Yasunobu, K. T. (1965), *in* Non-Heme Iron Proteins: Role in Energy Conversion, San Pietro, A., Ed., Yellow Springs, Ohio, Antioch.

Tanaka, M., Nakashima, T., Benson, A. M., Mower, H. F., and Yasunobu, K. T. (1964a), *Biochem. Biophys. Res. Commun. 16*, 422.

Tanaka, M., Nakashima, T., Mower, H. F., and Yasunobu, K. T. (1964b), *Arch. Biochem. Biophys. 105*, 570.

Valentine, R. C. (1964), *Bacteriol. Rev. 28*, 497.

Valentine, R. C., Mortenson, L. E., and Carnahan, J. E. (1963), *J. Biol. Chem. 238*, 1141.

Purification and Physical Characterization of Tyrosyl Ribonucleic Acid Synthetases from *Escherichia coli* and *Bacillus subtilis**

Richard Calendar and Paul Berg

ABSTRACT: Tyrosyl ribonucleic acid synthetases from *Escherichia coli* and *Bacillus subtilis* have been purified to near homogeneity. These enzymes have molecular weights of 95,000 and 88,000, respectively. They can be separated electrophoretically on starch or polyacrylamide gels. The *E. coli* enzyme contains 14 half-cystine residues/mole while the *B. subtilis* enzyme has 2–3.

Antiserum to the *E. coli* enzyme neutralizes and precipitates the *E. coli* enzyme but does not cross-react with the *B. subtilis* enzyme. Antiserum to the *B. subtilis* enzyme neutralizes and precipitates the *B. subtilis* enzyme and also shows partial cross-reaction with the *E. coli* enzyme.

Aminoacyl RNA[1] synthetases are one of a few types of known proteins which can discriminate between structurally similar nucleic acid molecules (Berg, 1961). How each aminoacyl RNA synthetase distinguishes the correct t-RNA from all others is not known; moreover, virtually nothing is known about the type of interactions which occur between the protein and the polynucleotide chain during the formation of an aminoacyl RNA.

Several examples are known in which aminoacyl RNA synthetases from one species fail to utilize the corresponding t-RNA's from another species; complete cross-reaction between enzymes and t-RNA's from different species is also well documented (Benzer and Weisblum, 1961; Yamane and Sueoka, 1963; Doctor and Mudd, 1963). Quite possibly, studies of one of the aminoacyl RNA synthetases, isolated from species which do and do not cross-react with their respective t-RNA's, could shed some light on the nature of the interaction between the enzyme and the t-RNA. Our first approach was to compare two enzymes from species which show complete cross-reaction with their corresponding t-RNA's (Calendar and Berg, 1966). Later studies will extend the comparison to the same enzyme from species which fail to react with the heterologous t-RNA's.

In this paper we report the isolation and a comparison of several physical and chemical properties of tyrosyl RNA synthetases from *Escherichia coli* and *Bacillus subtilis*, two enzymes which cross-react completely with their heterologous t-RNA's. Although the two enzymes have similar molecular weights, they are readily distinguishable by their electrophoretic mobility, amino acid composition, and immunological reaction. In the next paper (Calendar and Berg, 1966) the catalytic properties of the two enzymes are described.

Experimental Section

Materials. L-Tyrosine was purchased from California Corp. for Biochemical Research and gave only a single peak when chromatographed on an amino acid analyzer (Spackman *et al.*, 1958).

ATP and glutathione were purchased from Sigma Chemical Corp. ^{32}P-Labeled pyrophosphate ($^{32}PP_i$) was synthesized from ^{32}P-labeled orthophosphate (Oak Ridge National Laboratories) as described by Bergmann *et al.* (1961). Crystalline bovine plasma albumin was purchased from Armour Pharmaceutical Co., Kankakee, Ill. Sucrose, Baker analyzed grade, was recrystallized from ethanol and filtered through a 0.45 μ Millipore filter before use in sucrose-gradient centrifugation. Fluorochemical FC43 was obtained

* From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California. *Received February 15, 1966.* This work was supported in part by teaching and research grants from the National Institutes of Health.

[1] Abbreviations used: RNA, ribonucleic acid; t-RNA, transfer RNA; ATP, adenosine triphosphate.

from Beckman and Co., Palo Alto, Calif. Superbrite glass beads, 200-μ average diameter, were from Minnesota Mining and Manufacturing Co., and were washed before use with 1 N HCl, then with water. Whatman phosphocellulose P11 was obtained from BKH Division of Van Waters and Rogers Corp., San Francisco, Calif., and DEAE-cellulose came from Brown and Co., Berlin, N. H., and from Bio-Rad Laboratories, Richmond, Calif. Phosphocellulose and DEAE-cellulose were prepared for use by removal of the fines through repeated settling, suspension in 0.25 M NaOH at room temperature for 10 min, and finally washing with water on a Büchner funnel. Both resins were stored in 0.1 M KH_2PO_4 to maintain the pH below 5. Hydroxylapatite was obtained from Clarkson Chemical Corp., Williamsport, Pa., alumina Cγ gel was from Sigma Chemical Corp., St. Louis, Mo., and was also prepared according to Willstätter and Kraut (1923). Hydrolyzed starch was purchased from Connaught Medical Research Laboratories, Toronto, Canada; acrylamide and methylene bisacrylamide were from Eastman Chemical Laboratories, Rochester, N. Y., and were recrystallized from acetone before use. Virgin female rabbits were used for antibody production. Freund's adjuvant and Noble agar were the products of Difco Laboratories, Detroit, Mich. Normal rabbit γ-globulin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and was dialyzed *vs.* distilled water before use.

E. coli B or B/1 (a T1 phage-resistant mutant isolated from prototrophic *E. coli* B) and *B. subtilis* SB19 (a prototrophic strain obtained from Dr. Esther Lederberg and described by Nester *et al.*, 1963) were the starting materials for the enzyme isolation. *E. coli* was grown in large quantities in a 120-l. biogen apparatus (American Sterilizer Corp.) at 37° with forced aeration. M56 medium as described by Weismeyer and Cohn (1960) was used with 1% glucose as carbon source. The cells were removed in late log phase, quick cooled to 5° by passage through a cooling coil immersed in a −15° ethylene glycol bath, centrifuged in a Spinco Model 170 continuous flow centrifuge at 29,000*g*, and frozen in thin layers at −20°. Cells stored in a closed container were satisfactory for at least 6 months.

B. subtilis SB19 were grown in large quantities using the same procedure as described for *E. coli* B, except that the medium was Spizizen (1958) minimal +1% glucose, plus 0.1% casein amino acids (acid hydrolyzed, Nutritional Biochemicals) and 40 μg/ml of L-tryptophan (B grade, Calbiochem). Isoleucyl RNA synthetase was a gift from Dr. Anne N. Baldwin.

Methods. ENZYME ASSAY. The activity of the enzymes were measured by the L-tyrosine-dependent exchange of ATP with $^{32}PP_i$. The reaction mixture (1 ml) contained: 100 mM sodium cacodylate buffer, pH 7.0, 5 mM magnesium chloride ($MgCl_2$), 2 mM adenosine triphosphate (ATP), 2 mM sodium pyrophosphate (10^4–10^5 cpm/μmole), 10 mM 2-mercaptoethanol, 10 mM potassium fluoride (KF) (for crude fractions only), 0.1 mg of bovine plasma albumin, 2 mM L-tyrosine, and enzyme (0.01 to 0.1 unit) in a solution usually containing 50 mM potassium phosphate, pH 6.5, 10 mM 2-mercaptoethanol, and 0.1 mg/ml of bovine plasma albumin.

After 15 min at 37°, the reaction was stopped by the addition of 0.7 ml of cold 0.4 M sodium pyrophosphate in 15% perchloric acid. The ATP was adsorbed to charcoal by the addition of 0.1 ml of a 15% suspension of acid-washed Norit A. The charcoal suspension was filtered through a GF/C glass filter, and the filter was washed five times with 5-ml portions of cold distilled water. The filters were dried under a heat lamp and counted in an end-window counter. The values were corrected for a blank which was run without tyrosine; no self-absorption correction was made. One unit of enzyme activity is defined as that amount incorporating 1 μmole of $^{32}PP_i$ into ATP in 15 min, under the conditions described, and the specific activity is defined as enzyme units per milligram of protein. The exchange of $^{32}PP_i$ with ATP was proportional to the amount of enzyme added (up to 0.15 unit) and to time for periods up to 20 min.

PROTEIN ASSAY. Protein was determined by the method of Lowry *et al.* (1951). In crude fractions, protein was precipitated with cold 5% trichloroacetic acid before assay.

ELECTROPHORESIS. Starch gel electrophoresis was performed as described by Smithies (1959). Gels (15%) (w/v) were prepared in 20 mM potassium phosphate, pH 8.0, containing 10 mM 2-mercaptoethanol. Each slit was filled with 60 μl of enzyme solution (360 μg) and after 36 hr at 200 v the gel was sliced in half horizontally, and one-half was stained with amido black, while the other half was cut transversely into strips, eluted with 100 mM potassium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol, and assayed for enzyme activity.

Electrophoresis on polyacrylamide gels was performed as described by Jovin *et al.* (1964). Separating gels were prepared containing 6% acrylamide and 0.12% bisacrylamide, while stacking gels were 2.5% acrylamide and 0.6% bisacrylamide. The Tris buffer system was used. Two milliamperes per gel was applied at 5° until a brom phenol blue marker had reached the end of the gels (*ca.* 3 hr), and then parallel gels were stained or sliced, eluted, and assayed for enzymatic activity.

SUCROSE GRADIENT CENTRIFUGATION was carried out after Martin and Ames (1961). Gradients (4.8 ml) from 20 to 5% sucrose in 20 mM potassium phosphate, pH 6.5, 100 mM KCl, 10 mM 2-mercaptoethanol, were prepared. Purified enzyme (1 mg) in the same buffer was layered on top of the gradient. Centrifugation was at 39,000 rpm in a Spinco Model L ultracentrifuge for 16 hr at 3°. Two or three-drop fractions were collected from the bottom of the tube and assayed for protein content and enzyme activity.

IMMUNOLOGICAL TECHNIQUES. Rabbits were immunized by a primary subcutaneous injection of 100 μg (1 ml) of enzyme in an equal volume of adjuvant, followed by a secondary intravenous injection 6 weeks later with 10 μg of enzyme in isotonic saline. Blood

was collected by heart puncture and allowed to clot, and the serum was collected arter centrifugation of the clot. γ-Globulin was purified by precipitation of whole serum with ammonium sulfate to 50% saturation at 5°. The precipitate was dissolved in 0.016 M potassium phosphate, pH 6.5, dialyzed *vs.* the same buffer, and chromatographed on a column of DEAE-cellulose at the same buffer concentration. The material not adhering to the column (γ-globulin) was concentrated by pervaporation (Richardson and Kornberg, 1964).

Studies of antigen–antibody precipitin reactions were carried out by double-diffusion in agar (Ouchterlony, 1948), using gels of 2% Noble agar in 50 mM sodium barbital buffer, pH 8.6. Acid fixing of the precipitin lines with 5% acetic acid was done after the gel had been washed for a day with 150 mM NaCl. We are indebted to Dr. Jack Remington and Mr. Hilbert Morales for advice and aid in the immunological work.

Molecular Weight Studies. Sedimentation studies were done in a Spinco Model E analytical ultracentrifuge, equipped with temperature control and Schlieren optics, using both titanium and black anodized aluminum rotors. A 12-mm 2.5° Epon double-sector centerpiece with quartz windows was used for all studies, except that a 2.5° single-sector centerpiece was used for determinations of the sedimentation coefficient with the *B. subtilis* tyrosyl RNA synthetase. Diffusion coefficients were determined in boundary-forming runs, using a capillary-type, synthetic-boundary, double-sector cell. All centrifugal studies were performed in solutions containing 20 mM potassium phosphate, pH 6.5, 100 mM KCl, and 3 mM 2-mercaptoethanol. The rotor temperature was maintained at a constant value, which for most runs was between 5 and 10°. The rotor speed for sedimentation velocity runs was 50,740 rpm, while speeds of 8225–19,160 rpm were used for sedimentation equilibrium runs. Photographic plates were measured on a Gaertner M2001 RS two-dimensional microcomparator.

Calculations of $s_{20,w}$ and $D_{20,w}$ were according to Schachman (1957). Molecular weight was calculated from the equilibrium data by method I and method II of Van Holde and Baldwin (1958), and by plotting ln c (c = protein concentration) *vs.* r^2 (radius) after Schachman (1957). Method I utilized $\Delta c/c_0$ to give a weight-average molecular weight, while method II utilizes a graph of $(1/r)(dc/c_a)$ *vs.* $c - c_a$, which should be a straight line for homogeneous proteins, and whose slope defines an M_Z. The plot of ln c *vs.* r^2 gives the molecular weight (M^*) if the protein is homogeneous. Molecular weights were also obtained from $s_{20,w}$ and $D_{20,w}$, by use of the Svedberg equation (Svedberg and Pedersen, 1940).

AMINO ACID ANALYSES. Protein (1 mg) was dialyzed *vs.* 100 mM KCl, and then *vs.* distilled water. This material was hydrolyzed *in vacuo* at 110° for 22 hr in 6 N HCl, with a crystal of phenol to protect tyrosine from oxidation (Sanger and Thompson, 1963). The resultant hydrolysate was analyzed on a Beckman amino acid analyzer, Model 120B (Spackman *et al.*, 1958). Half-cysteine residues were determined as cysteic acid after performic acid oxidation according to Moore (1963), and tryptophan was determined by spectral analysis in 100 mM NaOH (Beaven and Holiday, 1952) on a Beckman PMQ II spectrophotometer. Since all hydrolyses were for 22 hr, no corrections could be made for destruction of amino acids or for incomplete hydrolysis.

Results

A. Purification of Tyrosyl RNA Synthetase from E. coli (a summary is given in Table I.) All operations were carried out at 4° and all centrifugations were in the GSA rotor of a Sorvall centrifuge at 8000 rpm.

TABLE I: Purification of Tyrosyl RNA Synthetases from *E. coli*.[a]

Fraction	Vol (ml)	Enzyme Sp Act (units/ml)	(units/mg)	Yield (%)
I. Crude extract	2225	32	1.5	100
II. Autolyzed extract	4225	15	2.1	89
III. Ammonium sulfate	324	142	6.5	65
IV. Alumina C_γ gel	750	39	24	41
V. DEAE-cellulose, pH 6.5	324	60	57	27
VI. Hydroxylapatite	150	154	294	32
VII. DEAE-cellulose, pH 8.0	50	142	910	10

[a] Seven preparations yielded material with final specific activities ranging from 500 to 910 units/mg, with an average of 660.

I. PREPARATION OF EXTRACT. Cells (500 g) (wet weight), 500 ml of 25 mM K_2HPO_4, and 1 kg of glass beads were blended for 10 min at low speed in a stainless-steel Waring Blendor maintained below 5° by circulating ethylene glycol at −15° through a cooling jacket. After 10 min, 1 l. of 25 mM K_2HPO_4 was added, and blending was continued for 3 min. The supernatant fluid was decanted and the beads were washed twice with 500-ml aliquots of 25 mM K_2HPO_4. The supernatant fluid and the two washes were pooled and centrifuged for 40 min, and the supernatant fluid (fraction I) was used in succeeding steps.

II. AUTOLYSIS. Fraction I was adjusted to 4.75 l. (*ca.* 10 mg of protein/ml) and the phosphate concentration was adjusted to 100 mM by addition of 80 g of solid K_2HPO_4. During 2 hr at 37° the acid-soluble A_{260} rose to 70% of the total A_{260} with little loss in enzyme activity. The material was cooled to 5° and a precipitate was eliminated by centrifugation (fraction II).

III. AMMONIUM SULFATE FRACTIONATION. Ammonium sulfate (34 g) was added for every 100 ml of fraction II

TABLE II: Purification of Tyrosyl RNA Synthetase from *B. subtilis*.[a]

Fraction	Vol (ml)	Enzyme (units/ml)	Sp Act (units/mg)	Yield (%)
I. Crude extract	3380	3.5	0.6	100
II. Autolyzed extract	3500	4.3	0.8	128
III. Ammonium sulfate, 60–75%	173	72	3.2	111
IV. Aumina C_γ gel	346	25	6.2	74
V. DEAE-cellulose, pH 6.0	340	14	15	42
VI. Cellulose phosphate	350	6	58	20
VII. Hydroxylapatite	240	8	180	18
VIII. DEAE-cellulose, pH 8.0	38	25	720	8

[a] Four preparations yielded final fractions with specific activities of 440, 510, 500, and 720.

and after 5 min the suspension was centrifuged for 30 min. Additional ammonium sulfate (12.9 g/100 ml of fraction II) was added to the supernatant fluid, and after 5 min the mixture was centrifuged for 30 min. The precipitate was dissolved in *ca.* 250 ml of 10 mM potassium succinate buffer, pH 6.0, containing 3 mM 2-mercaptoethanol and the solution was dialyzed overnight *vs.* two successive 30-l. aliquots of the same buffer. Any precipitate which appeared during dialysis was removed by centrifugation (fraction III). From this point all solutions contained 3 mM 2-mercaptoethanol.

IV. ALUMINA C_γ GEL FRACTIONATION. Alumina C_γ gel[2] was added to fraction III (58 mg of dry weight gel/mg of protein) and the suspension was centrifuged immediately. The gel was washed twice with 375-ml aliquots of 20 mM potassium phosphate buffer, pH 7.5, and then eluted twice with 375-ml aliquots of 110 mM potassium phosphate buffer, pH 7.5. The pooled supernatant fluids were fraction IV.

V. DEAE-CELLULOSE FRACTIONATION. Fraction IV was diluted threefold with 3 mM 2-mercaptoethanol and applied to a column of DEAE-cellulose[3] (20 cm × 3.1 cm^2) which had been previously equilibrated with 50 mM potassium phosphate, pH 6.5. Elution was effected with a linear gradient between 50 and 300 mM potassium phosphate, pH 6.5, the total volume of the eluent being 1 l. Fractions (20 ml) were collected and fractions containing enzyme having a specific activity at least twice that of fraction IV were pooled (fraction V).

VI. HYDROXYLAPATITE FRACTIONATION. Fraction V was diluted threefold with 3 mM 2-mercaptoethanol and applied to a column of hydroxylapatite (25 cm × 0.75 cm^2) previously washed with 1 l. of 50 mM potassium phosphate, pH 6.5. The column was then washed with 200 ml of 90 mM potassium phosphate, pH 6.5, and the enzyme was eluted (1 ml/min, 2 psi of pressure) with a linear gradient from 90 to 250 mM potassium phosphate, pH 6.5, the total volume of the eluent being 400 ml. Fractions (7 ml) were collected and fractions containing enzyme with a specific activity at least twice that of fraction V were pooled (fraction VI).

VII. SECOND DEAE-CELLULOSE FRACTIONATION. Fraction VI was diluted threefold with 3 mM 2-mercaptoethanol and applied to a column of DEAE-cellulose (30 cm × 0.75 cm^2) previously equilibrated with 50 mM potassium phosphate, pH 8.0. The enzyme was eluted with a linear gradient between 50 and 300 mM potassium phosphate, pH 8.0, the total volume of the gradient being 500 ml. Fractions (10 ml) were collected, and the fractions of highest specific activity were pooled (fraction VII). For storage, fraction VII was concentrated to 1 ml by pressure dialysis *vs.* 20 mM potassium phosphate, pH 6.5, 3 mM in 2-mercaptoethanol, or glutathione. Concentrated fractions containing mercaptoethanol were stable at 0° for several months. Fractions with glutathione could be frozen indefinitely in a liquid nitrogen refrigerator. Upon thawing, addition of 2-mercaptoethanol was necessary to restore full activity. Minor electrophoretic bands were present at this stage in the preparation and the following additional procedure was carried out on one sample to remove these contaminants.

VIII. PREPARATIVE ELECTROPHORESIS. Fraction VII was subjected to preparative electrophoresis on polyacrylamide gel as described by Jovin *et al.* (1964), using acrylamide–bisacrylamide (6–12%) separation gel, acrylamide–bisacrylamide (2.5–0.6%) concentration gel, and the Tris buffer system. The recovery of enzymatic activity was 75% and there was a 25% increase in specific activity.

B. Purification of Tyrosyl RNA Synthetase from B.

[2] This particular enzyme preparation was made with C_γ gel prepared according to Willstätter and Kraut (1923); commercially obtained C_γ gel also gave good fractionation, but the buffer concentrations used for washing and elution vary somewhat from those given here, and should be checked for each preparation of gel.

[3] With DEAE-cellulose purchased from Brown Paper Co. (0.9 mequiv/g) the enzyme was eluted at 120 mM phosphate, while with DEAE-cellulose purchased from Bio-Rad Labs (0.8 mequiv/g) a higher phosphate concentration (180 mM) was needed to elute the enzyme.

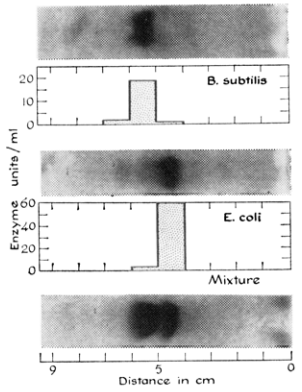

FIGURE 1: Electrophoresis of purified tyrosyl RNA synthetases on starch gel (Smithies, 1959). *B. subtilis* enzyme fraction VIII and *E. coli* enzyme fraction VII were used.

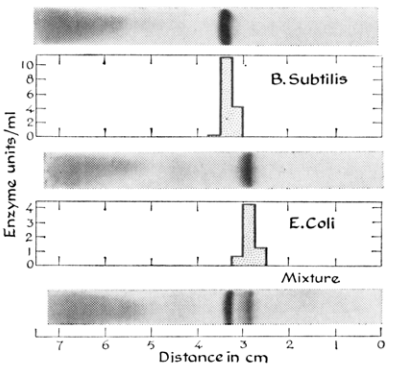

FIGURE 2: Electrophoresis of purified *B. subtilis* (fraction VIII) and *E. coli* (fraction VIII) enzymes on polyacrylamide gel (Jovin *et al.*, 1964)

subtilis SB19 (see Table II for summary.) Temperatures below 5° were maintained throughout, and centrifugations were as described in the previous purification.

I. CRUDE EXTRACT. Crude extract (fraction I) was prepared as in the previous purification, except that 50 mM potassium phosphate was the buffer used, and the whole procedure was scaled up for 800 g of cells.

II. AUTOLYSIS. Fraction I was diluted to contain 5 mg of protein/ml and 100 mM potassium phosphate by the addition of potassium phosphate, pH 7.5. After incubating the extract for 150 min at 37°, the amount of acid-soluble A_{260} rose to 70% of the total A_{260}, with an increase of approximately 20% in enzyme activity (fraction II).

III. AMMONIUM SULFATE FRACTIONATION. Ammonium sulfate (38 g) was added/100 ml of fraction II and after 5 min the suspension was centrifuged for 20 min. An additional 11 g of ammonium sulfate was added to the supernatant fluid/initial 100 ml of fraction II and after 5 min the suspension was centrifuged for 20 min, the precipitate was dissolved in *ca.* 300 ml of 10 mM potassium succinate buffer, pH 6.0, containing 3 mM 2-mercaptoethanol, and the solution was dialyzed overnight *vs.* two successive 30-l. aliquots of the same buffer (fraction III). From this point all solutions contained 3 mM 2-mercaptoethanol.[4]

IV. ALUMINA C_γ GEL FRACTIONATION. Alumina C_γ gel[5] (50 mg of dry weight gel/mg of protein) was added to fraction III and the suspension was centrifuged immediately. The gel was washed twice with 250-ml aliquots of 20 mM potassium phosphate buffer, pH 7.0, then eluted twice with 250-ml aliquots of 75 mM potassium phosphate, pH 7.5 (fraction IV).

V. DEAE-CELLULOSE FRACTIONATION. Fraction IV was diluted twofold with 3 mM 2-mercaptoethanol and applied to a DEAE-cellulose column (30 cm × 7.1 cm²) which had been equilibrated previously with 40 mM potassium phosphate, pH 6.0. The enzyme was eluted with a linear gradient between 40 and 300 mM potassium phosphate, pH 6.0, the total volume of the gradient being 2 l. Fractions of 30 ml were collected and those having a specific activity at least twofold greater than fraction IV were pooled (fraction V).

VI. PHOSPHOCELLULOSE FRACTIONATION. Fraction V was dialyzed *vs.* two successive 30-l. portions of 10 mM succinate buffer, pH 6.0, and then applied to a column of phosphocellulose (30 cm × 7.1 cm²) which had been equilibrated previously with 10 mM potassium succinate, pH 6.0.[6] The column was washed with *ca.* 600 ml of the 10 mM succinate, pH 6.0, and then the enzyme was eluted with a linear gradient between 10 mM potassium phosphate, pH 6.0, and 200 mM potassium phosphate, pH 8.0, the total volume of the gradient being 1.5 l. The fractions, 25 ml each, containing enzyme activity were pooled (fraction VI).

VII. HYDROXYLAPATITE FRACTIONATION. Fraction VI was diluted with 3 mM 2-mercaptoethanol to reduce the concentration of phosphate to *ca.* 50 mM. The diluted fraction was adsorbed to a hydroxylapatite column (15 cm × 3.1 cm²) previously washed with 1 l. of 50 mM potassium phosphate, pH 6.5. The enzyme was eluted with a linear gradient between 50 and 300 mM potassium phosphate, pH 6.5, the total volume of the eluent being 1 l. The fractions containing enzyme with a specific activity at least two times greater than fraction VI were pooled (fraction VII).

VIII. SECOND DEAE-CELLULOSE FRACTIONATION. Fraction VII was diluted twofold with 3 mM 2-mercaptoethanol and passed over a DEAE-cellulose column (25 cm × 0.75 cm²) previously equilibrated with 50 mM potassium phosphate, pH 8.0. Enzyme was eluted

[4] There is at present no evidence that SH reagent is required for the maintenance of enzyme activity.

[5] The gel used was that purchased from Sigma Chemical Co.

[6] It is important to check the pH and conductivity of both the dialyzed enzyme and column wash fluid before carrying out this step, since the enzyme will not be quantitatively adsorbed at higher pH.

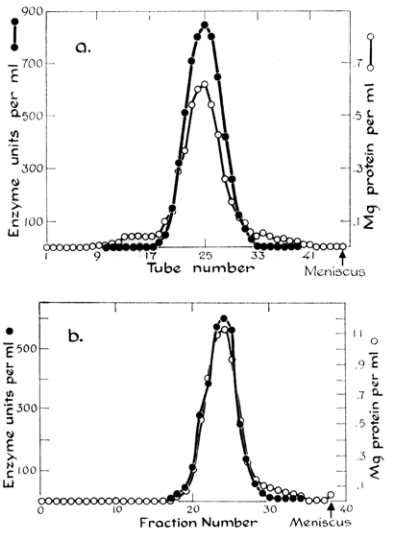

FIGURE 3: Sucrose gradient centrifugation (Martin and Ames, 1961) of purified tyrosyl RNA synthetases: (a) *E. coli* fraction VII; enzyme recovery 87%; protein recovery 66%; (b) *B. subtilis* fraction VIII; enzyme recovery 90%; protein recovery 105%.

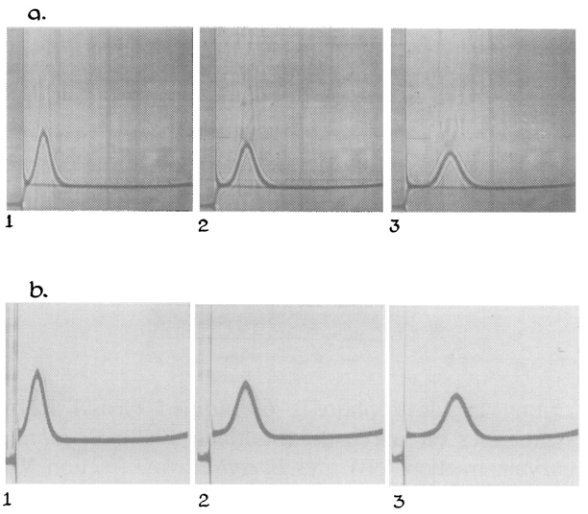

FIGURE 4: Schlieren photographs of sedimentation patterns of purified tyrosyl RNA synthetases. (a) *E. coli* enzyme at 7.5mg/ml. The rotor speed was 50,740 rpm at 6.2° with a schlieren angle of 75°. Times of photography were 32, 48, and 68 min. (b) *B. subtilis* enzyme at 6 mg/ml. The rotor speed was 50,740 rpm at 6.7° with the schlieren angle of 75°. Times of photography were 36, 56, and 80 min.

with a linear gradient between 50 and 250 mM potassium phosphate, pH 8.0, the total volume of the eluent being 500 ml. The peak fractions were pooled (fraction VIII). Concentration and storage at 0° were as described for the *E. coli* enzyme. The purification can also be carried out successfully by reversing steps VI and VII, with appropriate modification of the volume of the columns and gradients.

C. Characterization of Purified Enzyme Preparations. I. ELECTROPHORETIC BEHAVIOR. Electrophoresis of the *B. subtilis* tyrosyl RNA synthetase on starch gel (Figure 1) or polyacrylamide gel (Figure 2) revealed primarily one protein-containing band which coincided with the position of the enzymatic activity. The enzyme from *E. coli* contained additional minor protein bands (Figure 1), but in both the starch and polyacrylamide gel separations the activity migrated only with the major component. Photodensitometer tracings of the photographs shown in Figure 1 and integration of the protein peaks indicate that the maximum purities of the *B. subtilis* (fraction VIII) and *E. coli* (fraction VII) preparations are 90 and 70%, respectively. Electrophoresis of a mixture of the two tyrosyl RNA synthetases on starch gel (Figure 1) or polyacrylamide gel (Figure 2) shows that the two enzymes are readily separated; the *B. subtilis* enzyme is clearly more anionic at the pH values of 8 and 10 used in the two procedures.

II. SEDIMENTATION BEHAVIOR AND MOLECULAR SIZE. Sedimentation of the *B. subtilis* and *E. coli* tyrosyl RNA synthetases through a sucrose gradient showed that in both cases the enzymatic activity and the protein boundary sedimented together (Figure 3a and 3b). Lighter and heavier impurities were noted in the *E. coli* enzyme (fraction VII), and the specific activity in fractions 21–29 varied from 1120 to 1520 units/mg. The *B. subtilis* enzyme (fraction VIII) showed an enzyme peak of nearly constant specific activity (500–600 units/mg), although there was a small amount of lighter protein.

If the specific activity at the peak is taken as the specific activity of pure enzyme, the upper limits of the purities of the *E. coli* (fraction VII) and *B. subtilis* (fraction VIII) enzymes are 89 and 94%, respectively. The coincidence of protein and enzymatic activity in these sedimentation studies suggest that these enzyme preparations are suitable for the determination of the enzyme molecular weights in the analytical ultracentrifuge.

Measurement of the sedimentation coefficients of the two enzymes were also made at several protein concentrations using schlieren optics. Figure 4 (a and b) shows the Schlieren pattern for the *E. coli* and *B. subtilis* enzymes and Figure 5 shows the dependence of $s_{20,w}$ on protein concentration. Extrapolation to zero protein concentration yielded $s_{20,w}$ values of 5.2 and 5.2 for the *E. coli* and *B. subtilis* tyrosyl RNA synthetases, respectively. Little or no aggregation is seen with the *B. subtilis* enzyme, but there is less certainty of this with the *E. coli* enzyme.

The diffusion coefficient, $D_{20,w}$, was determined in one experiment as 4.7×10^{-7} cm²/sec for the *E. coli* tyrosyl RNA synthetase, at an initial concentration of 6.0 mg/ml. The value of $D_{20,w}$ for the *B. subtilis* enzyme was measured in three different experiments as 5.3, 5.2,

TABLE III: Molecular Weight Values of Tyrosyl RNA Synthetases from *E. coli* and *B. subtilis*.[a]

Enzyme	Protein Concn (mg/ml)	Rotor Speed (rpm)	M_w	M_z	M^*
a. *E. coli* tyrosyl RNA synthetase	3.7	19,160		95,000	95,000
	5.0	17,250		98,000	96,000
b. *B. subtilis* tyrosyl RNA synthetase	6.0	9,341	85,000	90,000	86,000
	6.0	15,220		92,000	91,000
	3.0	8,225		88,000	

[a] Equilibrium centrifugation results. M_w and M_z were determined using method I and method II (Van Holde and Baldwin, 1958) as described earlier. M^* was obtained from the slope of a plot of ln c *vs.* r^2 (Schachman, 1957).

TABLE IV: Amino Acid Composition of Tyrosyl RNA Synthetases of *E. coli* and *B. subtilis*.

Amino Acid	Approx Residues/Mole *E. coli*	*B. subtilis*
Aspartic acid + asparagine	90	91
Threonine	40	48
Serine	38	50
Glutamic acid + glutamine	111	102
Proline	37	21
Glycine	79	61
Alanine	77	55
Valine	51	39
Methionine	18	10
Isoleucine	51	54
Leucine	84	85
Tyrosine	26	31
Phenylalanine	41	39
Lysine	62	64
Histidine	13	10
Arginine	45	39
Tryptophan	≤ 18	14
Half-cystine	15	2–3
Total	896	815

and 5.8×10^{-7} cm²/sec at 6.0, 3.8, and 3.0 mg of protein/ml, respectively.

When $s_{20,w}$ and $D_{20,w}$ are combined in the Svedberg equation (Svedberg and Pedersen, 1940), molecular weight values of 97,000 and 87,000 are found for the *E. coli* and *B. subtilis* enzymes, respectively.

Sedimentation equilibrium studies were carried out using short columns of solution (1.5–3 mm). Plots of $1/r(dc/dr)$ *vs.* $(c - c_a)$ obtained from measurements of Schlieren photographs (method II, Van Holde and Baldwin, 1958) are shown in Figure 6. The fact that the plots are straight lines indicates reasonable homogeneity and lack of aggregation. The values determined for

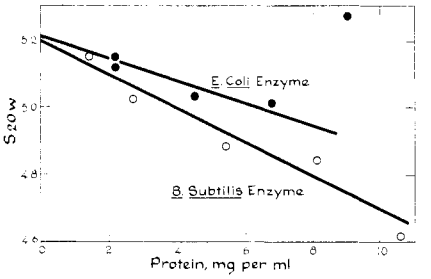

FIGURE 5: The dependence of sedimentation coefficient on protein concentration. The conditions of sedimentation are given in the legend to Figure 4 and in Methods.

molecular weight are given in Table III; the agreement of M_w and M_z further indicates the homogeneity of these preparations. The *E. coli* enzyme has a molecular weight of about 96,000, while the *B. subtilis* enzyme is *ca.* 88,000.

III. AMINO ACID COMPOSITION. Table IV presents the results of amino acid analyses carried out on the tyrosyl RNA synthetases of *E. coli* and *B. subtilis*. The values are presented as residues per molecule of enzyme. The most distinctive difference in amino acid composition of these two enzymes is their half-cystine content. The enzyme from *E. coli* appears to have 15 half-cystine residues, while the *B. subtilis* enzyme has only two or three. It is possible that some or all of the cysteic acid in the *E. coli* preparation comes from contaminating proteins. The value for two half-cystines/mole for the *B. subtilis* enzyme is not due to incomplete oxidation of cysteine and cystine, since the value for methionine (determined in the oxidized sample as methionine sulfone) is the same for oxidized and unoxidized samples of the protein.

The partial specific volumes estimated from the amino acid composition (Cohn and Edsall, 1943) were 0.730 for the *E. coli* enzyme and 0.735 for the *B. subtilis* enzyme.

IV. IMMUNOLOGIC CHARACTERIZATION OF *E. coli* AND

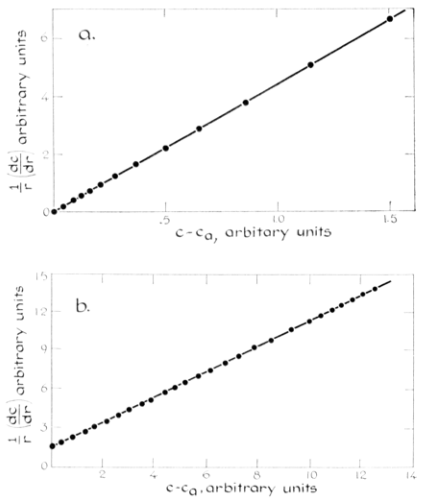

FIGURE 6: Method II plots (Van Holde and Baldwin, 1958) of sedimentation equilibrum data for purified tyrosyl RNA synthetases. A. *E. coli* enzyme at 3.7 mg/ml initial concentration. The rotor speed was 19,160 rpm for 33 hr at 6.2°. The column height was 1.88 cm. B. *B. subtilis* enzyme at 6.0 mg/ml initial concentration. The rotor speed was 9341 rpm for 53 hr at 6.7°. The column height was 3.25 cm.

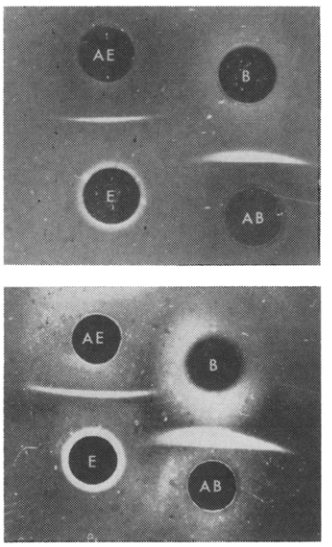

FIGURE 7: Immunodiffusion using antisera against purified tyrosyl RNA synthetases. The holes contained 0.1 ml of solution as follows: (AE) undiluted antiserum to *E. coli* enzyme; (E) *E. coli* enzyme (fraction VIII), 1.8 mg/ml; (B) *B. subtilis* enzyme (fraction VIII), 1.5 mg/ml; (AB) undiluted antiserum to *B. subtilis* enzyme; (a) before acid fixing; (b) after acid fixing.

B. subtilis TYROSYL SYNTHETASES. The *E. coli* (fraction VIII) and *B. subtilis* (fraction VIII) tyrosyl RNA synthetases were tested also for immunologic cross-reaction. Figure 7 shows that antiserum prepared against the *E. coli* enzyme (AE) formed a single precipitin line with the *E. coli* enzyme but no detectable precipitate with the *B. subtilis* enzyme. Antiserum to the *B. subtilis* enzyme precipitated the *B. subtilis* enzyme and gave a reaction of partial identity with *E. coli* enzyme. This latter cross-reaction was seen after acid fixation of the gel by the faint precipitin band between the wells marked E and AB (Figure 7, B).

A more quantitative estimate of the immunologic cross-reactivity was obtained by studying neutralization of enzyme activity by γ-globulin isolated from the antisera. γ-Globulin directed against the purified *E. coli* enzyme could neutralize (>99%) the tyrosine-dependent ATP–$^{32}PP_i$ exchange activity of purified *E. coli* enzyme or of an *E. coli* extract; however, there was no significant inhibition of activity of the purified *B. subtilis* enzyme when compared to a normal nonimmune γ-globulin (Figure 8A). With γ-globulin from animals immunized with the *B. subtilis* enzyme, the *B. subtilis* enzyme (purified or crude) was neutralized, but there was definite indication of some neutralization of the heterologous *E. coli* enzyme (Figure 8B). The heterologous reaction was, however, only *ca.* 10% that of the homologous reaction. Neither immune γ-globulin preparation neutralized the activity of purified isoleucyl RNA synthetase (Baldwin and Berg, 1966). Thus, by immunologic criteria, as well as electrophoretic behavior, the tyrosyl RNA synthetase from *E. coli* and *B. subtilis* are readily distinguishable.

Discussion

Although the two tyrosyl RNA synthetases catalyze the identical reaction and can be used interchangeably with t-RNA from *E. coli* or *B. subtilis* (Calendar and Berg, 1966) they are distinguishable by several criteria. The two enzymes have different electrophoretic mobilities in starch or polyacrylamide gels, the *B. subtilis* enzyme being more anionic. Of considerable interest is the striking difference in the half-cystine content of the two enzymes. The *E. coli* enzyme contains 15 half-cystine residues/mole, an amount similar to that found for *E. coli* isoleucyl RNA synthetase (Baldwin and Berg, 1966), while the *B. subtilis* enzyme has two or three. The tyrosyl RNA synthetases from *E. coli* and *B. subtilis* are also differentiated by immunologic criteria. Anti-*E. coli* tyrosyl RNA synthetase precipitates and completely neutralizes the *E. coli* enzyme but has no effect on the *B. subtilis* activity; anti-*B. subtilis* tyrosyl RNA synthetase precipitates and neutralizes the homologous enzyme but cross-reacts with the *E. coli* enzyme. The analysis of this partial cross-reaction in terms of the two protein structures needs further study.

Based upon the amount of purified protein recovered in each purification procedure one cannot determine if only a single tyrosyl RNA synthetase exists in the organisms examined. However, the neutralization of 95–100% of the tyrosyl RNA synthetase activity of crude extracts by antisera prepared against the most

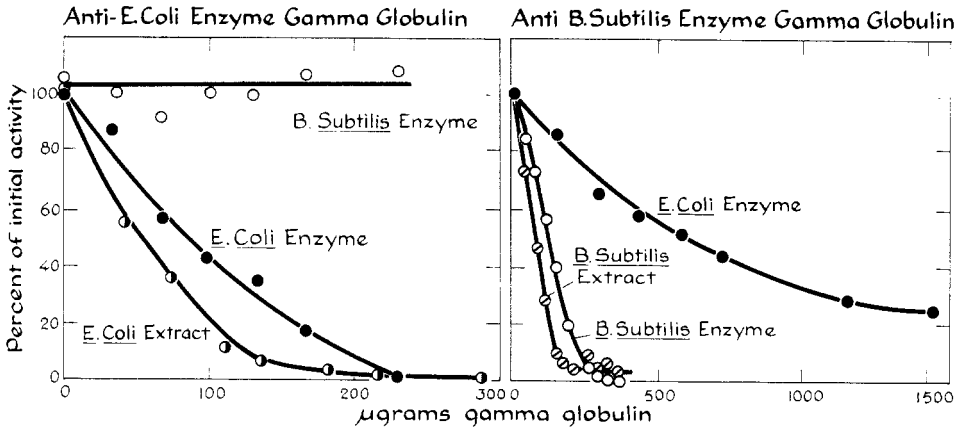

FIGURE 8: Neutralization of tyrosine-dependent ATP-$^{32}PP_i$ exchange activity with immune γ-globulin. Mixtures of 0.33 units of enzyme (0.6 μg of protein) and antiserum in 0.20 mM potassium phosphate, pH 6.5, 1 mM 2-mercaptoethanol, 250 μg/ml of bovine plasma albumin, were incubated at 37° for 5 min, kept at room temperature for 45 min, and stored at 4° overnight before centrifugation at 13,000*g* for 20 min. A. Reaction of γ-globulin directed against purified *E. coli* tyrosyl RNA synthetase with: purified *B. subtilis* enzyme; purified *E. coli* enzyme; *E. coli* dialyzed autolyzed extract (Table I, fraction II). B. Reaction of γ-globulin directed against purified *B. subtilis* tyrosyl RNA synthetase with: purified *E. coli* enzyme; purified *B. subtilis* enzyme; *B. subtilis* dialyzed, autolyzed extract (Table II, fraction II).

purified preparations (Figure 8) suggests that the enzymes isolated are probably the exclusive species present in the cell. Any multiple forms of the enzyme would have to be extremely labile or immunologically cross-reacting.

If the proteins isolated to represent the total complement of these enzymes in the cell, then there are *ca.* 1400 and 2000 molecules of enzyme/cell in *E. coli* and *B. subtilis*, respectively.[7] This is of the same order as that necessary to assure generation times of 19 and 40 min for *E. coli* and *B. subtilis*, respectively.[8] It is also of interest that based on the yield of t-RNA_{tyr} obtained from these bacteria grown under the same conditions (R. Calendar, unpublished data, 1966) there are *ca.* 1000 molecules of t-RNA_{tyr}/*E. coli* cell and 5000/*B. subtilis* cell.[9] Thus, it may be that the ratio of enzyme to t-RNA is near 1.

References

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241*, 831.

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Benzer, S., and Weisblum, R. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1149.

Berg, P. (1961), *Ann. Rev. Biochem. 30*, 293.

Bergmann, F., Berg, P., and Dieckmann, M. (1961), *J. Biol. Chem. 236*, 1735.

Calendar, R., and Berg, P. (1966), *Biochemistry 5*, 1690.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 375.

Doctor, B. P., and Mudd, J. A. (1963), *J. Biol. Chem. 238*, 3677.

Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9*, 351.

Long, C. (1961), Biochemists' Handbook, Princeton, N. J., D. Van Nostrand Co.

Lowry, O., Rosebrough, N., Farr, N., and Randall, R.

[7] 10^{12} *E. coli* cells (1-g wet weight) contain 150 units of enzyme; pure enzyme contains 7.0×10^{15} enzyme molecule (750 units)/mg of enzyme. Therefore, if enzyme molecules in the crude and purified states have the same activity, then 10^{12} cells contain 1.4×10^{15} enzyme molecules or 1400/cell. The pertinent values for *B. subtilis* are: 10^{11} *B. subtilis* cells (1 g wet weight) contain 20 units of enzyme; pure enzyme contains 6.5×10^{15} enzyme molecules (750 units)/mg of enzyme. Therefore, 10^{11} cells contain approximately 1.7×10^{14} enzyme molecules or 1700/cell.

[8] 10^{12} *E. coli* cells (1-g wet weight) contain 150 units of enzyme. One unit of enzyme will esterify 0.05 μmole of tyrosine to t-RNA (*E. coli*) in 15 min at 37°; 9.6 μmoles of L-tyrosine are contained in the protein of 1 wet g of *E. coli* (Sueoka, 1961; Long, 1961), and this amount of tyrosine can be esterified to t-RNA in 19 min by the tyrosyl RNA synthetase of a crude *E. coli* extract.

10^{11} *B. subtilis* cells (1-g wet weight) contain 20 units of enzyme; one unit of enzyme can esterify 0.15 μmole of tyrosine to t-RNA (*E. coli*). L-Tyrosine, 8 μmoles, is contained in the protein of 1 wet g of *B. subtilis* (Sueoka, 1961; Long, 1961), and this amount of tyrosine can be esterified to t-RNA in 40 min by the tyrosyl RNA synthetase of a crude *B. subtilis* extract. In these calculations it is assumed that enzyme in the intact cell has the same activity as that in a crude extract.

[9] *E. coli* cells (1-g wet weight) contain 1.7 mμmoles of t-RNA_{tyr}, *i.e.*, 1020 molecules of t-RNA_{tyr}/*E. coli* cell; 10^{11} *B. subtilis* cells (1-g wet weight) contain 0.8 mμmole of t-RNA_{tyr}, *i.e.*, 4800 molecules of t-RNA_{tyr}/*B. subtilis* cell. In these calculations it is assumed that assays using purified tyrosyl RNA synthetase and whole t-RNA (Calendar and Berg, 1966) define the correct amount of tyrosine acceptance in the cell.

(1951), *J. Biol. Chem. 193*, 265.
Martin, R., and Ames, B. (1961), *J. Biol. Chem. 236*, 1372.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Nester, E. W., Schafer, M., and Lederberg, J. (1963), *Genetics 48*, 530.
Ouchterlony, O. (1948), *Acta Pathol. Microbiol. Scand. 25*, 186.
Richardson, C. C., and Kornberg, A. (1964), *J. Biol. Chem. 239*, 242.
Sanger, F., and Thompson, E. O. P. (1963), *Biochim. Biophys. Acta 71*, 468.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Smithies, O. (1959), *Biochem. J. 71*, 585.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spizizen, J. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 1072.
Sueoka, N. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1141.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford Press, New York, N. Y.
Van Holde, K. W., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Weismeyer, H., and Cohn, M. (1960), *Biochim. Biophys. Acta 39*, 417.
Willstätter, R., and Kraut, H. (1923), *Ber. Deut. Chem. Ges. 56*, 1117.
Yamane, T., and Sueoka, N. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 1093.

The Catalytic Properties of Tyrosyl Ribonucleic Acid Synthetases from *Escherichia coli* and *Bacillus subtilis**

Richard Calendar and Paul Berg

ABSTRACT: A comparison of the substrate specificity of tyrosyl RNA synthetases from *Escherichia coli* and *Bacillus subtilis* has been made. Of the four common ribonucleoside triphosphates only adenosine triphosphate is utilized by both enzymes. Several alterations in the L-tyrosine structure have been examined for their effect on the formation of aminoacyl adenylate. Removal of the carboxyl group (tyramine) or its reduction (L-tyrosinol), amidation (L-tyrosine amide), or esterification (L-tyrosine methyl ester) yielded inactive substrates although each of these analogs was a competitive inhibitor. Modification of the α-amino group (*N*-acetyl or *N*-glycyl) or substitution of the α hydrogen by a methyl group yielded compounds which were neither substrates nor inhibitors. Quite unexpectedly, D-tyrosine was activated and transferred to t-RNA$_{tyr}$ by both enzymes. But in each case for the exchange reaction the V_{max} was lower and the K_m was higher than with L-tyrosine. A shift of the ring hydroxyl group from the 4 (*para*) to the 3 (*meta*) or 2 (*ortho*) position yielded inactive substrates with both enzymes. Although substitution of chloro, iodo, amino, or nitro groups in position 3 of L-tyrosine eliminated the substrate activity, after introduction of a fluoro or hydroxy group in the same position the derivatives were still active, but the V_{max} was lower and the K_m was higher. Conversion of the benzene ring to a pyridine structure (5-hydroxy-2-(3-DL-alanyl)pyridine) decreased the V_{max} to about half and increased the K_m about 30-fold with both enzymes. The *B. subtilis* and *E. coli* tyrosyl RNA synthetases utilize the t-RNA$_{tyr}$ from either bacterial source; the same amount of either tyrosyl RNA is formed with either enzyme.

In the previous paper (Calendar and Berg, 1966) tyrosyl RNA[1] synthetases from *E. coli* and *B. subtilis* were isolated and several of their physical and chemical properties were compared. These two enzymes have also been examined with respect to several catalytic parameters, particularly those related to the specificity for the substrates and analogs of the substrates, and these results are reported here.

Experimental Section

Materials. Glycyl-L-tyrosine, *N*-acetyl-L-tyrosine, 3-hydroxyl-L-tyrosine, and natural L-amino acids were purchased from California Corp. for Biochemical Research. D-Tyrosine, 3-iodo-L-tyrosine, 2-hydroxy-L-phenylalanine, 3-hydroxy-L-phenylalanine, 3-amino-L-tyrosine, 3-hydroxy-DL-tyrosine, and 4-fluoro-DL-phenylalanine were purchased from Nutritional Biochemicals, Inc., Cleveland, Ohio. 4-Chloro-DL-phenyl-

* From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California. *Received February 15, 1966.* This work was supported in part by teaching and research grants from the National Institutes of Health.

[1] Abbreviations used: RNA, ribonucleic acid; t-RNA, transfer RNA; F-tyrosine, 3-fluorotyrosine; DOPA, 3-hydroxytyrosine; HPA, 5-hydroxy-2-(3-DL-alanyl)pyridine; ATP, GTP, CTP, and UTP, adenosine, guanosine, cytidine, and uridine triphosphate; ADP, adenosine diphosphate; dATP, deoxy ATP; dTTP, deoxythymidine triphosphate.