, 1969; Castles and Wool, 1970; Falvey and Staehelin, 1970; Terao and Ogata, 1970) by treatment with 0.5–1.0 M KCl. In all these experiments much of the bound magnesium was probably displaced by monobasic cations, which permitted dissociation but prevented the electrostatic repulsion which ordinarily causes the subunits to unfold. The nature of the strong attachment between the subunits of the animal ribosome is not understood. Although the 28S RNA is larger than the bacterial 23S RNA, their compositions are similar; the tRNAs, which help to hold the subunits together (Lawford, 1969), are also similar. One striking difference is in the amount of protein. An animal ribosome contains over twice as much protein as a bacterial ribosome, and protein–protein interactions are important for the maintenance of subunit structure (Cox, 1969; Mizushima and Nomura, 1970). The possibility that animal ribosomes are stabilized by protein–protein interactions is supported by their dissociation in the presence of

urea. Sulfhydryl groups also appear to play some role in subunit interaction; without DTT, dissociation in 2 M urea requires the removal of more magnesium (Petermann *et al.*, 1969). Direct linkage through disulfide bonds does not seem to occur, however, since the subunits do separate on the addition of urea.

The 59S subunit has a molecular weight of 3.0×10^6 and a protein content of 45% (Hamilton *et al.*, 1971). It contains the theoretical amount of 5S RNA. In buffer G $s^0_{20,w}$ is 58.7 S (Haga *et al.*, 1970). For the 2 M urea large-subunit fraction, although electron microscopy showed uniform ovoidal particles, with no sign of whole ribosomes (Haga *et al.*, 1970), the presence of small subunits was revealed by ultracentrifugal analysis and by the amino acid incorporating activity. By both these criteria the 2.7 M urea large subunits were almost free of small ones. With 2 M urea about 5% of the large subunits we converted to inactive 40S–50S material that sedimented with the small subunits.

The small subunits were found in three forms. The 28S particle did not reassociate with the 59S subunit under conditions where the 40S subunit did, so was not tested for activity. It resembled the 30S small subunit obtained by EDTA dissociation, which had a molecular weight of 1.2×10^6 and a protein content of 50% (Hamilton and Ruth, 1969). The active small subunit, 40 S, had a higher molecular weight, 1.5×10^6, and a protein content of 55% (Hamilton *et al.*, 1971). It bound Phe-tRNA in the presence of poly(U) (Dr. D. Grünberger, personal communication). The subunit contained a small amount of rapidly labeled RNA that could be detached by EDTA treatment (Hamilton *et al.*, 1971). The nature of this RNA is now being investigated. It is not functional mRNA; the mixed subunits show no endogenous incorporation, either with phenylalanine or with a mixture of labeled amino acids (A. Pavlovec, unpublished data). The third form of small subunit, found in the 59S fraction, appeared to be a dimer of 40 S. Our isolated 40S subunits were converted to 60S particles in high-magnesium buffers, and similar 60S particles have been described by Tashiro and Morimoto (1966) and by Terao and Ogata (1970). Electron micrographs of such dimers show two small subunits in close association (Nonomura *et al.*, 1971). Adjusting the pH, KCl, or Mg content of the solvent, within the narrow limits where both subunits were stable, did not prevent dimer formation. Increasing the urea concentration to 2.7 M did dissociate the dimers, but converted most of the small subunits into 28S. Some of the forces that hold these dimers together may be electrostatic, since small subunits obtained by the method of Martin and Wool (1969), most of which sedimented as 40S particles in 0.88 M KCl (Castles and Wool, 1970), also dimerized in dilute buffers (M. L. Petermann, unpublished results). Our experience, however, agrees with that of Tashiro and Morimoto (1966), that more dimers are found at intermediate that at low KCl concentrations. When liver ribosomes dissociated to subunits on treatment with the sulfhydryl reagent *p*-mercuribenzoate (Incefy and Petermann, 1969) small-subunit dimerization appeared to be minimal. The bulky *p*-mercuribenzoate groups may have sterically hindered the subunits from dimerizing.

Although bentonite was used in all our ribosome preparations, and the control N ribosomes were highly active, traces of RNase were still present. To reduce damage from RNase all procedures were carried out rapidly, and the solutions were kept as cold as possible, with particular care to avoid warming in the zonal rotor. The 2 M urea 59S fraction was active, but when the 40S fraction was combined with 2.7 M urea large subunits the activities were only 20–40% of the control values. In the experiment shown in Table I the small subunits decreased the activity of the pH 5 fraction. Phenobarbital treatment leads to a reduction in liver RNase (Louis-Ferdinand and Fuller, 1970; Mycek, 1971), and the subunits from P ribosomes were highly active with poly(U). Liver homogenate from the treated animals requires more deoxycholate to dissolve the endoplasmic reticulum, but if the deoxycholate concentration is increased above 0.6 the large subunit becomes much less stable.

Progress in elucidating the structure of animal ribosomes depends on the availability of active subunits. The KCl dissociation methods give subunits that are suitable for some purposes, but high-salt washes may detach specific mRNA binding factors (Heywood, 1970; Arnstein, 1970). If the urea subunits retain these factors they should be of particular value for the study of initiation.

Acknowledgments

We thank Dr. I. B. Weinstein for very helpful collaboration in the early phases of this work; Dr. D. Grünberger for measuring the binding of poly(U); Dr. Mary G. Hamilton for many helpful discussions; and Dr. T. E. Martin and Dr. M. J. Mycek for allowing us to read their papers in advance of publication. Mrs. Concepcion Kane and Miss Cecile Ortigas contributed valuable technical assistance.

References

Arnstein, H. R. V. (1970), *Biochem. J. 117*, 55 P.
Barden, N., and Korner, A. (1969), *Biochem. J. 114*, 30 P.
Bonanou, S., Cox, R. A., Higginson, B., and Kanagalingam, K. (1968), *Biochem. J. 110*, 87.
Britten, R. J., and Roberts, R. B. (1960), *Science 131*, 32.
Castles, J. J., and Wool, I. G. (1970), *Biochemistry 9*, 1909.
Christman, J. K., and Goldstein, J. (1969), *Biochim. Biophys. Acta 179*, 280.
Cox, R. A. (1969), *Biochem. J. 114*, 769.
Falvey, A. K., and Staehelin, T. (1970), *J. Mol. Biol. 53*, 1.
Haga, J. Y., Hamilton, M. G., and Petermann, M. L. (1970), *J. Cell Biol. 47*, 211.
Hamada, K., Yang, P., Heintz, R., and Schweet, R. (1968), *Arch. Biochem. Biophys. 125*, 598.
Hamilton, M. G., Pavlovec, A., and Petermann, M. L. (1971), *Biochemistry 10* (in press).
Hamilton, M. G., and Ruth, M. E. (1967), *Biochemistry 6*, 2585.
Hamilton, M. G., and Ruth, M. E. (1969), *Biochemistry 8*, 851.
Heywood, S. M. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1782.
Incefy, G. S., and Petermann, M. L. (1969), *Biochemistry 8*, 1482.
Knight, E., Jr., and Darnell, J. E. (1967), *J. Mol. Biol. 28*, 491.
Lawford, G. R. (1969), *Biochem. Biophys. Res. Commun. 37*, 143.
Louis-Ferdinand, R. T., and Fuller, G. C. (1970), *Biochem. Biophys. Res. Commun. 38*, 811.
Martin, T. E., and Wool, I. G. (1969), *J. Mol. Biol. 43*, 151.
Mizushima, S., and Nomura, M. (1970), *Nature (London) 226*, 1214.
Mycek, M. J. (1971), *Biochem. Pharmacol. 20*, 325.
Nonomura, Y., Blobel, G., and Sabatini, D. (1971), *J. Mol. Biol.* (in press).
Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6*, 1818.

Petermann, M. L. (1964), The Physical and Chemical Properties of Ribosomes, Amsterdam, Elsevier.
Petermann, M. L. (1971), *Methods Enzymol. 20*, 429.
Petermann, M. L., and Pavlovec, A. (1963), *J. Biol. Chem. 238*, 3717.
Petermann, M. L., and Pavlovec, A. (1967), *Biochemistry 6*, 2950.
Petermann, M. L., Pavlovec, A., and Weinstein, I. B. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 725.
Staehelin, T., and Meselson, M. (1966), *J. Mol. Biol. 16*, 245.
Stahl, J., Lawford, G. R., Williams, B., and Campbell, P. N. (1968), *Biochem. J. 109*, 155.
Tashiro, Y., and Morimoto, T. (1966), *Biochim. Biophys. Acta 123*, 523.
Terao, K., and Ogata, K. (1970), *Biochem. Biophys. Res. Commun. 38*, 80.
Weinstein, I. B., Ochoa, M., Jr., and Friedman, S. M. (1966), *Biochemistry 5*, 3332.

Characterization of Sea Urchin Ribosomal Satellite Deoxyribonucleic Acid*

James B. Patterson† and Darrel W. Stafford‡

ABSTRACT: Ribosomal satellite DNA from the sperm of the sea urchin *Lytechinus variegatus* has been examined by means of saturation hybridization with rRNA, denaturation analysis, and renaturation analysis. The saturation level of hybridization with homologous rRNA was about 0.37 μg of rRNA/μg of rDNA. Denaturation analysis indicated that the ribosomal satellite DNA was composed of two nearly equal components of 59.5% G + C and 65.2% G + C content. Renaturation kinetics yielded a $C_0t_{1/2}$ of 1.7×10^{-2} mole sec l.$^{-1}$ under conditions equivalent to 0.12 M phosphate buffer (pH 7.0). Renaturation studies further indicated that the satellite DNA behaved as a single family of ribosomal genes of identical or very similar repeating base sequences of about 5.2×10^6 amu.

The genes for ribosomal RNA in *Lytechinus variegatus* occur in a satellite DNA with a density in CsCl of 1.722 g/cm^3. This ribosomal satellite (rDNA)[1] comprises about 0.02–0.03% of the total DNA of the sea urchin sperm. In a previous paper (Patterson and Stafford, 1970) we showed that the rDNA could be isolated from the main band DNA by virtue of the differences in their G + C content, 63 and 35%, respectively. The rDNA isolation was achieved by selective denaturation of the main band DNA followed by separation of the native and denatured DNA in a polyethylene glycol–dextran two-phase system. It was further demonstrated by hybridization of sea urchin rRNA to DNA that the genes for rRNA were contained in the satellite DNA.

The isolation of a specific portion of the sea urchin genome should allow us to ask specific questions on the control of gene expression in development. Before the relevant questions could be asked, however, further physical and chemical characterization of the satellite were necessary. We report here the analysis of satellite DNA by means of denaturation, renaturation, and saturation hybridization experiments.

Materials and Methods

Reagents. All reagents used in these experiments were previously described (Patterson and Stafford, 1970), except for the following additional reagents. CTAB was purchased from Matheson Coleman & Bell. Cesium chloride was purchased from Varlacoid Chemical Co.

DNA Isolation. Sea urchin sperm DNA was isolated as previously described (Patterson and Stafford, 1970). Satellite DNA was isolated using the polyethelene glycol–dextran two-phase system followed by preparative CsCl density gradient centrifugation as previously described (Patterson and Stafford, 1970). Each preparation of satellite DNA was analyzed by CsCl analytical centrifugation at a concentration of 15 μg/cell. There was no detectable main band in any preparation, indicating that the satellite DNA used for these experiments was at least 99.8% satellite DNA.

Sea urchin ^{32}P-labeled rRNA was isolated from ribosomes of larvae at the pluteus stage of development. The larvae were grown in Millipore-filtered sea water made 50 μg/ml with respect to streptomycin and 300 units/ml with respect to penicillin (Glišin and Glišin, 1964). The larvae were labeled at the prism stage with 5 μCi/ml of [^{32}P]monosodium for 20 hr. Larvae from the eggs of one gravid female were washed once in Millipore-filtered sea water, once in Hultin's homogenization buffer (Hultin, 1961), resuspended in 15 ml of Hultin's homogenization buffer, and homogenized in a Dounce homogenizer with tight-fitting pestle. No whole cells were observed by phase microscopy. The postmitochondrial supernatant was made 1% with respect to Triton X-100, pelleted through 40% sucrose in Hultin's buffer, and resuspended in 5 ml of pH 5.1 buffer (1 M NaCl–0.01 M sodium acetate) and 1% SDS. The rRNA was prepared by extracting the ribosomes four times

* From the Department of Zoology, the University of North Carolina, Chapel Hill, North Carolina 27514. *Received January 25, 1971.* The work was supported by a predoctoral traineeship from the National Science Foundation (J. B. P.) and Grant GB-8334 from the National Science Foundation (D. W. S.).

† Present address: Cell Biology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, N. C. 27709.
‡ To whom to address correspondence.

[1] Abbreviations used are: SSC, standard saline citrate; MAK, methylated albumin kieselguhr; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; rRNA, ribosomal ribonucleic acid; rDNA, deoxyribonucleic acid containing the base sequences coding for rRNA; amu, atomic mass units.