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Purification and Characterization of Yeast Nucleotidyl Transferase and Investigation of Enzyme–Transfer Ribonucleic Acid Complex Formation*

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ABSTRACT: Nucleotidyl transferase has been purified more than 1000-fold from yeast. Autolysis of yeast is followed by ammonium sulfate precipitation, gel filtration, DEAE-cellulose chromatography and, finally, hydroxylapatite chromatography. These last three steps are performed in 40% (v/v) glycerol. After the last step, the enzyme is essentially free of ribonucleases, phosphomonoesterases, phosphodiesterases, pyrophosphatases, and aminoacyl-tRNA synthetases.

The optimum concentrations for the components of the reaction are: ATP, 2 mM; CTP, 150 μM; and Mg^{2+}, 10 mM. The optimum pH is 9.5. When the hydroxylapatite fraction is sedimented in a sucrose density gradient, the enzymatic activity is found as a single symmetric peak sedimenting slightly more slowly than homoglobin (4.3 S). However, in the presence of tRNA, the enzymatic activity sediments 50% faster (~6 S). Disc gel electrophoresis patterns of the hydroxylapatite fraction show two bands of approximately equal density. The faster moving of these bands predominates in disc gel patterns of fractions from the enzyme region (~6S material) of a sucrose density gradient containing enzyme and tRNA, indicating that the enzyme is in the faster moving gel band. Two kinds of evidence are presented to show that a stable complex is formed between the transferase and tRNA. The first is sedimentation patterns in sucrose density gradients like those described above, and the second is enzyme-dependent binding of radioactive tRNA to nitrocellulose filters. Enzyme-dependent binding occurs in the presence or absence of ATP, CTP, or Mg^{2+} and shows little dependence on pH in the range 6.5–7.5. The enzyme binds very tightly to any tRNA that is missing part of the terminal pCpCpA triplet, even if a 3′-phosphate group is present. If, however, the terminal triplet is complete, the complex is very much weaker. The transferase cannot bind small fragments resulting from T_1 RNase digestion, nor can it bind polyuridylic or polycytidylic acids. While the last two polynucleotides do not bind, they do inhibit the enzyme-dependent binding of tRNA.

Nucleotidyl transferase catalyzes the addition of AMP and CMP to tRNA, forming the terminal pCpCpA triplet (Starr and Goldthwait, 1963; Preiss *et al.*, 1961; Hecht *et al.*, 1958; Herbert, 1959). This reaction has been formulated as follows

$$\text{tRNA} + \text{CTP} + \text{ATP} \xrightarrow{Mg^{2+}} \text{tRNApCpCpA} + PP_i$$

In order to study this reaction, the nucleotidyl transferase from yeast has been purified free of enzymes that degrade or modify the substrates or products of this reaction.

Two major difficulties in the previous studies of this reaction have been that the tRNA used as the substrate was inhomogeneous and of unknown and presumably inhomogeneous end-group composition. That is, bulk tRNA is a mixture of many amino acid acceptor species with at least three different end groups: tRNApXpC, tRNApXpCpC, and tRNApXpCpCpA,[1] where X is the undefined fourth nucleotide.

In the present study, advantage was taken of recent advances in tRNA purification by using tRNAs enriched for a single amino acid acceptor tRNA. The second difficulty mentioned above was overcome by the use of the periodate–amine degradation technique developed by Khym and Uziel (1968) which permits stepwise removal of nucleotides from the pCpCpA end of tRNA.

The use of yeast as the source of enzyme and tRNA pro-

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[1] Abbreviations used are: tRNApYpXpCpCpA is tRNA with the complete pCpCpA triplet and X and Y are the fourth and fifth nucleotides, respectively.

vides further advantages in that the system is homologous and that the complete nucleotide sequences of many yeast tRNA species are now known.

In this paper, we describe the purification of the nucleotidyl transferase from yeast, and we report some of its properties.

Recently the formation of complexes between tRNA and the aminoacyl-tRNA synthetases has been reported (Yarus and Berg, 1967). In these studies it has been shown that $tRNA_{Ile}$ will bind to a nitrocellulose filter when isoleucyl-tRNA synthetase is present. The following forms of $tRNA_{Ile}$ were able to bind to the filter under conditions described: tRNApCpCpA-Ile, tRNApCpCpA, and tRNApCpC.

In light of these results, it became of interest to determine whether or not complex formation occurs between tRNA and nucleotidyl transferase. In this paper we demonstrate formation of a complex between purified transferase and highly enriched amino acid acceptor tRNA fractions by measuring enzyme-dependent binding of radioactive tRNA to nitrocellulose filters and by examining the behavior of the enzyme and tRNA in sucrose density gradients.

In the next paper, the effects on nucleotide acceptor activity of consecutive removal of nucleotides from the CpCpA end of rabbit liver, yeast, and *Escherichia coli* tRNA are reported.

Materials and Methods

Yeast tRNA (Boehringer-Mannheim Co.) accepts 0.5–0.7 μmole of AMP/μmole of tRNA if CTP is also present in the incubation, and this can be increased to 0.8–1.0 μmole of AMP/μmole of tRNA by use of the periodate–amine degradation method of Khym and Uziel (1968). This procedure, more fully described in the succeeding paper, results in shortening of the tRNA by one nucleotide from the acceptor end of the chain.

tRNA specific for phenylalanine ($tRNA_{Phe}$) was prepared according to the method of Litt (1968). It was incubated for 3 hr or more with transferase, ATP and CTP to complete the terminal pCpCpA triplet of the tRNA. The triplet was then removed by three rounds of periodate–amine degradation. This tRNA was incubated with [^3H]CTP (Schwarz BioResearch Inc.) and nucleotidyl transferase under standard conditions for 3 hr, and the tRNA was recovered by ethanol precipitation. This yielded [^3H]tRNApCpC$_{Phe}$ with a specific activity of 110 cpm/pmole.

A second portion of the $tRNA_{Phe}$ was incubated for 1 hr in 50 mM potassium phosphate buffer (pH 7.5), containing 4 mM potassium pyrophosphate and 8 mM Mg^{2+}. This treatment removes essentially all terminal AMP residues. After reisolation, the tRNA was incubated in the presence of the transferase under standard conditions for 3 hr with nonradioactive CTP and [^{14}C]ATP (Schwarz BioResearch Inc.). The $tRNA_{Phe}$ was isolated by ethanol precipitation and had a specific activity of 250 cpm/pmole.

For the nitrocellulose binding studies, the periodate–amine degradation was used in conjunction with the transferase to prepare a series of $tRNA_{Phe}$ fractions with different end groups. These are referred to in Tables IV and V.

[^{14}C]Poly UMP and [^3H]poly CMP were purchased from Miles Laboratories and had specific activities of 2000 and 300 cpm per μg, respectively. Carrier-free [^{32}P]PP_i was purchased from New England Nuclear Corp. T_1 $RNase_1$ was purchased from Calbiochem and pancreatic RNase was purchased from Worthington Biochemical Corp.

Enzyme Assay. The standard assay mixture contained in an incubation volume of 0.125 ml: 25 mM glycine–NaOH buffer (pH 9.5), 10 mM $MgSO_4$, 0.15 mM CTP, 2 mM ATP, 32 A_{260}/ml of tRNA (approximately 50 μM), and 10–200 μg of protein. (The amount of protein used depended on the stage of the purification and was adjusted in each case to make the amount of incorporation proportional to the amount of protein.) This reaction mixture was incubated for 20 min at 37°. The reaction was stopped by the addition of 20 μl of 3 M perchloric acid and the incubation tube was placed on ice. After standing for at least 20 min on ice, the precipitate was plated, quantitatively, with suction, onto a glass fiber filter (Whatman, GF/C). The filter was washed with 25 ml of 0.35 M perchloric acid and allowed to dry at room temperature. The filters were either glued to planchets and counted in a thin-window counter (Beckman Instruments, Inc. Lowbeta II) or placed in scintillation vials with scintillation fluid (5 g of 2,5-diphenyloxazole, Pilot Chemicals, Inc., per l. of toluene) and counted in a liquid scintillation spectrometer (Nuclear-Chicago Model 720).

Plating efficiency—that is, that fraction of a known amount of radioactive tRNA which precipitated and was retained by the filter—was consistently between 60 and 70%. The counting efficiencies for ^{14}C were 10–20% in the planchet counter and 40–60% in the scintillation counter. Quenching, due to the nonvolatile perchloric acid on the filter disks, was observed and was corrected for by internal standardization. Duplicate samples agreed within 10%.

Binding Assay. Enzyme-dependent binding of radioactive tRNA to nitrocellulose filters was measured using a modification of the method of Yarus and Berg (1967). A mixture was prepared which contained 12.5 μmoles of potassium phosphate buffer (pH 6.5), 0–1.5 μmoles of ATP, 0–1.5 μmoles of CTP, 0–5 μmoles of $MgSO_4$, and 10 μg of bovine serum albumin. To this was added 0.36 A_{260} of radioactive tRNA and 0.03 A_{280} of the hydroxylapatite fraction. The final volume was 250 μl. After mixing, the samples were plated, quantitatively, by suction, onto nitrocellulose filters (Schleicher und Schuell, B-6) which had been soaked in 50 mM potassium phosphate buffer (pH 6.5). The filter was washed with 3 ml of the buffer, dried, and counted in a liquid scintillation spectrometer. In every case, a control sample containing bovine serum albumin instead of enzyme protein was run in parallel with the enzyme sample.

Sucrose Density Gradient Centrifugation. The enzyme was sedimented in a 15–30% (w/v) sucrose gradient (13 ml) which contained 50 mM potassium phosphate buffer (pH 7.5). The sample volumes were 0.6 or 1.2 ml and contained 10–12% glycerol and 150 mM potassium phosphate buffer (pH 7.5). The centrifugation runs were performed in a Spinco ultracentrifuge using an SW-40 or SW-41 rotor at a speed of 40,000 rpm for 40–45 hr. After the run, the tubes were punctured in the bottom and fractions of approximately 0.25 or 0.4 ml were collected. The optical density of each fraction was measured in a Zeiss spectrophotometer.

A 50-μl aliquot was used to determine the level of enzymatic activity of each sample. A 100-μl aliquot was taken from each fraction for determination of counts per minute in tRNA. This was added to 20 μl of a tRNA solution (8 A_{260}) and precipitated with perchloric acid. This precipitate

was plated onto glass fiber filters, dried, and counted as described in the enzyme assay.

Purification of the Nucleotidyl Transferase. PREPARATION OF THE CRUDE EXTRACT (J. Ivey, 1968, personal communication). Fresh baker's yeast (3 kg; Williams Bakery, Eugene, Ore.) was crumbled into 9 l. of toluene which had been cooled to −40° with Dry Ice. This mixture was kept at −40° for 4 hr by further additions of Dry Ice. The toluene was removed and the frozen yeast was allowed to thaw on ice for approximately 16 hr. The remaining toluene was then removed by aspiration, and 600 ml of 1 M Tris–0.5 M HCl was added. This mixture was allowed to autolyze on ice for 24 hr. Cellular debris was removed by centrifugation at 15,000*g* for 1 hr at 4°. The supernatant solution, approximately 700 ml/kg, had an A_{280} of 200 and was defined as crude extract.

AMMONIUM SULFATE FRACTIONATION AND DESALTING. The crude extract was brought to 58% saturation with ammonium sulfate by addition of an unbuffered saturated solution. (This and all following procedures were carried out at 4°.) This suspension was stirred 15–20 min, and the precipitate was recovered by centrifugation at 15,000*g* for 40 min. The precipitate was resuspended in 40% saturated ammonium sulfate and stirred for several hours. The suspension was clarified by 30-min centrifugation at 15,000*g*. To the clarified supernatant was added one volume of saturated ammonium sulfate. This suspension was stirred 15–20 min and the precipitate was collected by centrifugation for 2 hr at 15,000*g*. This pellet was redissolved in 40% glycerol–0.1 mM potassium phosphate buffer (pH 7.4) and further purified and desalted by gel filtration. (From this point on, all solutions were 40% (v/v) in glycerol unless otherwise stated.) The enzyme solution, about 600 ml, was placed on a G-25 coarse Sephadex column (10 × 50 cm) and the enzyme was recovered in about 1 l. of 40% glycerol–0.1 mM potassium phosphate buffer (pH 7.4). This desalted ammonium sulfate fraction contained 5–10% of the 280-nm-absorbing material and essentially all of the transferase activity placed upon the column.

DEAE-CELLULOSE CHROMATOGRAPHY. The desalted ammonium sulfate fraction was diluted to an A_{280} of less than 1 and a conductivity of less than 30 μmho, cm and placed on a 10 × 8 cm DEAE-cellulose column (microcrystalline DE-52, Reeve Angel). The column had been washed previously with 1 M potassium phosphate buffer (pH 7.4) and then equilibrated with 0.1 mM potassium phosphate buffer (pH 7.4). After the sample had entered the column, 5 mM potassium phosphate buffer (pH 7.4) was added, and the conductivity of the effluent was monitored. The effluent was collected until the conductivity rose sharply, indicating that the 5 mM buffer had passed through the column. All the protein eluted prior to the rise in conductivity was pooled and designated the DEAE-cellulose fraction. In this step, more than 95% of the A_{280} and less than 10% of the transferase activity remained bound to the column.

HYDROXYLAPATITE CHROMATOGRAPHY. The DEAE-cellulose fraction was then applied to a hydroxylapatite (calcium phosphate gel, Bio-Rad Laboratories, Inc.) column (5 × 12 cm). The protein was eluted with a 500-ml concave gradient from 0.08 to 1.5 M potassium phosphate buffer (pH 7.4). (The ratio of the cross section of the mixing chamber cylinder (480 ml) to the cross section of the reservoir cylinder (20 ml) was 25.) Transferase activity was eluted as a single peak at approximately 0.5 M buffer. This material was stored at −20° in 40% glycerol and has been stable for at least 18 months with no measurable loss of activity.

TABLE I: Typical Purification of Nucleotidyl Transferase from Yeast.[a]

Fraction	Total OD_{280}/kg	Sp Act.	OD_{280}/OD_{260}	% Recov
Crude extract	130,000	1[b]	0.6	
Ammonium sulfate	10,000	10	0.6	
Desalted ammonium sulfate	650	160	0.9	(100)
DEAE-cellulose	13	7700	0.9	87
Hydroxylapatite	11	3900	0.9	46

[a] This preparation was made using 5 kg of yeast. It was found that this was the largest amount which could be handled conveniently. The specific activity is reported as cpm of [^{14}C]CMP incorporated in the standard assay by 0.05 A_{280} of enzyme protein. The specific activity of the CTP was 2 Ci/mole. [b] Because this fraction is contaminated by nucleases, assays were not considered to be accurate reflections of the level of nucleotidyl transferase and were not used as references for the purification. However, estimates suggest that the ammonium sulfate fractionation results in a 10–20-fold purification. These estimates are based on the ≥10-fold reduction in total A_{280} and the fact that transferase activity could not be observed in the other (<40 and >58%) ammonium sulfate fractions.

Table I shows a typical purification. It is noted that there is a decrease in specific activity at the hydroxylapatite step. The nature of this is not known, but it appears to be a loss of activity rather than a loss of enzyme protein. This step is useful, even though it reduces the activity of the product, because it removes contaminating enzymes (Table II) which would interfere with further studies, and also because it gives approximately 5–10-fold concentration of the enzyme. To test the reproducibility of the method, six aliquots of yeast were carried through this scheme. The resulting enzyme preparations showed only minor variations in the properties shown in Tables I and II.

Results

Assays for Contaminant Enzymes. At various stages of purification, transferase preparations were checked very carefully for the presence of phosphomonoesterases, phosphodiesterases, pyrophosphatases, ribonucleases, and aminoacyl-tRNA synthetases. To provide sensitivity, the amount of enzyme used in these assays was many times that used in the standard assay for transferase activity.

The esterases were assayed using *p*-nitrophenyl phosphate and bis(*p*-nitrophenyl) phosphate, measuring the appearance of nitrophenol spectrophotometrically. The reaction conditions were those reported by Garen and Levinthal (1960) for the monoesterase, and by Koerner and Sinsheimer (1957) for the diesterase.

TABLE II: Levels of Contaminating Enzymatic Activities at Different Stages in the Purification of Nucleotidyl Transferase.

Stage	Phosphomonoesterase[a]	Phosphodiesterase[b]	Ribonuclease[c]	Aminoacyl-tRNA Synthetase	Pyrophosphatase
Desalted ammonium sulfate	3.2 mU/mg	12 U/mg	~0.5 mg/g	10 nmoles/μg	Slightly detectable
DEAE-cellulose	Undetectable	Undetectable	Barely detectable <1 mg/g	2 nmoles/μg	Undetectable
Hydroxylapatite	<0.5 mU/mg	Undetectable	Undetectable ≪0.1 mg/g	70 pmoles/μg	Undetectable

[a] Unit = 1 μmole nitrophenol liberated/min. [b] Unit as defined in Koerner and Sinsheimer (1957). [c] As pure pancreatic ribonuclease.

TABLE III: Effect of Co^{2+} and Mg^{2+} on Nucleotide Incorporation.[a]

Ion	Mg^{2+}					Co^{2+}	Mg^{2+} + Co^{2+}	
Concentration (mM)	0	0.1	5	10	50	10	10	2.5
Amount incorporated (cpm)	11	13	27	140	65	18	20	

[a] Assay conditions are those described in the text. Precursor specific activity: 200 nCi/μmole.

Pyrophosphatase activity was measured according to the method of Herbert and Wilson (1962).

The method of Kalnitsky *et al.* (1959) was used to determine ribonuclease activity. The enzyme is incubated in 0.1 M potassium acetate buffer (pH 5.0) at 37° with either polyuridylic acid, polycytidylic acid, or tRNA to assay for several kinds of RNase (T_1 RNase, pancreatic RNase, T_2 RNase, etc.). At the end of the incubation, the unreacted polynucleotide is precipitated by the addition of 0.75 M uranyl acetate in 25% perchloric acid. The supernatant is diluted and its absorbance at 260 nm is measured.

Aminoacyl-tRNA synthetase levels were measured by ATP–PP_i exchange. The preparation was incubated with ATP, [^{32}P]PP_i, Mg^{2+}, and potassium phosphate buffer (pH 7.5) in the presence or absence of a mixture of 19 amino acids. At the end of the incubation the [^{32}P]ATP was recovered by adsorption to charcoal and was counted. This assay does not take into account the fact that all the synthetases do not have maximal activity at the same pH, ATP, and Mg^{2+} concentrations. Thus, it gives only a rough estimate of the levels of these enzymes.

Table II shows the results of typical assays for the above activities at various stages in the purification. The values given are upper boundaries; that is, the maximum possible levels of the enzymes present. Thus, the observation that the DEAE-cellulose fraction has a higher value for ribonuclease than does the desalted ammonium sulfate fraction is merely a reflection of the fact that it was far more dilute and therefore the assay was less sensitive.

It is worthwhile to restate these values in terms of the enzymatic assay. Thus, the phosphodiesterase level in the hydroxylapatite fraction is an order of magnitude lower than that which would cause one break per tRNA chain under standard assay conditions. The amount of ATP–PP_i exchange in this fraction indicates that less than 0.005% of the protein is aminoacyl-tRNA synthetase with the proviso noted above. The ribonuclease present in the hydroxylapatite fraction would cause less than one break per chain under standard reaction conditions. This is supported by the observation that the tRNA can be isolated from the reaction mixture and shows little loss of activity.

Characterization of the Purified Yeast Transferase. The optimum conditions for the enzyme assay were determined by standard techniques. Care was taken to ensure that rates, and not plateau levels of incorporation, were being measured. Unless otherwise indicated, the ratio of enzyme to tRNA was adjusted to give linear incorporation for longer than 20 min. Further, because of high background when ATP was the radioactive substrate, CTP was used as the radioactive substrate in most cases. However, when ATP was used as substrate, it gave the same results as when CTP was used (Figure 1).

Table III shows the dependence of the enzyme activity on divalent cations, particularly Mg^{2+}. Co^{2+} was used because Starr and Goldthwait (1963) reported a Co^{2+} stimulation of incorporation with the transferase from rabbit muscle. That is, in the presence of Co^{2+} the enzyme was 1.6 times as active as in the presence of Mg^{2+}. Clearly, that is not the case with the yeast enzyme. This enzyme is strongly inhibited by Co^{2+} alone or in the presence of Mg^{2+}.

Figure 1 shows some of the characteristics of the nucleotidyl transferase reaction.

Figure 1A shows that the optimum concentration of the ATP is 2 mM, and that for CTP is 150 μM. The presence of

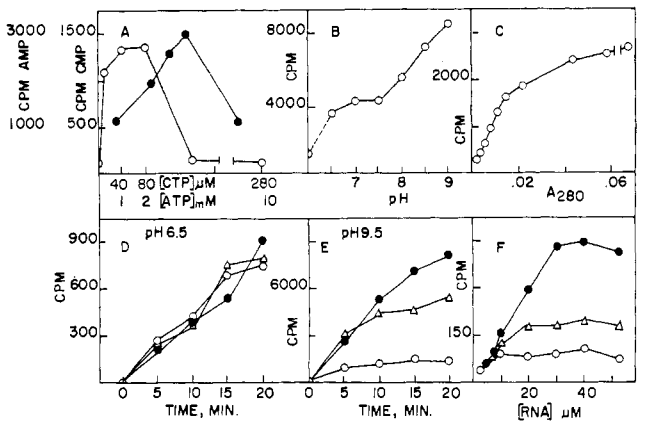

FIGURE 1: Characteristics of the transferase reaction. (A) Incorporation as a function of the concentration of respective ribonucleoside triphosphate. The assay conditions were as described in Materials and Methods; 0.015 A_{280} of enzyme was used. The specific activities of the precursors were ATP (○), 600 cpm/nmole; CTP (●), 1200 cpm/nmole. (B) CMP incorporation as a function of pH. The assay conditions were as described in Materials and Methods except that at pH 8.0 and below, 25 mM potassium phosphate buffer was used. The value at pH 8.0 was obtained with either buffer. CTP specific activity: 3000 cpm/nmole. (C) CMP incorporation as a function of protein concentration. CTP specific activity: 1200 cpm/nmole. (D, E) CMP incorporation as a function of tRNA concentration and time. CTP specific activity: 3000 cpm/nmole. tRNA concentrations 10 μM (○), 25 μM (△), 50 μM (●). (F) CMP incorporation at 20 min as a function of tRNA concentration and pH. CTP specific activity: 3000 cpm/nmole. pH 6.5 (○), 7.0 (△), 7.5 (●).

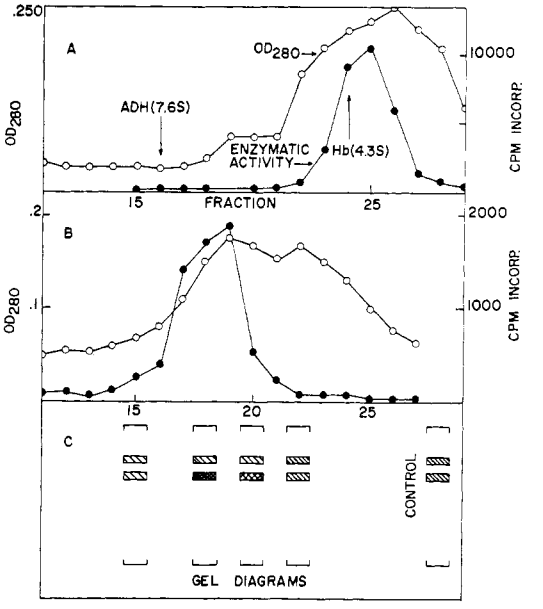

FIGURE 2: Sucrose density gradient centrifugation and disc gel electrophoresis patterns of nucleotidyl transferase. The gradients were run as described in Materials and Methods. The disc gel electrophoreses were performed on a Canalco Model 12 (Canal Instrument Co.) using the standard formulation, *i.e.*, stack pH 7.5, 3% gel; run pH 9.5, 7.5% gel. (A) Transferase alone. A_{280} (○), enzymatic activity (●). The gradient was heavily loaded (0.5 A_{280} of enzyme) to emphasize the heterogeneity of the enzyme. (B) 0.15 A_{280} of transferase plus 0.4 A_{280} of tRNA. A_{280} (○), enzymatic activity (●). (C) Gel patterns of the corresponding fractions. Control: unfractionated transferase. All samples except fraction 15 had approximately the same A_{280}.

either triphosphate had no noticeable effect on the rate of incorporation of the other (not shown here). However, the presence of CTP increased the extent of AMP incorporation.

The pH measurements (Figure 1B) were not extended above pH 9.5 because denaturation of the tRNA would make interpretation of such data very difficult. It is, or course, not clear whether the observed changes in the activity are due to acid or base involvement in catalysis or to changes in secondary or tertiary structure of the enzyme or the tRNA. Such changes are known to occur in tRNA over this pH range, but their effect on acceptor capacity is unknown. It is clear, however, that the observed changes are in the rate and not the stability of the enzyme. Enzyme preincubated at different pH's over this range and assayed at pH 9.5 showed no loss in enzymatic activity.

The amount of CMP incorporation increases (Figure 1C) linearly at low levels of enzyme protein, and the slope of the curve approaches zero at high levels. The amount of incorporation at high protein levels indicates saturation of the tRNA with CMP. In all enzyme assays, several levels of protein were used in order to find the linear portion of the curve.

The time course of CMP incorporation into tRNA at three different tRNA concentrations at pH 6.5 and 9.5 is shown (Figure 1D,E). At the lower pH, all three RNA concentrations show linear incorporation of CMP over the 20-min incubation, while at the higher pH only the highest tRNA concentration shows linearity for as long as 10 min. The extent of incorporation after 20 min into different amounts of tRNA at different pH's is also displayed in Figure 1F. The three plateaus indicate the maximum amount of CMP which can be incorporated in 20 min at the given pH by a constant amount of enzyme. The rising line shows the amount of CMP that the tRNA will accept, and the intersections of the plateau with this line show the maximum amount of tRNA which can be fully labeled with CMP in 20 min at the indicated pH. That is, the amount of enzyme required to fully label 7 μmoles of tRNA with CMP in 20 min at pH 6.5 will label 30 μmoles of tRNA in the same time at pH 7.5.

All of these studies were done using unfractionated tRNA, care being taken to assure that the rate of reaction was independent of tRNA concentration. Repetition of the kinetic measurements using purified, single tRNA species (Phe, Ala, Arg) showed no significant variation from the above.

Physical Characteristics of the Enzyme. Centrifugation of the hydroxylapatite fraction in sucrose density gradients gives single peaks of A_{280} and activity. Examination of Figure 2 clearly shows that this fraction is not homogeneous. That is, the enzyme activity shows a symmetric peak which is not coincident with the less symmetric A_{280} peak. Since the presence of hemoglobin and yeast alcohol dehydrogenase do not perturb the sedimentation of the enzyme, we were able to use them as marker proteins and estimate the sedimentation coefficient of the transferase. Figure 2 indicates that transferase sediments slightly more slowly than hemoglobin (4.3 S).

In contrast to the marker proteins, the presence of tRNA causes a large shift in the sedimentation velocity of the transferase, *i.e.*, from *ca.* 4 to *ca.* 6 S. This suggests that tRNA

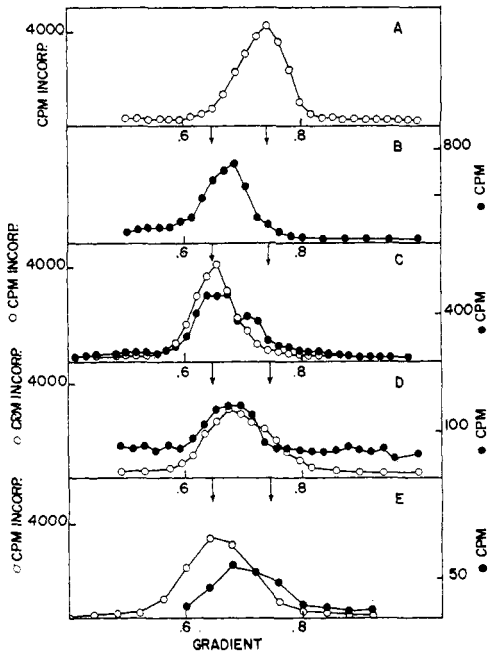

FIGURE 3: Sucrose density gradient patterns of nucleotidyl transferase in the presence of various forms of $tRNA_{Phe}$. The gradients were run as described in Materials and Methods. Each gradient contained 0.3 A_{280} of enzyme and/or 0.4 A_{280} of tRNA. The small arrow on the left is in the position of the tRNApCpC–enzyme complex and that on the right is in the position of the enzyme alone. (A) Enzyme alone. (B) [^3H]tRNApCpC and [^{14}C]tRNApCpCpA (total counts per minute are plotted). There was no separation of count. (C) Enzyme plus [^3H]tRNApCpC. (D) Enzyme plus [^{14}C]-tRNApCpCpA. (E) Enzyme plus 1:1 [^{14}C]tRNApCpCpA and tRNApCpC. Enzymatic activity (○), tRNA radioactivity (●). The specific activities of the tRNA were [^3H]tRNApCpC, 1000 cpm/nmole; [^{14}C]tRNApCpCpA, 2000 cpm/nmole.

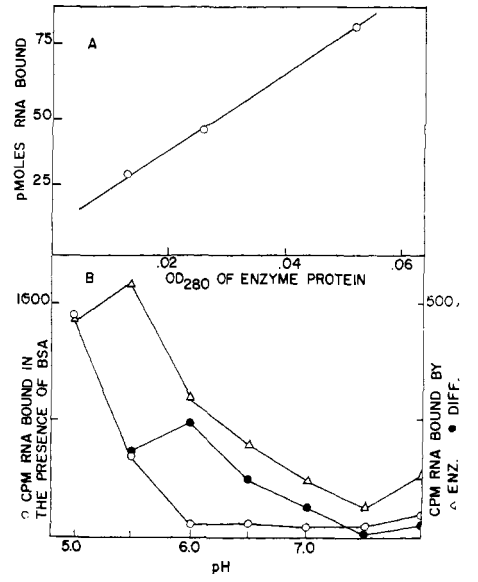

FIGURE 4: Characteristics of binding of radioactive tRNA to nitrocellulose filters. The binding assays were done as described in Materials and Methods. The specific activity of the [^3H]tRNApCpC was 200 cpm/pmole. (A) Enzyme dependence of binding. (B) pH dependence of binding. Right ordinate: enzyme binding (△), difference (●). Left ordinate: bovine serum albumin binding (○).

either forms a complex with the transferase or induces aggregation or a change in conformation of the enzyme, or some combination of these.

Whatever its cause, this increase in sedimentation velocity, coupled with the observation (not shown) that the presence of tRNA does not affect the pattern on disc gel electrophoresis, enabled us to further characterize the transferase. Disc gel patterns of the hydroxylapatite fraction and of fractions from a sucrose gradient containing enzyme and tRNA are shown in Figure 2. Note that, as indicated by the degree of cross-hatching, the faster band intensifies relative to the slower band as one moves into the region of the gradient containing enzymatic activity. This suggests that the enzyme is in the faster moving band.

The material from the upper part of the gradient which is in the faster moving band may well be enzyme protein which is not shifted by the tRNA and which is inactive in the assay. That such inactive enzyme copurifies with active enzyme is suggested by the results of hydroxylapatite chromatography described in Materials and Methods. This hypothesis is also supported by an observation (R. W. Morris, 1968, unpublished data) that a highly purified preparation of rabbit muscle transferase gives a single band on disc gel electrophoresis even after it has been allowed to become partially or completely inactive. Further, the band obtained with the active enzyme is in the same position as the band obtained with inactive enzyme, and protein in this band accounts for almost all of the protein placed on the gel.

Complex Formation in Sucrose Density Gradients. The data in Figure 3 indicate that the most likely explanation of the increase in the sedimentation velocity of the enzyme in the presence of tRNA is the formation of a complex between the enzyme and tRNA. Nucleotidyl transferase, [^3H]-$tRNApCpC_{Phe}$, [^{14}C]$tRNApCpCpA_{Phe}$, and a 50–50 mixture of [^{14}C]$tRNApCpCpA_{Phe}$, and $tRNApCpC_{Phe}$ were sedimented individually and in combination. Note that the [^3H]$tRNApCpC_{Phe}$ sediments more rapidly in the presence of the enzyme and that the enzyme peak retains its sharpness. Further, the position of the tRNA coincides with that of the enzyme, as though the enzyme and tRNA are present as a single molecular species or complex. On the other hand, the [^{14}C]$tRNApCpCpA_{Phe}$ tends to broaden the peak of enzymatic activity and not to shift the activity peak down the gradient as much as [^3H]tRNApCpC does (three-tube shift for the former and five- or six-tube shift for the latter). Also, note that the tRNA, itself, is not shifted from its original position. When $tRNApCpC_{Phe}$ and [^{14}C]$tRNApCpCpA_{Phe}$ are present in the same gradient with the enzyme, the [^{14}C]-$tRNApCpCpA_{Phe}$ is not shifted while the enzyme is shifted to the position corresponding to the [^3H]$tRNApCpC_{Phe}$–enzyme peak.

These data suggest that a complex is formed between the transferase and tRNApCpC. They further indicate that if a complex is formed between tRNApCpCpA and the enzyme, it is considerably weaker than the transferase–tRNApCpC complex. Further support for the notion that tRNA lacking its terminal adenosine is more tightly bound than tRNA with its terminal adenosine intact is presented in the following sections.

One can make a rough estimate of the relative amounts

TABLE IV: Enzyme-Dependent Binding of Polynucleotides to Nitrocellulose Filters.

tRNA Used	Sp Act. (cpm/pmole)	pmoles Bound/ 30 μg of Protein
[^3H]tRNApC	56	85
[^3H]tRNApCp	50	92
[^3H]tRNApCpC	114	60
[^3H]tRNApCpCp	109	102
[^3H]tRNApCpCp[a]	109	<2
[^{14}C]tRNApCpCpA	250	<1
[^{14}C]Poly UMP	2.0 cpm/ng	2[b]
[^3H]Poly CMP	0.3 cpm/ng	8[b]

[a] Digested for 1 hr with T_1 RNase or pancreatic RNase. [b] Approximate number of picomoles of polynucleotide assuming an average chain length of 200 nucleotides. The binding conditions were those described in Materials and Methods.

TABLE V: Competition Among Polynucleotides for Enzyme-Dependent Binding to Nitrocellulose Filters.[a]

A. Unfractionated tRNA

tRNA Used	Competitor	pmoles Bound/ 40 μg of Protein ^3H	^{14}C
[^3H]tRNApCpC	0	180	
	[^{14}C]tRNApCpCpA	180	
	Poly CMP	50	
	Poly UMP	0	

B. $tRNA_{Phe}$

tRNA Used	Competitor	pmoles Bound/ 30 μg of Protein ^3H	^{14}C
[^3H]tRNApCp	0	92	
	tRNApYpXp	53	
	tRNApY	45	
	[^{14}C]Poly UMP	9	3[b]
[^3H]tRNApCpCp	0	102	
	[^{14}C]tRNApCpCpA	49	<1

[a] The binding conditions were those described in Materials and Methods. When a competitor was used, it was present in an amount (A_{260}) equal to the other tRNA. The polynucleotides used and their specific activities (counts per minute per picomole) were: unfractionated [^3H]tRNApCpC, 4; unfractionated [^{14}C]tRNApCpCpA, 20; $tRNApCp_{Phe}$, 50; $tRNApCpCp_{Phe}$, 109; $tRNApCpCpA_{Phe}$, 250; [^{14}C]poly UMP, 2 cpm/ng. The nonradioactive poly UMP and poly CMP were purchased from Miles Laboratories and had $s_{20,w}$ of 3.3 and 6.7 S, respectively. [b] Approximate number of picomoles of polynucleotide assuming an average chain length of 200 nucleotides.

of tRNA and transferase in the shifted peak. First, one must assume molecular weights and $\epsilon_{280\ nm}^{1\ mg/ml}$ for the enzyme and tRNA of 60,000 and 25,000, and 1 and 12 (Starr and Goldthwait, 1963), respectively. The molecular weight of the enzyme was estimated at 60,000 by referring it to hemoglobin and assuming that the two proteins have similar shapes. Secondly, one must assume that the transferase in the shifted position is pure and that all of the [^3H]$tRNApCpC_{Phe}$ in that position is in the complex. Given these assumptions, one may calculate that there are approximately two tRNA molecules for every transferase molecule in the complex.

Determination of Conditions for Enzyme-Dependent Binding of tRNA to Nitrocellulose Filters. Because the density gradient technique does not allow screening of many variables, the binding technique of Yarus and Berg was employed. Figure 4A shows that unfractionated tRNApCpC binds to nitrocellulose filters in the presence of the enzyme. Heating the enzyme at 100° for 5 min destroys its capacity to bind tRNA. ATP, CTP, and Mg^{2+} were varied over the ranges indicated in Materials and Methods with no effect on the extent of binding (not shown here). The pH dependence of the binding is shown (Figure 4B). A pH of 6.5 was chosen for later experiments because the bovine serum albumin controls rise sharply below this point, reducing the precision of the measurement. This rise may be due to a decrease in the solubility of bovine serum albumin as its isoelectric point is approached. This insolubility could lead to trapping of the tRNA on the filter. The observation (not shown) that the binding of tRNA to the enzyme in sucrose gradients occurs at pH 7.5 and is not affected by a shift in pH to 7.0 suggests that the pH dependence observed in binding to nitrocellulose filters is some function of the nitrocellulose filter system and not of the complex. Nevertheless, as will be shown in the next section, the binding to nitrocellulose filters shows the same specificity as complex formation in the sucrose density gradients.

Specificity of Binding to Nitrocellulose Filters. The principle concern in these studies was the specificity of the binding reaction. Table IV shows the binding of radioactive $tRNA_{Phe}$ of various end-group compositions and of [^{14}C]poly UMP and [^3H]poly CMP. In addition, [^3H]$tRNApCpCp_{Phe}$ was treated, under conditions which ensure quantitative release of the terminal fragment (Herbert and Smith, 1967), with T_1 RNase to determine if the fragment [^3H]CpApCpCp would be bound with the enzyme to the filter. It can be seen that only tRNA is bound. Further, if the tRNA has its terminal adenosine to complete the triplet, it is not bound.

The data in Figure 4A and Table IV allow one to calculate the relative amounts of tRNA and transferase bound to the nitrocellulose filters. One assumes an enzyme molecular weight of 60,000 and a purity of approximately 20%;[2] then

[2] This estimate was obtained by assuming that the protein in the tRNApCpC–enzyme peak (Figure 3C) is pure transferase and subtracting the contribution of the tRNA to the A_{280}. The ratio of the remaining A_{280} in that peak to the total A_{280} due to protein on the gradient is 0.2.

TABLE VI: Optimal Conditions for the Activity of Nucleotidyl Transferase from Yeast, Rabbit Muscle, and Rat Liver.

Parameter	Yeast	Rabbit Muscle[b]	Rat Liver[c]
pH			
Forward	9.5	9.5	9.5
Reverse	7.5	*a*	7.5
Mg^{2+} (mM)	10	5	10
Co^{2+} (mM)	*d*	5 ($1.6 \times Mg^{2+}$ activity)	*a*
CTP (μM)	150	200	100
ATP (mM)	2	2	0.5

[a] Not reported. [b] After Starr and Goldthwait (1963). [c] After Daniel and Littauer (1963). [d] Strongly inhibitory even in the presence of Mg^{2+}.

one obtains a value of between 1 and 2.5 molecules of tRNA per molecule of transferase. This is in agreement with the value calculated from Figure 3.

It was also of interest to examine the competition for binding with the enzyme between tRNAs of different end-groups. These data are shown in Table V. In the first part of the table, unfractionated [^3H]tRNApCpC is used with poly UMP, poly CMP, and unfractionated [^{14}C]tRNApCpCpA. Note that the [^3H]tRNApCpC was bound even in the presence of [^{14}C]tRNApCpCpA which was not bound. In the second part of the table, $tRNA_{Phe}$s of different end groups were used with very similar results. This is in agreement with the conclusion drawn from data in Figure 3 that tRNApCpCpA binds less tightly to the enzyme than tRNApCpC does.

The nature of the effect of the synthetic polynucleotides is not clear. They strongly inhibit enzyme-dependent binding of tRNA to nitrocellulose filters, but are not bound themselves to any extent. This may be an effect on the filter rather than on the complex or either component thereof.

Discussion

Purification. The purification scheme outlined yields a reasonable amount of enzyme in a form that is stable for at least 18 months at −20° with repeated warming and cooling. It suffers from the disadvantages that are inherent in working in 40% glycerol; that is, the increased viscosity makes all of the column procedures slower than they would be in the absence of glycerol. The glycerol, however, is essential to the stability of the enzyme. In its absence, for example, chromatography on the DEAE-cellulose column results in a 50% loss in total enzyme activity.

The final product of this method is a preparation that is essentially free of nuclease, esterase, and aminoacyl-tRNA synthetase activities.

Comparison of Yeast and Mammalian Enzymes. The general characteristics of three enzyme systems are shown in Table VI. As can be seen in this table, the three enzymes are remarkably similar with regard to optimal assay conditions. The high pH optima are surprising in view of the fact that tRNA begins to change secondary structure around pH 9. In view of this high pH optimum, one must be very cautious in drawing any conclusions about the situation *in vivo*. The difference in ATP and CTP optima may reflect the relative intracellular abundance of the two nucleoside triphosphates to which the enzyme system has adapted. The effect of Co^{2+} ion is the only radical difference which has been observed between the yeast and rabbit muscle transferases.

Complex Formation. Bluestein *et al.* (1968) demonstrated that a complex forms between aminoacyl adenylate and aminoacyl-tRNA synthetase, the enzyme which catalyzes amino acid activation. The complex binds to nitrocellulose filters and is stable during gel filtration. Yarus and Berg (1967) also used nitrocellulose filters to examine complex formation between the synthetase and tRNA. Honda (1969) reported a shift in the sedimentation velocity of the transferase activity in a crude state in the presence of bulk tRNA but not in the presence of rRNA. In the present work, complex formation between nucleotidyl transferase and various purified tRNA fractions with different end-group compositions has been demonstrated by the binding technique and by density gradient centrifugation.

Two observations supply critical evidence of a specific complex between tRNA and nucleotidyl transferase. The density gradient studies indicate that the enzyme and tRNApCpC are both moved in the gradient to a new position and that this is not the case with tRNApCpCpA. This suggests that tRNApCpC is more tightly bound than tRNApCpCpA. This second point is supported by the observation in the nitrocellulose binding studies that tRNApCpCpA is not bound at all and that tRNApCpC is strongly bound. The only other class of enzyme likely to make this distinction is the aminoacyl-tRNA synthetases which are essentially absent from the preparation. The indication in Table V that tRNApCpCpA reduces the amount of tRNApCpCp bound although it is not itself bound is most probably due to the presence, in the tRNApCpCpA preparation, of some chains (*ca.* 30%) which had not accepted an AMP residue.

The binding of tRNA–transferase complexes to nitrocellulose filters shows interesting pH dependence. First, the optimum pH for this binding is more than 3 units below the optimum for enzymatic activity. This is in accord with the report of Yarus and Berg (1967) on the binding of isoleucyl-tRNA to isoleucyl-tRNA synthetase. The latter enzyme has a pH optimum of 7.5 for catalysis, and of 5.5 for binding.

The binding and density gradient data indicate that the enzyme can distinguish, for purposes of complex formation, only between the presence and absence of the terminal adenosine. The final product, tRNApCpCpA, is bound loosely if at all; the known precursors of tRNApCpCpA and some incomplete forms of tRNA which are not precursors are bound with facility. This is in contrast to the observations of Yarus and Berg (1967) that aminoacyl-tRNA, the product, and tRNApCpCpA, a substrate, complex equally well with the aminoacyl-tRNA synthetase.

An interesting question arising from the binding studies relates to the change in the tRNA–transferase complex when the terminal AMP residue is added to the tRNA. Apparently any tRNA molecule of end-group composition from pY to pYpXpCpCp exhibits enzyme-dependent binding to nitrocellulose filters. This array includes tRNA-pYpX, -pYpXpC, and -pYpXpCpC, all of which are natural substrates for the en-

zyme; and tRNA-pY, -pYpXp, -pYpXpCp, and -pYpXpCpCp, none of which will accept AMP or CMP. However, with the addition of the terminal adenosine, the tRNA, which would be an active substrate for the reverse reaction and could therefore interact with the enzyme, loses all ability to bind with the enzyme on nitrocellulose filters and complexes only weakly with the enzyme in density gradients. Three possible hypotheses explaining these observations are: (1) a length requirement in the tRNA, (2) a change in the secondary and tertiary structure of the tRNA, or (3) a conformational change in the enzyme induced by tRNApCpCpA which inhibits complex formation. The first hypothesis is that the enzyme recognizes a region of specific length in the tRNA from some internal point to the end. Thus, when the terminal AMP is added, this region becomes too long to specify binding. A related observation is that the 3′-terminal oligonucleotide, CpApCpCp, from T_1-ribonuclease-digested phenylalanine [^3H]tRNApCpCp is not bound by the enzyme. This leads one to suspect that either there is a minimum as well as a maximum length, or that some structural parameter in addition to length is necessary to determine binding.

The second hypothesis would suggest that the conformation of the tRNA is different when the terminal adenosine is missing than when this residue is present. That is, some part of this riboside interacts with another region of the tRNA to maintain or prevent some conformer. This is unlikely in light of the observed lability and nuclease sensitivity of this residue which indicate that it does not participate in structure maintenance.

The third hypothesis is that the presence of the terminal adenosine on the tRNA induces a conformational change in the enzyme which prevents complex formation. It would be of interest to measure the binding of the dialdehyde form of tRNApCpCpA resulting from periodate oxidation. If this form does complex with the enzyme, it would suggest that either the terminal ribose is involved in the conformational change in the enzyme, or that the ring opening changes the location of the terminal base which makes it unable to modify the enzyme.

Attempts to correlate inhibition of the binding by synthetic polynucleotides with inhibition of the catalytic activity of the enzyme met with no success. The enzyme was assayed at pH 6.5, in that range of tRNA concentrations where the amount of incorporation in 20 min is tRNA concentration dependent, and where the rate of incorporation has not fallen to zero. To these incubations, poly UMP or poly CMP, far in excess of the amount required to inhibit the binding, was added. These reactions were followed from 0 time to 0 plus 30 min. The presence of either synthetic polynucleotide, even in as much as equimolar concentrations, had no effect on either the rate or the plateau level of incorporation.

The complex is apparently stable enough to be used as a final purification step. This would enable one to remove inactive enzyme and other proteins so that one could use analytical ultracentrifugation to determine accurately the size, subunit structure, and interactions of this most intriguing enzyme.

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