is fortuitous. The next paper in this series is concerned with this latter question; data will be presented which strongly suggest that there is no functional relationship between the poly A polymerase and the ribosomes.

Acknowledgment

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The Relationship between Poly A Polymerase and the Ribosomes*

S. J. S. Hardy† and C. G. Kurland

ABSTRACT: The structural and functional relationship between polyriboadenylic acid (poly A) polymerase and the ribosomes of *Escherichia coli* was studied. Four experimental criteria to decide whether or not an enzyme is a structural element of the ribosomes were applied. These are: (1) that there is some functional relationship between the enzyme and the ribosomes; (2) that the enzyme remains associated with the ribosomes under conditions which remove strongly adsorbed supernatant proteins and yield active ribosomes; (3) that the enzyme is specifically associated with either the 30- or 50S ribosomal subunit; (4) that the number of ribosomal binding sites for the enzyme is well defined. Poly A polymerase fails to meet each of these criteria, and it is concluded that the enzyme is an adsorbed contaminant of the ribosomes. A survey of other putative ribosomal enzymes suggests that none of them has been convincingly identified as a structural element of the ribosomes and, therefore, that they may also be contaminants.

It is as yet impossible to assign a function to any particular molecular component of the ribosomes. Indeed, the task is a formidable one since the ribosomes of *Escherichia coli* contain between 20 and 40 electrophoretically distinct protein components (Waller and Harris, 1961; Waller, 1964; E. C. Cox and J. G. Flaks, personal communication, 1966), as well as at least two ribonucleic acid (RNA) molecules (Littauer and Eisenberg, 1959; Kurland, 1960). In addition to these physically separable components there have been a number of reports of ribosomal enzymes (Elson, 1958, 1959; Bolton and McCarthy, 1959; Matheson and Tsai, 1965; Tsai and Matheson, 1965; Wade and Lovett, 1961; August *et al.*, 1962; McCorquodale, 1963). If some of the ribosomal proteins could be shown to have an enzymatic activity which could be assayed independently of the ribosome function in polypeptide

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synthesis, the analysis of ribosome structure and function would be greatly facilitated. However, a problem arises in determining whether or not an enzyme activity which is apparently associated with the ribosomes is really an activity of a ribosomal structural element or that of a strongly adsorbed contaminant.

The primary criterion for the identification of a ribosomal enzyme has been that such an enzyme is tightly bound to and predominantly associated with the ribosomes. However, Neu and Heppel (1964) have shown for ribonuclease that these criteria can be misleading. The present study was initiated to provide other simple but more stringent criteria for the identification of a ribosomal protein which has some assayable property such as an enzyme activity. The relationship of polyriboadenylic acid (poly A) polymerase to the structure and function of the ribosomes has been analyzed. The data show that this enzyme, which under some conditions is tightly bound to the ribosomes, fails to qualify as a ribosomal structural element. The application of the present criteria to other putative ribosomal enzymes has led to the conclusion that there are apparently no enzymes that can be convincingly identified as structural components of the ribosomes.

Materials and Methods

All of the procedures and materials used in the experiments described in this paper have been described in detail in the accompanying paper (Hardy and Kurland, 1966), except as follows.

Assay of Polyphenylalanine Synthesis. Reaction mixtures contained in a volume of 0.05 ml: 0.01 M Tris, pH 7.8, 0.06 M KCl, 0.011 M $MgSO_4$, 1 mM ATP,[1] 0.4 mM GTP, 4 μg of polyribouridylic acid (poly U) (Miles Laboratories, Elkhart, Ind.), 0.01 M β mercaptoethanol, 0.16 A_{260} unit of ribosomes, 0.9 A_{260} unit of [^{14}C]Phe soluble ribonucleic acid (s-RNA) (4150 cpm/A_{260} unit of RNA, 350 μc/μmole of Phe prepared as described in Kurland, 1966), and 1 μl of crude transfer factors (prepared by the procedure of Allende *et al.* (1964) through the ammonium sulfate fractionation). At the end of the incubation period the mixtures were quickly chilled, and 100 μg of bovine serum albumin and 3 ml of 5% trichloroacetic acid were added. The tubes were immersed in a boiling water bath for 15 min and the hot acid-precipitable material was collected by filtration and counted as described for the poly A polymerase assay (Hardy and Kurland, 1966). In this reaction mixture the concentration of ribosomes is the rate-limiting factor.

[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; A_{260} unit, unit of material which in a 1-ml volume and light path of 1 cm will have an optical density of 1 at 260 mμ; TSM, 0.01 M Tris–0.003 M succinic acid–0.01 M $MgSO_4$, pH 8.0; ADP, adenosine diphosphate; UDP, uridine diphosphate; AMP, adenosine monophosphate.

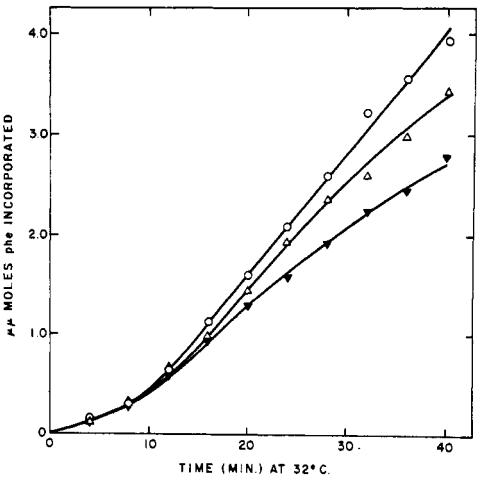

FIGURE 1: Various quantities of purified poly A polymerase were added to ribosomes and the mixtures were dialyzed overnight against TSM to remove the enzyme buffer. These ribosomes were used to mediate polyphenylalanine synthesis at 32°. The specific poly A polymerase activity of the ribosomes measured as mμmoles of AMP incorporated by a 30-min incubation at 32° per A_{260} unit of ribosomes was: O———O, <0.01; △———△; 1.15, ▼———▼, 2.06.

Results

The Effect of Poly A Polymerase on Polypeptide Synthesis. It has been previously shown that ribosomes which are free of detectable poly A polymerase activity are active in specific s-RNA binding (Kurland, 1966). Therefore, if poly A polymerase is a structural element of the ribosome, its presence on the ribosome is not necessary for either messenger ribonucleic acid (m-RNA) or s-RNA binding. Experiments were performed to determine whether the presence or absence of poly A polymerase influences the ability of the ribosomes to mediate specific polypeptide synthesis.

A limiting concentration of ribosomes (prepared as described in Kurland, 1966) and excess amounts of partially purified transfer enzyme (Allende *et al.*, 1964), both of which are free of detectable poly A polymerase activity, were used to mediate the synthesis of polyphenylalanine from [^{14}C]Phe s-RNA in the presence of poly U. The kinetics of polyphenylalanine synthesis were compared in the presence and absence of poly A polymerase (Figure 1). Two concentrations of poly A polymerase were employed in this experiment: (a) an amount of enzyme per ribosome equivalent to 0.72 times that obtained with the ribosome-bound poly A polymerase preparation (Hardy and Kurland, 1966), and (b) an amount of enzyme per ribosome equivalent to 1.4 times that obtained with the ribosome-bound poly A polymerase preparation. As can be seen from Figure 1, there is no significant effect on the initial rate of polyphenylalanine synthesis when poly A polymerase is added to the incubation mixtures. At later times of incubation there is a significant but small

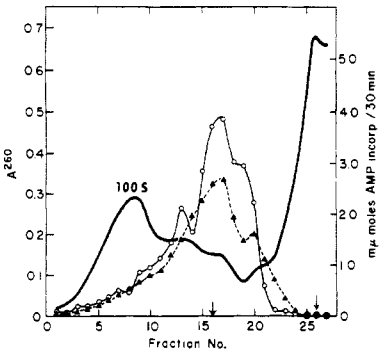

FIGURE 2: A crude extract (175 A_{260} units) of *E. coli* was subjected to sucrose density gradient zone centrifugation in TSM for 5 hr at 25,000 rpm in a Spinco SW 25.1 rotor. The A_{260} (———), poly A polymerase (▲——▲), and polynucleotide phosphorylase activities (O——O) of each fraction were measured. Polynucleotide phosphorylase activity was measured in a 0.2-ml reaction mixture containing 0.5 mM [8-^{14}C]ADP and 0.15 ml of the sucrose gradient fraction.

inhibition of peptide synthesis (less than 30%) in the presence of excess amounts of poly A polymerase. This inhibition can be attributed to a variety of effects, *e.g.*, complex formation between the poly U messenger and the poly A synthesized by the enzyme, or the inactivation of s-RNA by the poly A polymerase. The data suggest that poly A polymerase is not a necessary structural element of the ribosome since polypeptide synthesis can be mediated equally well in its presence or absence.

It is of course possible that there are functions for the ribosome which are not detectable in an experiment like that shown in Figure 1. For example, such an experiment does not exclude a function for poly A polymerase in polypeptide chain termination. Therefore, it was necessary to turn to more indirect methods to decide whether or not the poly A polymerase is a structural element of the ribosomes.

Association of Poly A Polymerase with Ribosomes. It has been shown previously by August *et al.* (1962) that most of the *E. coli* poly A polymerase is associated with the ribosomes. If the poly A polymerase were a structural element of the ribosomes, not only should this be the case but in addition it might be anticipated that the poly A polymerase would be associated with the various classes of ribosome aggregates (70, 100 S, etc.). However, when crude extracts of *E. coli* are fractionated by zone centrifugation and the individual fractions are assayed for poly A polymerase activity, this is not observed (Figure 2). Instead, it is seen that the major fraction of ribosomes sedimenting at 70 S or greater has little or no enzyme activity and the minor fraction sedimenting at less than 70 S has most of the enzyme activity. This experiment suggests that only a minor fraction of the ribosomes has poly A polymerase activity and that the aggregates which should be active in protein synthesis are essentially free of the enzyme. Such a result is consistent with the notion that the poly A polymerase is a contaminant adsorbed to the ribosomes.

It has been reported that up to 40% of the polynucleotide phosphorylase of *E. coli* extracts is associated with the ribosomes (Grunberg-Manago, 1963). The presence of this enzyme on the ribosomes is also shown in Figure 2. In order to be certain that the poly A polymerase assays are not distorted by the presence of polynucleotide phosphorylase, the Mn^{2+} dependence of poly A synthesis by selected sucrose gradient fractions was tested since it is known that polynucleotide phosphorylase activity is inhibited by Mn^{2+} (Littauer and Kornberg, 1957; Levin *et al.*, 1963; Babinet *et al.*, 1965). It was observed that the polynucleotide phosphorylase activity was inhibited by the conditions of the poly A polymerase assay (high Mn^{2+} and Mg^{2+}) and that the incorporation with ATP as substrate was stimulated at least threefold by the presence of Mn^{2+}. Therefore, it is concluded that the profile of poly A polymerase activity seen in Figure 2 is not significantly altered by the presence of polynucleotide phosphorylase in these fractions.

The observation (Grunberg-Manago, 1963) that a substantial fraction of polynucleotide phosphorylase is in the supernatant fractions seems to be contradicted by the data of Figure 2. Here, almost all of the polynucleotide phosphorylase appears to be associated solely with the ribosomes. This could be explained by the presence of an inhibitor in the slowly sedimenting fractions of the extract which antagonizes the polynucleotide phosphorylase assay. Such an inhibitor might also be responsible for the apparent absence of poly A polymerase activity in the nonribosomal fractions. Therefore, assays of poly A polymerase and polynucleotide phosphorylase were carried out with mixed aliquots of the highly active fraction 16 and fraction 26 near the gradient meniscus. The results (Table I) show

TABLE I: Supernatant Inhibitor of Enzymes.[a]

	Incorporation of AMP/30 Min			
	[^{14}C]ATP Substrate		[^{14}C]ADP Substrate	
Fraction	Cpm	mμmoles	Cpm	mμmoles
16	225	0.73	660	1.88
26	55	0.16	0	0
16 + 26	80	0.23	5	<0.02

[a] For the ATP substrate, standard poly A polymerase reaction mixtures (0.25 ml) contained 0.1 ml of each fraction or 0.1 ml of one fraction and 0.1 ml of a compensating sucrose solution. Reaction mixtures and the procedure were the same for the ADP substrate except that Mn^{2+} was omitted and the Mg^{2+} concentration was 8 mM.

that both of the enzyme activities present in fraction 16 are greatly inhibited by the presence of fraction 26 in the assay mixture. Because of the presence of this inhibitor it cannot be concluded from the experiment of Figure 2 that a majority of poly A polymerase activity is associated with the ribosomes. All attempts to inactivate the inhibitor without inactivating the poly A polymerase or polynucleotide phosphorylase have met with failure.

Although it has proved difficult to ascertain unambiguously whether most of the poly A polymerase of an *E. coli* extract is associated with the ribosomes, it is possible to determine whether or not the ribosome-bound enzyme is tightly bound. This was done by subjecting ribosome-bound poly A polymerase to zone centrifugation through a sucrose gradient in TSM. The A_{260}, poly A polymerase activity, and polynucleotide phosphorylase activity of each fraction were measured. While more than one-third of the very low polynucleotide phosphorylase activity is dissociated from the ribosomes in the course of centrifugation, all of the poly A polymerase activity remains tightly bound to them (Figure 3).

A further attempt was made to dissociate poly A polymerase from ribosomes by washing them repeatedly in TSM. In this experiment a crude extract of *E. coli* was made and the ribosomes were isolated and washed by five successive differential centrifugations in TSM. After each centrifugation an aliquot of the resuspended ribosome pellet was removed and assayed for poly A polymerase activity (Table II). After an initial increase,

TABLE II: Specific Enzyme Activity of Repeatedly Washed Ribosomes.[a]

Fraction	mμmoles of AMP Incorp in 30 Min/A_{260} Unit of Ribosomes
1st ribosomal pellet	1.35
2nd ribosomal pellet	1.81
3rd ribosomal pellet	1.59
4th ribosomal pellet	1.81
5th ribosomal pellet	1.70

[a] A crude extract of *E. coli* was centrifuged at 150,000*g* for 2 hr. The ribosomal pellet was resuspended in TSM and recentrifuged at 150,000*g* for 90 min. This cycle was repeated three more times. The A_{260} and poly A polymerase activity of each resuspended ribosome pellet were measured.

the specific enzyme activity of the ribosomes remains constant. Therefore, it is concluded that the poly A polymerase activity associated with ribosomes is firmly bound under these conditions.

Localization of Poly A Polymerase on 30- and 50S Subunits. When 70S ribosomes are dialyzed into buffer containing between 0.1 and 1 mM magnesium, a majority of the particles dissociate into 30- and 50S subunits (Tissières *et al.*, 1959). Therefore, experiments were performed to determine whether or not the poly A polymerase is specifically associated with one of the ribosomal subunits.

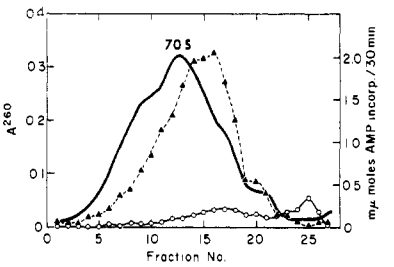

FIGURE 3: Ribosome-bound poly A polymerase (100 A_{260} units) was subjected to zone centrifugation as in Figure 2. The A_{260} (———), poly A polymerase (▲—▲), and polynucleotide phosphorylase activities (○——○) of each fraction were measured as in Figure 2.

Ribosome-bound enzyme was dialyzed overnight against 0.01 M Tris–0.003 M succinic acid–0.001 M $MgSO_4$, pH 8.0, and was then subjected to zone centrifugation. The A_{260} and poly A polymerase activity of each fraction were measured (Figure 4a). The poly A polymerase activity is associated with both the 30- and 50S ribosomal subunits as well as with the undissociated 70S ribosomes. As in the experiments reported in Figures 2 and 3, the specific enzyme activity of the subunits is greater than that of the intact 70S ribosomes. A control assay for poly A polymerase in the absence of manganese was carried out on each fraction (Figure 4a). The results show that all the AMP-incorporating activity associated with the 260-mμ absorbing material is stimulated by manganese and is, therefore, poly A polymerase and not polynucleotide phosphorylase. A small amount of activity, sedimenting more slowly than the 30S particles, is inhibited by manganese and is, therefore, probably polynucleotide phosphorylase. As a further control, a similar experiment was carried out to show the location of polynucleotide phosphorylase on the 30- and 50S ribosomal subunits. Each fraction of this gradient was assayed for polynucleotide phosphorylase with substrates of [^{14}C]ADP and [^{14}C]-UDP (Figure 4b). Approximately two-thirds of the polynucleotide phosphorylase activity sediments more slowly than the 30S ribosomal subunit. Comparison of Figures 4a and b shows that the presence of polynucleotide phosphorylase does not interfere significantly with the assay for poly A polymerase in these gradients. It can be concluded, therefore, that poly A polymerase is not specifically associated with one of the ribosomal subunits, but is present on both.

The Number of Poly A Polymerase Molecules per Ribosome. The data in Figure 2 clearly show that only a small fraction of the ribosomes in a crude bacterial

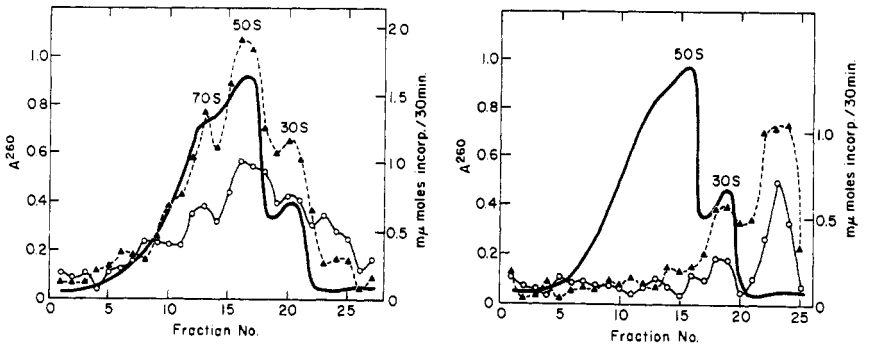

FIGURE 4: Ribosome-bound poly A polymerase was dialyzed overnight against TSM buffer containing 1 mM Mg^{2+}. Zone centrifugation was carried out as in Figure 2. (a) The A_{260} (———) and poly A polymerase activity of each fraction in the presence (▲—▲) and absence (○———○) of Mn^{2+} were measured. (b) The A_{260} (———) and polynucleotide phosphorylase activity with [^{14}C]ADP (○———○) and [^{14}C]UDP (▲—▲) as substrates were measured. Reaction mixtures contained 1 mM Mg^{2+}.

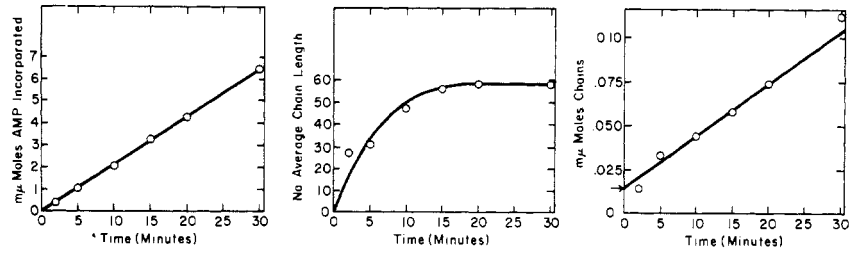

FIGURE 5: At zero time 80 A_{260} units of ribosome-bound poly A polymerase were added to a prewarmed reaction mixture (1.6 ml) containing 8.0 A_{260} units of s-RNA. At various times thereafter 0.2-ml aliquots were withdrawn and quickly chilled. Each (0.1 ml) was subjected to the standard enzyme assay procedure and the chain length of the product in the remaining portion was measured. (a) Shows the rate of AMP incorporation into acid-precipitable material. (b) Shows the rate of increase in the chain length. (c) Calculated from the data in a and b; shows the rate of increase of the number of poly A chains in 0.1 ml of reaction mixture.

extracts are associated with an active poly A polymerase molecule. This by itself would suggest that the enzyme was an adsorbed contaminant. However, it is possible that a significant fraction of the ribosome-bound poly A polymerase molecules are inactivated. Therefore, it seemed necessary to obtain a quantitative estimate of the fraction of ribosomes with an active poly A polymerase molecule and then to determine if this fraction is a unique one, *i.e.*, whether the number of ribosomal binding sites for the enzyme was well defined.

The number of active poly A polymerase molecules was estimated indirectly. This was done by determining the initial number of nascent poly A chains which should be equal to the minimum number of active enzyme molecules in the reaction mixture.

Two assumptions must be made for this analysis. First, it must be assumed that the rate of growth of any given chain of poly A is independent of its length. Second, the probability of the chain terminating with each new residue is also assumed to be independent of length. Under these two conditions, the number of poly A chains will increase linearly with time. If it is also assumed that all the molecules of enzyme are functioning all of the time, then the number of poly A chains initiated at time zero is equal to the number of enzyme molecules present in the reaction mixture. This naive model provides a method for estimating the *minimum* number of enzyme molecules per ribosome.

For measurement of the chain length, the poly A product of the reaction was hydrolyzed and subjected to electrophoresis as described previously (Hardy and Kurland, 1966). The average chain length of the product is taken to be the ratio of the radioactivity in 2′(3′)-AMP to that in adenosine. Contamination of the ribosome-bound poly A polymerase with nucleases could lead to a false estimate of the chain length of the reaction product. In particular, an endonuclease which cleaves a chain of poly A to give a 5′-phosphate end would lead to an underestimate of the average chain length while an exonuclease which removes 3′-AMP residues starting at the 3′-hydroxyl end would lead to an overestimate of the average chain length. However, contamination of the ribosome-

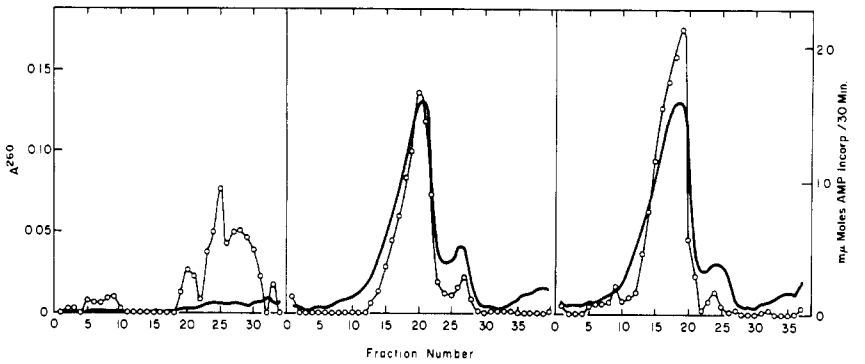

FIGURE 6: Zone centrifugation (5–20% sucrose in 0.1 M Tris–0.03 M succinic acid–0.01 M $MgSO_4$) for 2 hr at 35,000 rpm in the Spinco SW 39 rotor of poly A polymerase in the presence and absence of ribosomes: (a) 100 enzyme units of purified enzyme dialyzed for 2 hr against TSM containing 0.6 M ammonium sulfate; (b) 100 units of purified enzyme and 9 A_{260} units of ribosomes dialyzed for 2 hr against TSM; (c) 100 units of purified enzyme and 9 A_{260} units of ribosome-bound enzyme dialyzed for 2 hr against TSM. (———) A_{260}; (O———O) poly A polymerase activity.

bound enzyme preparation by such enzymes is probably minimal for the following reasons. (1) There are no nucleotides, released by hydrolysis of the poly A product of the reaction, characteristic of free 5′-phosphorylated ends (Hardy and Kurland, 1966). (2) The number of 5′-phosphorylated ends of the poly A detected by nearest neighbor analysis is approximately equivalent to the number of free 3′-hydroxyl ends (Hardy and Kurland, 1966). (3) When 40 μg of radioactive poly A was incubated with ribosome-bound poly A polymerase for 1 hr at 32° in a standard reaction mixture, less than 3% of the radioactivity became acid soluble.

An experiment was carried out to measure the rate of increase in the number of poly A chains. At time zero, a limiting quantity of ribosome-bound poly A polymerase was added to a standard reaction mixture containing saturating quantities of [^{14}C]ATP substrate and s-RNA primer. At various times thereafter aliquots were removed, chilled quickly, and divided into two portions. One of these portions was assayed for acid-precipitable radioactivity, and the chain length of the poly A present in the other portion was measured. The number of AMP residues incorporated into acid-precipitable material at any given time divided by the average number of residues per chain at that time gives the number of the poly A chains present in the reaction mixture. Figure 5a shows the linear time course of AMP incorporation into acid-precipitable material and 5b the increase of chain length with time. Figure 5c calculated from the data in 5a and b shows the increase in the number of poly A chains with time. The straight line drawn through the points in Figure 5c intercepts the zero time axis at a point corresponding to 0.014 mμmole of chains. However, other straight lines can be drawn which intercept between 0.023 and 0.010 mμmole of chains. Since these values are calculated for an amount of reaction mixture containing 0.33 mg of ribosomes or 0.12 mμmole (mol wt 2.7 × 10^6) (Tissières *et al.*, 1959), it is concluded that between 8 and 19% of the 70S ribosomes of this preparation carry an active enzyme molecule.

Before it can be concluded that the majority of ribosomes lack a poly A polymerase molecule, the possibility that enzyme molecules have been extensively lost or inactivated during the involved preparation of ribosome-bound enzyme must be examined. To do this, the poly A polymerase activity of ribosomes prepared from a crude *E. coli* extract by several cycles of differential centrifugation in TSM was measured (Table II) and compared with the activity of a normal preparation of ribosome-bound enzyme. The data in Table II show no evidence for loss of activity of the poly A polymerase associated with the ribosomes, during five cycles of centrifugation. The average specific activity of the ribosomes (last four cycles) is 1.73 mμmoles of AMP incorporated in 30 min/A_{260} unit, which is slightly greater than the 1.60 of the usual preparation of ribosome-bound enzyme. The ribosome-bound enzyme used for the experiments summarized in Figure 5 had a specific activity of 1.30 mμmoles of AMP incorporated in 30 min/A_{260} unit or 75% of the average specific activity of ribosomes in Table II. A corresponding adjustment of the results calculated from Figure 5c leads to the conclusion that between 11 and 25% of the 70S ribosome equivalents have an active enzyme molecule. It is, of course, still possible that poly A polymerase inactivation occurred while the *E. coli* cells were stored frozen or were being thawed prior to preparation of the enzyme. However, preparations of ribosome-bound enzyme from different batches of cells, stored for different periods of time, had about the same activity.

Since it appears that approximately four-fifths of the ribosomes do not carry an active poly A polymerase molecule, this enzyme cannot be part of the ribosome unless there are different classes of ribosomes. If this is the case, poly A polymerase should be present on

only a defined fraction of the ribosomes and any attempts to firmly bind more of the enzyme to ribosomes should be unsuccessful.

Such an attempt was made in the following way. Ribosomes and ribosome-bound poly A polymerase were treated with an excess of purified poly A polymerase. The ammonium sulfate present from the purified enzyme preparation was removed by dialysis for 2 hr against TSM, and the mixtures were subjected to zone centrifugation. A control of purified enzyme alone was treated in the same way except that dialysis was against TSM containing 0.6 M ammonium sulfate. The A_{260} and the poly A polymerase activity of each fraction were measured. The results (Figures 6b and c) show clearly that all of the active enzyme of the gradients is bound to ribosomal material. In the control (Figure 6a), three-quarters of the enzyme activity sediments in the upper third of the gradient while only 2% of the activity in the gradients containing ribosomes sediments in this region. The specific activity of the ribosome peaks in Figures 5b and c are 6.3 and 8.2 mμmoles of AMP incorporated in 30 min/A_{260} unit, respectively, or about four and five times that of a normal ribosome-bound enzyme preparation. Similar experiments have been carried out which give ribosome peaks with a specific activity of 24 mμmoles of AMP incorporated in 30 min/A_{260} unit of ribosomes or about 14 times that of the most active ribosome-bound enzyme preparation. Therefore, poly A polymerase binds firmly to ribosomes up to a level which corresponds to a minimum of between two and three molecules of active enzyme per 70S ribosome particle. Furthermore, in all the reconstruction experiments reported above, poly A polymerase was considerably inactivated and the yield of enzyme activity from the gradient was usually about 25%. If inactive enzyme also binds to ribosomes, then the value for the number of enzyme molecules per ribosome is as large as 10 enzyme molecules for each 70S ribosome.

These results show that the number of ribosomal binding sites for poly A polymerase is not well defined. Therefore, it is concluded that the enzyme is a firmly adsorbed contaminant of the ribosomes.

Discussion

The large number of proteins as well as enzymes which have previously been implicated as ribosomal elements present a bewildering complexity. The rigorous identification of these proteins is necessary if the detailed structure and function of the ribosomes is to be made intelligible. There are two gross techniques which can be employed for a preliminary analysis of this problem. First, for proteins which are known to be present in nonribosomal fractions, such as the supernatant proteins, it is possible to purify ribosomes and determine the extent of contamination by such proteins (Kurland, 1966). Second, for proteins which are thought to be ribosomal elements and which have some assayable property, such as an enzymatic activity, it is possible to purify the ribosomes and determine whether or not such proteins remain tightly bound to the ribosomes in the course of purification. In both cases, the final ribosome preparation must be functional, that is, capable of mediating polypeptide synthesis. This is necessary because at present the ribosome is most rigorously defined by its function in protein synthesis rather than as a physical entity. When it is possible to obtain functional ribosomes with a minimum contamination by supernatant proteins, then the conditions for the positive identification of a suspected ribosomal protein are most favorable. This has been attempted in the present study with the suspected ribosomal enzyme, poly A polymerase.

Ribosomes which are sedimented through buffer containing 0.6 M ammonium sulfate can be recovered in a fully active state (Kurland, 1966). However, the poly A polymerase is dissociated from the ribosome by this treatment (Hardy and Kurland, 1966). Furthermore, this procedure removes supernatant proteins which are normally recovered with the ribosomes (Kurland, 1966). Since the poly A polymerase is removed from the ribosomes under the same conditions that remove strongly adsorbed supernatant proteins, it would appear that the poly A polymerase is an adsorbed contaminant of the ribosomes.

This conclusion is underscored by the repeated failure to obtain any functional relationship between the ribosomes and poly A polymerase. Thus, messenger RNA and s-RNA binding is mediated by ribosomes which have no detectable poly A polymerase activity (Kurland, 1966). The data presented in this paper suggest that ribosome-mediated polypeptide synthesis does not require the presence of poly A polymerase. Conversely, the enzymatic properties of partially purified poly A polymerase are virtually identical with those of the ribosome-bound enzyme (Hardy and Kurland, 1966).

Most of the electrophoretically identifiable proteins of the ribosomes from *E. coli* are specific for either the 30- or 50S subunits (Waller, 1964; E. C. Cox and J. G. Flaks, personal communication, 1966; Traub *et al.*, 1966). Furthermore, the two subunits appear to be functionally distinct (Gilbert, 1963a; Okamoto and Takanami, 1963; Watson, 1963). From these observations it would be anticipated that a ribosomal enzyme should be specifically associated with either the 30- or the 50S subunit but not, in general, with both subunits. Therefore, the observation that poly A polymerase is associated with both the 30- and 50S subunits suggests that the enzyme is a contaminant of the ribosomes.

It is generally assumed that all of the ribosomes in *E. coli* are the same. If this is the case, it would follow that there would be some integral number of poly A polymerase molecules for each 70S ribosome. However, it is observed that only a minor fraction of the ribosomes has an active poly A polymerase molecule bound to them and control experiments show that this small fraction of ribosome-bound poly A polymerase is not the result of extensive loss of enzyme during purification. In addition, purified

TABLE III: Other Ribosomal Enzymes.[a]

Enzyme	Criteria				Ref
	Functional Relationship	Firm Assocn with Ribosomes	30 or 50 S	Well-Defined No. of Binding Sites	
Poly A polymerase	−	−	−	−	This paper
Ribonuclease I	−	+	+	−	Elson and Tal (1959); Neu and Heppel (1964); Spahr and Hollingworth (1961)
Ribonuclease II	0	−	0	0	Spahr (1964)
Deoxyribonuclease	0	−	0	0	Tal and Elson (1961, 1963)
Polynucleotide phosphorylase	0	−	0	0	This paper; Wade and Lovett (1961)
Acid phosphatase	0	+	−	0	Spahr and Hollingworth (1961)
Amino peptidase	0	+[b]	−	0	Bolton and McCarthy (1959); Matheson and Tsai (1965); Tsai and Matheson (1965); McCorquodale (1963)

[a] + = meets criterion; − = fails to meet criterion; 0 = no data. [b] A. T. Matheson (personal communication) reports that washing ribosomes in Tris buffer containing 0.6 M ammonium sulfate almost completely removes their leucylglycinase.

enzyme can be firmly adsorbed to the ribosomes in amounts far exceeding that normally found associated with the ribosomes isolated from crude extracts. Since the number of ribosomal binding sites for poly A polymerase does not seem to be well defined, it is concluded that the enzyme is a contaminant.

In summary, four reasonable criteria have been used to test the notion that poly A polymerase is a structural element of the ribosomes: (1) that there is some functional relationship between the enzyme and the ribosomes; (2) that the enzyme remains associated with the ribosomes under conditions which remove strongly adsorbed supernatant proteins and yield active ribosomes; (3) that the enzyme is specifically associated with either the 30- or 50S subunit; (4) that the number of ribosomal binding sites for the enzyme is well defined. The enzyme has failed to meet each of these criteria. It is, therefore, concluded that poly A polymerase is not a ribosomal enzyme. When these four criteria are applied to other putative ribosomal enzymes (Table III), these, too, fail to qualify as structural elements of the ribosomes. We are, therefore, forced to the conclusion that there are no enzymes which have been convincingly identified as ribosomal structural elements.

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Inhibition by Actinomycin D of Valine Incorporation into Specific Proteins of Rat Pancreas *in Vivo**

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ABSTRACT: Valine incorporation was used to study protein biosynthesis in rat pancreas. Incorporation into pancreas proteins was reduced to 50% by actinomycin D within 4 hr; meanwhile incorporation into nuclear proteins was drastically diminished. Cytoplasmic material was separated into acidic and basic proteins. Inhibition by actinomycin D of valine incorporation into acidic proteins (chymotrypsinogen and trypsinogen 2) was two to three times as great as the inhibition of incorporation into basic proteins (amylase and ribonuclease). Results were interpreted in terms of messenger ribonucleic acid stability.

Ribonucleic acid biosynthesis in bacterial and animal cells has been shown to be inhibited by actinomycin D (Reich *et al.*, 1961; Levinthal *et al.*, 1962; Revel and Hiatt, 1964; Penman *et al.*, 1963; Scott and Bell, 1964). This antibiotic appears to react by forming a stable complex with some of the guanosine groups of deoxyribonucleic acid (DNA) (Goldberg and Reich, 1964), thereby impairing the transcription process. In bacteria, the inhibition of ribonucleic acid (RNA) synthesis produces a rapid decrease in protein biosynthesis. The messenger ribonucleic acid (m-RNA) that governs this synthesis is quite unstable; its half-life is measured in minutes (Levinthal *et al.*, 1962; Kepes, 1963). It soon becomes the limiting factor in protein biosynthesis, which stops after about 15 min in *Bacillus subtilis*. In mammals, RNA biosynthesis is also blocked by actinomycin D, but protein biosynthesis is less rapidly affected. The biosynthetic system (polysomes) is much more stable and can, in certain instances, remain operative for several days.

In order to study polysome stability in the absence of RNA synthesis, two types of experiment have been performed using actinomycin D. In the first set of experiments (Penman *et al.*, 1963; Staehelin *et al.*, 1963) polysomes were isolated by sucrose gradient centrifugation at various times after injection of antibiotic, and the ratio of polysomes to ribosomes was measured. In the second type of experiment (Revel and Hiatt, 1964; Bloom *et al.*, 1965), the amount of polysomes was determined by the incorporation of labeled amino acids into proteins.

Ribosomal and transfer ribonucleic acids (t-RNA's) are stable both in bacterial and animal cells (Levinthal *et al.*, 1962; Scherrer *et al.*, 1963). The amount of polysomes in such cells is thought to be determined by the amount of available m-RNA that serves as a connecting link between the ribosomes. Penman *et al.* (1963) working with HeLa cell cultures and Staehelin *et al.* (1963) with rat liver have shown that the heavy polysome fraction decreases after exposure to actinomycin D. This is accompanied by an increase in the 73S ribosome peak, suggesting the breakdown of polysomes into ribosomes. In HeLa cells, m-RNA is unstable; its half-life is about 3–4 hr. The m-RNA of liver is reported to have a longer half-life.

The experiments using amino acid incorporation

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