Polyuridylic Acid Binding by Protein from Ehrlich Ascites Cell Ribosomes and Its Inhibition by Aurintricarboxylic Acid*

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ABSTRACT: An assay method has been developed, based upon filtration through cellulose acetate membranes at relatively high ionic strength, which permits the detection of poly(U)-binding protein removed from Ehrlich ascites cell ribosomes by salt washing. Ribosomes from which the binding protein has been removed retain some ability to bind poly(U) but only at low ionic strengths. Poly(U) binding to both the binding protein and the ribosomes was inhibited by low concentrations of aurintricarboxylic acid (ATA), providing additional evidence that this compound inhibits protein synthesis by interfering with the attachment of mRNA to the ribosomes. Poly(U) binding to proteins washed from 40S and 60S ribosomal subunits was investigated. In terms of specific binding activity, the 40S subunits were seven times more effective than 60S subunits in promoting the binding of poly(U). Furthermore, binding to the 40S subunit proteins was three times more sensitive to inhibition by ATA than binding to the 60S subunit proteins. These results indicate that the primary ribosomal poly(U)-binding site is on the 40S subunit. A variety of basic proteins and other compounds were assayed in the binding reaction. Protamine, DEAE-dextran, and poly-L-lysine all bound poly(U) very effectively and in a manner that was readily detected by attachment of the complex to cellulose acetate membranes; less than 0.01 μg of these compounds could be assayed using this procedure. Basic proteins such as egg-white lysozyme, cytochrome *c*, and chymotrypsinogen showed little affinity for poly(U) when assayed in this manner. The binding of poly(U) by all compounds tested was inhibited by very low concentrations of ATA, indicating that this compound is a general inhibitor of complex formation between poly(U) and polycations.

Despite the fact that the binding of polyribonucleotides to ribosomes is the necessary first step in protein synthesis little is known at the molecular level concerning the attachment process. It is recognized that the secondary structure of the polynucleotide plays an important role in attachment (Takanmi and Okamoto, 1963; Okamoto and Takanmi, 1963) but beyond a requirement for divalent cations it has been generally agreed that no other cell-soluble factors are necessary for binding (Moore, 1966). Recently, however, evidence has been presented which suggests that binding factors may be involved in the attachment of mRNA to ribosomes from bacteria (Greenshpan and Revel, 1969), plant (Marcus *et al.*, 1970), and animal cells (Heywood, 1970).

Studies on the attachment of polynucleotides to ribosomes usually involve binding of radioactive polynucleotides to ribosomes and detection of the complex by zonal centrifugation or adsorption to membrane filters. Of these two assay procedures the latter is the simplest, most rapid, and the method of choice for multiple quantitative determination of binding. It has been used to study the binding to ribosomes of tRNA (Nirenberg and Leder, 1964), viral RNA (Dahlberg and Haselkorn, 1967), pulse-labeled RNA (Naora and Kodaira, 1969), and poly(U) (Moore, 1966; Smolarsky and Tal, 1970).

We report here a modification of the usual membrane filter binding assay. By using cellulose acetate Millipore filter membranes and performing the assays at relatively high ionic strength, we are able to measure the binding of [^{3}H]-poly(U) to ribosomal protein and follow quantitatively the removal of the protein from the ribosomes by salt washing. This procedure also provides a sensitive assay for the binding of poly(U) to certain polycationic compounds. The removal of poly(U)-binding protein from Ehrlich ascites cell ribosomes, and a demonstration of the inhibition of poly(U) binding to this protein and other compounds by an inhibitor of protein synthesis, aurintricarboxylic acid (ATA),[1] form the basis of this report.

Materials and Methods

The [^{3}H]poly(U) (78.1 μCi/μmole of P, >50,000 molecular weight) was obtained from Miles Laboratories. The filter membranes used in routine assays were 0.2 μ pore size cellulose acetate membranes (EGWPO2500) from Millipore. Aurintricarboxylic acid was a product of K & K Laboratories, Inc. Spermidine diphosphate, spermine diphosphate, putrescine dihydrochloride, trypsin (type III, bovine pancreas, twice crystallized), poly-L-lysine (type II, approximate mol wt 1000–4000), protamine chloride (Grade V, salmon sperm), cytochrome *c* (type VI, horse heart), and bovine albumin (fraction V) were all obtained from Sigma Chemical Co. Chymotrypsinogen A (bovine pancreas, five-times crystallized) and egg-white lysozyme (twice crystallized) were from Schwarz BioResearch. Chymotrypsin (three-times crystallized) was obtained from Worthington Biochemical Corp. DEAE-dextran (approximate mol wt 2×10^6) was from Pharmacia. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Preparation of Cells. Ehrlich ascites cells were propagated and washed as previously described (Roberts, 1965). To reduce the proportion of polyribosomes in the cells, cells were taken from advanced 8- to 9-day tumors (Frindel *et al.*, 1970). In

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[1] Abbreviation used is: ATA, aurintricarboxylic acid.

some cases the polyribosome concentration was reduced further by preincubating the cells in glucose-free medium (Kerr *et al.*, 1966) containing puromycin (Hogan, 1969). Neither the age nor the preincubation of the cells affected significantly poly(U) binding by the ribosomes, but these treatments did reduce the backgrounds in the polyphenylalanine synthesis experiments.

Preparation of Ribosomes. Ribosomes were isolated as described by Kerr *et al.* (1966), with the following exceptions. After centrifugation of cell sap at 105,000*g* for 2 hr at 0°, the ribosome pellets were washed three times vigorously in a Vortex mixer with 0.25 M sucrose. This treatment removed a white precipitate from the top of the ribosome pellet, leaving a pellet that was completely transparent. There was no deoxycholate treatment. Ribosomes were resuspended in 0.25 M sucrose by using a Duall glass homogenizer, and they were stored at −75°. The average concentration of stored ribosomer was 250 A_{260} units/ml.

Sucrose-washed ribosomes were prepared by layering 5 ml of ribosomes suspended in 0.25 M sucrose onto 4 ml of 15% (w/v) sucrose containing 0.05 M Tris-acetate buffer (pH 7.8), 0.005 M $MgCl_2$, and 0.05 M KCl. The ribosomes were then recovered by centrifugation at 105,000*g* for 2 hr at 0° and resuspended in 0.25 M sucrose.

Preparation of NH_4Cl Wash Factors and Extensively Washed Ribosomes. For each 15 ml of ribosomes suspended in 0.25 M sucrose, 5 ml of a solution containing 2 M NH_4Cl, 0.04 M $MgCl_2$, and 0.2 M Tris-acetate buffer (pH 7.4) were added slowly in the cold with stirring. This extraction buffer mixture was frozen at −75° and thawed rapidly under running water at 37°. Aliquots (5 ml) of the extraction mixture were each layered onto 4 ml of 15% (w/v) sucrose containing 0.05 M Tris-acetate buffer (pH 7.8), 0.005 M $MgCl_2$, and 0.05 M KCl. These discontinuous sucrose gradients were centrifuged at 105,000*g* for 2 hr at 0°. The top 4 ml of each tube was carefully removed, and this was the source of NH_4Cl wash factors. The remaining supernatant was discarded, and the pelleted ribosomes (NH_4Cl-1 ribosomes) were resuspended in 0.25 M sucrose. This procedure was repeated twice more in order to prepare extensively washed ribosomes (NH_4Cl-3 ribosomes).

Poly(U)-Binding Assay. Suitable dilutions of the material to be assayed were made into 3.0-ml aliquots of assay buffer containing 0.05 M potassium phosphate (pH 7.4), 0.05 M KCl, and 5 mM $MgCl_2$. To each assay mixture was added 0.1 ml of a [^{3}H]poly(U) solution which had been diluted in distilled water to a concentration of 0.25 μCi/ml. After 5 min the mixtures were placed on top of cellulose acetate membrane filters, previously washed with 5 ml of assay buffer, and the vacuum in the manifold filtration apparatus regulated such that approximately 10 min was required for passage of the samples through the membranes. The membranes were washed twice with 5-ml portions of assay buffer, glued to planchets, and counted in a Nuclear-Chicago windowless gas-flow counter. All operations were performed at 22°.

Inhibition of Poly(U) Binding by ATA. The material to be tested for inhibition of poly(U) binding was titrated in the binding assay above and a concentration selected which gave approximately 1000 cpm bound in the standard assay. To samples containing this concentration of poly(U) binding material, was added varying concentrations of ATA 5 min prior to the addition of [^{3}H]poly(U). The [^{3}H]poly(U) was then added and the assay carried out as described above.

Preparation of pH 5 Enzymes. The supernatant from the centrifugation of cell homogenate at 105,000*g* for 2 hr at 0° was used to prepare pH 5 enzymes. This was accomplished by the dropwise addition of cold 1 M acetic acid to the high-speed supernatant fraction with stirring to pH 5. The precipitate was centrifuged at 7710*g* for 8 min and the pellets dissolved in a solution containing 0.02 M Tris-HCl (pH 7.8), 5 mM β-mercaptoethanol, and 1 mM EDTA. The enzyme preparation, generally containing about 5 mg/ml of protein, was stored at −75°. Protein concentration was estimated by the method of Lowry *et al.* (1951).

Preparation of tRNA. The 105,000*g* supernatant was the source of tRNA as well as pH 5 enzymes. The supernatant was phenol extracted by the high pH–deoxycholate procedure of Roberts *et al.* (1966). This RNA was resuspended in 1 M NaCl containing 0.05 M sodium acetate buffer (pH 5.3) at 4° for 16 hr to precipitate any high molecular weight species of RNA. The solution was centrifuged at 20,000*g* for 30 min and tRNA was recovered from the supernatant by the addition of two volumes of ethanol, placing the solution at 0° for 4 hr and removing the tRNA precipitate by centrifugation at 20,000*g* for 30 min.

In Vitro Polyphenylalanine Synthesis Assay. The incorporation of phenylalanine into polyphenylalanine was assayed using the procedure of Kerr *et al.* (1966). The standard reaction mixture had a final volume of 1.0 ml and contained (final concentrations): GTP (0.25 mM), ATP (1.0 mM), phosphoenolpyruvate (5.0 mM), pyruvate kinase (40 μg/ml), magnesium acetate (5.0 mM), potassium chloride (50 mM), β-mercaptoethanol (20.0 mM), Tris-acetate buffer (pH 7.8, 100 mM), 1 μCi of L-[^{3}H]phenylalanine (New England Nuclear Corp., 8.3 mCi/μmole), 0.5 A_{260} unit of tRNA, 12 A_{260} units of ribosomes, and 0.5 mg of pH 5 enzymes, unless otherwise indicated in the text. Reactions were stopped by adding 0.2 ml of nonradioactive phenylalanine (1 mg/ml), incubating at 37° for 5 min, then adding 1 ml of 10% trichloroacetic acid and cooling for at least 5 min. Pellets were washed with 5% trichloroacetic acid dissolved in 1 ml of 1 N NaOH in the presence of 0.2 ml of nonradioactive phenylalanine (1 mg/ml) and incubated 1 hr at 37°. The NaOH was neutralized with 0.2 ml of 100% trichloroacetic acid and an additional 1 ml of 10% trichloroacetic acid was added to precipitate the protein. The recovered precipitate was washed with 5% trichloroacetic acid, acetone, 95% ethanol, and ethyl ether, in succession. The dried pellets were dissolved in 0.5 ml of 98% formic acid and dried on copper planchets. Samples were counted in a Nuclear-Chicago low-background windowless gas-flow counter.

Results

Assay. The usual membrane filter binding assay makes use of the standard MF-type (mixed esters of cellulose) Millipore filters. In this assay ribosomes and any radioactive polynucleotides which may be attached to them in the assay mixture are bound to the membrane. The binding of the ribosomes to the membranes appears to be an adsorption phenomenon, since the mean pore size of the MF-type HA Millipore membrane is 0.45 μ yet it quantitatively binds ribosomes with diameters of 30 mμ and less. In addition to binding ribosomes, MF membranes are known to bind a variety of proteins and polynucleotides.

MF membranes alone will not bind tRNA (Nirenberg and Leder, 1964), viral RNA (Dahlberg and Haselkorn, 1967), or pulse-labeled RNA (Naora and Kodaira, 1969). However, they will bind poly(U) and in order for this technique to be used to study the binding of poly(U) to ribosomes, methods

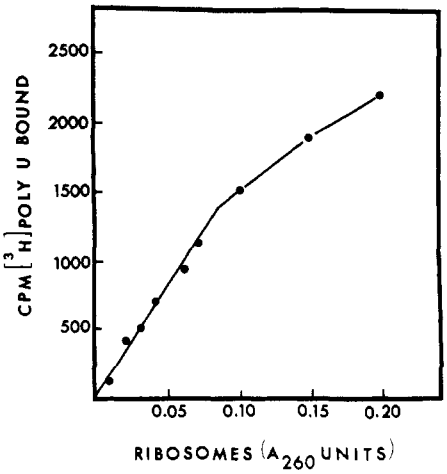

FIGURE 1: Dependence of [^{3}H]poly(U) binding upon ribosomes. Varying amounts of sucrose-washed ribosomes were added to the reaction mixture and assayed as described in Materials and Methods.

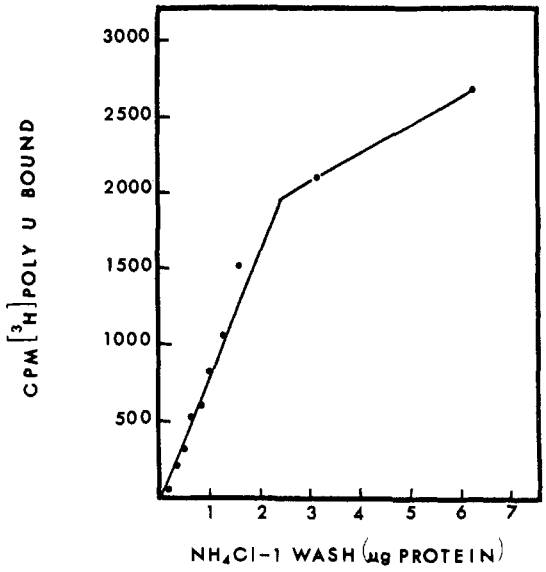

FIGURE 2: Dependence of [^{3}H]poly(U) binding upon NH_4Cl-1 ribosome wash. The NH_4Cl-1 wash was diluted and aliquots (containing the amounts of protein shown) removed, added to the reaction mixture and assayed as described in Materials and Methods.

must be used to prevent poly(U) binding to the membrane. These methods include saturating the membrane-binding sites with preadsorbed albumin (Moore, 1966) and destroying the sites by pretreatment of the membrane with alkali (Smolarsky and Tal, 1970).

The binding of poly(U) to filter membranes and ribosomes is affected by the ionic strength of the assay buffer. We investigated a number of types of Millipore membranes for their ability to bind [^{3}H]poly(U) at ionic strengths which were similar to that found in animal cells but were higher than the ionic strengths usually employed in binding assays. One of the membranes EGWP "cellotate" (cellulose acetate), was found to bind essentially no poly(U) by itself at these ionic strengths but to bind it quite efficiently when the polynucleotide was mixed with ribosomes. At lower ionic strengths, [^{3}H]poly(U) binding in the presence of ribosomes was more efficient but a small background binding of poly(U) to the filter itself appeared. Therefore, a relatively high ionic strength buffer (0.05 M potassium phosphate (pH 7.4)–0.05 M KCl–0.005 M $MgCl_2$) was used routinely for assays, since it eliminated the background without pretreatment of the filter membrane, retained sufficient sensitivity for our studies, and reduced the possibility of poly(U) binding to spurious basic proteins in the assay. Concerning this last point, later studies showed that ribosomes from which poly(U)-binding protein had been removed by salt washing still bound [^{3}H]poly(U) effectively in low ionic strength buffer but not in our standard assay buffer (Table II).

Using the standard assay procedure given under Materials and Methods the binding of [^{3}H]poly(U) to the filter membranes was dependent upon ribosome concentration (Figure 1). At limiting ribosome concentrations the curve is linear and can be used to assay the poly(U)-binding capacity of a given ribosome preparation. The curve is largely unaffected by the time and temperature of binding within the limits 0.5–30 min and 0–37°. The apparent binding capacity of the sample is reduced slightly by increasing the volume of the assay mixture and it is lowered approximately 50% by reducing the time of filtration from 10 min to 10 sec.

Removal of Binding Activity from Ribosomes by Washing with NH_4Cl. Ribosomes were washed with 0.5 M NH_4Cl (Felicetti and Lipmann, 1968) as described in Materials and Methods and then assayed for poly(U)-binding activity. It was found that most of the ribosomal binding activity was lost by this procedure and that three successive NH_4Cl washes removed over 95% of the binding activity from the ribosome preparations.

The NH_4Cl washes were analyzed for poly(U)-binding activity using the standard assay procedure. This procedure also proved to be a sensitive assay for a binding factor present in these washes. The binding activity was not lost by heating the washes at 95° for 15 min. However, it was destroyed by incubating the NH_4Cl wash samples, diluted for assay, with 0.05 µg/ml of trypsin or chymotrypsin at 37° for 20 min.

The binding of poly(U) by increasing concentrations of NH_4Cl-1 wash is shown in Figure 2. The binding response is linear at low protein concentrations and can be used to assay the amount of binding protein present in the wash. Binding protein corresponding to less than 0.1 µg of NH_4Cl wash protein can be detected using this procedure.

Table I summarizes the removal of poly(U)-binding factor from ribosomes by three successive NH_4Cl washes. The numbers in this table vary slightly from preparation to preparation, but they are essentially unaffected by such factors as the age of the ascites tumor and prior *in vitro* incubation of the cells (Materials and Methods). Over 95% of the binding activity is removed from ribosomes by three NH_4Cl washes. All of this activity is accounted for in the NH_4Cl washes.

Requirement of the Binding Reaction for Magnesium Ions. The binding of [^{3}H]poly(U) to both ribosome preparations and the binding protein in the NH_4Cl washes requires magnesium ions (Figure 3). The plateau for optimum binding beginning at about 10 mM Mg^{2+} is similar to that observed for the attachment of poly(U) to the ribosomes from *Escherichia coli* (Moore, 1966). The similarity between the magnesium response curves in Figure 3A,B suggests that the binding of poly(U) to binding protein in the washes is similar in nature to the binding that occurs with ribosome preparations.

Inhibition of the Poly(U)-Binding Reaction by ATA. ATA has been shown to inhibit the attachment of mRNA to ribosomes in protein-synthesizing systems from bacteria

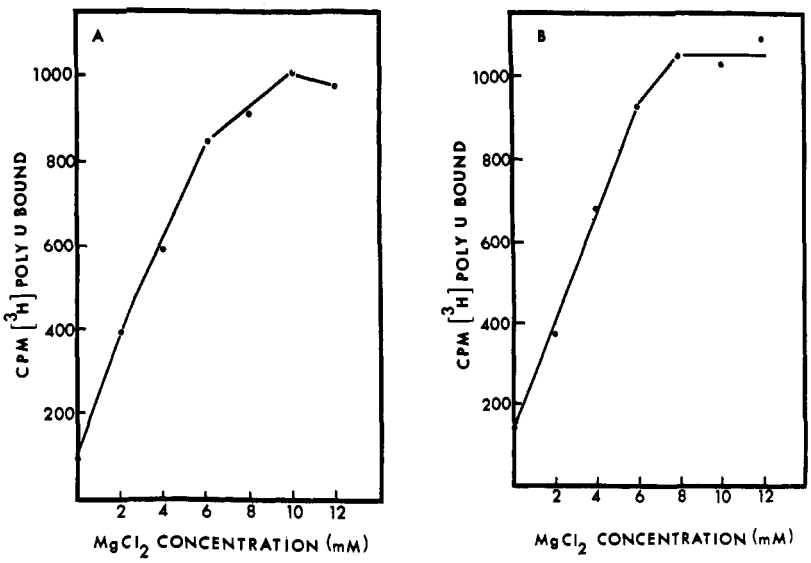

FIGURE 3: Magnesium dependence of the poly(U)-binding reaction. Samples of sucrose-washed ribosomes (0.05 A_{260} unit) or NH_4Cl-1 wash (1 μg of protein) were added to reaction mixtures containing assay buffers of varying $MgCl_2$ concentrations. The binding to the ribosome preparation (A) and to NH_4Cl-1 wash (B) was assayed in the usual manner.

(Grollman and Stewart, 1968), plants (Marcus *et al.*, 1970) and animal cells (Stewart *et al.*, 1971). We have used this compound to study its inhibition of [^{3}H]poly(U) binding to ribosome preparations and binding protein in our assay system.

Figure 4 shows the inhibition of poly(U) binding to both a ribosome preparation and to binding protein by low concentrations of ATA. The extreme sensitivity of both poly(U)-binding systems to ATA demonstrates again the similarity between poly(U) binding to samples containing ribosomes and poly(U) binding to samples containing NH_4Cl wash protein.

Nature of the Poly(U) Binding by Ribosome Preparations. Similar results to those summarized in Table I were obtained when ribosomes or NH_4Cl washes were assayed in our assay buffer on MF-type filter membranes according to the procedures of Moore (1966) and Smolarsky and Tal (1970). One fundamental difference between the procedures, however, is that ribosomes bind almost quantitatively to MF membranes, whereas approximately 90% of the ribosomes pass through EGWP membranes under our assay conditions. The fact that ribosomes themselves do not attach to these membranes suggests that perhaps it is a ribosome–poly(U) complex which binds to the filter. However, the possibility also exists that dilution of the ribosomes into assay buffer washes poly(U)-binding protein from the ribosomes, and it is poly(U) binding to the dissociated protein that is assayed in the ribosomes preparations. This second possibility would explain the similarities in poly(U)-binding properties between preparations of ribosomes and NH_4Cl washes (magnesium ion dependence, ATA sensitivity, one to one correspondence between binding units lost from the ribosomes with salt washing and binding units appearing in the wash).

To examine this question, ribosomes were passed through an EGWP filter membrane, the membrane washed and then poly(U) passed through the filter. The results show that it makes little difference in the assay whether poly(U) and ribosomes are filtered together or in stepwise fashion (Table II, EGWP experiment). This suggests that the high dilution of ribosomes into buffer washes from the ribosomes protein which attaches to the filter membrane and binds poly(U).

The ribosomes which pass through the cellulose acetate membrane were assayed for poly(U)-binding activity according to the procedure of Moore (1966), since this procedure utilizes MF-type membranes which will bind these ribosomes. Binding assays utilizing either cellulose acetate or MF membranes were found to be influenced strongly by the

TABLE I: Removal of [^{3}H]Poly(U)-Binding Protein from Ribosomes.[a]

Preparation	μg of Protein/ A_{260} Unit	Sp Act.[b]	Binding Units Recovered: Total ($\times 10^{-5}$)	Binding Units Recovered: From Washing Step (%)[c]
Sucrose-washed ribosomes	47.8	360	100	
NH_4Cl-1 ribosomes	37.3	200	31.0	88
NH_4Cl-1 wash		920	57.1	
NH_4Cl-2 ribosomes	32.2	56	7.1	118
NH_4Cl-2 wash		1650	29.6	
NH_4Cl-3 ribosomes	30.4	20	1.8	111
NH_4Cl-3 wash		740	6.1	

[a] Poly(U)-binding protein was removed from ribosomes by successive washes with buffered NH_4Cl solution (Materials and Methods). A binding unit is defined as that amount of binding protein which binds 1 cpm of [^{3}H]poly(U) under the standard assay conditions. [b] Specific activity is defined as binding units per microgram of protein. [c] Per cent recovery compares the total binding units in the ribosomes and wash from a washing step with the total units available in the ribosomes from the previous step.

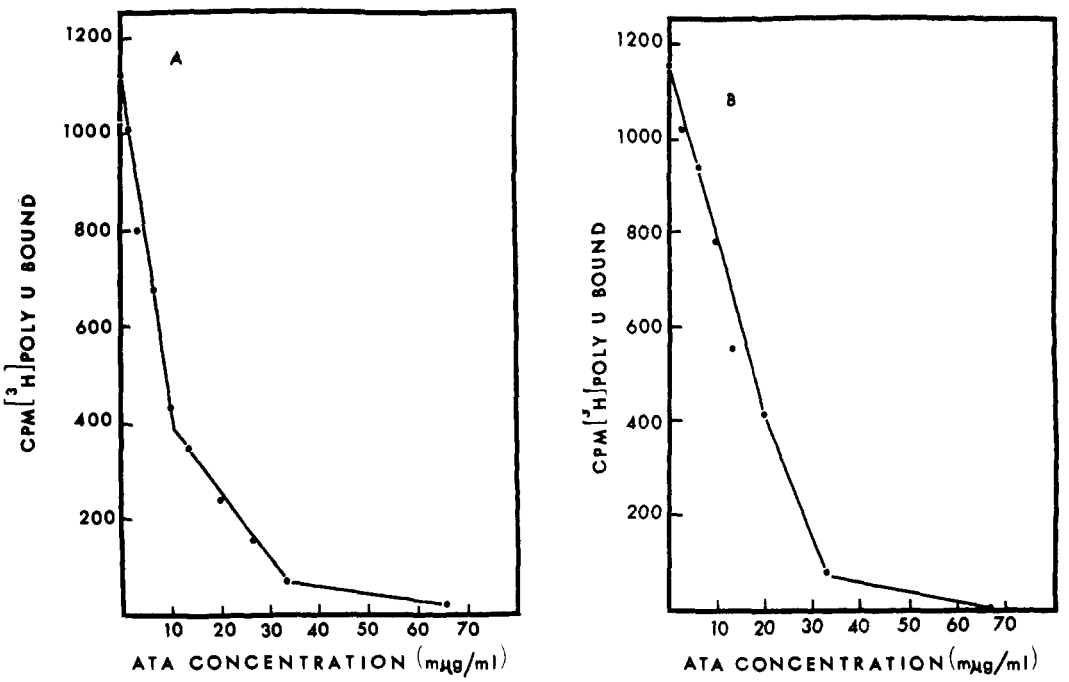

FIGURE 4: Inhibition of [^{3}H]poly(U) binding by ATA. The inhibition by ATA of poly(U) binding to sucrose-washed ribosomes (A) and NH_4Cl-2 wash (B) was assayed as described in Materials and Methods.

ionic strength of the buffers used. In assays carried out in a stepwise manner, the buffer used for the addition of ribosome or NH_4Cl washes to the filter membrane had little effect on the assay; however, the use of a low ionic strength buffer for the addition of poly(U) and subsequent washings produced a significantly higher specific binding activity than that observed with our standard PKM buffer (see Table II for definition of various buffers). Presumably this is simply a reflection of higher ionic strengths masking the ionic interactions between poly(U) and the binding protein, since raising the KCl concentration in PKM gradually reduces binding activity until it essentially disappears at about 0.5 M KCl.

The filtered ribosomes and the NH_4Cl-3 ribosomes were found to contain almost no binding activity when assayed in PKM but bind poly(U) well in TM buffer (Table II). Sucrose washed ribosomes and the NH_4Cl-2 wash, however, retain significant poly(U)-binding activity in PKM buffer. We interpret these results to mean that washing ribosomes with NH_4Cl or diluting them in buffer and filtering through EGWP membranes removes protein which has a strong affinity for poly(U) and therefore is able to bind poly(U) in PKM buffer. The core ribosomes which remain contain a separate capacity to bind poly(U), but the affinity for poly(U) is weaker and the binding can only be detected in low ionic strength buffer.

Binding of Poly(U) by Ribosomal Subunit Preparations. Sucrose washed ribosomes were dissociated in magnesium-free buffer and the 40S and 60S subunits separated by zonal centrifugation as described by Bielka *et al.* (1968). This procedure gave an excellent separation of the ribosomal subunits as shown by the absorbance profiles in Figure 5. For purposes of comparison an equal amount of ribosomes was centrifuged in a sucrose gradient containing Mg^{2+} and the 80S absorbance profile plotted on the same graph with the magnesium-free centrifugation.

Fractions from the zonal centrifugation were assayed for their ability to bind [^{3}H]poly(U) (Figure 5). The 40S subunit region was found to contain approximately 70% of the total binding activity found in the 40S plus 60S regions. The trailing of activity from the 40S region toward the top of the gradient may reflect a washing of binding protein from the subunits during centrifugation. In terms of binding units/A_{260} unit, the 40S subunit fractions are seven times more effective than the 60S subunit fractions in binding poly(U).

Recovery from the zonal centrifugation was 96% of the A_{260} units and 72% of the binding units present in the ribosomes added to the gradient. This suggests that under our assay conditions the efficiency of poly(U) binding to ribosomal subunit preparations is similar to the efficiency of binding to samples of intact ribosomes. Probably this is because what is being assayed in each case is poly(U)-binding protein washed from the sample upon its dilution into assay buffer.

The binding of poly(U) to the ribosomal subunit preparations also is similar to the binding to samples of intact ribosomes and binding protein in its dependence upon Mg^{2+} and its inhibition by ATA. The omission of Mg^{2+} from the assay buffer inhibits the binding of poly(U) to both the 40S and the 60S subunit fractions by over 95%. ATA will also inhibit the binding reaction (Figure 6) but poly(U) binding to the 40S subunit fractions is approximately three times more sensitive to ATA than binding to the 60S fractions, indicating a qualitative difference in the binding to the two subunits.

Binding of Poly(U) to Various Proteins and Polycations. A preliminary study of the specificity of the binding reaction was made by assaying the binding of [^{3}H]poly(U) in the presence of a number of proteins and polycations. Table III summarizes these results together with the ATA sensitivities of the different binding reactions. The high-speed supernatant, pH 5 enzymes, and basic proteins such as egg-white lysozyme, chymotrypsinogen, and cytochrome *c* were all ineffective in the binding reaction except at high concentrations. However, the large polycations (protamine, DEAE-dextran, and poly-L-lysine) formed complexes with poly(U) at low concentrations which were retained on the membrane filters, resulting

TABLE II: Effects of Varying Conditions on the [^{3}H]Poly(U)-Binding Assay.

Preparation	Filter Membrane[a]	Assay Conditions[b]	Sp Act.[c] (× 10^{-2})
Sucrose-washed ribosomes	EGWP	Sample and poly(U) added together in PKM	2.9
Sucrose-washed ribosomes	EGWP	Sample added in PKM; poly(U) added in PKM	2.7
Filtered ribosomes[d]	HAWP	Sample added in PKM; poly(U) added in TM	15
Filtered ribosomes	HAWP	Sample added in PKM; poly(U) added in PKM	0.1
NH_4Cl-3 ribosomes	HAWP	Sample added in PKM; poly(U) added in TM	29
NH_4Cl-3 ribosomes	HAWP	Sample added in PKM; poly(U) added in PKM	0.1
Sucrose-washed ribosomes	HAWP	Sample added in PKM; poly(U) added in TM	46
Sucrose-washed ribosomes	HAWP	Sample added in PKM; poly(U) added in PKM	1.5
NH_4Cl-2 wash	HAWP	Sample added in PKM; poly(U) added in TM	57
NH_4Cl-2 wash	HAWP	Sample added in PKM; poly(U) added in PKM	11

[a] HAWP is the Millipore designation for 0.45 μ pore size mixed ester membranes; EGWP for 0.22 μ pore size cellulose acetate membranes. [b] Assays using HAWP were performed according to Moore (1966) using bovine albumin to mask the poly(U)-binding sites on the membranes. TM is the buffer used by Moore (0.005 M Tris-HCl (pH 7.4)–0.01 M $MgCl_2$); PKM is the buffer used in our routine binding assays (0.05 M potassium phosphate (pH 7.4)–0.05 M KCl–0.005 M $MgCl_2$). All samples were added in 3.0 ml of buffer; the [^{3}H]poly(U) was added separately in 3.0 ml of buffer after the sample had passed through the membrane or together with the sample as indicated; membranes were washed with two 5-ml portions of the buffer in which the [^{3}H]poly(U) was added. [c] Specific activity is calculated as counts per minute of [^{3}H]poly(U) bound/μg of protein. [d] Sucrose-washed ribosomes (2 ml) diluted in PKM (0.200 A_{260} unit/ml) were passed through an EGWP filter membrane, the membrane washed with another 2 ml of the buffer, and the filtrates collected and pooled. Each filtrations step took about 5 min and approximately 90% of the ribosomes (A_{260} units) were in the filtrate.

in very high specific activities for these compounds. A correlation is seen between the specific binding activities of the compounds in Table III and the concentrations of ATA needed for 50% inhibition. The compounds fall into two broad categories: compounds with high affinities for poly(U) in the binding reaction and high sensitivities to inhibition by ATA, and compounds with low affinities for poly(U) and low sensitivities to ATA.

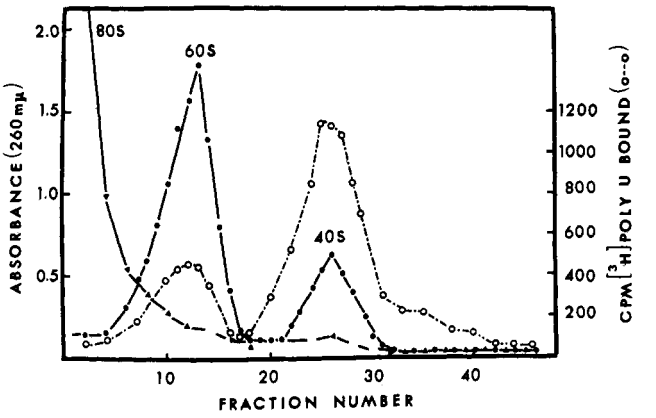

FIGURE 5: Binding of [^{3}H]poly(U) to ribosomal subunit fractions. Sucrose washed ribosomes (14 A_{260} units) were centrifuged in sucrose gradients according to Grummt and Bielka (1970) and 6 drop fractions collected. (▲) A_{260} of fractions from undissociated ribosomes centrifuged through a gradient containing 1.5 mM Mg^{2+}; (●) A_{260} of fractions from dissociated ribosomes centrifuged through a gradient containing no Mg^{2+}; (○) counts per minute of [^{3}H]poly-(U) bound to 0.05-ml samples from the dissociated ribosome fractions.

A number of small organic cations were tested for their ability to bind poly(U). Tris, imidazole, lysine, and arginine showed no binding at 100 μmoles/assay. The polyamines, however, showed a differential effect in their ability to bind poly(U) in the assay. Low concentrations of spermidine caused binding of significant amounts of [^{3}H]poly(U) to the membrane (Table III), whereas spermine was approximately one-tenth as effective and putrescine promoted no binding at concentrations up to 100 μmoles/assay. In connec-

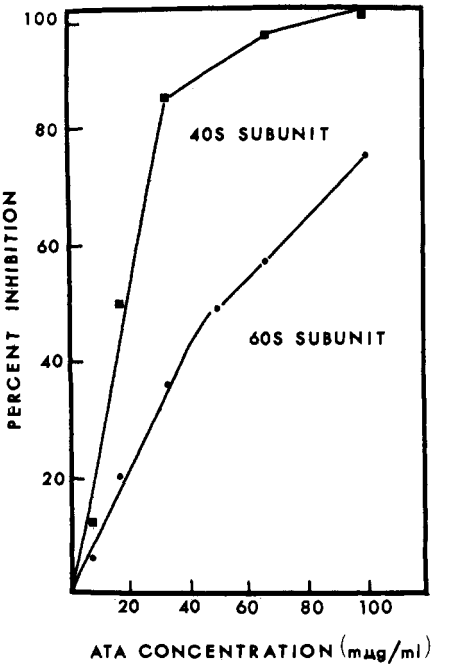

FIGURE 6: ATA inhibition of [^{3}H]poly(U) binding to ribosomal subunit fractions. The four peak fractions were combined from each of the 40S and 60S subunit regions shown in Figure 5. Aliquots from the 60S fractions (0.1 ml) and the 40S fractions (0.05 ml) were assayed for [^{3}H]poly(U)-binding activity in the presence of varying concentrations of ATA as given in Materials and Methods.

TABLE III: Inhibition of [^{3}H]Poly(U) Binding by ATA.

Compound Assayed	Sp Act.[a]	mμg/ml[b,c]
Protamine	28,000	11
DEAE-dextran	22,000	11
Poly-L-lysine	6,000	4
NH_4Cl-2 ribosome wash	1,650	16
40S ribosomal subunit	528	19
Sucrose-washed ribosomes	204	8
60S ribosomal subunit	72	52
pH 5 enzymes	60	120
Chymotrypsinogen	36	80
High-speed supernatant	33	190
Cytochrome *c*	23	70
Egg-white lysozyme	22	110
Spermidine	5	100
Chymotrypsin	5	
Trypsin	5	
Bovine serum albumin	<1	

[a] Specific activities are expressed as counts per minute of [^{3}H]poly(U) bound per microgram of compound. In the case of ribosomes and ribosomal subunits the approximation is made that 12 A_{260} units = 1 mg. With the NH_4Cl-2 wash, pH 5 enzymes, and high-speed supernatant the specific activity is based upon μg of assayed protein. [b] Binding inhibition assays were performed as described in Materials and Methods using just sufficient binding compound to bind approximately 1000 cpm in the absence of ATA. [c] ATA needed for 50% inhibition of poly(U) binding.

TABLE IV: Effects of Washing Ribosomes with NH_4Cl on Polyphenylalanine Synthesis.[a]

Ribosome Preparation	Change in Assay System	Cpm of [^{3}H]Phe Polymerized ($\times 10^{-2}$)
Sucrose-washed ribosomes	None	800
Sucrose-washed ribosomes	Poly(U) omitted	2
Sucrose-washed ribosomes	pH 5 enzymes omitted	500
NH_4Cl-1 ribosomes	None	100
NH_4Cl-1 ribosomes	0.1 mg of NH_4Cl-1 wash protein added	80
NH_4Cl-1 ribosomes	pH 5 enzymes omitted	1
NH_4Cl-3 ribosomes	None	3
NH_4Cl-3 ribosomes	0.1 mg of NH_4Cl-1 wash protein added	20
NH_4Cl-3 ribosomes	pH 5 enzymes omitted	1

[a] The assays were performed as described in Materials and Methods using 12 A_{260} units of a given ribosome preparation and adding or omitting factors in the standard assay system as indicated.

tion with this, it should be noted that Moore (1966) has shown that spermidine is much more effective than putrescine in promoting the binding of poly(U) to Millipore HA filter membranes.

Effects of NH_4Cl Washing on Polyphenylalanine Synthesis. An *in vitro* poly(U)-dependent polyphenylalanine-synthesizing system was prepared from Ehrlich ascites cells to compare the effects of NH_4Cl washing and ATA treatment on poly(U) binding with their effects on polyphenylalanine synthesis. The synthesizing system was isolated essentially according to the procedure of Kerr *et al.* (1966). Endogeneous incorporation of phenylalanine in the absence of poly(U) was reduced by using old tumors in which the cells were no longer rapidly dividing (Frindel *et al.*, 1970), and by preincubating the cells in a glucose-free medium (Kerr *et al.*, 1966) which contained puromycin (Hogan, 1969). Synthesizing systems isolated in this manner routinely showed an approximate 500-fold increase in polyphenylalanine synthesis upon the addition of 20 μg of poly(U).

Animal cell ribosomes washed once with NH_4Cl lose their ability to carry out polyphenylalanine synthesis, but this ability can be partially restored by the addition of pH 5 enzymes to the system (Fellicetti and Lipmann, 1968). Since the pH 5 enzyme fraction from ascites cells contains little poly(U)-binding protein (Table III), ribosomes washed repeatedly with NH_4Cl to free them from binding protein should require both pH 5 enzymes and NH_4Cl wash to restore polyphenylalanine-synthesizing activity. To test this possibility, several ribosome preparations were assayed in the polyphenylalanine-synthesizing system in the presence and absence of pH 5 enzymes and NH_4Cl-1 wash (Table IV).

Sucrose-washed ribosomes did not require pH 5 enzymes for polyphenylalanine synthesis, but had bound to them all of the protein factors, including Phe-tRNA synthetase, necessary for the polymerization of phenylalanine. Washing the ribosomes once with NH_4Cl made them dependent upon pH 5 enzymes for activity, but the activity could only be partially restored. The addition of the NH_4Cl-1 wash had little effect on this system. Additional ribosome washings caused further irreversible losses of polyphenylalanine-synthesizing activity. However, a small but significant amount of activity could be restored to the NH_4Cl-3 ribosome system by the addition of the NH_4Cl-1 wash. This suggests that repeated washing removes from the ribosomes a factor necessary for polyphenylalanine synthesis which is not present in significant amounts in the pH 5 enzyme fraction. This factor might be the same as the poly(U)-binding protein; however, purification of the poly(U)-binding protein will be necessary to establish this since a variety of ribosome-bound proteins necessary for polypeptide synthesis have recently been identified (see Discussion).

Inhibition of Polyphenylalanine Synthesis by ATA. Synthesis of polyphenylalanine in the ascites cell system was found to be inhibited by low concentrations of ATA (Figure 7). This synthesis was inhibited 50% at approximately 0.5 μg/ml of ATA (1.2×10^{-6} M). This is similar to a 50% inhibition by 3×10^{-6} M concentration of ATA in a bacterial *in vitro* protein-synthesizing system as originally reported by Grollman and Stewart (1968). A 50% inhibition of poly(U) binding to ribosome samples is achieved with approximately 8 mμg of ATA/ml (Figure 4). If we assume that the concentration of ATA needed for inhibition is proportional to the amount of ribosomes in each assay mixture (Grollman and Stewart, 1968), then the binding of poly(U) to ribosome preparations (about 0.1 A_{260} unit of ribosomes/assay) and the synthesis of polyphenylalanine (about 12 A_{260} units of ribosomes/assay) are roughly equivalent in their sensitivity to ATA. This

similarity between the concentration of ATA (per unit of ribosomes) necessary for 50% inhibition of binding and 50% inhibition of polyphenylalanine synthesis, provides additional evidence that the primary site of ATA inhibition of protein synthesis is the binding of mRNA to ribosomes.

Discussion

The poly(U)-binding assay used throughout most of this investigation contains two modifications of earlier assay procedures (Moore, 1966; Smolarsky and Tal, 1970); it utilizes cellulose acetate filter membranes instead of mixed ester membranes, and the assay is performed in phosphate buffer at a relatively high ionic strength. Cellulose acetate membranes by themselves show little capacity for binding poly(U), compared to mixed ester membranes, thus permitting their use in the assay without further treatment to mask or alter poly(U)-binding sites on the membranes. Assays performed in a low ionic strength buffer (TM, Table II) require correction for a moderate amount of binding to the membrane alone, whereas assays in the standard assay buffer (PKM) show virtually no background binding. The PKM buffer, although less efficient than TM buffer in promoting binding to preparations of ribosomes and ribosomal protein, does permit one to distinguish between poly(U) binding to ribosomal-binding protein and to ribosomes from which the binding protein has been removed by salt washing; poly(U) binding to the binding protein takes place effectively in PKM buffer, binding to washed ribosomes is effective only in TM buffer. This property, together with the observation that ribosomes at moderate concentrations in PKM buffer can be filtered through cellulose acetate membranes, has been used to obtain evidence that poly(U)-binding protein is washed from ribosomes upon their dilution in PKM buffer. This cautions against facile interpretations of ribosome-binding reactions unless it can be shown that no protein is washed from the ribosome by dilution into the assay buffer.

The use of a relatively high ionic strength buffer, such as PKM, in the binding reaction should prevent weak poly(U) attachments and detect binding only under ionic conditions roughly equivalent to those existing for *in vivo* and *in vitro* protein synthesis. For this reason the binding of poly(U) to salt-washed ribosomes, which occurs in TM but not PKM buffer, is probably fortuitous and does not represent the binding that occurs during polyphenylalanine synthesis. The interaction between poly(U)- and ribosomal-binding protein appears to be quite strong, however, since many basic proteins will not bind poly(U) under our standard assay conditions (Table III). We feel that the binding protein (or proteins) in the ribosome washes functions to bind poly(U) to the ribosome and that it may well be a very basic protein. Of the compounds we have examined, the NH_4Cl wash binding protein is most similar in its binding properties to protamine and poly-L-lysine. All of these compounds have high specific activities of binding, require magnesium ions for optimal binding, are very sensitive to inhibition by ATA, are inhibited only partially (approximately 50%) when the KCl concentration in the assay buffer is raised to 0.2 M, and are not inhibited in their binding properties by boiling for 15 min.

It can be postulated that the binding protein in the NH_4Cl washes functions on the ribosome to bind poly(U) during polyphenylalanine synthesis and perhaps bind mRNA during protein synthesis. However, it must be remembered that a number of ribosome-bound proteins have been identified which might be expected to be found in our NH_4Cl washes.

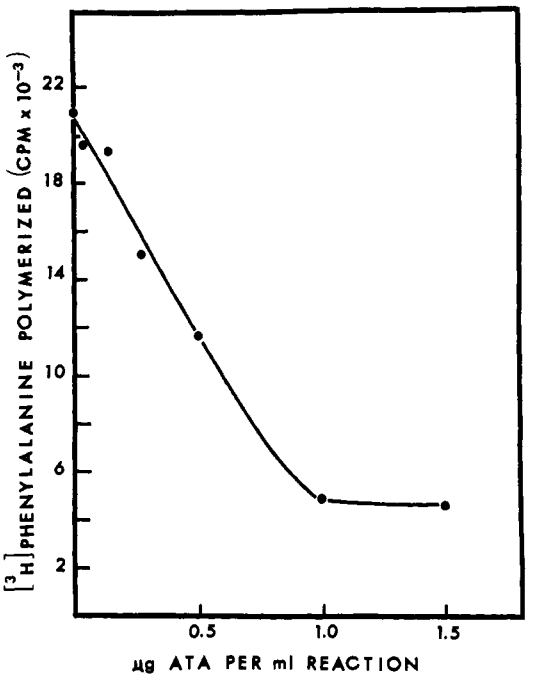

FIGURE 7: Inhibition of polyphenylalanine synthesis by aurintricarboxylic acid (ATA). ATA (K & K Laboratories, Inc., Plainview, N. J.) was added to standard assay mixtures at the concentrations shown.

These would include transfer factor T_2, which is tightly bound to tumor ribosomes (Krisko *et al.*, 1969; Griffin and Black, 1971); the magnesium shift factor which is needed for initiation of hemoglobin synthesis (Miller and Schweet, 1968); M_1, M_2, and M_3 (Prichard *et al.*, 1970), two of these factors (M_1 and M_2) functioning in the binding of an initiator Met-tRNA to ribosomes (Shafritz and Anderson, 1970); a factor necessary for the reassociation of ribosomal subunits (Godin *et al.*, 1969); and a factor necessary for the binding of mRNA to ribosomes (Heywood, 1970). In addition, we have recently found that very little Phe-tRNA synthetase is present in the pH 5 fraction; over 80% of this enzyme activity in the ascites cell is ribosome associated and repeated salt washings are required to remove all the activity from the ribosome fraction. Any one of these factors might bind poly(U) when free in the NH_4Cl wash and, of course, it might also serve to bind poly(U) on the ribosome. Testing of this possibility must await purification of the poly(U)-binding protein and testing for associated factor activities. The poly(U)-binding protein, whatever its normal function on the ribosome may be, should prove relatively easy to purify because of the simplicity and sensitivity of the assay procedure (less than 0.1 µg of NH_4Cl wash protein is readily detectable).

It has been demonstrated that in *E. coli* poly(U) binds to the 30S ribosomal subunit (Takanami and Okamoto, 1963). In animal cells it is known that the binding of mRNA occurs at the 40S ribosomal subunit (Joklik and Becker, 1965; Henshaw *et al.*, 1965) as does the poly(U)-directed binding of Phe-tRNA (Castles and Wool, 1970). Our results also indicate that the 40S subunit is the primary binding site for poly(U) but, in addition, they show a small but significant amount of binding to the 60S subunit fractions. Assuming that there is only one poly(U)-binding site per ribosome (this has been shown for ribosomes from *E. coli* (Takanami and Zubay, 1964; Moore, 1966) but not for ribosomes from animal cells),

then the simplest explanation for the poly(U) binding to the 60S subunit fraction is that it is nonspecific binding as indicated by its relative insensitivity to inhibition by ATA.

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Biosynthesis of Dimethylarsine by Methanobacterium*

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ABSTRACT: Cell extracts and whole cells of *Methanobacterium* strain M.o.H. reduce and methylate arsenate to dimethylarsine under anaerobic conditions. Methylcobalamin is the methyl donor of choice. Adenosine triphosphate and hydrogen are essential for the formation of dimethylarsine by cell extracts. In the pathway arsenate is reduced to arsenite which is methylated to form methylarsonic acid. Dimethylarsinic acid, which is formed by the reductive methylation of methylarsonic acid, is reduced to dimethylarsine. Selenate and tellurate also inhibit methane formation by serving as methyl traps.

The first systematic study of the biological formation of arsines was provided by Gosio (1897), who reported that a number of fungi gave off a strong garlic-like odor when they were grown in the presence of sodium arsenite. Bignelli (1900) erroneously concluded that the gas was diethylarsine. Challenger *et al.* (1933) proved that the gas was trimethylarsine, and investigated the ability of a number of arsenic derivatives to serve as prescursors in arsine synthesis (Challenger, 1945). Arsenic, selenium, and tellurium are metabolized by a number of fungi in a similar manner. Challenger and North (1934) showed that *Scopulariopsis brevicaulis* formed dimethyl selenide from sodium selenate and dimethyl telluride from sodium tellurate (Bird and Challenger, 1939). Although bacteria are known to reduce selenate to selenide and tellurate to telluride, we are not aware of references to bacterial synthesis of arsine or its alkylated derivatives. Recently Wood *et al.* (1968) described the biosynthesis of dimethylmercury by extracts of *Methanobacterium* strain M.o.H. We present evidence now that this organism can synthesize dimethylarsine from a variety of arsenic derivatives; the identifica-

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