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Protein Biosynthesis in the Spleen. V. Increase in Poly(uridylic acid) Binding Factor Following Primary Immunization†

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ABSTRACT: Ribosomes extracted from the spleens of immunized rats are more active in an *in vitro* protein synthesizing system than similar extracts from spleens of unimmunized controls. Part of this enhanced activity can be explained by increased amounts of the elongation factor EF-1 and by decreased amounts of ribosomal-bound ribonuclease in the immunized preparations. This communication describes the partial purification of a poly(uridylic acid) binding factor from a KCl extract of rat spleen microsomes that is present in greater amounts in immunized spleens than in controls. The major poly(U) binding protein of spleen does not appear to be identical with the major poly(U) binding protein of liver. Reconstitution of "stripped" ribosomes with microsomal KCl washes restores some of the poly(U) binding activity, but has no apparent effect on the polymerization ability of the ribosomes.

The initial step in the process of protein biosynthesis is the binding of messenger RNA to the small ribosomal subunit, yet little is known about the attachment process at the molecular level. In bacteria, the binding of natural messenger RNA containing the initiator codon AUG at 4 mM Mg^{2+} to the native 30S ribosomal subunit is catalyzed by initiation factor IF-3. The binding of *N*-formylmethionyl-tRNA is promoted by IF-2. The 50S subunit is joined to the tRNA–messenger–30S complex for the initiation of protein synthesis (Pestka and Nirenberg, 1966). The importance of initiation factors in this process is well documented, for ribosomes lacking initiation factors are incapable of binding natural mRNA (Brown and Doty, 1968; Brawerman *et al.*, 1969).

Despite the fact that the synthetic polyribonucleotide, poly(uridylic acid), contains no initiating codon, its reaction

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with ribosomes has been used as a model system for elucidating some of the characteristics of, and requirements for, binding of mRNA to ribosomes (Moore, 1966a,b; Takanami and Okamoto, 1963; Castles, 1969a,b; Roberts and Coleman, 1971). The binding of poly(U) is greatest at elevated Mg^{2+} concentrations and at lower temperatures (Krahn and Paranchyh, 1970). Poly(U) binds to 70S ribosomes as well as 30S subunits and does not require any of the three known initiation factors at 8–20 mM Mg^{2+} (Brown and Doty, 1968; Roberts and Coleman, 1971). However, at lower levels of Mg^{2+} (2–4 mM) at least two of the initiation factors, as well as *N*-acetylphenylalanyl-tRNA, are required for poly(U)-directed synthesis of polyphenylalanine (Lucas-Lenard and Lipmann, 1967). Recently, factors that promote the binding of poly(U) to *Escherichia coli* ribosomes at high Mg^{2+} concentrations have been discovered (Smolarsky and Tal, 1970a,b; Brot *et al.*, 1970; Kan *et al.*, 1971; Van Duin and Kurland, 1970). The relationship of these factors to initiation factors is uncertain.

Initiation of protein synthesis in eukaryotic systems, at least in reticulocytes, has been shown to be quite similar to that in bacteria. Three analogous initiation factors, extracted from reticulocyte ribosomes with high concentrations of monovalent cations, have been described (Prichard *et al.*, 1970). There is, in addition, a methionyl-tRNA species specific for the initiating codon (Smith and Marcker, 1970). As in bacteria, the Mg^{2+} optimum for poly(U)-dependent polypeptide synthesis with salt-washed ribosomes is 8–12 mM Mg^{2+}. The (Mg^{2+}) optimum shifts to lower values in the presence of a salt wash from the crude ribosomes. This wash contains initiation and other factors (Miller and Schweet, 1968). No requirement for an N-blocked phenylalanyl-tRNA has been demonstrated, probably because the formylation of the amino group of mammalian initiator methionine-tRNA is unnecessary (Brown and Smith, 1970).

The control of protein synthesis at the translational level has been demonstrated in a wide variety of eukaryotic cells. Ribosomal particles interact not only with specific mRNA but also with tRNAs and many initiation, elongation, and termination factors. The complexity of these events provides many possible regulatory steps. The model system that has been used in this laboratory for studying the factors responsible for increased efficiency of translation of genetic information has been the primary immune response in the rat spleen following an injection of *Salmonella* flagellar antigen. We have previously shown that ribosomes from immunized rat spleens contain decreased amounts of ribosomal-bound ribonuclease (Willis and Starr, 1972). In addition, pH 5 supernatants from immunized rat spleens contain appreciably more of the polypeptide chain elongation factor transferase I (EF-1)[1] than identical supernatants prepared from control spleens (Willis and Starr, 1971). In the present study we investigated the initial step in posttranscriptional events, *i.e.*, the interaction of ribosomes with template RNA.

Materials and Methods

Triton X-100 was obtained from the Sigma Chemical Corporation, St. Louis, Mo. [^{3}H]Poly(uridylic acid) (sp act. 8.1 μCi/mmol of P) was purchased from Schwarz/Mann, Orangeburg, N. Y. NCS reagent was a product of Amersham Searle, Chicago, Ill. Omnifluor and L-[^{3}H]phenylalanine were purchased from New England Nuclear Corp., Boston, Mass. Puromycin dihydrochloride was a product of Nutritional Biochemicals, Cleveland, Ohio. Millipore filters (type HAWP, 0.45 μ pore size) were obtained from the Millipore Filter Corporation, Bedford, Mass. Poly(uridylic acid) and poly(adenylic acid) were the products of Miles Laboratories, Kankakee, Ill. Carboxymethylcellulose was purchased from Bio-Rad Laboratories, Richmond, Calif. Stripped *Escherichia coli* B tRNA was obtained from General Biochemical Inc., Chagrin Falls, Ohio.

Immunization of Animals. Male Sprague-Dawley rats (150–200 g) received a primary immunization of a single intravenous injection of 20 μg of *Salmonella oranienberg* flagella. The animals were killed 72 hr after immunization and the spleens were excised and placed in cold homogenizing buffer which consisted of 0.1 M Tris-HCl (pH 7.8), 5 mM magnesium acetate, 0.06 M KCl, 0.25 M sucrose, and 1 mM dithiothreitol. Standard buffer has the same composition as homogenizing buffer, but with sucrose omitted. Spleens from unimmunized rats were used as controls (Lazda and Starr, 1965).

Preparation of Ribosomes. Approximately 5 g of spleen or liver tissue (wet weight) was minced and homogenized in two volumes of homogenizing buffer. Nuclei and cellular debris were removed by a 5-min centrifugation at 1500*g*. Postnuclear supernatant was then centrifuged for 10 min at 7000*g*, a force sufficient to pellet mitochondria and lysosomes (Burghouts *et al.*, 1970). One-tenth volume of a mixture of 1% Triton X-100 and 5% sodium desoxycholate was added to the postmitochondrial supernatant. The crude ribosomes were collected by centrifugation for 1 hr at 65,000 rpm in the type 65 rotor of the Spinco Model L2-65B ultracentrifuge. The crude ribosomal pellets were resuspended in 9 ml of homogenizing buffer and layered over a 0.5-ml cushion of standard buffer containing 60% sucrose. After a 1-hr spin at 4° at 65,000 rpm, the ribosomes formed a loosely packed pellet under the 60% sucrose layer. The upper 9 ml was removed by aspiration and the ribosomes gently resuspended in the bottom 0.5 ml plus an additional 1 ml of standard buffer. A portion of the ribosomes was frozen at −70° as a source of "buffer-washed ribosomes." The KCl concentration of the remainder of the ribosomes was adjusted to 1 M and the total volume of the ribosomal suspension was brought to 9 ml with 1 M KCl in homogenizing buffer. The ribosomes were pelleted into a sucrose cushion as before. All centrifugation steps after increasing the salt concentration were carried out at 25° to prevent the aggregation of ribosomal subunits which occurs at lower temperatures (Martin *et al.*, 1970). The salt-washed ribosomes were resuspended in 5 ml of buffer with 0.5 M NH_4Cl and 1 mM puromycin dihydrochloride. The ribosomes were incubated for 30 min at 37° to strip them of any peptidyl-tRNA. The ribosomal suspension was then layered over a discontinuous sucrose gradient consisting of 5 ml of 1 M sucrose–0.5 M NH_4Cl in standard buffer and 20 ml of 0.5 M sucrose–0.5 M NH_4Cl in standard buffer, and centrifuged 16–18 hr at 22,500 rpm in the SW-25 rotor at 25°. The ribosomal pellets were resuspended in 0.5 ml of homogenizing buffer and frozen at −70°. RNA concentrations were determined by the method of Fleck and Munro (1962).

Preparation of Microsomal Washes. The postmitochondrial supernatants of control and immunized spleen homogenates were centrifuged for 1 hr at 65,000 rpm to pellet the microsomes. Microsomes rather than ribosomes were used because sodium deoxycholate and Triton are inhibitory to many RNA

[1] Abbreviations used are: IF, initiation factor; EF-1, elongation factor transferase I; hign binding buffer, 0.05 M potassium phosphate (pH 7.4)–0.05 M KCl–0.006 M $MgCl_2$; low binding buffer, 0.01 M Tris (pH 7.5)–0.05 M KCl–0.008 M $MgCl_2$.

binding factors (Baltimore and Huang, 1970; Schweiger and Hanning, 1971). The microsomal pellet was resuspended in 10 ml of buffer M (0.25 M sucrose–50 mM Tris-HCl (pH 7.5)–35 mM KCl–1 mM dithiothreitol–0.1 mM $MgCl_2$) and washed by a second ultracentrifugation. The microsomes were then suspended in 5 ml of buffer M containing 1 M KCl and stirred at 0° for 15 min. The microsomal suspension was then spun at 4° for 1 hr at 50,000 rpm in the SW-50 rotor. The resulting KCl supernatant was dialyzed overnight at 4° against 0.1 M sodium acetate buffer (pH 4.4) containing 0.2 M KCl. The dialyzed KCl washes were centrifuged 15 min at 10,000*g* to remove the precipitate and then concentrated to 2 ml in an Amicon ultrafiltration apparatus with a PM10 filter. Protein concentrations of the supernatant were determined by the Lowry method (Lowry *et al.*, 1951) and the wash fractions were frozen at −70°.

Measurement of Poly(uridyiic acid) Degradation. Ribosomes (100 μg of ribosomal RNA) or ribosomal wash (50 μg of protein) was incubated in 0.5 ml of high binding buffer with 0.1 μCi of ^{3}H-labeled poly(U) (~2 μg) for 10 min at 37°. The tubes were chilled to 0° and 500 μg of stripped *E. coli* B tRNA was added as a marker. The incubation mixtures were layered on top of an 11.5-ml 5–20% linear sucrose gradient in buffer high B. The gradients were spun for 24 hr at 40,000 rpm in the SW-41 rotor of a Spinco Model L2-65B ultracentrifuge. The centrifuged gradients were analyzed by passing through the flow cell of a Gilford recording spectrophotometer with the wavelength set at 260 nm. Approximately twenty 0.6-ml samples were collected from each gradient. Each sample was mixed with 15 ml of a scintillation cocktail consisting of Omnifluor in 90% toluene–10% Triton X-100, and was counted in a Nuclear-Chicago Mark I liquid scintillation spectrometer equipped with an external standard. The pellets were dissolved in 0.5 ml of NCS and counted in 15 ml of an Omnifluor–toluene solution. Ribonuclease activity of the preparation was also measured by degradation of purified yeast RNA (Shortman, 1962).

Carboxymethylcellulose Column Purification of Binding Factors. Plastic or Siliclad-coated glassware was used in the preparation of factors from microsomal washes because of the tendency of such factors to stick to glass (Rourke and Heywood, 1972). One–two milliliters of concentrated microsomal wash containing 2–10 mg of protein in 0.1 M sodium acetate and 0.2 M KCl buffer (pH 4.4) was placed on a 1.5 × 25 cm carboxymethylcellulose column previously equilibrated with the same buffer. The column was washed with 50 ml of this starting buffer and the column was then eluted with a 500-ml linear gradient of 0.2–0.8 M KCl in 0.1 M sodium acetate buffer (pH 4.4). Five-milliliter fractions were collected, pooled in groups of ten, and concentrated to 2 ml. The fractions were then dialyzed overnight against 200 volumes of high B binding buffer (see below), and 0.5 ml of the dialyzed fractions was used in the binding assay.

[^{3}H]Poly(uridylic acid) Binding Assay. The binding of radioactive poly(U) was measured by the alkali Millipore filter method of Smolarsky and Tal (1970a,b). The reaction mixture consisted of 0.5 ml of high binding buffer (0.05 M potassium phosphate (pH 7.4)–0.05 M KCl–0.006 M $MgCl_2$) (Roberts and Coleman, 1971) to which were added 0.005 ml of [^{3}H]poly(U) (50 μCi, ~2 μg), and ribosomal wash protein or ribosomes as indicated. In some experiments, a low binding buffer containing 0.01 M Tris (pH 7.5), 0.05 M KCl, and 0.008 M $MgCl_2$ was used (Smolarsky and Tal, 1970a,b). Samples were incubated for 10 min in the presence of [^{3}H]-poly(U), diluted with 5 ml of cold binding buffer, and filtered through alkali-treated Millipore filters. The filters were washed with 10 ml of binding buffer, allowed to dry, and then counted in 15 ml of an Omnifluor–toluene scintillation solution in a Nuclear-Chicago Mark I liquid scintillation spectrometer. A blank sample without ribosomes or protein was routinely prepared and the counts subtracted from all values reported.

[^{3}H]Polyphenylalanine Synthesis. The preparation of the liver elongation factors (EF-1 and EF-2) and the charging of *Escherichia coli* B tRNA with phenylalanine have been previously described (Willis and Starr, 1971). Ribosomes (50 μg of rRNA) were preincubated at 0 or 37° with 50 μg of poly(U) and 5 μg of stripped *E. coli* B tRNA (Culp *et al.*, 1970) in 0.2 ml of a buffer consisting of 0.005 M Tris-HCl (pH 7.5), 0.07 M KCl, and 8 or 4 mM $MgCl_2$. After 10 min the tubes were chilled and 100 μg of poly(adenylic acid) was added to prevent further functional binding of poly(U) (Williamson, 1969). A solution (0.2 ml) containing 0.05 M Tris-HCl (pH 7.5), 0.07 M KCl, 2 mM dithiothreitol, 200 μg/ml of [^{3}H]-phenylalanyl-tRNA, 100 μg/ml of transferase I protein, 300 μg/ml of transferase II protein, 0.2 mM GTP, and $MgCl_2$ as indicated was then added to each reaction tube. The samples were incubated for an additional 10 min after which the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. The samples were heated at 90° for 10 min, washed twice with 5 ml of 5% trichloroacetic acid, and once with 5 ml of 70% ethanol. The precipitates were dissolved in 0.6 ml of NCS reagent. A 0.5-ml aliquot of this solution was mixed with 15 ml of Omnifluor in toluene and counted in a Nuclear-Chicago Mark I liquid scintillation spectrometer equipped with an external standard.

Reconstitution of Salt-Washed Ribosomes. The following method was adopted to reconstitute "stripped" ribosomes with the protein that had been removed by the high-salt wash. The washes were dialyzed overnight in the cold against standard buffer and the precipitate which formed was removed by spinning 10 min at 10,000*g*. The dialyzed wash proteins were concentrated tenfold in an Amicon ultrafiltration apparatus using a PM10 filter. The proteins were incubated for 10 min at 37° with "stripped" ribosomes at approximately the same ratio at which they were eluted (0.5–0.6 mg of protein/mg of ribosomal RNA). The "reconstituted" ribosomes in a 1-ml volume were layered atop 9 ml of homogenizing buffer and sedimented by spinning 1 hr at 65,000 rpm. The pellets were carefully rinsed off and resuspended in homogenizing buffer at a concentration of approximately 2–3 mg of RNA/ml.

Results

Dependence of [^{3}H]Poly(U) Binding on Ionic Strength and [Mg^{2+}]. The physical binding of radioactive poly(uridylic acid) to ribosomes is influenced by a large number of factors. Table I demonstrates the binding of labeled poly(U) to rat liver ribosomes as a function of ionic strength of the assay buffer, magnesium concentration, and the state of ribosomal purification. Binding to ribosomes washed in standard buffer was identical in both assay buffers in the absence of added magnesium. Binding increased in both buffers with increasing magnesium concentration, but, generally, binding was greater in the buffer of lower ionic strength. It is interesting to note that in low binding buffer the binding of poly(U) to stripped ribosomes was considerable in the presence of added Mg^{2+}, and the stripped ribosomes bound [^{3}H]poly(U) more avidly than the buffer-washed ones. In high binding buffer, which

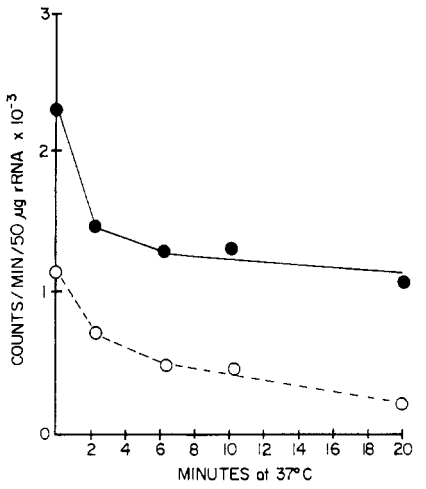

FIGURE 1: Kinetics of [^{3}H]poly(uridylic acid) binding to buffer-washed ribosomes; 50 μg of rRNA was incubated with 0.05 μCi of [^{3}H]poly(U) in 0.5 ml of high binding assay buffer for 10 min at 37°. Samples were filtered and washed as described under Materials and Methods: (●—●) immunized; (○- - -○) control.

more nearly approximates the conditions of *in vivo* and *in vitro* protein synthesis (Roberts and Coleman, 1971), stripped ribosomes bound insignificant amounts of poly(U) at low magnesium concentrations and, at higher concentrations, were able to bind only about 10% of that bound by ribosomes washed only with standard buffer. Because high binding buffer discriminated between ribosomes which still retained binding factors and ribosomes which had been washed free of such factors, it was used in the remainder of the experiments to be described.

Binding of [^{3}H]Poly(U) to Control and Immunized Spleen Ribosomes. The binding of [^{3}H]poly(uridylic acid) to spleen ribosomes was measured as a function of both ribosome purification and temperature (Table II). At 0°, the temperature at which the interaction between poly(U) and ribosomes is usually studied, buffer-washed ribosomes from immunized spleens bound about 50% more poly(U) than similar preparations from control spleens. Stripping the ribosomes of peptidyl-tRNA by incubating with puromycin, plus washing them with 1 M KCl, caused binding of poly(U) to be reduced to negligible levels in both control and immunized ribosomes. When ribosomes and poly(U) were incubated at 37°, there was a decrease in the amount of poly(U) bound by both kinds of ribosomes. The decrease was much greater for the control ribosomes than for the immunized, resulting in an apparent fourfold greater binding activity of the immunized ribosomes. The decreased binding of poly(U) to control ribosomes at 37° could be attributed to the greater ribonuclease content of the control ribosomes. We have previously reported that ribosomes from control spleens have a higher ribonuclease activity than those from immunized spleens (Willis and Starr, 1972). Ribonuclease was measured by the appearance of acid-soluble fragments from yeast RNA (Shortman, 1962). There was no ribonuclease activity or breakdown of poly(U) at 0°.

TABLE I: Dependence of [^{3}H]Poly(uridylic acid) Binding to Liver Ribosomes on Ionic Strength and Mg^{2+} Concentration.[a]

	ng of [^{3}H]Poly(U) Bound/50 μg of rRNA			
	Low-Binding Buffer		High-Binding Buffer	
[Mg^{2+}] (mM)	Std Buffer-Washed Ribosomes	Stripped Ribosomes	Std Buffer-Washed Ribosomes	Stripped Ribosomes
0	145 ± 6	6 ± 1	154 ± 3	2 ± 0.3
4	432 ± 16	315 ± 5	229 ± 5	7 ± 1
8	475 ± 17	439 ± 8	265 ± 10	19 ± 2
12	417 ± 16	605 ± 15	332 ± 7	43 ± 3

[a] Liver ribosomal RNA (50 μg) was incubated for 10 min at 37° with 0.05 μCi of [^{3}H]poly(uridylic acid) in 0.5 ml of assay buffer containing the concentrations of Mg^{2+} indicated. The samples were diluted with 5 ml of cold assay buffer adjusted to the proper [Mg^{2+}], filtered through KOH-treated Millipore filters, and washed with 10 ml of assay buffer. A blank value for the [^{3}H]poly(U) bound to the filters in the absence of ribosomes was subtracted from each sample.

TABLE II: Binding of [^{3}H]Poly(uridylic acid) to Control and Immunized Spleen Ribosomes.[a]

	ng of [^{3}H]Poly(U) Bound/50 μg of rRNA			
	0°		37°	
Ribosomes	Control	Immunized	Control	Immunized
Standard buffer washed	342 ± 10	499 ± 15	78 ± 6	303 ± 4
Stripped	16 ± 4	18 ± 3	17 ± 2	20 ± 3

[a] Assay was performed in high binding assay buffer as described under Materials and Methods and in the legend to Table I. The magnesium concentration was 6 mM.

The kinetics of binding at 37° tend to confirm that both preparations were contaminated with nuclease (Figure 1). At the end of 20-min incubation at 37°, binding to immunized ribosomes was reduced to approximately half that bound at the first time interval measured. In contrast, binding to control ribosomes was reduced more than 80%.

It should be noted that the initial binding activity of control ribosomes was only 50% of that of immunized ribosomes. The binding recorded at time zero took place at 0°, where ribonuclease activity is undetectable. Therefore, the differences observed under these conditions suggest the presence of larger amounts of a poly(U) binding factor on the immunized ribosomes. The negligible binding of poly(U) to salt-washed ribosomes, which contain no measurable nuclease at any temperature (Willis and Starr, 1972), must also be explained on the basis of the absence of binding factors rather than digestion of bound polynucleotide.

Binding of [^{3}H]Poly(uridylic acid) to Microsomal Wash Protein. Since ribosomes washed with high concentrations of KCl were unable to bind [^{3}H]poly(U) (Table II), the KCl washes themselves were analyzed for poly(U) binding activity. Figure 2 illustrates that the KCl washes did form complexes with [^{3}H]poly(U) which were retained on Millipore filters. The reaction was linear at low concentrations of wash protein, and, per milligram of protein, the immunized microsomal wash contained more binding activity than the control. Although the ribosomal-bound ribonuclease could be recovered in a semiquantitative manner from the 1 M KCl washes of the ribosomes (Willis and Starr, 1972) there was no nuclease found in the soluble extract after overnight dialysis

TABLE III: [^{3}H]Poly(uridylic acid) Degradation by Ribosomes and Microsomal Washes.[a]

Preparation	% Total Radioactivity	
	Smaller than 3 S	Pellet
Blank (no ribosomes or wash)	8.0 ± 0.02	12.2 ± 0.2
Buffer-washed control ribosomes	38.3 ± 1.4	6.0 ± 0.4
Buffer-washed immunized ribosomes	13.6 ± 0.9	17.4 ± 0.9
Stripped control ribosomes	8.7 ± 0.7	10.6 ± 0.8
Stripped immunized ribosomes	9.2 ± 1.1	11.1 ± 0.9
Control microsomal wash (undialyzed)	53.1 ± 6.1	5.3 ± 0.8
Immunized microsomal wash (undialyzed)	17.5 ± 3.4	9.6 ± 1.1
Control microsomal wash (dialyzed)	9.2 ± 0.3	12.0 ± 0.3
Immunized microsomal wash (dialyzed)	8.6 ± 0.4	13.9 ± 0.5

[a] [^{3}H]Poly(uridylic acid) (0.1 μCi/2 μg) was incubated for 10 min at 37° in 0.5 ml of high binding buffer with ribosomes (100 μg of ribosomal RNA) or wash (50 μg of protein). Conditions are as described under Materials and Methods. *E. coli* B stripped tRNA was used as a size marker.

against buffers at lower ionic strength. The nuclease probably precipitated during dialysis, but this possibility has not been tested due to the extreme insolubility of the precipitate, even in 1 M KCl (unpublished observations). Nuclease contamination of the dialyzed extracts also appeared unlikely since higher concentrations of control protein did not result in a leveling off of the curve, as might be expected if the concentration of an inhibitor of the reaction were increased. The curve representing the binding of [^{3}H]poly(U) by immunized microsomal wash proteins did become nonlinear at higher concentrations of protein, undoubtedly because the concentration of [^{3}H]poly(U) was limiting in this region.

Previous analysis by sucrose gradient velocity sedimentation had demonstrated that our [^{3}H]poly(U) preparation consisted of a mixture of polynucleotides of heterogeneous molecular weights, with the predominant species slightly smaller than that of the 4S tRNA marker. Approximately 12% of the input radioactivity of the poly(U) was of large enough size to pellet through the gradient after 24 hr, but only 8% was smaller than the estimated 3S position of the gradient (Willis and Starr, 1972). Table III shows the percentages of radioactive poly(U) which were degraded to species smaller than 3 S after incubation with the various preparations, as well as the percentage found in the pellet. The counts in the pellet represent a combination of undegraded, unbound large molecular weight poly(U) as well as ribosomal bound polynucleotide of undetermined size. While the buffer washed ribosomes and the undialyzed washes contained poly(U) degrading activity (control more than immunized), incubation of [^{3}H]poly(U) with stripped ribosomes or dialyzed microsomal KCl washes did not result in the appearance of small molecular weight species greater than the blank value.

Binding of [^{3}H]Poly(uridylic acid) to Reconstituted Ribosomes. An attempt was made to determine if the protein removed from spleen microsomes with high concentrations of salt could be reassociated with stripped, salt-washed liver ribosomes and thereby stimulate poly(uridylic acid) binding. Liver ribosomes were chosen to avoid any influence on the reaction by the state of immunization of the ribosomes. Preliminary experiments indicated that the amount of protein removed by 1 M KCl from ribosomes per milligram of ribosomal RNA was between 0.5 and 0.6 mg and was independent of the animals' condition of immunization. Therefore, between 0.5 and 0.6 mg of wash protein was incubated with an equivalent amount of stripped liver ribosomes for 10 min at 37°. The ribosomes were subsequently reisolated by ultracentrifugation. A "blank" sample of ribosomes was incubated with the same volume of standard buffer and reisolated to indicate the effect of the treatment alone on poly(U) binding. As can be seen in Table IV, incubation with

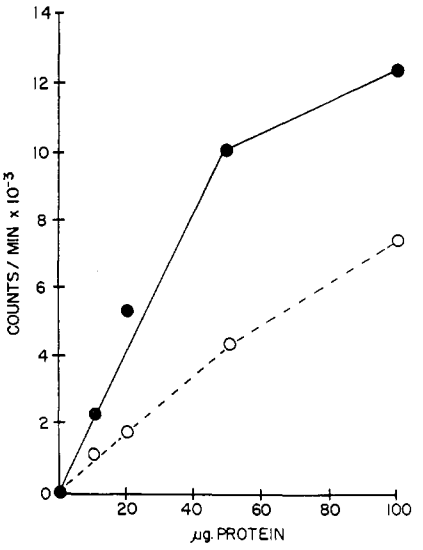

FIGURE 2: [^{3}H]Poly(uridylic acid) binding to microsomal wash protein at 37°. Microsomal salt washes were dialyzed against 0.2 M acetate buffer containing 0.2 M KCl at pH 4.4. The supernatants were concentrated to contain 5 mg/ml of protein. Various concentrations of wash protein were incubated with 0.05 μCi of [^{3}H]poly(U) in 0.5 ml of high binding assay buffer for 10 min at 37°. The samples were adjusted to the same total volume with acetate buffer: (●—●) immunized; (○- - -○) control.

TABLE IV: [^{3}H]Poly(uridylic acid) Binding to Stripped Liver Ribosomes Preincubated with Spleen Microsomal Washes.[a]

Microsomal Wash	ng of [^{3}H]Poly(U) Bound/ 50 μg of rRNA
None (buffer)	58 ± 3
Control spleen	103 ± 3
Immunized spleen	179 ± 35

[a] Two–three milligrams of stripped liver ribosomes were combined with 0.2 ml of standard buffer or microsomal washes (0.5 mg of protein/mg of rRNA). The total volume was brought to 1 ml with standard buffer (pH 7.8) and the [Mg^{2+}] was adjusted to 5 mM. Incubation was for 10 min at 37°. The ribosomes were reisolated by centrifugation at 65,000 rpm for 1 hr; 50 μg of these "reconstituted" ribosomes was then incubated for 10 min at 37° with 0.05 μCi of [^{3}H]poly(U) in 0.5 ml of high binding assay buffer. Filtration was carried out as previously described.

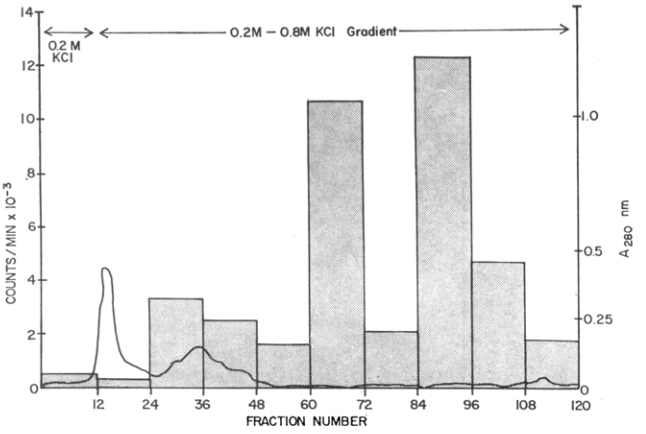

FIGURE 3: Carboxymethylcellulose purification of poly(U) binding factors from liver microsomal wash. The 1 M KCl wash from liver microsomes was dialyzed overnight against 0.1 M acetate buffer (pH 4.4) containing 0.2 M KCl; 10 mg of supernatant protein in 1.2 ml was layered on a 25 × 1.5 cm column of CM-cellulose previously equilibrated in the same buffer. The column was washed with 50 ml of the buffer followed by a 500-ml KCl gradient of 0.2–0.8 M in acetate buffer (pH 4.4); 4–5-ml fractions were collected, pooled in groups of 12, concentrated to 3 ml, and analyzed for poly(U) binding activity as described under Materials and Methods: (line) absorbance at 280 nm; (filled bar) counts per minute.

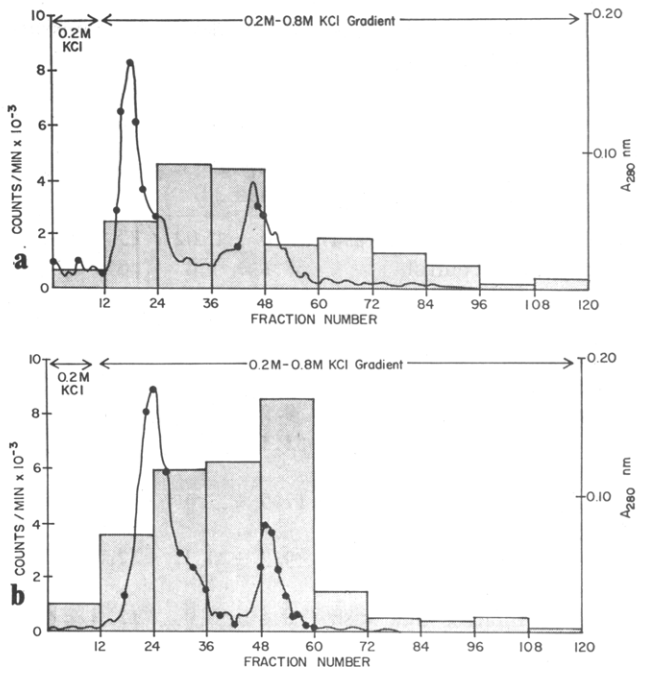

FIGURE 4: CM-cellulose purification of poly(U) binding factors from control and immunized spleens. Conditions are identical with those described for Figure 3, except that 2.6 and 3.0 mg of microsomal wash protein were layered on the column for the control and immunized systems, respectively: (line) absorbancy at 280 nm; (filled bar) counts per minute; (a) control; (b) immunized.

the spleen microsomal washes enabled the ribosomes to bind poly(U) to a greater extent than if the ribosomes were incubated with buffer alone. In addition, the immunized wash conferred greater binding activity to the ribosomes than did the controls. Subsequent experiments with stripped spleen ribosomes gave virtually identical results.

Partial Purification of Poly(U) Binding Factor. In order to determine whether or not the immunized microsomal wash contained greater quantities of a specific poly(U) binding protein, partial purification of the washes on carboxymethylcellulose was carried out. We reasoned that a protein which bound a polyanion such as poly(uridylic acid) at high ionic strength would be extremely basic in character. Therefore, greater resolution of activity should be obtained on carboxymethylcellulose at acidic pH than on DEAE-cellulose at neutral pH, the conditions which have been used to separate initiation factors (Prichard *et al.*, 1970) or poly(U) binding factors active at low ionic strengths (Smolarsky and Tal, 1970a,b). A 1 M KCl extract was prepared from both liver and spleen microsomes and dialyzed against 0.1 M acetate buffer (pH 4.4) containing 0.2 M KCl. Approximately 5 mg of protein was placed on the column.The column was washed with 50 ml of 0.2 M KCl and the protein was eluted with a linear 0.2–0.8 M KCl gradient in 0.1 M acetate buffer (pH 4.4). Five-milliliter fractions were collected. Fractions were pooled in groups of ten and concentrated to a 3-ml volume. After dialysis against high binding buffer, aliquots of each concentrated fraction were tested for [^{3}H]poly(U) binding activity. Figure 3 illustrates the elution pattern of microsomal wash from liver. The majority of the protein adhered to the column at 0.2 M KCl, but was eluted as the salt concentration was slightly increased. There were three peaks of poly(U) binding activity—a small one eluting at approximately 0.32 M KCl and two larger ones eluting at 0.42 and 0.6 M KCl. The protein elution pattern of spleen microsomal wash protein was essentially the same (Figure 4). The poly(U)-binding activity in the spleen washes, on the other hand, was in a single broad peak eluting between 0.3 and 0.4 M KCl. The binding activity did not correspond with the fraction which contained the majority of the protein, although it did overlap a second, smaller protein peak. The elution patterns of control and immunized washes were essentially the same. The amount of binding activity found in the microsomal washes from immunized spleens was 50–100% greater than that from control spleens. There was no temperature effect on poly(U) binding to the purified protein, and binding was just as avid at 37° as at 0°. It is unlikely, therefore, that these preparations contain nuclease which might account for the differences. We postulated that there was a real difference between control and immunized spleens in the amount of a poly(U) binding protein associated with their microsomes.

Physical vs. Functional Binding of Poly(U). Most of the experiments reported in the literature on the binding of poly(U) to ribosomes have been performed at 0° and at high magnesium concentrations, apparently because optimal physical binding was obtained under these conditions. That the physical binding may not be related to functional binding is shown by the data presented in Table V. In this experiment, patterned after a similar study by Williamson (1969), buffer-washed ribosomes were preincubated with poly(U) under conditions in which a minimum amount of peptide synthesis could occur, *i.e.*, with no added GTP or elongation factors. Excess poly(adenylic acid) was then added where indicated to complex all unbound poly(U) (Williamson *et al.*, 1967). Ribosomes that had been exposed to poly(U) at 37° were quite active in the polymerization assay even in the presence of an excess of poly(A), while those preincubated with poly(U) at 0° only polymerized phenylalanine to a slight extent. Nonpreincubated samples containing both poly(U) and poly(A) and nonpreincubated samples without poly(U) (endogenous incorporation) all displayed the same minimal degree of polymerizing activity.

TABLE V: Polymerization of [^{3}H]Phenylalanine after Preincubation of Ribosomes with Poly(uridylic acid).[a]

Preinc Temp (°C)	Cpm of L-[^{3}H]Phenylalanine/50 μg of rRNA			
	Control		Immunized	
	+ Poly(A)	− Poly(A)	+ Poly(A)	− Poly(A)
0	1095	8,027	1,069	16,273
37	8240	10,351	15,915	18,854
No preinc	1054	6,665	1,380	11,163
No poly(U)	1013	1,275	1,298	1,406

[a] Buffer-washed ribosomes (50 μg of rRNA) were preincubated for 10 min at the indicated temperatures with poly(U) as described under Materials and Methods. A twofold excess of poly(A) was added to one-half of the preincubated samples and to one-half of the unpreincubated samples; poly(U) was then added to the unpreincubated sample. An endogenous control was prepared without poly(U) or poly(A). All samples were then incubated an additional 10 min at 37°. Preincubations were at 8 mM Mg^{2+}; polymerization was at 4 mM Mg^{2+}.

Although it is clear that poly(A) prevented functional binding of the poly(U), we could demonstrate no influence whatever on the physical binding. Ribosomes incubated at 37° with a mixture of [^{3}H]poly(U) and cold poly(A) demonstrated an increased physical binding of [^{3}H]poly(U) to KOH-treated Millipore filters (unpublished observations). This may be due to retention of the poly(U)–poly(A) complex by the filter, for such preparations are known to bind to untreated filters (Mendecki *et al.*, 1972). Poly(A) did not affect any steps subsequent to message binding in protein synthesis since its presence had little influence on polymerization once poly(U) had been functionally bound.

The rate of the protein synthesis after a 37° preincubation with poly(U) was greater than the rate without preincubation both in the presence and absence of poly(A). The immunized buffer-washed ribosomes polymerized phenylalanine to a greater extent than the controls. The polymerization portion of the assays was performed in the presence of saturating concentrations of transferases I and II to eliminate differences due to the presence of higher concentrations of transferase I bound to immunized ribosomes. The results of an identical assay performed with stripped spleen ribosomes are presented in Table VI. With a preincubation at 0°, where physical binding of poly(U) is minimal, subsequent polymerization with added poly(A) is absent. With preincubation at 37°, where apparent physical binding is also minimal (Table II), some polymerization occurred even in the presence of poly(A). This indicated that functional as well as physical binding had occurred. The decreased polymerization, and therefore decreased functional binding, by the stripped ribosomes suggested that factors required for such binding had been removed by the salt wash. In any event, functional binding by immunized stripped ribosomes was greater than that by control ribosomes, indicating either that the stripping procedure did not completely wash off the factors, or that the stripped ribosomes were intrinsically changed following immunization.

TABLE VI: Polymerization of L-[^{3}H]Phenylalanine after Preincubation of Stripped Spleen Ribosomes with Poly(uridylic acid).[a]

Preinc Temp (°C)	Cpm of L-[^{3}H]Phenylalanine/50 μg of rRNA			
	Control		Immunized	
	+ Poly(A)	− Poly(A)	+ Poly(A)	− Poly(A)
0	75	1680	89	3001
37	1096	3220	2698	5127
None	62	1225	42	2458
No poly(U)		53		106

[a] Conditions the same as in Table V, except for the use of stripped ribosomes in place of buffer-washed ones.

Discussion

Most of the literature concerning the binding of poly(U) to ribosomes has described the physical interaction between poly(U) and ribosomes at 0° (Castles, 1969a,b; Moore, 1966a,b; Salas and Bollum, 1969; Krahn and Paranchych, 1970; Roberts and Coleman, 1971). At this temperature there is little nuclease activity associated with the ribosomes (Willis and Starr, 1972) and poly(U) has a more compact secondary structure (Salas and Bollum, 1969). Binding is markedly stimulated by an increase of magnesium concentrations up to 40 mM at 0° (Krahn and Paranchych, 1970), but under these nonphysiological conditions, as many as 2.5 chains of poly(U) can be bound per ribosome (Castles, 1969). As the temperature is raised, not only is nuclease activity enhanced, but the poly(U)–ribosome complex becomes much less stable due to the breaking of hydrogen bonds (McLaughlin *et al.*, 1966).

The physical binding of poly(U) also depends on the ionic strength of the binding buffer. Our data, and those of Roberts and Coleman (1971), demonstrate that stripped ribosomes do not bind as much [^{3}H]poly(U) as buffer-washed ribosomes in high ionic strength buffers, while at lower ionic strengths the opposite effect is seen. In low salt concentrations, the net positive charge of the stripped ribosome and the net negative charge of the poly(U) cause the two to aggregate nonspecifically. Higher concentrations of salt tend to neutralize the charges so that the ribosome or protein must have an extremely high affinity for poly(U) in order to achieve binding. These conditions are attained only with buffer-washed ribosomes which have not been stripped of bound proteins and with isolated wash proteins. Higher ionic strength buffers more closely approximate the situation *in vivo* as well as in most *in vitro* protein synthesizing systems (Roberts and Coleman, 1971). Still, physical measurement of bound labeled polynucleotide offers no indication of how much poly(U) is bound in a functional manner. The results reported here demonstrate that physical binding is extensive at 0°, but little functional binding, as evidenced by subsequent polypeptide synthesis, occurs at this temperature. We have demonstrated the existence of factors in the 1 M KCl wash from both liver and spleen microsomes which avidly bind [^{3}H]poly(U). Such results must be interpreted with caution, considering the well-known affinity of polynucleotides for any basic proteins (Baltimore and Huang, 1970). These factors may only be concerned with nonenzy-

matic attachment, but the fact that the binding activity of the factors from immunized spleen microsomes is greater than that from controls argues for a physiological role, at least for the splenic factor.

The experiments in which ribosomes were preincubated with poly(U) and treated with excess poly(A) provide further evidence for initiation and/or binding factors associated with the high-salt washes of the ribosomes. Poly(A) had little influence on elongation once poly(U) attachment had taken place at 37°. Since all the factors necessary for elongation were present in saturating quantities during the second incubation, any differences noted between control and immunized preparations must be due to increased efficiency in some phase of initiation. Stripped ribosomes were unable to initiate protein synthesis as well as buffer-washed ones, suggesting removal of an essential factor from the ribosomes. The fact that differences were still observed between control and immunized ribosomes after stripping probably indicates incomplete removal of such factors, although there may be alterations in the ribosomes themselves. Ideally, reconstitution of the stripped ribosomes with the factors removed by the salt wash should restore the original activity. We have shown that reassociating spleen wash factors with liver or spleen ribosomes partially restored binding activity, but we have thus far been unable to demonstrate restoration of translational activity. The reason for this discrepancy is not clear.

The relationship between the poly(U) binding factor we have observed and the known mammalian initiation factors is uncertain. The separability of this activity into three subfractions in liver whereas only one fraction is found in spleen is interesting and suggests that the poly(U) binding proteins are a heterogeneous mixture as is IF-3 in bacteria (Dube and Rudland, 1970). Since our factor binds the artificial messenger poly(U), it is probably not IF-M3, which is specific for natural messages (Prichard *et al.*, 1970). The bacterial factor IF-2 is known to promote the binding of both natural and synthetic messenger RNAs (Revel *et al.*, 1968). IF-M1 may be functionally identical with IF-2, for, in addition to having sulfhydryl groups, the two factors can substitute for each other in heterologous systems (Noll *et al.*, 1972; Zasloff and Ochoa,1972). Reticulocyte IF-M1 is not retained by DEAE at 0.1 M KCl (Prichard *et al.*, 1970), indicating that it is a basic protein as is our splenic factor. Experiments currently in progress in our laboratory suggest that the concentration of IF-M1 (as measured by [^{3}H]phenylalanyl-tRNA binding to the ribosomal subunit–poly(U) complex) is indeed greater in immunized spleen cytoplasm than in controls. If IF-M1 is not of sufficient concentration in the supernatant to saturate all the ribosomes, then salt washes of the ribosomes might be deficient in this factor.

The spleen consists of a mixture of cell types, and a shift in the predominant cell population of this tissue could result in considerable enzymic variation. Following an antigenic challenge, the primary cell type of the spleen changes from small lymphocytes to lymphoblasts and plasma cells (Kraft and Shortman, 1970). It is probably these newly activated cells which account for the changes observed in the protein synthesizing mechanism of the immunized spleen. Reproducibility of data was variable, but was noted to depend on the degree of proliferation of lymphoid tissue in the spleen, arbitrarily measured by increase in spleen weight (Willis and Starr, 1971). In every instance in which the average weight of the immunized spleens was 50% or greater than that of controls, equivalent differences were seen in the activity of the factors associated with protein synthesis. With the increasing number of factors that we have found to be altered following immunization, it now becomes of interest to separate the various classes of spleen cells and identify the responsible cell types.

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Independent Protein Synthesis in Isolated Rat Tumor Nucleoli. Aminoacylation of Endogenous Transfer Ribonucleic Acid†

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ABSTRACT: Nucleoli isolated from the Novikoff ascites tumor of the rat incorporate all the commonly labeled amino acids, added singly without other factors, into protein *in vitro*, and are capable of forming hydroxamates from most of the amino acids when hydroxylamine and ATP are added. The present work shows that the nucleoli are able to transfer each of the common amino acids into a complex with endogenous transfer RNAs. The activated complex was extractable in phenol–water, precipitable in cold trichloroacetic acid, labile to mild alkali and hot trichloroacetic acid, nondialyzable, and reactive chemically with hydroxylamine to yield hydroxamates. The complex was isolated and shown to contain RNA and to transfer its amino acids to protein in a ribosomal system purified to require added aminoacyl-tRNAs. Endogenous ATP has been detected in the isolated nucleoli by exchange with [^{32}P]pyrophosphate. The results show that these nucleoli contain an integrated, self-sufficient system capable of genuine protein synthesis and that this system resembles the mammalian cytoplasmic ribosomal system in that amino acids are activated and transferred by means of tRNAs.

In recent years the possibility that cell nucleoli contain a separate capacity for the synthesis of protein has been examined by a number of laboratories. Early reports were directed to the intense labeling of the nucleolus observed in autoradiography studies (Sirlin and Waddington, 1956; Waddington and Sirlin, 1959; Errera *et al.*, 1961) and, in addition, Birnstiel *et al.* (1961, 1962) and Zimmerman *et al.* (1969) showed that amino acid incorporation by pea seedling nuclei and HeLa cell nuclei, respectively, was primarily into a nucleolar fraction. Several other investigators, in addition to the ones cited here, have shown uptake of several single amino acids into protein of isolated nucleoli. Although incorporations proven to represent complete and genuine protein synthesis endogenous to the nucleolar organelle were not fully demonstrated, the existence of such a system has become an important consideration because of the possibility that the proteins synthesized therein serve specialized functions, such as initiators or repressors of RNA synthesis, or as structural or enzymatic components of nucleolar activities.

Lamkin and Hurlbert (1972) showed that nucleoli isolated from the Novikoff ascites tumor of the rat were able to utilize essentially all of the common amino acids for protein synthesis, and that the nucleoli contained a complete complement of activating enzymes. The purpose of the present paper is to extend these observations with proof of the existence and formation of aminoacyl-tRNAs, and demonstration of the presence of another essential reaction component, ATP, in isolated Novikoff nucleoli. A detailed comparison of the nucleolar and cytoplasmic systems with respect to amino acid incorporation and the effect of various known inhibitors of cytoplasmic protein synthesis upon the nucleolar system are also presented.

Experimental Procedure

Materials

Reconstituted protein hydrolysates containing 13 uniformly ^{14}C-labeled L-amino acids (average specific activities, 123 and 283 mCi/mmol) were obtained in separate shipments from Schwarz BioResearch. The amino acids contained were: alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine. The ^{3}H-reconstituted protein hydrolysate (Schwarz BioResearch) contained glycine and histidine in addition to the amino acids cited above and had an average specific activity of 16.13 Ci/mmol. Individual ^{14}C-labeled L-amino acids, each possessing a specific activity of 50 mCi/mmol, were also purchased from Schwarz BioResearch. $NaH_2{}^{32}PO_4$ in water (500 mCi/mmol) and $Na_4{}^{32}P_2O_7$ in water (4580 mCi/mmol) came from New England Nuclear Corp. Crystalline RNase and electrophoretically pure DNase I were products of Worthington Biochemical Corp. Puromycin (dihydrochloride) was obtained from Nutritional Biochemicals Corp.; chloramphenicol was obtained from Parke, Davis and Co.

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