Interaction of Transferase II with Polynucleotides and Inhibition of the Interaction by Guanosine Nucleotides*

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ABSTRACT: Transferase II, the peptidyl-tRNA translocation factor from rabbit reticulocytes, has been shown to interact with RNA and DNA. Complexes of the transferase with reticulocyte 18S and 28S rRNA were detected by sedimenting mixtures of purified rRNA and reticulocyte supernatant fraction in sucrose gradients and assaying the fractions for the transferase. The complexes, which contained less than one transferase molecule per molecule of RNA, were found to be sensitive to salt but were stable for at least several hours at 0–4° in buffer of low ionic strength. Divalent cations were not required for their formation. Stable complexes were also formed with *Escherichia coli* rRNA, R17 bacteriophage RNA, and various homoribopolymers. Transferase II, either free or bound to RNA, was assayed by measuring transfer of the adenosine diphosphate ribose moiety of radioactive NAD^+ into covalent linkage with the transferase, a reaction catalyzed by a fragment of diphtheria toxin. RNA was found to inhibit the rate but not the extent of transfer of adenosine diphosphate ribose to transferase II. This method was used to demonstrate binding of the transferase to tRNA and DNA although stable complexes containing these polynucleotides were not isolated. Interaction of transferase II with most types of RNA and with DNA was inhibited by GDP and GTP, and to a lesser extent by 5′-guanylyl methylenediphosphonate, an analog of GTP. GDP ($K_i = 1.5 \times 10^{-6}$ M) was about tenfold more effective than GTP ($K_i = 1.6 \times 10^{-5}$ M) in inhibiting complex formation. GMP and the mono-, di-, or triphosphates of other nucleotides had little if any effect. The inhibition is interpreted as resulting from a conformational change in transferase II induced by GDP and GTP. The activity of GTP may have been due to GDP present as a contaminant or generated by hydrolysis during assay. The results suggest that GTP and RNA may be ligands of the transferase on the ribosome complex during translocation. Because of the lack of specificity of the transferase for individual RNAs no conclusions can be drawn concerning the particular species of RNA to which the transferase may bind *in vivo*. However, evidence is presented which suggests that the transferase binds preferentially to single-stranded regions of polynucleotides.

Little information is available concerning the molecular events which occur during translocation of peptidyl-tRNA on ribosomes. The simplest models of protein synthesis (two-site models) assume that peptidyl-tRNA is translocated from the acceptor site to the donor site after formation of each peptide bond (see Lengyel and Söll, 1969). Deacylated tRNA remaining in the donor site must be removed before or at the same time that peptidyl-tRNA is translocated, and mRNA must be shifted by one codon relative to the ribosome at some stage during the cycle of peptide bond formation. However, the relationships between these processes are not clear.

Equally unclear are the roles of the translocation factor and GTP. Translocation of peptidyl-tRNA to the donor site, which can be assayed by its increased reactivity with puromycin (Brot *et al.*, 1968; Skogerson and Moldave, 1968a,b), and release of deacylated tRNA (Hardesty *et al.*, 1969; Kuriki and Kaji, 1968; Lucas-Lenard and Haenni, 1969) both require the presence of transferase II[1] and GTP in mammalian cell-free systems or G factor plus GTP in bacterial systems. GTP is believed to be hydrolyzed to GDP + P_i in both systems (Nathans *et al.*, 1962; Raeburn *et al.*, 1968; Felicetti and Lipmann, 1968; Nishizuka and Lipmann, 1966; Skogerson and Moldave, 1968a). Evidence that partially purified transferase II from rat liver binds GTP has been reported (Raeburn *et al.*, 1968), but precise data concerning possible ligands of the translocation factors on the functioning ribosome complex are lacking.

Knowledge of the enzymic activity of diphtheria toxin which has accumulated in recent years has rendered the toxin valuable in investigating the role of transferase II in protein synthesis in eucaryotic systems. A 24,000 fragment (fragment A) of the toxin catalyzes transfer of the adenosine diphosphate ribose moiety (ADPR) of NAD^+ into covalent linkage with transferase II, thereby producing an inactive derivative of the factor (Honjo *et al.*, 1968; Gill *et al.*, 1969, 1971; Collier and Kandel, 1971; Collier and Traugh, 1969).

$$\text{transferase II} + NAD^+ \rightleftharpoons \text{ADPR–transferase II} + \text{nicotinamide} + H^+$$

The reaction is specific for transferase II, and the equilibrium is far to the right at neutral pH or above (Gill *et al.*, 1969). Also, each molecule of the transferase has been shown to accept a single ADPR group (Raeburn *et al.*, 1971; Collins

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[1] Abbreviations used are: transferase II, for aminoacyl transferase II; ADPR, adenosine diphosphate ribose; GMPPCP, 5′-guanylyl methylenediphosphonate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

et al., 1971).[2] Thus if NAD^+ radioactively labeled in the ADPR moiety is employed, the transferase may be estimated by allowing the reaction to go to completion and measuring incorporation of label into acid-insoluble material. Alterations in the kinetics of the reaction have also been used to detect interactions of the transferase and its ligands (Goor and Maxwell, 1970).

Here we report experiments in which the toxin-catalyzed reaction has been used to demonstrate binding of transferase II from the supernatant fraction of reticulocytes to a variety of types of RNA and to DNA. The binding to RNA is relatively nonspecific, although significant differences were observed among various RNAs. The interaction in most cases is strongly inhibited by GDP or GTP, but not by GMP or other nucleotides. GDP is shown to be about tenfold more active than GTP. The results suggest that GTP and one or more species of RNA are ligands of the transferase on the ribosome complex during translocation, and the relative activities of GDP and GTP indicate a possible effect of hydrolysis of GTP during translocation.

Materials and Methods

Materials. GTP, GMP, 2′,3′-cyclic GMP, UDP, UMP, CDP, and CMP were obtained from Calbiochem; dGTP, ADP, AMP, and 3′,5′-cyclic AMP from Sigma Chemical; GDP and GMPPCP from Miles Laboratories; ATP and UTP from P-L Biochemicals. Fusidic acid was a gift from Leo Pharmaceutical; Sephadex G-25 and G-100 were from Pharmacia. The homopolymers of A, C, G, and I were obtained from Miles Laboratories; poly(U) was from Schwartz BioResearch. Yeast tRNA was from Boehringer und Soehne, and *Escherichia coli* tRNA from Schwartz BioResearch; *E. coli* $tRNA^{Arg}$ (70% pure) and $tRNA^{Glu}$ (78% pure) were gifts from Oak Ridge National Laboratories; RNA from bacteriophage R17 was a gift of Ray Gesteland. Calf thymus and salmon sperm DNA were obtained from Worthington; *E. coli* DNA was a gift of Donald Kaplan.

Diphtheria Toxin. Diphtheria toxin was prepared and purified by methods reported elsewhere (Collier and Kandel, 1971). The final product contained 2700 enzyme units/μg of protein.

Radioactive NAD^+. NAD^+, labeled uniformly with ^{14}C in the adenosine moiety, was prepared enzymically from [^{14}C]ATP and NMN, and purified according to methods previously described (Collier and Kandel, 1971). The specific activity of the final product was approximately 419 μCi/μmole.

Transferase II. Reticulocytes from phenylhydrazine-poisoned rabbits were lysed according to methods previously described (Collier, 1967). The extract was centrifuged at 15,000*g* for 15 min to remove unlysed cells and membrane fragments. The supernatant was then centrifuged at 60,000 rpm in a Spinco Ti-60 rotor at 4° for 3 hr, which removed ribosomal subunits as well as ribosomes and polysomes. Finally, the supernatant was centrifuged again for 5 hr at 50,000 rpm in a Spinco 65 rotor, passed through Sephadex G-25, equilibrated with buffer 1 (10 mM Tris-HCl (pH 7.6), 25 mM KCl, and 1.5 mM $MgCl_2$), and stored at −70°.

For studies on the kinetics of inhibition of the ADP-ribosylation reaction, a preparation of partially purified transferase II was used which was obtained by collecting that fraction of the ribosome-free supernatant which precipitated between 40 and 60% saturation $(NH_4)_2SO_4$ at 4° (Collier, 1967). This preparation (AS 40–60), which contained both transferases I and II, was passed through a column of Sephadex G-25 equilibrated with 10 mM Tris-HCl (pH 7.5), 1 mM DTT, and 0.1 mM EDTA, and stored at −70°.

rRNA was prepared from washed reticulocyte ribosomes by extraction with phenol in the presence of SDS. All operations were carried out at 0–4° unless otherwise indicated.

Reticulocyte ribosomes from the first high speed centrifugation of the whole cell extract were resuspended in buffer 1 to a concentration of 10 mg/ml using a Dounce homogenizer. The ribosomes were washed twice by centrifugation for 5 hr at 50,000 rpm in a Spinco 65 rotor at 4° with resuspension to a concentration of 10 mg/ml in buffer 1. The final pellet was resuspended in buffer 1 to a concentration of 30 mg/ml and stored at −70°.

rRNA was extracted from the ribosomes by adding 10% SDS to a final concentration of 0.5% and shaking vigorously at room temperature with an equal volume of redistilled phenol saturated with buffer 3 (50 mM NaAc (pH 5.1)–10 mM EDTA). After centrifugation at 1500*g* for 10 min, about 90% of the aqueous layer was removed and the phenol was reextracted with an equal volume of buffer 3. The combined aqueous phases were extracted again with buffer-saturated phenol after addition of solid NaCl to 3% (w/v). Two volumes of ethanol were added to the final aqueous phase and the solution was allowed to stand overnight at −20°. The precipitate was collected by centrifugation and was resuspended in 10 mM Tris-HCl (pH 8.2) to a concentration of 2–3 mg/ml (25 absorbance units at 260 nm is assumed to correspond to 1 mg of rRNA) and extracted three times with ether to remove traces of phenol. Nitrogen gas was bubbled through the preparation before storing at −70°.

ADP Ribosylation of Transferase II. The NAD-dependent ADP ribosylation of transferase II was conducted under two different sets of conditions. System A was used to estimate the concentrations of transferase present in specific samples such as fractions from sucrose gradients, while system B was employed in studies of the kinetics of the ADP-ribosylation reaction.

System A. Each sample was reacted in a final volume of 0.25 ml containing 40 mM Tris-HCl (pH 7.6), 40 mM DTT (pH 8.2), 4 mM EDTA, 11,000 eu of diphtheria toxin (4.1 μg of protein), 50 nM [^{14}C]NAD^+, and variable concentrations of transferase II. All components except transferase II were mixed and kept at 0–4° until the reaction was initiated by addition of the transferase. Samples were incubated at 25° for 20 min, at which time the reactions were terminated by addition of 5 ml of 5% trichloroacetic acid. The precipitates were collected on 2.4-cm glass filter disks (Whatman GF/C), and washed four times with 5 ml of 5% trichloroacetic acid before adhering the disks to aluminum planchets with milk. The radioactivity was determined with a low-background Nuclear-Chicago counter (efficiency 30%).

Under these conditions transferase II present in the supernatant fraction was reacted completely within 5 min, while maximal incorporation of label in the presence of RNA or ribosomes required 15 min. The radioactivity incorporated was proportional to the amount of transferase added up to at least 10 pmoles/sample. Routinely our samples were limited to 5 pmoles of transferase or less to ensure a large excess of unreacted NAD^+.

System B: The concentration of diphtheria toxin in system

[2] We have found that the ADP-ribosyl derivative of transferase II migrates on SDS–acrylamide gels according to a molecular weight of about 105,000, in agreement with the results of Collins *et al.* (1971).

TABLE I: Effects of Salts on the Binding of Transferase II to rRNA.[a]

	Transferase II Bound to rRNA (% of Total)
10 mM KCl plus	
1 mM EDTA	82
1 mM $MgCl_2$	76
5 mM $MgCl_2$	51
10 mM $MgCl_2$	41
1 mM $MgCl_2$ plus	
10 mM KCl	76
50 mM KCl	12
100 mM KCl	1

[a] Each sample (0.5 ml) containing 40 mM Tris-HCl (pH 7.6), 40 mM DTT, reticulocyte supernatant (6.6 mg of protein), rRNA (0.35 mg), and KCl, $MgCl_2$, or EDTA at the concentrations stated, was layered on a 10–30% sucrose gradient containing the same salt concentrations and 1 mM sodium phosphate (pH 7.8). Centrifugation was for 9.5 hr at 41,000 rpm in a Spinco SW-41 rotor. The gradient was fractionated and the samples were assayed as described in Figure 1.

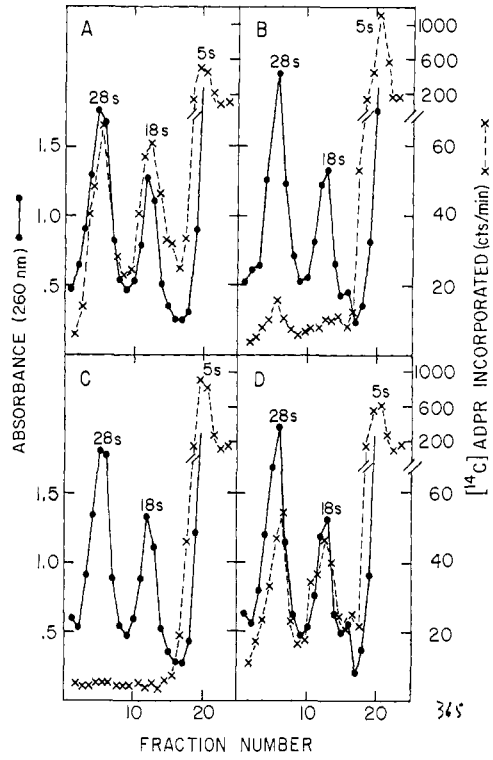

FIGURE 1: Sucrose gradient centrifugation of mixtures of transferase II and reticulocyte rRNA in the presence of various nucleotides. Each sample (0.5 ml), containing 40 mM Tris-HCl, (pH 7.6), 2 mM $MgCl_2$, 40 mM DTT, rRNA (0.32 mg), reticulocyte supernatant (6.6 mg of protein), and 0.4 mM nucleotide, was layered on a 15–30% sucrose gradient (12.7 ml in buffer 2) and centrifuged at 41,000 for 9.5 hr at 4° in a Spinco SW-41 rotor. Samples of 0.24 ml were collected, and alternate tubes reacted with toxin and NAD^+ as described under Materials and Methods using system A. The remaining samples were used for determination of absorbance at 260 nm. (A) No nucleotide, (B) GTP, (C) GDP, and (D) ATP. [^{14}C] ADPR incorporated per fraction (×---×); absorbance at 260 nm (●—●).

B was approximately 3000-fold less than the toxin concentration in system A. Under these conditions the rate of reaction was greatly reduced for studies on the kinetics of the ADP-ribosylation reaction. Reaction mixtures (0.25 ml) contained 40 mM Tris-HCl (pH 8.2), 40 mM DTT (pH 8.2), 0.4 mM EDTA, 3–5 eu of diphtheria toxin, 50 nM [^{14}C]NAD^+, and 50 nM transferase II in the form of AS 40–60. All components except NAD^+ were mixed and kept at 0° until the reaction was initiated by the addition of [^{14}C]NAD. After incubation for 15 min at 25°, the reactions were terminated by addition of 0.25 ml of 10% trichloroacetic acid. Samples were filtered and counted as in system A.

Sucrose Gradients. Linear sucrose gradients (12.6 ml) were prepared in buffer 2 (10 mM KCl, 1 mM KH_2PO_4, and 50 μM $MgCl_2$) and were cooled to 4° before use. Centrifugation was performed in a Spinco rotor SW-41 at 4°. The gradients were collected by inserting a 50-μl capillary pipet through the gradient to the bottom of the tube, and pumping the contents directly to a fraction collector at a flow rate of 47 ml/hr. Fractions of 0.24 ml were collected. Alternate tubes contained assay mixture A in 40 μl, and immediately after the gradient was collected these samples were assayed as described above (assay A). The remaining fractions were used for determination of absorbance at 260 nm.

Results

Formation of Complexes of Transferase II with 18S and 28S rRNA from Reticulocytes. rRNA purified by phenol extraction from reticulocyte ribosomes was combined with unfractionated reticulocyte supernatant containing transferase II, and the mixture was incubated at 0° for 20 min in the presence of 40 mM dithiothreitol and layered on a sucrose gradient. After centrifugation the gradient was fractionated and alternate samples were assayed for transferase II using diphtheria toxin and radioactive NAD^+. The absorbance profile at 260 nm was determined with the remaining samples. Figure 1A shows transferase II bound to both the 28S and 18S species of rRNA. A portion of the transferase remained near the top of the gradient at a position corresponding to about 5 S. In the absence of added rRNA all the transferase sedimented at 5 S. The same amount of transferase was associated with rRNA regardless of whether the mixture was incubated at 0° for 20 min or at 25° for 5 min prior to sedimentation; thus formation of the complex was not highly temperature dependent.

The extent of complex formation was measured in the presence of various concentrations of either $MgCl_2$ or KCl. The salts were added to transferase II before mixing with rRNA, and after incubation at 0° the mixtures were layered on sucrose gradients containing identical salt concentrations and centrifuged. The results (Table I) showed that increasing the concentration of either salt decreased the proportion of transferase II bound to RNA, but $MgCl_2$ was more effective than KCl. The binding to both 18S and 28S rRNA was inhibited approximately equally. Addition of 1 mM EDTA did not significantly affect the binding, which indicated that divalent cations are probably not required for complex formation. Further experiments were conducted in 40 mM Tris buffer (pH 7.6) containing 40 mM DTT, without additional salt unless otherwise stated. The gradients were rou-

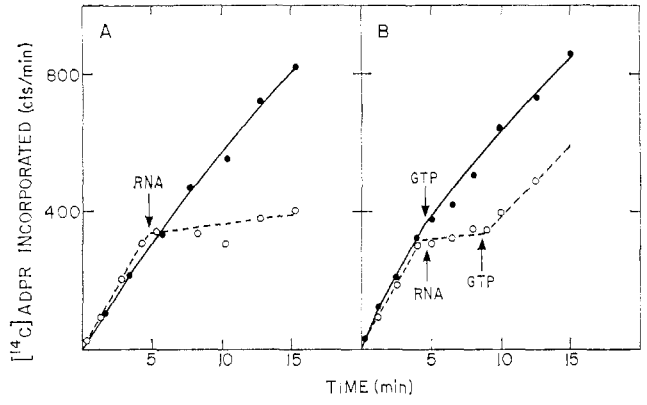

FIGURE 2: Effects of reticulocyte rRNA and GTP on the kinetics of ADP ribosylation of transferase II. Each reaction was conducted in a single tube containing sufficient volume of the reaction mixture for the number of points to be measured, plus one. Each tube was warmed to 25° and [^{14}C]NAD was added to initiate the reaction. Samples of 0.25 ml were removed at the times indicated and pipetted into an equal volume of 10% trichloroacetic acid. (A) Control, no additions (●—●); reticulocyte rRNA was added at 4:40 min (112 μg in 54 μl for each milliliter of reaction mixture) (○---○). (B) GTP was added at 4.5 min (0.3 μmole in 28 μl for each ml of reaction mixture) (●—●); reticulocyte rRNA was added at 4.5 min (112 μg in 54 μl to each milliliter of reaction mixture) and GTP was added at 8.5 min (0.3 μmole in 28 μl to each milliliter of reaction mixture) (○---○).

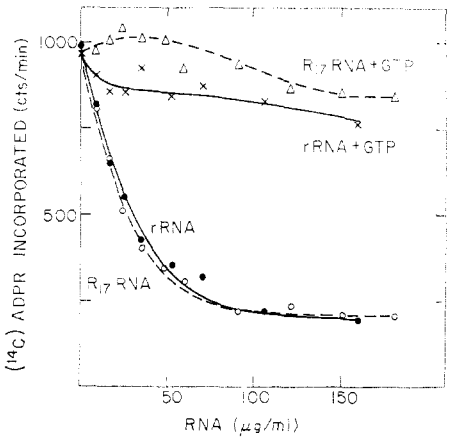

FIGURE 3: Inhibition of the ADP-ribosylation reaction by bacteriophage R17 RNA and reticulocyte rRNA in the presence and absence of GTP. The assay conditions (system B) were as described under Materials and Methods. The RNA and nucleotide were mixed and added to reaction mixtures shortly before the reaction was initiated with [^{14}C]NAD$^+$. R17 RNA (○---○), R17 RNA plus 0.3 mM GTP (Δ---Δ), rRNA (○—○), and rRNA plus 0.3 mM GTP (×---×).

tinely prepared in the following buffer: 1 mM K_2HPO_4, 10 mM KCl, and 50 μM $MgCl_2$.

GDP and GTP Inhibit Binding of Transferase II to rRNA. When either GTP or GDP (0.4 mM) was added to mixture of transferase II and rRNA before incubation at 0°, only a small fraction of the total enzyme bound to either 28S or 18S rRNA (Figure 1B,C). ATP (0.4 mM) caused only slight inhibition of binding (Figure 1D). In each case the nucleotide was present only in the incubation mixture; no additional nucleotide was present in the sucrose gradient. In other experiments it was shown that GMP and GMPPCP, the β,γ-methylene analog of GTP, each decreased the amount of bound enzyme by about 40%, while UTP and CTP had no significant effect on the binding. In all cases the relative inhibition of binding of the transferase to either 18S or 28S rRNA was about the same.

The experiments above showed that GTP and GDP prevented formation of a complex between transferase II and rRNA. If the nucleotides were added *after* incubation of transferase II with rRNA at 0°, the degree of inhibition observed was the same, which suggested that these nucleotides might dissociate the complexes into their component parts as well as inhibit their formation. Dissociation by GTP was demonstrated by isolating the complexes containing rRNA and transferase II from an initial 5–20% sucrose density gradient and resedimenting them in a second 15–30% gradient after incubation either in the presence or absence of GTP. In the presence of GTP approximately 80% of the transferase was released from rRNA, whereas only very slight dissociation occurred in the control sample. GMPPCP released about half of the bound transferase. The released transferase was recovered at the top of the gradient in each case. These results indicate that the RNA–transferase complex is stable over a period of at least several hours at 0° and can be dissociated by GTP.

ADP-Ribosyl Derivative of Transferase II Does Not Bind to rRNA. The ADP-ribosyl derivative of transferase II resulting from treatment of reticulocyte supernatant with toxin and NAD$^+$ was found not to bind to rRNA as shown by sucrose gradient centrifugation. The lack of binding to rRNA probably is not due to competition between toxin and rRNA for common or overlapping binding sites on the transferase since we showed in a separate experiment that in the absence of NAD$^+$ toxin does not affect the amount of transferase bound to rRNA. The inability of the ADP-ribosyl transferase to bind rRNA may either result from a conformational change in the enzyme or because the site of attachment of the ADP-ribosyl group may overlap that portion of the transferase directly involved in binding RNA.

Inhibition of ADP Ribosylation by rRNA and Release of the Inhibition by GTP. It has been reported that transferase II bound to ribosomes reacts with toxin and NAD$^+$ at a lower rate than free transferase (Gill *et al.*, 1969). We have found that the reactivity of transferase II is similarly affected by binding to rRNA (Traugh and Collier, 1970). As shown in Figure 2, ADP ribosylation of the transferase was inhibited immediately and almost completely upon addition of rRNA (112 μg/ml). However, at high concentrations of toxin the extent of the reaction was unaffected by rRNA. Treatment of the RNA with pancreatic ribonuclease (10 μg/ml for 30 min at 37°) prior to addition abolished its inhibitory activity.

GTP (0.3 mM) was found to counteract the inhibition by rRNA (Figure 2b), as expected from our earlier results in sucrose gradients. The reaction rate increased immediately upon addition of GTP. In the absence of rRNA GTP produced a very slight inhibition of the ADP-ribosylation reaction, which is consistent with the findings of Goor and Maxwell (1970), who showed that GTP inhibits the reaction competitively with respect to both the transferase and NAD$^+$.

ADP ribosylation of the transferase as a function of the concentration of rRNA is shown in Figure 3. This and all subsequent experiments of this type were conducted under conditions similar to those described under Figure 2. The extent of the reaction in 15 min at 25° was measured in the presence of low concentrations of toxin. The reaction was almost linear, and less than 40% of the total transferase or NAD$^+$ was reacted.

Under these conditions inhibition of ADP ribosylation

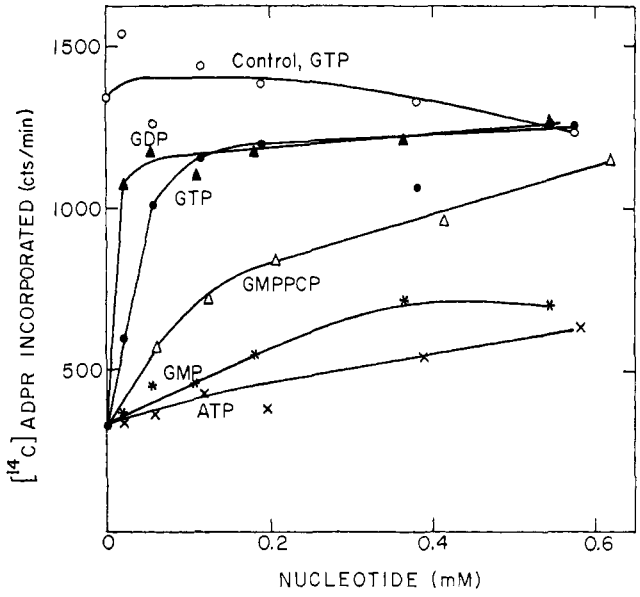

FIGURE 4: Effects of various nucleotides on inhibition of the ADP-ribosylation reaction by rRNA from reticulocytes. Each reaction mixture (system B), except for the control, contained 33 μg of reticulocyte rRNA and nucleotide as indicated. Additions to the reaction tubes were made in the following order; RNA, nucleotide, components of system B except NAD^+, and [^{14}C]NAD^+. Other conditions of the assay were as described under Materials and Methods. RNA plus GTP (●—●), plus GDP (▲—▲), plus GMPPCP (Δ—Δ), plus GMP (*—*), and plus ATP (×—×). Control with GTP and no RNA (○—○).

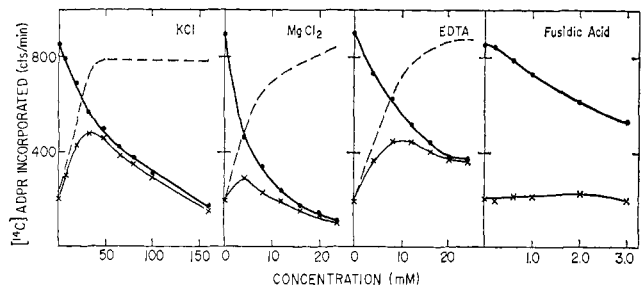

FIGURE 5: Effects of increasing salt concentrations and fusidic acid on the ADP-ribosylation reaction in the presence and absence of reticulocyte rRNA. The assays were performed in system B as described under Materials and Methods with components of the reaction mixture combined in the following order at 0°; salt or fusidic acid, rRNA (30 μg), components for assay B except NAD^+, and [^{14}C]NAD^+ to initiate the reaction. The dashed lines represent ADP ribosylation in the presence of rRNA corrected for the inhibition of the rate of ADPR incorporation by each salt measured in the absence of RNA. Each corrected point, R_0, was calculated by the following formula: $R_0 = R_s (A_0/A_s)$, where A_0 is the radioactivity incorporated in the absence of RNA or added salt, As is that incorporated at salt concentration s in the absence of RNA, and R_s is the incorporation in the presence of RNA at salt concentration, S. [^{14}C]ADPR incorporation in the presence of RNA (×—×), [^{14}C]-ADPR incorporation in the absence of RNA (●—●), and [^{14}C]ADPR incorporated in the presence of RNA corrected for the inhibition by salt (---).

was almost complete at a concentration of 160 μg/ml of rRNA, and 50% inhibition occurred at 33 μg/ml of rRNA. For unknown reasons complete inhibition of the reaction has not been observed even at very high concentrations of rRNA. The kinetics of the reaction were linear at each of the various concentrations of rRNA. In the presence of 0.3 mM GTP only slight inhibition of ADP ribosylation was observed even at high concentrations of rRNA.

GDP Is More Effective than GTP in Releasing the Inhibition by rRNA. As shown in Figure 4, both GTP and GDP are highly effective at concentrations well below 0.1 mM in blocking the inhibition of the ADP-ribosylation reaction by rRNA. GMPPCP showed significant activity also, but much higher concentrations required than with GDP or GTP; GMP or ATP showed little activity even at concentrations as high as 0.5 mM. These findings are in general accord with the relative activities of these nucleotides observed in earlier studies with sucrose gradients. For unknown reasons even very high concentrations of GDP or GTP failed to counteract the inhibition by rRNA completely.

Comparison of the titration curve for GDP with that for GTP (Figure 4) indicated that GDP may be the more efficient in releasing inhibition by rRNA. This fact was confirmed by careful titration of four different commercial preparations of GTP and of GDP over the concentration range of 0.5–400 μM. The curves obtained with the various preparations of GDP were almost identical; half-maximal release of inhibition was observed at 1.5 μM. Those with the different preparations of GTP were also almost identical, and half-maximal release of inhibition occurred at 16 μM nucleotide. dGTP had approximately the same activity as GTP. dGTP has been shown to substitute for GTP in stimulating translocation in bacterial systems (Pestka, 1969).

The mono-, di-, and triphosphates of guanosine, adenosine, cytosine, and uridine, as well as dGTP, 2′,3′-cyclic GMP, and 3′,5′-cyclic AMP, were examined at concentrations of 4 μM to determine the effectiveness of each in releasing the inhibition of rRNA. Assigning maximal release of inhibition by high concentrations of GDP (50 μM) a value of 100, the following values were obtained for release by various nucleotides at 4 μM: GDP, 74; GTP, 29; dGTP, 20; all other nucleotides, less than 11. Control samples containing 4 μM concentrations of each nucleotide in the absence of RNA showed no inhibition of the ADP-ribosylation reaction. In subsequent experiments the mono-, di-, and triphosphates of inosine were shown to lack activity.

Effects of Salts and Fusidic Acid on the ADP-Ribosylation Reaction in the Presence and Absence of rRNA. The effects of increasing concentrations of KCl, $MgCl_2$, EDTA, and the antibiotic, fusidic acid, on the ADP-ribosylation reaction were examined in the presence and absence of rRNA (Figure 5). In the absence of RNA, the reaction was inhibited by each of these. Magnesium chloride is more effective than either KCl or EDTA on a molar basis, suggesting that divalent cations may be very effective in inhibiting the reaction. These results confirm previous findings that the ADP-ribosylation reaction is sensitive to increases in ionic strength (Collier and Kandel, 1971).

Low concentrations of each of the salts, but not of fusidic acid, partially counteracted the inhibition due to rRNA. At higher salt concentrations the inhibitory effects became predominant and the final level of radioactivity incorporated was lower. Since it was previously shown by studies in sucrose gradients that KCl and $MgCl_2$ dissociate the complex between transferase II and rRNA, the initial rise in reaction rates at low concentrations of the salts is apparently due to release of the transferase from RNA, the free transferase being more reactive with toxin and NAD^+ than that bound to RNA. The dashed lines in each frame show the curves calculated by correcting the data obtained with rRNA for inhibition

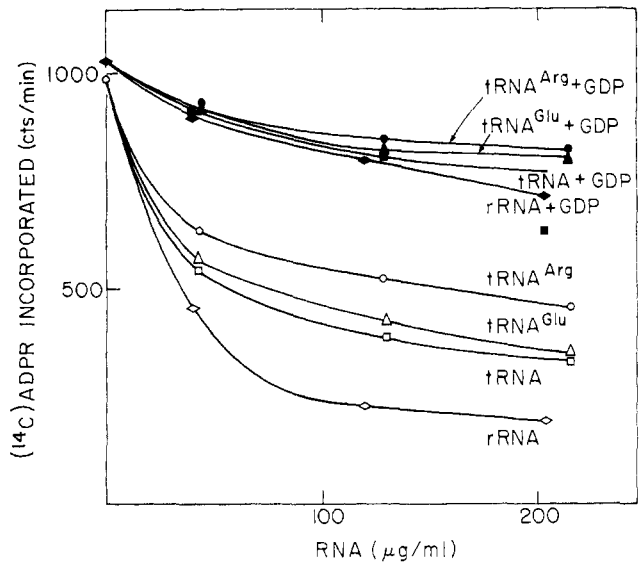

FIGURE 6: Inhibition of ADP-ribosylation by deacylated tRNA from *E. coli* in the presence and absence of GDP. Assay conditions (system B) were as described under Materials and Figure 4. GDP was added to a final concentration of 0.1 mM where indicated. The concentrations of RNA were measured by reaction with orcinol. *E. coli* $tRNA^{Arg}$ (○—○), $tRNA^{Arg}$ plus GDP (●—●), *E. coli* $tRNA^{Glu}$ (△—△), $tRNA^{Glu}$ plus GDP (▲—▲), bulk *E. coli* tRNA (□—□), bulk tRNA plus GDP (■--■), reticulocyte rRNA (◊—◊), and reticulocyte rRNA plus GDP (◆—◆).

TABLE II: Inhibition of the ADP-Ribosylation Reaction by Mixtures of Homopolymers.[a]

	Poly(C)	Poly(A)	Poly(U)	Poly(G)	Poly(I)
Poly(I)	22	79	79	74	84 (78)
Poly(G)	50	57	55	64 (54)	
Poly(U)	37	22	51 (38)		
Poly(A)	25	31 (19)			
Poly(C)	12 (7)				

[a] Mixtures of two homopolymers (100 μg/ml each) were heated to 60° for 1 min in 40 mM Tris-HCl and slowly cooled. Control samples of individual homopolymers at 200 and 100 μg per ml were treated identically. The components for system B were added to the nucleic acid mixtures in the order indicated in Figure 4, and the assay was performed as usual. Where the polynucleotide is the same in both horizontal and vertical columns the values shown in parentheses are for final polynucleotide concentrations of 100 μg/ml, and those without parentheses are for 200 μg/ml. Inhibition of ADP-ribosylation (%) by mixtures of homopolymers listed in horizontal and vertical columns (100 μg/ml each polymer; 200 μg/ml total).

of the ADP-ribosylation reaction by salts. Half-maximal release of the inhibition by RNA occurred in the presence of 18 mM KCl, 5 mM $MgCl_2$, or 7 mM EDTA.

The antibiotic fusidic acid, at a concentration of 0.4 mM, has been shown by Malkin and Lipmann (1969) to inhibit by more than 90% the translocation and GTPase activities in a rabbit reticulocyte system. Fusidic acid inhibited the ADP-ribosylation reaction very slightly at this concentration but did not appear to effect release of the transferase from rRNA (Figure 5). In a separate experiment (data not shown) fusidic acid (1.6 mM) was found not to prevent release of transferase II from rRNA by GTP and GDP.

Interaction of Transferase II with Other Polynucleotides. rRNA FROM *Escherichia coli.* Transferase II in unfractionated reticulocyte supernatant was shown in sucrose gradients to bind to both the 16S and 23S species of rRNA from *E. coli.* Under the conditions employed, which were similar to those described for Figure 1, the transferase bound to the bacterial RNA with approximately the same efficiency as to rRNA from reticulocytes, and GTP released the transferase.

BACTERIOPHAGE R17 RNA. Binding of transferase II to bacteriophage R17 RNA was demonstrated in sucrose gradients and by inhibition of the ADP-ribosylation reaction between 16 and 28 S, with or without bound transferase. GTP (0.3 mM) released the transferase from the viral RNA.

Titration of R17 RNA in the ADP-ribosylation reaction produced an inhibition curve which was almost coincident with that obtained with reticulocyte rRNA (Figure 3). At low concentrations of R17 RNA (70 μg/ml or less) the inhibition was completely reversed by 0.3 mM GTP, and even at higher concentrations of the RNA the release by GTP was almost complete.

tRNA. We have not been able to demonstrate a complex of the transferase with deacylated tRNA by sucrose gradient centrifugation, acrylamide gel electrophoresis, or chromatography on Sephadex G-100. However, tRNA inhibits the ADP ribosylation of transferase II, and the inhibition is efficiently released by GTP or GDP, thus indicating that the transferase does interact with tRNA (Figure 6). The efficiency of binding of tRNA to transferase II is approximately equal to that of rRNA on a weight basis or about 2% as efficient on a molar basis as judged by comparison of the initial slopes of the inhibition curves. The maximal inhibition by tRNA was lower than that by rRNA, while the release by GDP was equally efficient as with RNA.

Commercial preparations of tRNA from *E. coli* and yeast and tRNA from rabbit liver prepared in our laboratory all gave approximately the same results. The possibility that the inhibition by these preparations might be due to contaminating species or fragments of RNA is unlikely for the following reasons. (1) When tRNA from rabbit liver was chromatographed on Sephadex G-100, inhibition of the ADP-ribosylation reaction by the individual fractions followed the absorbance profile; thus strong inhibitory activity was found in the peak of tRNA, which contained over 80% of the total RNA (data not shown). (2) Two highly purified species of tRNA from *E. coli* $tRNA^{Glu}$ (78% pure) and $tRNA^{Arg}$ (70% pure) were found to give inhibition curves similar to those obtained with unfractionated tRNA both in the presence and absence of GDP (Figure 6).

INDIVIDUAL SYNTHETIC HOMOPOLYMERS. Interactions of transferase II with individual homopolymers of the four most common ribonucleotides were examined. The homopolymers sedimented at 5–12 S in sucrose gradients under the conditions employed, which permitted them to be resolved from free transferase. With either poly(U) or poly(G) (500 μg), all of the transferase (30 pmoles) sedimented as a complex with the homopolymer. Under the same conditions approximately one-half of the transferase bound to poly(C). No binding to poly(A) was detected. For unknown reasons GTP or GDP (0.4 mM) produced little or no inhibition of binding.

TABLE III: Effects of Double-Stranded and Single-Stranded DNA on the Rate of the ADP-Ribosylation Reaction in the Presence and Absence of GTP.[a]

Polymer (160 μg/ml)	No GTP		Plus GTP (0.3 mM)		% Inhibn[b] Released by GTP
	Cpm Incorp	% Inhibn (*a*)	Cpm Incorp	% Inhibn (*b*)	
No addition	992	0	966	0	
Calf thymus					
Double stranded	769	23	701	27	0
Single stranded	565	43	750	22	49
Salmon sperm					
Double stranded	757	24	904	6	73
Single stranded	722	27	885	8	70
Escherichia coli					
Double stranded	846	15	924	4	79
Single stranded	758	25	900	7	72
rRNA	203	80	822	15	81

[a] DNA samples were incubated with 10 μg/ml of RNase overnight at 0°. Single-stranded DNA was obtained from double-stranded DNA by incubation at 85° in 1 N NaOH for 1 hr. The double-stranded and single-stranded DNA samples were dialyzed separately against two changes of 10 mM Tris-HCl (pH 8.2). The assays were performed using system B, with the order of addition of components as indicated in Figure 5. Each tube contained 40 μg of nucleic acid and 0.3 mM GTP where indicated. [b] [((*a*) − (*b*))/(*a*)]100.

Inhibition of the ADP-ribosylation reaction by the individual homopolymers and release of the inhibition by GTP correlated with the results obtained in sucrose gradients. In Figure 7 it is shown that the relative order of inhibitory activity of the homopolymers was poly(G) ≥ rRNA > poly(U) > poly(A) ≅ poly(C). Poly(I) was later shown to be a more potent inhibitor than poly(G).

GTP was relatively inefficient in releasing the inhibition of the ADP-ribosylation reaction by the homopolymers. The release by GTP was greatest with those polymers which were least effective in inhibiting the reaction (50–75% of the inhibition with poly(A) was released; 30–50% with poly(G)). In contrast GTP released the maximal inhibition by rRNA by about 90%. GDP was about as effective as GTP in relieving inhibition by the homopolymers.

MIXTURES OF SYNTHETIC HOMOPOLYMERS. Inhibition of the ADP ribosylation of transferase II by mixtures of various pairs of homopolymers was measured to determine whether the transferase might bind preferentially to single- or double-stranded polynucleotides. With those pairs in which double-stranded structures would not be expected to form, the inhibition was about the same as that produced by the more potent individual nucleotide alone (Table II). Thus, poly(I) (100 μg/ml) inhibited the reaction by 78%, and mixtures of this concentration of poly(I) with the same concentration of poly(G), poly(A), or poly(U) produced 74, 79, and 79% inhibition, respectively. In contrast, a mixture of poly(I) and poly(C), in which double-stranded structures are formed, produced only 22% inhibition of the reaction. A similar decrease was observed with poly(A) plus poly(U). This mixture inhibited the reaction 22% as contrasted with 38% for poly(U) alone or for poly(U) plus poly(C). These results suggest that transferase II has a higher affinity for single-stranded than for double-stranded polymers.

DNA. Inhibition of the ADP-ribosylation reaction by double-stranded and single-stranded DNA from various sources (Table III) was measured at a single concentration of polynucleotide (160 μg/ml). All the samples had been treated with RNase (10 μg/ml overnight at 2°). Single-stranded DNA was obtained by treatment of double-stranded DNA with 1 N NaOH, which also degraded any traces of RNase-resistant RNA. Double-stranded DNA from calf thymus, salmon sperm, or *E. coli* inhibited the reaction about 15–25% compared to 80% for the same concentration of rRNA. Single-stranded DNA derived from each of the preparations was found to be slightly more inhibitory than an equivalent amount of double-stranded DNA, which supports the suggestion that the transferase binds to single-stranded regions of polynucleotides. GTP (0.3 mM) released the inhibition by single-stranded or double-stranded DNA by 50–75%.

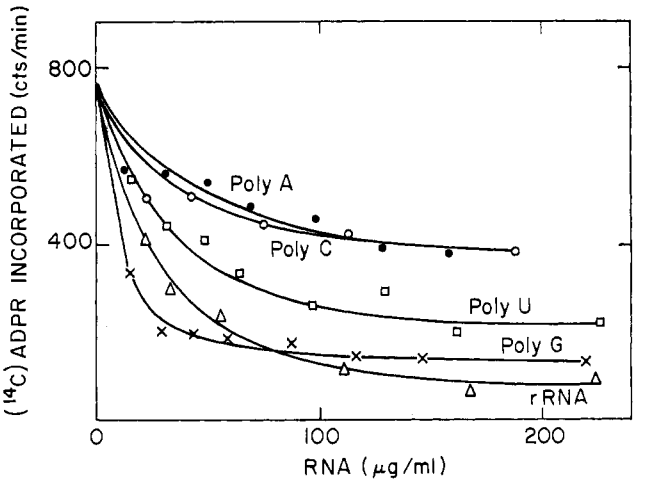

FIGURE 7: Inhibition of ADP ribosylation of transferase II by homoribopolymers. Assay conditions (system B) were as described under Materials and Methods and Figure 4. Poly(A) (●—●), poly(C) (○—○), poly(U) (□—□), poly(G) (×—×), and rRNA from reticulocytes (△– △).

Discussion

Transferase II from the supernatant fraction of reticulocytes is capable of binding to a variety of RNAs, both natural and synthetic, and DNA. Stable complexes were detected with all of the species of natural RNA tested except tRNA. Our inability to isolate such complexes with tRNA may have resulted from possible instability or difficulties in resolving the complexes from free transferase. tRNA clearly interacts with the transferase, however, as shown by its inhibition of the ADP-ribosylation reaction. All of the natural RNAs tested had about the same affinity for the transferase on a weight basis as judged by their capacities to inhibit the ADP-ribosylation reaction. On a molar basis the affinity of tRNA was about 50 times lower than rRNA or R17 RNA. It is uncertain which of these is more significant.

The experiments with reticulocyte rRNA indicate that interaction of the transferase with RNA does not require divalent cations and is sensitive to ionic strength. We interpret the inhibition of complex formation by EDTA (Figure 5) as resulting from its contribution to the ionic strength and not from its capacity to chelate divalent cations, since only partial inhibition of binding occurred at concentrations of EDTA as high as 5–10 mM. Since the transferase is negatively charged at neutral pH (Raeburn *et al.*, 1970) and since it interacts with RNA at pH's above neutrality it is probable that the site of interaction is a positively charged region on the surface of the enzyme. This would explain the inhibitory effects of salts on the interaction, although effects of ionic strength on the secondary structure of RNA may be of importance.

In most of the experiments with sucrose gradients a saturating amount of RNA was employed, which resulted in a low molar ratio of bound transferase to RNA (about 1:10); at limiting concentrations of rRNA the ratio increased to approximately 1:3. An independent estimate of this ratio has been calculated from curves of inhibition of the ADP-ribosylation reaction as a function of the concentration of RNA (Figure 3) assuming a very high affinity constant under the conditions of our assay.[3] The figures obtained were one transferase per 1.1 molecules of either 18S or 28S rRNA, or per 1.0 molecules of R17 RNA. For tRNA the ratio was 1 transferase/50 molecules of RNA; it is possible, however, that a difference in affinity for tRNA may account for this low ratio. Although further work must be done to obtain precise estimates of the number of binding sites for transferase II per molecule of RNA, these figures indicate that the number is not large and may be near one.

Although further studies will be necessary to determine which properties of polynucleotides influence their affinity for transferase II, our results contain some information regarding this question. (1) Data from experiments with mixtures of homoribopolymers and with double- and single-stranded DNA suggest that the transferase interacts preferentially with single-stranded polynucleotides. The findings that rRNA, R17 RNA, and tRNA interact with the transferase about equally on a weight basis would be consistent with this since they all have similar contents of secondary structure. Apparent binding by double-stranded polynucleotides may have resulted from exposed single-stranded regions. (2) The differences in affinity of the various homoribopolymers for the transferase (poly(I) > poly(G) > poly(U) > poly(C) $\geq$ poly(A)) may reflect specificity of the binding site on the transferase for certain bases. However factors such as secondary structure or average molecular weight of the RNA (for instance, if the transferase binds to one or both ends of RNA) clearly may be of equal or greater importance. Since R17 RNA does not contain modified bases these can be eliminated as being essential for binding of the transferase or its susceptibility to release by GTP or GDP. A lower affinity of the transferase for polynucleotides containing deoxyribose than those containing ribose is suggested by the low inhibitory activity of DNA compared to RNA.

Inhibition of complexing of transferase II with RNA by GDP or GTP must result from interactions of the nucleotides with the transferase rather than with RNA, since (1) GMP is virtually inactive although it would be expected to bind to RNA with affinity at least equal to that of GDP or GTP, and (2) the values of K_i for GDP and GTP fall in the micromolar range, indicating a binding site of high affinity. It seems certain that the activity of GDP in inhibiting binding of transferase II to natural RNAs is not artifactual since GMP which may have been generated by hydrolysis of GDP during the assay and GTP which might have been produced by phosphorylation, were both much lower in activity. Other nucleotides except GMPPCP were almost completely inactive. It is less certain that GTP itself inhibits binding of transferase II to RNA, since low levels of GDP either present as contaminants or resulting from partial hydrolysis of the GTP by contaminating enzymes in the crude preparation of transferase might have produced the observed results. More highly purified preparations of transferase will be required before this possibility can be tested.

The possibility that GDP and GTP inhibit complex formation by competing with RNA for the same binding site on transferase II seems unlikely because of the specificity for the di- and triphosphates of guanosine for dissociation of RNA–transferase complex and also because of the lack of similar specificity for guanosine-containing polynucleotides. It is probable that RNA and guanosine nucleotides bind at different sites on the transferase and thus that the inhibitory effects of GDP and GTP result from conformational changes in the enzyme. The fact that GMPPCP is partially active presumably indicates that hydrolysis of phosphoanhydride linkages is not required for dissociation of the transferase–RNA complex.

It seems likely that the phenomena of transferase II binding to RNA and its release by guanosine nucleotides may be related to the mechanism of translocation, although the relationships are far from clear. The fact that the transferase is required for a step involving translocation of peptidyl-tRNA (and perhaps mRNA) and ejection of deacylated tRNA from the ribosome is certainly suggestive that it may interact directly with one or more species of RNA. Furthermore the correlation between the specific requirement for GDP or GTP in releasing transferase II from RNA with the

[3] The number of binding sites for transferase II per molecule of RNA was calculated from initial slopes of the inhibition curves using the formula, $(T_0/A_0)(dA/d(RNA))$, where T_0 is the total molar concentration of transferase II, A is the radioactivity incorporated at any given concentration of RNA, A_0 is the radioactivity incorporated in the absence of RNA, and (RNA) is the molar concentration of RNA. It is assumed that transferase bound to RNA is unreactive with toxin and NAD. The concentration of rRNA was calculated assuming that 18S and 28S rRNA were present in equimolar concentrations. The smaller 5S rRNA which may have been present was not included in these calculations. We have found that in the presence of saturating concentrations of rRNA, very little transferase sediments near the top of sucrose gradients at a position expected for a complex of 5S RNA and transferase II. Hence the transferase is assumed to have a lower affinity for 5S rRNA than the larger species of rRNA.

requirement for GTP in translocation (Skogerson and Moldave, 1968a) and the ribosome-dependent GTPase activity of the transferase (Felicetti and Lipmann, 1968; Raeburn *et al.*, 1968) would seem to be more than coincidental. Finally, it is clear that an interdependence exists between binding of RNA and guanosine nucleotides to the transferase, which might well be related to the mechanism of translocation. If there is no interchange or cleavage of covalent bonds during translocation besides hydrolysis of GTP, then the rearrangements occurring on the surface of ribosomes almost certainly must involve alterations in the conformation of certain proteins, possibly resulting in changes in affinity for RNA such as that observed.

It might be contended that the sensitivity of the RNA–transferase II complexes to KCl and $MgCl_2$ is inconsistent with the formation of such complexes *in vivo* since relatively high concentration of both K^+ and Mg^{2+} are required for protein synthesis. However, although it is true that such complexes would not be expected to form with RNA free in solution, they might well form in a sheltered ionic environment in which the transferase may function on the ribosome. Such complexes might be stabilized by secondary interactions of the transferase and RNA with other ribosomal components.

In addition the fact that transferase II binds to RNA with little apparent specificity might be interpreted as speaking against the relevance of our observations to its activity. The conditions employed here were clearly far from the optimal ionic conditions for protein synthesis, and the possibility that specificity for certain species of RNA may be expressed as optimal conditions for protein synthesis are approached remains to be tested. However the species of RNA to which the transferase binds *in vivo* might well be determined by secondary interactions of it and the RNA with other components of the ribosome complex. Thus specificity might be determined by relative positioning of the transferase and RNA on the ribosome, and not be an intrinsic affinity of the RNA binding site of the transferase for a specific sequence of bases or structural feature of RNA. This might be expected to be the case if for instance the transferase interacted with mRNA.

Assuming our results are relevant to the mechanism of translocation, the difference in activity between GDP and GTP in our assay system suggests a possible effect of GTP hydrolysis in translocation. Siler and Moldave (1969) have demonstrated in a cell-free system from rat liver that one molecule of transferase II can catalyze translocation of 34 peptidyl-tRNA molecules. Since only one translocation could occur per ribosome under the conditions employed the transferase must have been released from one ribosome before catalyzing translocation on a second. The results presented here suggest that GDP resulting from hydrolysis of GTP bound to the transferase may induce a conformational change in the transferase causing it to be released from RNA, and perhaps from the entire ribosome. A similar suggestion has been made by Gill *et al.* (1969). Also the inhibitory effect of GDP on translocation (Skogerson and Moldave, 1968c; Pestka, 1969) may result at least partially from release of transferase II or G factor from RNA to which it may be bound on ribosomes.

Gill *et al.* (1969) have shown that transferase II from reticulocytes binds to mammalian ribosomes and that the binding is partially inhibited by GTP, GDP, and GMPPCP. The binding to ribosomes was demonstrated directly by sucrose gradient sedimentation and indirectly by inhibition of the ADP-ribosylation reaction by ribosomes. By the latter method they estimated that approximately six molecules of transferase II may be bound per ribosome. It is possible that several species of RNA on the ribosomes may have interacted with transferase II to give the high value obtained. The percentage of the bound transferase which was functional was not tested. Raeburn *et al.* (1968) and Everse *et al.* (1970) have also reported inhibition of the ADP ribosylation of transferase II by ribosomes.

An apparent conflict exists between the requirement of GTP for transferase II binding to ribosomes in the rat liver system (Rao and Moldave, 1969) and our observation that GTP decreases the affinity of the transferase for RNA. As stated earlier, however, the effect of GTP in our system may have resulted from GDP due to contamination or hydrolysis of GTP. Moreover it is conceivable that the primary interaction of transferase II to ribosomes may involve its binding to protein, rather than RNA on the ribosome, an interaction which might depend on GTP, rather than being inhibited by it.

References

Brot, N., Ertel, R., and Weissbach, H. (1968), *Biochem. Biophys. Res. Commun. 31*, 563.

Collier, R. J. (1967), *J. Mol. Biol. 25*, 83.

Collier, R. J., and Kandel, J. (1971), *J. Biol. Chem. 246*, 1496.

Collier, R. J., and Traugh, J. A. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 589.

Collins, J. F., Raeburn, S., and Maxwell, E. S. (1971), *J. Biol. Chem. 246*, 1049.

Everse, J., Gardner, D. A., Kaplan, N., Galasinski, W., and Moldave, K. (1970), *J. Biol. Chem. 245*, 809.

Felicetti, L., and Lipmann, F. (1968), *Arch. Biochem. Biophys. 125*, 548.

Gill, D. M., and Pappenheimer, A. M., Jr. (1971), *J. Biol. Chem. 246*, 1492.

Gill, D. M., Pappenheimer, A. M., Jr., and Baseman, J. B. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 595.

Goor, R. S., and Maxwell, E. S. (1970), *J. Biol. Chem. 245*, 616.

Hardesty, B., Culp, W., and McKeehan, W. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 331.

Honjo, T., Nishizuka, Y., Hayahishi, O., and Kato, I. (1968), *J. Biol. Chem. 243*, 3553.

Kuriki, Y., and Kaji, A. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1399.

Lengyel, P., and Söll, D. (1969), *Bacteriol. Rev. 33*, 264.

Lucas-Lenard, J., and Haenni, A.-L. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 93.

Malkin, M., and Lipmann, F. (1969), *Science 164*, 71.

Nathans, D., von Ehrenstein, G., Munro, R., and Lipmann, F. (1962), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 21*, 127.

Nishizuka, Y., and Lipmann, F. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 212.

Pestka, S. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 395.

Raeburn, S., Collins, J. F., Moon, H. M., and Maxwell, E. S. (1971), *J. Biol. Chem. 246*, 1041.

Raeburn, S., Goor, R. S., Collins, J. F., and Maxwell, E. S. (1970), *Biochim. Biophys. Acta 199*, 294.

Raeburn, S., Goor, R. S., Schneider, J. A., and Maxwell, E. S. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1428.

Rao, P., and Moldave, K. (1969), *J. Mol. Biol. 46*, 447.

Siler, J., and Moldave, K. (1969), *Biochim. Biophys. Acta 195*, 138.

Skogerson, L., and Moldave, K. (1968a), *Arch. Biochem. Biophys. 124*, 497.
Skogerson, L., and Moldave, K. (1968b), *J. Biol. Chem. 243*, 5354.
Skogerson, L., and Moldave, K. (1968c), *J. Biol. Chem. 243*, 5361.
Traugh, J. A., and Collier, R. J. (1970), *Biochem. Biophys. Res. Commun. 40*, 1437.

Changes in Labeling Pattern of Ribonucleic Acid from Mammary Tissue as a Result of Hormone Treatment*

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ABSTRACT: RNAs produced in explants of mouse mammary glands maintained on an insulin (I) or insulin plus hydrocortisone (IF) containing medium in the presence or absence of prolactin (P) have been studied. Electrophoretic analysis of the RNAs demonstrated that addition of P for 24 hr to explants incubated previously on I or IF for 4 days produced no significant alteration in the distribution of radioactivity in RNA following 2- or 4-hr incubations with tritiated uridine.

In contrast, the RNAs isolated from explants which had been on IF medium, whether P was present or not, showed a greater percentage of slowly migrating RNA species following incubation with tritiated uridine than those which had been maintained on I or IP medium.

Explants of mouse mammary glands may be maintained on a synthetic medium for several days. Depending on which hormones are used to supplement the medium, the physiological and biochemical activity of the explants may be varied. In particular, the gland may be induced to form specific milk proteins.

Proliferation of alveolar epithelial cells of mammary glands of mice in midpregnancy occurs when these cells are cultured on synthetic medium containing insulin (Stockdale *et al.*, 1966). Supplements of hydrocortisone (Mills and Topper, 1970) and prolactin (Green and Topper, 1970) to this medium do not detectably alter the proliferative response to insulin. The addition of hydrocortisone to an insulin-containing medium results in an extensive increase of the rough endoplasmic reticulum of the alveolar epithelium (Mills and Topper, 1970) but has no detectable effect on incorporation of uridine into total RNA of the epithelial cells and does not increase milk protein production in the absence of added prolactin (Turkington *et al.*, 1967). The adipose cells, a normal component of mammary explants, incorporate less uridine into RNA as a result of hydrocortisone addition (Green and Topper, 1970).

The addition of prolactin to explants previously cultured on an insulin-containing medium produces only a transient increase in incorporation of uridine into RNA of epithelial cells, and in the absence of hydrocortisone is not effective in eliciting milk protein production (Green and Topper, 1970). The addition of prolactin to explants cultured on insulin plus hydrocortisone induces the alveolar epithelial cells to assume ultrastructural characteristics typical of secretory cells (Mills and Topper, 1970). Uridine incorporation into RNA and the amount of RNA present in epithelial cells increase, but RNA metabolism of the adipose cells is unaffected (Green and Topper, 1970). The production of the milk proteins, casein (Turkington *et al.*, 1967), and the two proteins of the lactose synthetase system (Turkington *et al.*, 1968; Palmiter, 1969) is markedly increased.

The present study is an electrophoretic analysis of RNAs produced in explants cultured in the presence of insulin or insulin plus hydrocortisone with and without the addition of prolactin.

Materials and Methods

Tissue. Mammary explants were prepared from Balb/C mice 10–12 days in their first pregnancy.

Reagents. Medium 199 was purchased from Microbiological Associates. Crystalline beef insulin was a gift from the Eli Lilly Co., and ovine prolactin was a gift from the National Institutes of Health Endocrinology Study Section. Hydrocortisone was purchased from Nutritional Biochemicals, Inc. The final concentration of each hormone, when used, was 5 μg/ml. Uridine-*5-t* (Schwarz BioResearch, Inc.; specific activity 8 Ci/mmole) was used at a level of 1 μCi/ml in 24-hr incubations or 5 μCi/ml for incubations of 4 hr or less. Tris (Schwarz BioResearch, Inc.), Na_2EDTA, sodium lauryl sulfate (SLS),[1] phenol, and boric acid were analytical grade. Acrylamide, *N,N*-methylenebisacrylamide, and 3-dimethylaminopropionitrile (DMAPN), as well as *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and Stains-all (Dahlberg *et al.*, 1969), were obtained from Eastman Organic Chemical Distillation Products Industries, Rochester, N. Y. SeaKem agarose was purchased from Marine Colloid, Inc., Bausch and Lomb, Distributors. NCS reagent was purchased from Amersham-Searle.

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[1] Abbreviations used are: SLS, sodium lauryl sulfate; I, insulin; IF, insulin plus hydrocortisone; P, prolactin; SDS, sodium dodecyl sulfate.