Guanylation of Transfer Ribonucleic Acid by a Cell-free Lysate of Rabbit Reticulocytes*

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SUMMARY

The guanylation of tRNA in a cell-free system has been achieved in a lysate of rabbit reticulocytes. The enzymatic nature of the reaction is indicated by sensitivity to trypsin, heat inactivation, and by the precipitation of the guanylating activity with ammonium sulfate. The lysate did not incorporate guanine after endogenous tRNA was removed by RNase, or by binding the tRNA to an anion exchange resin. Guanylation activity was restored only by adding back reticulocyte or yeast tRNA. Liver tRNA or the synthetic homopolymers poly(A), poly(U), poly(C), or poly(G) did not serve as substrates. The reaction requires a monovalent cation which is best met by Li⁺ or K⁺ and the Kₐ for guanine is 2.5 × 10⁻⁷ M. When uniformly labeled guanosine is the substrate the purine ring but not the ribose moiety is incorporated into tRNA.

Even though the mammalian reticulocyte has no nucleus and is incapable of synthesizing RNA (1, 2), rabbit reticulocytes incorporate guanine into tRNA (2–4). This was initially interpreted as being due to the ability of red cells to convert guanine to adenine (5) which was then incorporated into the 3′-terminal residue by tRNA-CMP-AMP pyrophosphorylase (2, 6). However, this simple explanation of the phenomenon is without validity since it was later shown that the guanine was not incorporated into the 3′ terminus but internally into the polynucleotide chain and the guanylated tRNA co-chromatographs with the minor reticulocyte tRNA₁ in a reversed phase column. When uniformly labeled guanosine is the substrate the purine ring but not the ribose moiety is incorporated into tRNA.

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The guanylation of tRNA in a cell-free system has been achieved in a lysate of rabbit reticulocytes. The enzymatic nature of the reaction is indicated by sensitivity to trypsin, heat inactivation, and by the precipitation of the guanylating activity with ammonium sulfate. The lysate did not incorporate guanine after endogenous tRNA was removed by RNase, or by binding the tRNA to an anion exchange resin. Guanylation activity was restored only by adding back reticulocyte or yeast tRNA. Liver tRNA or the synthetic homopolymers poly(A), poly(U), poly(C), or poly(G) did not serve as substrates. The reaction requires a monovalent cation which is best met by Li⁺ or K⁺ and the Kₐ for guanine is 2.5 × 10⁻⁷ M. When uniformly labeled guanosine is the substrate the purine ring but not the ribose moiety is incorporated into tRNA.

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MATERIALS AND METHODS

[²⁻¹⁴C]Guanine and [³H]histidine had specific activities of 10 mCi per mmole and 5 Ci per mmole, respectively, and were purchased from Schwarz-Mann Corp. [U⁻¹⁴C]Guanosine (413 mCi per mmole) was bought from New England Nuclear; the homoribopolynucleotides were from Miles; T₁ RNase was purchased from Calbiochem; matrix-bound RNase was from Nutritional Biochemicals. The resin used to remove endogenous tRNA from hemolysates was DE-32 from Whatman.

Preparation of Hemolysates—Reticulocytes from rabbits, rats, mice, sheep, and man were obtained, washed, and harvested as previously described (3, 4). The human reticulocytes were obtained from two patients with elevated (greater than 10%) reticulocyte counts. The first patient had hemoglobin SC disease. The second patient was admitted for acute alcoholism. The cells were lysed by the addition of an equal volume of cold glass-distilled water and unlysed cells and stroma were removed by centrifugation at 12,000 × g. The stroma from sheep reticulocytes did not pack tightly after centrifugation and only the upper one-third of the hemolysate was used. In some
experiments the lysate was used directly. We later found that ribosomes were not essential for the guanylation reaction and they were removed by centrifugation at 105,000 x g for 90 min. This lysate could be stored without loss of activity at 40°C for at least 1 month.

**Treatment of Lysate with Matrix-bound RNase—**A 1:1 lysate was dialyzed against 0.15 M NaCl for 5 hours, then against distilled water for 8 more hours. It was sedimented at 105,000 x g for 90 min and the precipitate removed. One milliliter of the supernatant was incubated with gentle agitation with 15 mg of matrix-bound RNase for 60 min in 0.025 M TES, pH 7.4. The bound RNase was removed by filtration and the tRNA-free filtrate frozen for future experiments. The recovered bound RNase was washed twice with 0.15 M NaCl, four times with H2O, and dried in vacuo for future use.

**Removal of tRNA from Hemolysates with DEAE-cellulose—**The tRNA was removed from the lysate by a modification of the method described by Gilbert and Anderson (10). A suspension of Whatman DE-32 was centrifuged in a graduated tube in a clinical centrifuge to a packed resin volume of 3.5 ml. The 1:1 reticulocyte hemolysate was freed of ribosomes by sedimentation at 105,000 x g and was then dialyzed against 0.30 M KCl. Six milliliters of the dialyzed hemolysate were added to the DE-32 and the mixture stirred for 5 min. The resin was removed by centrifugation. The tRNA-free hemolysate was now dialyzed against H2O for direct assay or against 0.01 M Tris, pH 7.4-0.01 M mercaptoethanol for enzyme purification. The tRNA was recovered from the DE-32 by first washing the resin with 0.30 M KCl to remove trapped solution and then eluting the tRNA with 1.0 M NaCl in 0.40 M ammonium acetate, pH 8.2. The eluted tRNA was extracted with an equal volume of phenol and the tRNA recovered from the aqueous phase after precipitation with 2 volumes of 95% EtOH.

**Assay of Guanylating Enzyme in Hemolysates—**A typical assay for guanylating enzyme activity in a lysate from which endogenous tRNA was removed was performed as follows. The reaction mixture contained 1 ml of lysate (82 mg of protein), 25 μmoles of TES, pH 7.4, 200 μmoles of KCl, 6.8 A260 units of reticulocyte tRNA, and 0.1 μmole of [14C]guanine in a volume of 1.41 ml. The solution was incubated at 37°C. At the end of the incubation period the tubes were chilled to 0°C and 2 ml of 0.1 M sodium acetate, pH 5.0-0.1 M NaCl-0.01 M EDTA was added followed by 2 volumes of water-saturated phenol. After shaking for 5 min the phases were separated and the aqueous phase collected by aspiration. The phenol phase was washed with the acetic buffer and the aqueous phases pooled. The tRNA was precipitated with 2 volumes of 95% ethanol. After storage at -15°C for at least 2 hours the tRNA was collected by centrifugation and dried in vacuo. The tRNA was dissolved in 0.40 M ammonium acetate, pH 8.2, and adsorbed to a DEAE cellulose column (3). The column was washed with the 0.40 M ammonium acetate buffer until all radioactive impurities had been eluted. The tRNA was then eluted with 1.0 M NaCl in 0.40 M ammonium acetate, pH 8.2. Poly(G) was not eluted with 1.0 M NaCl so 0.1 M HCl was used to elute this polynucleotide. The absorbance of the tRNA at 260 nm was determined and then the tRNA was precipitated by the addition of trichloroacetic acid to 7.5%. The tRNA was collected on glass filters and the radioactivity determined with a liquid scintillation counter (3, 4). Incorporation was expressed as counts per min per A260 units. Some of the earlier experiments were performed using lysates that contained ribosomes and endogenous tRNA. These experiments were run as described but no tRNA was added to the reaction mixture and prior to the DEAE-cellulose step the ribosomal RNA was removed by precipitation with 1 M NaCl (11). In some experiments the incubation mixtures were sterilized by Millipore filtration and the incubations run in sterile tubes. The results obtained under sterile conditions were so similar to those obtained without this measure that sterilization was not adopted as part of the routine guanylation enzyme assay.

**Removal of Radioactive Impurity from [14C]guanine—**Three samples of commercial [8-14C]guanine were found to be contaminated with a radioactive impurity by paper chromatography in EtOH-1 M ammonium acetate (70:30). The impurity which constituted as much as 4% of the radioactivity moved 33 cm whereas guanine moved 29 cm. The impurity was separated from [14C]guanine by paper chromatography and the guanine recovered from the chromatogram by elution with 0.1 M HCl.

**Incorporation of the purified guanine was no better or worse than incorporation using unpurified commercial [8-14C]guanine preparations.**

**Precipitation of Guanylating Enzyme with Ammonium Sulfate—**After removal of RNA with DE-32 the lysate was diluted with 0.01 M Tris, pH 7.4-1.0 M mercaptoethanol to give a solution containing 20 mg of protein per ml. To this solution a solid uniform mixture made by mixing 99 g of (NH4)2SO4 with 1 g of KHCO3 was added until 60% of saturation was reached, saturation being defined as 72 g of (NH4)2SO4 per 100 ml of H2O. Stirring was continued for 30 min. The precipitate was collected by centrifugation at 10,000 x g and the protein remaining in the supernatant was precipitated by further addition of the (NH4)2SO4-KHCO3 mixture to 90% of saturation. The precipitates were dissolved in 0.01 M Tris, pH 7.4-1.0 M β-mercaptoethanol. The volume of the buffer was equal to the volume of the DE-32-treated lysate. The solution was dialyzed against two changes of 100 volumes of the same buffer. The two fractions were assayed and 100% of the guanylation activity was recovered in the fraction precipitated by 50% ammonium sulfate. The ammonium sulfate treatment yielded a 5.5-fold purification over the lysate.

**Chromatography of [14C]Guanylated tRNA Produced in Vitro on Sephadex G-75—**A 1:1 lysate containing tRNA was incubated with [14C]guanine for 3 hours. The tRNA was extracted and chromatographed on a Sephadex G-75 column (2 x 50 cm) that had been equilibrated with 0.10 M sodium acetate buffer, pH 5.0, 0.01 M EDTA, and 0.10 M NaCl. The eluate from the column was monitored for absorbance at 260 nm. One-milliliter fractions were collected and 0.10 ml of 90% trichloroacetic acid was added to each fraction. The suspensions were filtered and the precipitated radioactivity determined. The volume at which 4 S RNA was eluted was determined.

**Preparation and Hydrolysis of [14C]GMP from Reticulocyte Incubated with [U-14C]Guanosine—**Cells were washed, incubated with [14C]guanosine, and lysed as previously described (3, 4). The tRNA was extracted and purified as described above and then hydrolyzed for 18 hours with 0.30 M KOH at 37°C. The solution was neutralized (3) and then chromatographed on Whatman No. 3MM paper with EtOH-1 M ammonium acetate (70:30) as the solvent. The GMP was eluted with 0.1 M HCl, and concentrated to 1 ml by lyophilization. The N-glycoside bond of 2',3'-GMP was hydrolyzed by a modification of the method described by Khym and Cohn (12). Dowex 50 (H+) (1 g) was added and the suspension sparged
with N₂ while immersed in a 100° bath for 3 min. The solution was cooled and the slurry poured into a small column (0.5 x 3 cm) containing more Dowex 50 (H⁺). The column was washed with H₂O until all ribose and ribose phosphate and no radioactivity was eluted. The guanine was then eluted with 2.0 M KOH.

RESULTS

Time Course and Dependence of Guanylation Reaction on Protein Concentration—The data plotted in Fig. 1 show that the incorporation of guanine by a cell-free lysate is linear for 1 hour and continues to increase for 3 hours.

The data plotted in Fig. 2 show that the rate of guanine uptake into tRNA catalyzed by the partially purified enzyme is proportional to enzyme concentrations in the range 0.6 to 3.8 mg of protein per ml.

Heat Inactivation and Trypsin Sensitivity of Guanylating Enzyme—To test if the guanylating enzyme is heat-labile, aliquots of the 50% ammonium sulfate precipitate were maintained at elevated temperatures for 7 min, cooled, and assayed at 37°C. The enzyme is stable at 55°C but labile at 65°C.

Incubation of 1.0 ml of the 1:1 lysate (50 mg of protein) with 7.5 mg of trypsin for 60 min at pH 7.4 completely inactivated the guanylation activity in the lysate.

Proof that Guanylated RNA Produced In Vitro Is tRNA—When the guanylated tRNA produced in vitro was chromatographed on a Sephadex G 75 column all of the radioactivity was eluted at the same position as tRNA.

In order to show that the guanylated tRNA produced in vitro is identical with reticulocyte tRNA, [14C]guanylated tRNA was charged with histidine (3) and analyzed on a reversed phase column (13). The results shown in Fig. 3 are identical with the pattern obtained in vivo (4). There are two isoaccepting tRNA in rabbit reticulocytes and the minor one is the one that serves as the substrate for the guanylating enzyme. As in the case for guanylated tRNA produced in vivo there is also a minor guanylated tRNA that is eluted from reversed phase columns earlier than tRNA (3, 4).

Guanine Residue Is Incorporated into Internal Position of tRNA without Modification—The [14C]guanylated tRNA was hydrolyzed with 0.3 M KOH for 18 hours at 37°C. The KOH hydrolysate was neutralized with Dowex 50 (H⁺) and chromatographed in a paper chromatographic system that separates nucleosides, mononucleotides, and nucleotides with more than 1 phosphate residue (3, 14). All of the radioactive material in the alkaline hydrolysate was found in the mononucleotide region of the chromatogram. The mononucleotide spot was eluted with 0.01 M HCl and subjected to high voltage electrophoresis in a system that separates the four major bases (15). All of the radioactivity was electrophoretically identical with 2'-3'-GMP.
in Aliquot III co-chromatographed with 2':3'-GMP again confirming the fact that the guanine was not incorporated at the 3' end of the oligonucleotide. All of the radioactivity was at the 3' end of the oligonucleotide. The [14C]guanylated tRNA was digested with T1 RNase and the products analyzed by paper chromatography (Fig. 4B). The results show that T1 RNase which attacks RNA at the 3' side of guanosine residues forms a radioactive oligonucleotide (Aliquot I). When this oligonucleotide is treated with alkaline phosphatase and then digested with dilute KOH (Aliquot II) the product is an internal position in the polynucleotide (Fig. 4A) thus confirming the fact that the cell-free system, just prior treatment with alkaline phosphatase (m--m). The soluble RNase guanine incorporation could be restored by the addition of reticulocyte tRNA.

Table I also shows that yeast and rabbit reticulocyte tRNA were good acceptors for the guanylating enzymes; Escherichia coli and rabbit liver tRNA were not as efficient. None of the synthetic homopolynucleotides served as substrates. We could not study whether poly(G) was a substrate in the usual manner because even after the extensive purification procedure in our lysate with matrix-bound RNase. After removal of the insoluble RNase guanine incorporation could be restored by the addition of reticulocyte tRNA.

Table I summarizes the data from an experiment showing that the capacity of a lysate to carry out the guanylation reaction is diminished after incubation of the lysate with matrix-bound RNase. After removal of the insoluble RNase guanine incorporation could be restored by the addition of reticulocyte tRNA.

Because of occlusion of guanine by poly(G) and the strong affinity of poly(G) for DEAE-cellulose the experiment with this polynucleotide was performed using the modification described in the text.

the amino acid acceptor end. If it were, the radioactivity would have been recovered as guanosine. Had the guanine been incorporated as the terminal nucleotide at the 5' end of tRNA the radioactivity would have been in 3':5' guanosine diphosphate.

The radioactive nucleotides obtained by alkaline hydrolysis of the [14C]guanylated tRNA formed by the cell-free lysate and by intact reticulocytes (3, 4) were compared. The nucleotides first hydrolyzed to the mononucleotides with alkaline phosphatase and the products analyzed by paper chromatography in four solvents. In each case the radioactive nucleosides formed in vivo and in vitro had identical RF values which also corresponded to the RF of guanosine. The solvents were: Solvent I, 0.5M ethanol 103 ammonium acetate, pH 7.2; Solvent II, isobutyric acid-H2O-NH4OH, 66:33:1; Solvent III, 88% formic acid-95% ethanol-t-butyl alcohol-H2O, 5:60:20:15; Solvent IV, isopropyl alcohol-concentrated HCl (180:42) brought to 250 ml with H2O.

The radioactive nucleotides recovered from an alkaline hydrolysate of [14C]guanylated tRNA produced in vitro. The solution containing the nucleotides was spotted on Whatman No. 3MM paper and subjected to electrophoresis at 2000 volts for 2 hours. The buffer was 0.40 M sodium acetate, pH 3.75. The position to which the four major 2',3'-mononucleotides migrated is indicated. The paper was dried, cut into 0.5-cm strips, and counted. Digestion of [14C]guanylated RNA with T1 RNase, [14C]guanylated tRNA (3.9 A260 units) was dissolved in 1.0 ml of 0.30 M ammonium acetate, pH 7.2, and digested with 0.3 mg of T1 RNase at 37° for 4 hours. The digest was divided into three parts. Aliquot I was not treated further (O--O); Aliquot II was treated with alkaline phosphatase, then digested with 0.3 mg of T1 RNase at 37° for 4 hours. The digest was divided into three aliquots. Aliquot I was not subject to further degradation, Aliquot II was treated with alkaline phosphatase, then digested with 0.3 M KOH, and Aliquot III was digested with 0.3 M KOH. The three samples were then analyzed by paper chromatography (Fig. 4B). The results show that T1 RNase which attacks RNA at the 3' side of guanosine residues forms a radioactive oligonucleotide (Aliquot I). When this oligonucleotide is treated with alkaline phosphatase and then digested with dilute KOH (Aliquot II) the product is radioactive guanosine indicating that the incorporated guanine was at the 3' end of the oligonucleotide. All of the radioactivity in Aliquot III co-chromatographed with 2':3'-GMP again confirming the fact that the guanine was not incorporated at the amino acid acceptor end. If it were, the radioactivity would have been recovered as guanosine. Had the guanine been incorporated as the terminal nucleotide at the 5' end of tRNA the radioactivity would have been in 3':5' guanosine diphosphate.

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Dependence of Guanylation Reaction on tRNA and Comparison of Polynucleotides as Substrates—Table I summarizes the data from an experiment showing that the capacity of a lysate to carry out the guanylation reaction is diminished after incubation of the lysate with matrix-bound RNase. After removal of the insoluble RNase guanine incorporation could be restored by the addition of reticulocyte tRNA.

Table I also shows that yeast and rabbit reticulocyte tRNA were good acceptors for the guanylating enzymes; Escherichia coli and rabbit liver tRNA were not as efficient. None of the synthetic homopolynucleotides served as substrates. We could not study whether poly(G) was a substrate in the usual manner because even after the extensive purification procedure in our assay, guanine was occluded to the poly(G). In order to show that these counts were due to occlusion of guanine and not guanylation, the poly(G) was digested for 18 hours with 0.3 M KOH and then subjected to high voltage electrophoresis (16). Radioactivity was detected in the guanine region but was not found associated with 2':3'-GMP.

Cofactor Requirements for Guanylation Reaction—The data in Table I show that dialysis of a 1:1 lysate brought about an 86%...
inhibition of guanylating enzyme activity. Most of the activity was restored by the addition of the concentrated dialysate. Various combinations of ATP, Mg²⁺, and K⁺ were tested to their ability to restore activity and, as seen in the table, all of the combinations that worked contained K⁺. The optimal K⁺ concentration is 0.107 M. The ability of different monovalent cations to substitute for K⁺ was also tested and the order in which the cations restored activity was Li⁺ > K⁺ > Cs⁺ > NH₄⁺ > Na⁺ > Rb⁺ while Mg²⁺ had no effect. The restoration of activity is not by the restoration of the ionic strength since MgCl₂ at an ionic strength equivalent to that at which the monovalent cations were tested was without effect.

Effect of pH—The activity of the guanylating enzyme at different pH values was measured in a buffer that was 0.05 M with respect to sodium formate, sodium acetate, maleic acid, and TES between pH 5.8 and 7.5, and a second buffer that was 0.20 M with respect to Tris, glycine, and boric acid between pH 7.1 and 9.3. The pH was adjusted by addition of HCl or NaOH. The optimal pH was 7.8. At pH 7.0 and 8.2 the enzyme was 50% as active as at the optimum. No guanylating activity was detected below pH 6.0 or above 9.0.

Effect of Variation of Guanine and tRNA Concentration on Guanylation Reaction—Lysates were incubated for 1 hour and the concentration of guanine (Fig. 5) or tRNA (Fig. 6) was varied. The incorporation of guanine is linear with time for up to 1 hour (Fig. 1). The guanylating enzyme obeys Michaelis-Menten kinetics for both substrates and apparent Kₘ values of 2.7 × 10⁻³ M for guanine and of 0.7 A₂₆₀ unit per ml for unfractionated reticulocyte tRNA were obtained.

Effect of Removing Ribosomes—Removal of ribosomes by sedimentation at 105,000 × g from lysates did not affect the ability of the supernatant to guanylate tRNA.

Comparison of Lysates from Different Species—Table III shows that lysates prepared from rabbit reticulocytes were able to carry out the guanylation reaction but those of mouse or rat were not. The table also shows that the rodent reticulocyte extracts contain an inhibitor of guanylation by the rabbit reticulocyte cell-free system.

Ribose Moiety of Guanosine Is Not Incorporated—GMP was recovered from a dilute KOH digest of [¹⁴C]guanylated tRNA that had been prepared by incubating reticulocytes with guanosine uniformly labeled with [¹⁴C]. The N-glycoside bond of GMP was hydrolyzed with Dowex 50 (H⁺) (12) and the resultant (2',3') ribose phosphate recovered from the resin by washing with H₂O.

**TABLE II**

Reconstitution of guanylating activity after dialysis
A 1:1 lysate was dialyzed for 5 hours against 0.15 M NaCl then against H₂O for 8 hours. The ATP was 82 μM; MgCl₂, 0.82 mM; KCl, 81 mM.

| Reaction components | [¹⁴C]Guanine cpm | A₂₆₀
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Undialyzed lysate</td>
<td>414</td>
<td>0.82</td>
</tr>
<tr>
<td>Dialyzed lysate</td>
<td>57</td>
<td>0.82</td>
</tr>
<tr>
<td>Dialyzed lysate + ATP, + Mg²⁺, + K⁺</td>
<td>455</td>
<td>0.82</td>
</tr>
<tr>
<td>Dialyzed lysate + ATP, + Mg²⁺</td>
<td>138</td>
<td>0.82</td>
</tr>
<tr>
<td>Dialyzed lysate + ATP, + K⁺</td>
<td>345</td>
<td>0.82</td>
</tr>
<tr>
<td>Dialyzed lysate + Mg²⁺, + K⁺</td>
<td>348</td>
<td>0.82</td>
</tr>
<tr>
<td>Dialyzed lysate + K⁺</td>
<td>390</td>
<td>0.82</td>
</tr>
</tbody>
</table>

**Fig. 5.** Dependence of the rate of guanylation on guanine concentration. A 1:1 lysate was incubated at the indicated concentrations of [¹⁴C]guanine for 1 hour and the extent of guanylation was determined.

**Fig. 6.** Dependence of the rate of guanylation on reticulocyte tRNA concentration. The tRNA was removed from a lysate with DE-32. The lysate was then incubated with the indicated concentrations of tRNA for 1 hour.

**TABLE III**

Species specificity for guanylation of tRNA

| Type of reticulocyte lysate | [¹⁴C]Guanine cpm | A₂₆₀
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>401</td>
<td>0.82</td>
</tr>
<tr>
<td>Human</td>
<td>317</td>
<td>0.82</td>
</tr>
<tr>
<td>Sheep</td>
<td>287</td>
<td>0.82</td>
</tr>
<tr>
<td>Rat</td>
<td>4</td>
<td>0.82</td>
</tr>
<tr>
<td>Mouse</td>
<td>2</td>
<td>0.82</td>
</tr>
<tr>
<td>Mouse + rabbit</td>
<td>2</td>
<td>0.82</td>
</tr>
<tr>
<td>Rat + rabbit</td>
<td>3</td>
<td>0.82</td>
</tr>
</tbody>
</table>

**TABLE IV**

Distribution of radioactivity between purine and ribose moieties after labeling with [U-¹⁴C]guanosine

<table>
<thead>
<tr>
<th>Compound hydrolyzed</th>
<th>Ribose cpm</th>
<th>Guanine cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁴C]GMP from tRNA</td>
<td>22</td>
<td>2418</td>
</tr>
<tr>
<td>[U-¹⁴C]Guanosine</td>
<td>2730</td>
<td>2760</td>
</tr>
</tbody>
</table>
The guanine was then eluted with 2.0 M KOH. The results in Table IV show that virtually all of the radioactivity was recovered in the purine ring and not in the ribose. Table IV also shows that when the uniformly $^{14}$C-labeled guanosine was hydrolyzed by the Dowex 50 (H$^+$) method the radioactivity was equally distributed between the purine ring and ribose moieties.

**DISCUSSION**

The results presented in this study indicate that the guanylation of tRNA is effected with a cell-free system as well as with intact cells. The assay for the guanylylating enzyme described in this report is lengthy which has hampered the purification of the enzyme. The presence of a radioactive impurity (1 to 4%) in several samples of commercial $[8-14$C]guanine is mentioned, even though its presence did not affect any of the studies described in this report. The product formed by the cell-free system and intact cells were identical. In both cases the same isoacceptor $tRNA^\star$ is guanylated and in both cases alkaline hydrolysis yields unmodified 2':3'-GMP, which is proof that the guanine was incorporated into an internal position in the polynucleotide chain. If the guanine were incorporated at the 3' end or 5' end of $tRNA$, alkaline hydrolysis would have produced radioactive guanosine or pGp, respectively.

The simplicity of the system that carries out guanylation is striking. There is no requirement for energy in the usual sense, as no nucleoside triphosphates are required. The only requirement is for the presence of a monovalent cation and all the monovalent cations activate the enzyme to some degree. There is no pattern in relation to size with which the ions activate the enzyme. The results presented in this study indicate that the guanylation of $tRNA$ is effected with a cell-free system as well as with intact cells. The assay for the guanylylating enzyme described in this report is lengthy which has hampered the purification of the enzyme. The presence of a radioactive impurity (1 to 4%) in several samples of commercial $[8-14$C]guanine is mentioned, even though its presence did not affect any of the studies described in this report. The product formed by the cell-free system and intact cells were identical. In both cases the same isoacceptor $tRNA^\star$ is guanylated and in both cases alkaline hydrolysis yields unmodified 2':3'-GMP, which is proof that the guanine was incorporated into an internal position in the polynucleotide chain. If the guanine were incorporated at the 3' end or 5' end of $tRNA$, alkaline hydrolysis would have produced radioactive guanosine or pGp, respectively.

The reaction is enzymatic as shown by heat lability, sensitivity to trypsin, the capacity of the system to be saturated by both substrates, guanine, and $tRNA$, and the precipitation of guanylation activity with 50% ammonium sulfate. The reaction is quite specific both for the $tRNA$ molecule and probably for a particular nucleotide sequence in that $tRNA$ molecule since the only $tRNA$ that appears to be guanylated is $tRNA^\star$ and when the guanylated $tRNA$ is degraded with either T$\alpha$ or pancreatic RNase and the resultant oligonucleotides separated by chromatography all of the $[4$C]guanine is found in a single oligonucleotide (4). The simplicity of requirements for the reaction and identification of unmodified GMP as the product seem to rule out all mechanisms other than a replacement of a specific base in the $tRNA^\star$ by a guanine residue. This is also indicated by the experiment in which guanosine uniformly labeled with $^{14}$C in the purine ring and ribose moieties was used as the precursor of guanylated $tRNA$. Only the purine ring and not the ribose moiety was incorporated into $tRNA$.

The reason why rodent reticulocytes contain an inhibitor of the guanylation reaction remains obscure. The inhibitor which is a heat-labile protein is presently being studied. The biological role of guanylated $tRNA$ is still obscure but a role for $tRNA$ in regulating the rate of translation of hemoglobin mRNA has been postulated by Itano (16) and by Winslow and Ingram (17). In fact Winslow and Ingram found that the rate of translation of the two globin chains was slowed down in the vicinity of the histidine residues $a$-87 and $b$-92 in human bone marrow cells. These $2$ histidine residues are linked to heme and it was postulated that the heme was inserted into the nascent globin chain at this point. More recent work indicates that heme does not bind to the polypeptide until the completed globin chain is released from the ribosomes (18). Heme exerts its control upon globin synthesis by stimulating initiation (19). The “control points” observed by Winslow and Ingram may therefore be due to $tRNA$ and since the guanylylation enzyme is specific for reticulocyte $tRNA^\star$, it may play a role in controlling globin chain synthesis. Clegg et al., however, were unable to detect a control point during the assembly of $\beta$ chains in normal reticulocytes (20).

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