The Tryptophanyl Transfer Ribonucleic Acid Synthetase of Escherichia coli

I. PURIFICATION OF THE ENZYME AND OF TRYPTOPHAN TRANSFER RIBONUCLEIC ACID*

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SUMMARY

Tryptophanyl-tRNA synthetase of Escherichia coli B was purified 1000-fold by MnCl₂ precipitation and column chromatography on DEAE-cellulose, hydroxylapatite, and Amberlite CG-50. The purified enzyme gave one peak of constant specific activity on Sephadex G-200, one protein band on disc gel electrophoresis, and contained no other aminoacyl-tRNA synthetases. The rate of tryptophanyl-tRNA formation was optimal at pH 8.8. The kinetic constants for tryptophan, ATP, and tRNA<sup>Ttp</sup> were determined at pH 7.0 and 8.8, and the turnover number was 1200 at pH 8.8. The amino acid composition is similar to the composition of other aminoacyl-tRNA synthetases, except for a low tryptophan and half-cystine content. tRNA<sup>Ttp</sup> was purified on benzoylated DEAE-cellulose and hydroxylapatite columns. The final product had a specific activity of 1.8 nmoles per A<sub>260</sub> unit and an estimated purity of 100%. The calculated ratio of tRNA<sup>Ttp</sup> to tryptophanyl-tRNA synthetase in vivo is 1:1.

Although many aminoacyl-tRNA synthetases and their cognate tRNAs have been purified to homogeneity (1–3), the mechanism involved in the recognition of the specific tRNAs by the aminoacyl-tRNA synthetases is not understood. We have approached that problem with a study of the tryptophanyl-tRNA synthetase (L-tryptophan + ATP + tRNA = L-tryptophanyl-tRNA synthetase) from beef pancreas (6), the enzyme from E. coli catalyzes the formation of a tryptophanyl-ATP ester (7). This reaction is analogous to the esterification of tryptophan to the terminal adenyclic acid residue of tRNA. Finally, tRNA<sup>Ttp</sup> from E. coli exists in active and inactive conformations (8–10), and only the active conformation is a substrate for tryptophanyl-tRNA synthetase.

The only other tryptophanyl-tRNA synthetase studied extensively is that from beef pancreas (11–13). That enzyme is of historical interest because it was the first aminoacyl-tRNA synthetase purified to homogeneity (14). We here report the purification and partial characterization of tryptophanyl-tRNA synthetase and the purification of tRNA<sup>Ttp</sup> from E. coli. The following communication (15) describes the molecular weight, sedimentation, and substrate-binding properties of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

E. coli cells harvested in logarithmic phase at 75% of maximum growth were purchased from Grinnell Processing Corporation, Muscatine, Iowa. E. coli B tRNA was prepared as previously described (20 pmoles of tRNA<sup>Ttp</sup> per A<sub>260</sub> unit) (16), or was obtained from Schwartz BioResearch (10 to 15 pmoles of tRNA<sup>Ttp</sup> per A<sub>260</sub> unit). One A<sub>260</sub> unit of tRNA has an A<sub>260</sub> absorbance of 2.0 when dissolved in 1.0 ml of 50 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM K<sub>2</sub>HPO<sub>4</sub>, buffer in a 1.0-cm optical path. L-[3-<sup>14</sup>C]Tryptophan from New England Nuclear Corporation, Boston, Mass., was purified as previously described (5) with spectrophotometric determination of its concentration (17). Chloroquine dihydrochloride was purchased from Winthrop Laboratories, New York, N.Y., as a sterile solution, 50 mg per ml. Its concentration was determined spectrophotometrically (18). Biene<sup>1</sup> and Taps buffers were obtained from Calbiochem, Los Angeles, Cal. Polyethylene glycol 6000 was from Dow Chemical Co., Midland, Mich., ammonium sulfate from Mann Research Laboratories.

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1 The abbreviations used are: Bicine, N,N-bis(2-hydroxyethyl)glycine; Taps, tris(hydroxymethyl)methylaminopropyl sulfonic acid; BD-cellulose, benzoylated DEAE-cellulose.
New York, N. Y., and crystalline ovalbumin and bovine serum albumin from Sigma, St. Louis, Mo. Hydroxylapatite was prepared as the "CPA" material of Main, Wilkins, and Cole (19). DE52-cellulose (Whatman DEAE-cellulose), GF/C and ACG/B glass fiber filters were from Whatman, Clifton, N. J., BD-cellulose from Schwarz BioResearch, Sephadex G-200 and blue dextran from Pharmacia Fine Chemicals, Piscataway, N. J. Amberlite CG-50 (100 to 400 mesh), polyethylene glycol, was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., and prepared as described by Hirs, Moore, and Stein (20). The materials for disc gel electrophoresis were purchased from Canaco, Rockville, Md. Other materials were from sources named previously (7, 10).

Methods

Assay of Tryptophanyl-tRNA Formation—The enzymatic activity was measured by the rate of tryptophanyl-tRNA formation at 37°C. The final 0.5-m1 reaction mixture contained 100 mM potassium Bicine buffer, pH 8.8, 5.0 mM MgCl₂, 4.0 mM reduced glutathione, 2.5 mM chloroquine, 1.0 mM ATP, 1-2 μm tRNA^{TTP} (45 to 50 A₂₆₀ units of tRNA), and 0.10 mM 1-L-[3⁻¹⁴C]tryptophan, 4 to 8 ppm per μmole. After these components in 0.45 to 0.49 ml were incubated at 37°C for 5 min, 0.05 to 0.2 unit of enzyme was added in 0.010 to 0.050 ml of 10 mM potassium phosphate buffer, pH 6.9, containing 20 mM 2-mercaptoethanol and 10% glycerol, and the complete mixture was incubated for 2 to 10 min at 37°C. The reaction was stopped by the addition of 1.3 ml of 12:1 (v/v) cold 95% ethanol-2 M sodium acetate buffer, pH 5. The tubes were placed at 0°C. The precipitates were collected on glass fiber filters, washed with 2 ml of 12:1 cold 95% ethanol-2 M sodium acetate buffer, pH 5, and then washed five times with 3 ml of 2 M HCl. In both methods the filters were dried and counted in a liquid scintillation counter. The two procedures for measuring tryptophanyl-tRNA give identical results. Under these assay conditions the amount of tryptophanyl-tRNA formed is proportional to time and to the amount of enzyme added. One unit of enzyme forms 1 nmole of tryptophanyl-tRNA per minute at 37°C. The final 0.5 ml reaction mixture contained 100 mM potassium Bicine buffer, pH 8.8, 5.0 mM MgCl₂, 20 mM 2-mercaptoethanol, and 15% glycerol, and the tryptophanyl-tRNA formed was separated from free tryptophan hydrolysis. At 0°C and pH 4.8 in 100 mM potassium acetate buffer, the tryptophanyl-tRNA was stable for at least 2 hours.

RESULTS AND DISCUSSION

Purification—The purification of the enzyme extends and modifies that previously published (7) and is summarized in Table I. All steps were at 0-4°C. All phosphate buffers were made by mixing equimolar amounts of K₂HPO₄ and KH₂PO₄.

Frozen E. coli cells (950 g) were suspended in a final volume of 3 liters by addition of 1 mM magnesium acetate buffer containing 10 mM MgCl₂, 20 mM 2-mercaptoethanol, and 10% glycerol. The cells were thawed and passed through a French press at 10,000 p.s.i. The suspension was centrifuged at 16,000 × g for 2 hours, and the turbid, supernatant fluid (2185 ml) was collected by decantation. The extract was placed in a 4-liter flask, and 100 ml of 1 M MnCl₂ (26) were added dropwise with stirring. After addition of the MnCl₂ the suspension was allowed to stand for 5 hours and then centrifuged as above. The cloudy, supernatant solution was dialyzed 24 hours against 12 liters of 5 mM phosphate buffer containing 20 mM 2-mercaptoethanol, 10% glycerol, and 15% polyethylene glycol 6000. The content of the dialysate was centrifuged as before; 500 ml of turbid supernatant fluid were collected and diluted to 1600 ml with Solution A (20 mM 2-mercaptoethanol, 10% glycerol), in order to decrease the conductivity to that of 10 mM phosphate buffer or less.

| Fraction | Total protein | Specific activity | Purification | Yield |
|----------|--------------|-------------------|--------------|-------|
| Extract  | 72,000       | 4,600             | 64           | 1.0   | 100 |
| MnCl₂    | 30,000       | 3,000             | 100          | 1.6   | 65  |
| DE52     | 1,000        | 1,400             | 1,400        | 21    | 30  |
| Hydroxylapitate | 39,1,100 | 28,000             | 440          | 24    |     |
| Amberlite CG-50 | 15,680     | 65,000             | 1,000        | 21    |     |

The purification procedure is described in the text.
FIG. 1. Isoelectric focusing. In a stepwise gradient from 0 to 70% glycerol a pH gradient was established with 1% ampholyte, pH range 5 to 8, at 0° by operation of the LKB model 8101 apparatus at 360 to 1270 volts for 28 hours. The higher glycerol concentration was at the higher pH. Then 350 units of hydroxylapatite fraction in 0.05 ml of 3 mM potassium phosphate buffer, pH 6.9, 10 mM 2- mercaptoethanol, 50% glycerol were placed at the 50% glycerol level (pH ~ 8) and focused for 17 hours at 0°, 900 volts. The pH (Q) was measured directly on 2-ml fractions at 0°. The activity (a) of tryptophanyl-tRNA synthetase was measured as under “Methods.” Recovery was 53% of input activity.

The 1600 ml were then placed over a DE52 column, 4 x 24 cm, previously equilibrated with 10 mM phosphate buffer in Solution A. The column was washed with 1000 ml of the buffer in Solution A, then developed at 60 ml per hour by a linear gradient from 10 to 100 mM phosphate buffer in 4000 ml of Solution A. The enzyme emerged as a sharp, symmetrical peak centered at a buffer concentration of 70 mM. Fractions comprising the peak were pooled and applied directly to a hydroxylapatite column, 2.5 x 30 cm, equilibrated with 100 mM phosphate buffer in Solution A. The column was washed with 450 ml of the buffer in Solution A, then developed at 70 ml per hour by a linear gradient from 100 to 200 mM phosphate buffer in 2000 ml of Solution A. The fractions containing the enzyme, centered at a buffer concentration of 150 mM, were pooled and dialyzed against 4000 ml of 10 mM phosphate buffer and 30% polyethylene glycol 6000 in Solution A. The content of the dialysis sacs was adsorbed on an Amberlite CG-50 column, 1.5 x 15 cm, equilibrated with 10 mM phosphate buffer in Solution A. The column was washed with 50 ml of the buffer in Solution A, then developed at 50 ml per hour by a linear gradient from 100 to 200 mM phosphate buffer in 2000 ml of Solution A. The fractions containing the enzyme, centered at a buffer concentration of 150 mM, were pooled and dialyzed against 4000 ml of 10 mM phosphate buffer and 30% polyethylene glycol 6000 in Solution A. The content of the dialysis sacs was adsorbed on a Amberlite CG-50 column, 1.5 x 15 cm, equilibrated with 10 mM phosphate buffer in Solution A. After a wash with 50 ml of the buffer in Solution A the column was developed at 50 ml per hour by a linear gradient from 100 to 300 mM phosphate buffer in 500 ml of Solution A. The enzyme appeared in a single, symmetrical peak centered at a buffer concentration of 150 mM. The purified enzyme was concentrated to 3 mg per ml by dialysis as before and stored at 2° in 20 mM phosphate buffer in Solution A.

Notes on Purification—Streptomycin sulfate treatment of the extract (1.3 g/62 ml) allowed recovery of 95% of the activity but removed less nucleic acid than did the MnCl₂ precipitation. Autolysis for 5 hours at 37° destroyed 67% of the extract activity. Fractional precipitation of the MnCl₂ fraction with polyethylene glycol 6000 or with ammonium sulfate provided little or no purification. Another purification from 40 g of cells produced a homogeneous enzyme after only a 330-fold purification. The difference is explainable, since a different batch of cells was used in each preparation, and the specific activities of the extracts differed by a factor of 4.

Isoelectric Focusing—As shown in Fig. 1 the enzyme has a pI of 6.2. The tryptophanyl-tRNA synthetase purified from human lymphocytes has a pI of 5.2 (27). The chromatographic properties of the two enzymes on DEAE-cellulose and on Amberlite CG-50 are in direct agreement with the pI values.

Polyacrylamide Disc Gel Electrophoresis—By disc gel electrophoresis the DE52 fraction exhibited at least 13 protein bands, and the hydroxylapatite fraction contained three bands, one major and two minor. The Amberlite fraction migrated as a single protein band (Fig. 2) corresponding with the major band of the hydroxylapatite fraction. Whereas 10 μg gave a clearly visible band, even 240 μg of protein (not shown) gave no trace of a second band. Moreover, the Amberlite fraction migrated as a single band under denaturing conditions (15). During electrophoresis of the hydroxylapatite fraction at 2° with 10% glycerol and 20 mM 2-mercaptopethanol in the buffers all of the tryptophanyl-tRNA synthetase recovered by crushing 1-mm gel slices in enzyme dilution buffer (see “Methods”) migrated with the major protein band.

Gel Filtration on Sephadex G-200—A sample of the Amberlite fraction was subjected to gel filtration on a Sephadex G-200 column (29). The enzyme emerged in a single, symmetrical peak with constant specific activity, equal to that of the input. The recovery of activity and protein was 90%. Tryptophanyl-tRNA synthetase of E. coli Vol. 246, No. 24
TABLE II

| Additions to reaction mixture | Amount ATP formed per µg of protein |
|------------------------------|-----------------------------------|
| 2 mM L-tryptophan and 0.13 µg of hydroxylapatite fraction | 160 µmoles |
| 2 mM each of 20 L-amino acids and 0.13 µg of hydroxylapatite fraction | 120 µmoles |
| 2 mM each of 19 amino acids (minus L-tryptophan) and 13 µg of hydroxylapatite fraction | 1.7 µmoles |
| 2 mM L-tryptophan and 0.13 µg of Amberlite fraction | 300 µmoles |
| 2 mM each of 20 L-amino acids and 0.13 µg of Amberlite fraction | 350 µmoles |
| 2 mM each of 19 amino acids (minus L-tryptophan) and 13 µg of Amberlite fraction | 2.3 µmoles |

Synthetase cochromatographed with E. coli alkaline phosphatase, molecular weight 74,000 (29). This apparent2 molecular weight was confirmed on calibrated Sephadex G-100 and G-150 columns (15).

Absence of Other Amino Acid-activating Enzymes—The absence of other amino acid-activating enzymes in the purified enzyme was determined by measuring the stimulation of ATP-PP i exchange by the other 19 coded amino acids (Table II). In the hydroxylapatite fraction the combined activities of all 19 other amino acid-activating enzymes added up to 1% of the activity of tryptophan-activating enzyme, and in the Amberlite fraction the sum was 0.6%.

Optimal Assay Conditions—The rate of tryptophanyl-tRNA formation as a function of pH was determined in 100 mM potassium Bicinie buffer, pH 8.1, 0.8 mM ATP, 4.0 mM MgCl2, 1.6 mM [32P]PPi (106 cpm per µmole), and the amino acids and enzyme fraction as indicated. The mixtures were incubated at 37° for 10 min, and the reaction was stopped by the addition of 0.5 ml of 10% per chloric acid containing freshly added 0.25 M sodium PPi at 0°. Aliquots of 0.2 ml were placed on ACG/filters, washed seven times with 12 ml of water, dried, and counted (7).

Additions to reaction mixture | Amount ATP formed per µg of protein |
|------------------------------|-----------------------------------|
| 2 mM L-tryptophan and 0.13 µg of hydroxylapatite fraction | 160 µmoles |
| 2 mM each of 20 L-amino acids and 0.13 µg of hydroxylapatite fraction | 120 µmoles |
| 2 mM each of 19 amino acids (minus L-tryptophan) and 13 µg of hydroxylapatite fraction | 1.7 µmoles |
| 2 mM L-tryptophan and 0.13 µg of Amberlite fraction | 300 µmoles |
| 2 mM each of 20 L-amino acids and 0.13 µg of Amberlite fraction | 350 µmoles |
| 2 mM each of 19 amino acids (minus L-tryptophan) and 13 µg of Amberlite fraction | 2.3 µmoles |

The optimal concentration of potassium Bicinie buffer, pH 8.8, for tryptophanyl-tRNA formation was 50 to 100 mM. The optimal Mg2+ concentration was 5 mM in the presence of 1 mM ATP and 100 mM potassium Bicinie buffer, pH 8.0. No activity was observed in the absence of Mg2+.

The optimal ATP concentration was 1 mM; 3 mM ATP decreased the rate by 40%, and 6 mM ATP by 80%. The enzyme synthesizes tryptophanyl-ATP ester in 6 mM ATP (7), and that

2 In this and the following paper the word "apparent" in connection with molecular weights determined by gel filtration of native proteins indicates our cognizance of the limitations of the method.

Fig. 3. The effect of pH on the rate of tryptophanyl-tRNA (Trp-tRNA) formation. Each reaction mixture contained 100 mM buffer, potassium Bicinie (●) or potassium cacodylate (▲), and the usual other components (see "Methods"). Each point represents an initial rate (2 and 4 min) at 37°.

Reaction may play a role in the inhibition of tryptophanyl-tRNA formation by ATP, although there are other possibilities, including simple chelation of Mg2+. Whole tRNA (50 A260 units, 10 to 20 pmoles of tRNATrp per A260 unit) and 2.5 mM chloroquine were routinely used in the enzyme assay (7). In the presence of 2.5 mM chloroquine, tRNATrp is totally in the active form at pH 7 (10). To be certain that chloroquine has the same effect on tRNATrp at pH 8.8 we measured the acceptor capacity of tRNATrp for [3H]tryptophan with and without chloroquine in sodium cacodylate buffer, pH 7, and potassium Bicinie buffer, pH 8.8. The specific acceptor activity was 20 pmoles of tryptophan per A260 unit of tRNA in the presence of chloroquine in both systems. However, in the absence of chloroquine, the specific activity was 6.0 µmoles per A260 unit at pH 7.0 and 9.5 µmoles per A260 unit at pH 8.8. The result suggests that at pH 8.8 tRNATrp was partially activated during the 30-min incubation at 37°. Chloroquine has pK values (30) of 8.1 (quinoline ring) and 10.2 (side chain tertiary amine). The pK of the ring of chloroquine bound to DNA is higher than 8.1 (18). Therefore, the chloroquine in the chloroquine-tRNATrp complex responsible for tRNATrp activation is probably divalent. Experiments with chloroquine analogs (31), for example 7-chloro-4-aminoquinoline, indicate the tertiary ammonium group on the side chain of chloroquine is not needed for activation of tRNATrp.

Kinetics—The apparent K m values for tryptophan, ATP, and tRNA^Trp in the charging reaction were determined as described in Fig. 4 and Table III. The 4-fold difference in V max at the two pH values agrees with the results soon on the pH curve (Fig. 3), which shows a 4-fold difference in the rate at pH 7.0 and 8.8. The apparent K m values for tryptophan, ATP, and tRNA^Trp are typical of those reported for other aminocyl-tRNA synthetases.

Turnover Number—In addition to the experiments presented in Table III and Fig. 4 the V max for tryptophanyl-tRNA formation at pH 8.8 was determined with the homogeneous enzyme (Amberlite CG-50 fraction). On the basis of a molecular weight of 75,000 the turnover number is 1200 moles tryptophanyl-tRNA per mole of enzyme per min. The values reported for other aminocyl-tRNA synthetases are lower, ranging from 8 to 640 (33-36). The values are difficult to compare, because in most
cases, the optimal rate conditions were not determined. However, the high turnover number of tryptophanyl-tRNA synthetase must partially account for its high activity in extracts of E. coli.

**Amino Acid Composition**—The amino acid composition of purified tryptophanyl-tRNA synthetase, expressed as residues per subunit of molecular weight 37,000 (19), is summarized in Table IV. This composition is similar to the composition of other aminoacyl tRNA synthetases (11, 33, 34, 41-45). The most unusual features of the amino acid composition are the low tryptophan and half-cystine contents. Spectrophotometric analysis (40) revealed a tyrosine to tryptophan ratio of 4.8. Tryptophanyl-tRNA synthetase contains only 2 residues of tryptophan per subunit, less than has been found for other aminoacyl-tRNA synthetases from E. coli. The minimum molecular weight of the enzyme, calculated from the amino acid analysis on the basis of 1 tryptophanyl residue, is 20,000.

The relatively high content of lysine and arginine agrees with the finding that this enzyme binds only weakly to DEAE-cellulose, being the first of all aminoacyl-tRNA synthetases of E. coli to emerge from columns developed by potassium phosphate buffer gradients at pH 6.9. Conversely, the enzyme binds strongly to Amberlite CG-50, an unusual property aiding purification.

The half-cystine content was not determined accurately, although the value was less than 7 residues per mole. Most other aminoacyl-tRNA synthetases have 8 to 15 half-cystine residues per subunit. After 20 min, the enzyme is stable for at least 20 min, according to the method of Selwyn (46). Thus, the same amount of tryptophanyl-tRNA was formed by 0.04 unit of enzyme in 20 min as by 0.5 unit of enzyme in 1.6 min.

Tryptophanyl-tRNA synthetase from E. coli is stabilized by glycero1 (7). The hydroxylapatite and Amberlite fractions stored at 2°C in 10% glyceral or at 20°C in 50% glyceral, both in 20 mM 2-mercaptoethanol, 20 mM potassium phosphate buffer, pH 6.9, had no detectable loss of activity in 1 year. Dilute solutions of enzyme (12 units per ml) in 50 mM potassium phosphate

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**Table IV**

| Amino acid | A | B | C | Approximate residues per subunit* |
|------------|---|---|---|-----------------------------------|
| Lysine     | 72 | 72 | e | 20 |
| Histidine  | 22 | 20 | e | 9  |
| Arginine   | 37 | 33 | e | 15 |
| Half-cystine | 0 | 0 | 0-7 | <3 |
| Aspartic acid and asparagine | 98 | 90 | 107 | 39 |
| Methionine | 0 | 18 | e | 7  |
| Threonine  | 36 | 36 | e | 14 |
| Serine     | 51 | 49 | e | 21 |
| Glutamic acid and glutamine | 166 | 90 | 104 | 43 |
| Proline    | 45 | 45 | e | 18 |
| Glycine    | 60 | 61 | 65 | 24 |
| Alanine    | 84 | 82 | 72 | 34 |
| Valine     | 49 | 68 | e | 27 |
| Isoleucine | 33 | 37 | e | 15 |
| Leucine    | 69 | 69 | e | 28 |
| Tyrosine   | 22 | 25 | e | 9  |
| Phenylalanine | 22 | 23 | e | 9  |
| Tryptophan | e | e | e | 2  |

* Molecular weight of 37,000.

* The values were normalized to the 24-hour hydrolysis by comparison with aspartic acid, glycine, and alanine in each run.

* Not determined.

* During analysis an unknown material had an Rf value similar to cysteic acid. Although only a trace of this material was found, it was enough to interfere with the calculation for the small amount of cysteic acid present.

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**Table III**

Kinetic constants for substrates of tryptophanyl-tRNA synthetase

Experiments were as in Fig. 4, with 0.10 mM L-[^14]C]tryptophan and 2.6 nM tRNA[^14]P present for the ATP determinations. Constants were determined from plots of v against v/s (32) by the method of least squares.

| Substrate | pH | Keq | Vmax |
|-----------|----|-----|------|
| Tryptophan | 7.0 | 3 x 10^-1 | 1.6 |
| ATP       | 7.0 | 9 x 10^-4 | 6.2 |
| tRNA[^14]P | 7.0 | 3 x 10^-7 | 1.6 |

* Not determined for the same enzyme preparation.
Acids provided pure tRNA on BD-cellulose at 23°C (Fig. 5). The 21-fold purified tRNATrp was 1.56. By that criterion (47) not more than 1 mole of nucleotidyl residue can be present per mole of the pure enzyme. Molecular weight was determined (see “Methods”) after ethanol precipitation. No tRNA\textsuperscript{Trp} appeared in the NaCl gradient. Fractions 18 to 21 were pooled. The tRNA was precipitated with ethanol, harvested by centrifugation, dissolved in water, and stored at -20°C.

Purification of tRNA\textsuperscript{Trp}. Roy and Sill (48) purified tRNAs from E. coli on BD-cellulose (49) and found that tRNA\textsuperscript{Trp} from E. coli had a high affinity for the resin. Tenen et al. (50) have purified yeast tRNA\textsuperscript{Trp} and tRNA\textsuperscript{Trp} on BD-cellulose by taking advantage of the lipophilic nature of the amino acids; chromatography on BD-cellulose before and after esterification with amino acids provided pure tRNAs. The tRNA\textsuperscript{Trp} from E. coli can be easily purified in this way.

Partial purification of tRNA\textsuperscript{Trp} was achieved by fractionating tRNA on BD-cellulose at 23°C (Fig. 5). The 21-fold purified tRNA\textsuperscript{Trp} was then charged with \textsuperscript{14}C]tryptophan and rechromatographed on the same column at 0°C (Fig. 6). Whereas the tRNA\textsuperscript{Trp} peak had appeared in 5% ethanol (Fig. 5), the tryptophanyl-tRNA appeared in 12% ethanol (Fig. 6). The shift in elution position resulted in a further 3.5-fold purification. About 2% of this material was further purified by hydroxylapatite column chromatography (Fig. 7). The results are summarized in Table V. The final preparation had an acceptor activity of 1800 pmoles per A\textsubscript{260} unit, a value equivalent to 100% purity if 1 pmole of tRNA nucleotidyl residue is 7.4 A\textsubscript{260} units at pH 7 (52), and if tRNA\textsuperscript{Trp} has a chain length of 76 nucleotidyl residues (53).

Spectral Properties. The pure enzyme was separated from 2-mercaptoethanol and glycerol by gel filtration, and the spectral properties were studied in 0.1 M Tris-HCl buffer, pH 8.0. The absorbance at 280 nm of a solution containing 1.0 mg per ml (Lowry) in a 1.0-cm light path was 1.0 at pH 8.0. The A\textsubscript{280}/A\textsubscript{600} of every other fraction was measured and the A\textsubscript{280} (0) was determined (see “Methods”) after ethanol precipitation. No tRNA\textsuperscript{Trp} appeared in the NaCl gradient. Fractions 18 to 21 were pooled. The tRNA was precipitated with ethanol, harvested by centrifugation, dissolved in water, and stored at -20°C.

Although five isoacceptors for tRNA\textsuperscript{Trp} have been resolved by gradient partition chromatography (16), we observed only one major isoacceptor by BD-cellulose and hydroxylapatite chromatography. Similarly, Hirsh (53) observed only one tRNA\textsuperscript{Trp}...
by BD-cellulose and reversed phase chromatography and discussed evidence for only one tRNA<TT> gene. The five isoacceptors resolved by gradient partition chromatography may differ only in base modifications not affecting acceptor activity or chromatographic characteristics in other systems.

In E. coli tRNA<TT> and other tRNAs recognizing codons beginning with U contain cytidinines (54). The presence of a lipophilic cytotokrin, however, is probably not responsible for the high affinity of tRNA<TT> for BD-cellulose (55).

State of tRNA<TT> and Tryptophanyl-tRNA Synthetase in Vivo-

A single E. coli cell (approximately 0.3 × 10<sup>6</sup> µg of protein) contains about 6000 molecules of phenylalanyl-tRNA synthetase (36). Calendar and Berg (33) calculated that there are 500 to 1100 molecules of enzyme per cell. Similarly, the tRNA<sub>Trp</sub> content is 800 molecules per cell. These concentrations of about 1400 molecules of tyrosyl-tRNA synthetase and 1000 molecules of tyrosyl-tRNA synthetase in E. coli is roughly 2 × 10<sup>6</sup> M.

The affinity of tRNA<sub>Trp</sub> for BD-cellulose (55). Calendar and Berg (33) calculated that there are approximately 1400 molecules per cell of E. coli. Thus the concentration of tyrosyl-tRNA synthetase in E. coli is roughly 2 × 10<sup>6</sup> M. DeLorenzo and Ames (50) found that in Salmonella typhimurium tRNA<sup>H</sup><sub>Trp</sub> is 2.3 × 10<sup>-4</sup> M and histidyl-tRNA synthetase is 2.2 × 10<sup>-4</sup> M. Because the K<sub>m</sub> for tRNA<sup>H</sup><sub>Trp</sub> is 1 × 10<sup>-7</sup> M, histidyl-tRNA synthetase and tRNA<sup>H</sup><sub>Trp</sub> may exist as a complex in vivo. If the tryptophanyl-tRNA synthetase isolated in the cell extract represents the total content of this enzyme in the cells, we can calculate with the assumptions used by Calendar and Berg (33) that there are 500 to 1100 molecules of enzyme per cell. Similarly, the tRNA<sup>H</sup><sub>Trp</sub> content is 800 molecules per cell. Cell extract is 800 molecules per cell. These concentrations, both near 10<sup>-6</sup> M, and the K<sub>m</sub> of 3 × 10<sup>-7</sup> M suggest that the enzyme and tRNA<sup>H</sup><sub>Trp</sub> exist as a complex in vivo, as already indicated for the histidine system (56).

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### TABLE V

| Fraction     | Ass units | tRNA<sub>Trp</sub> | Recovery Ass | Recovery tRNA<sub>Trp</sub> | Specific acceptor activity | Purification |
|--------------|-----------|---------------------|--------------|-----------------------------|-----------------------------|--------------|
| Input on BD-cellulose I                        | 34,000    | 1080                | 100          | 100                         | 20             | 1            |
| Total eluate from salt gradient                | 45,000    | 0                   | 84           | 0                           | 0              | 0            |
| Total eluate from ethanol gradient             | 10,000    | 1000                | 18           | 93                          | 3              | 2            |
| Pooled fractions from ethanol gradient (BD-cellulose I fraction) | 1,700 | 700 | 3.1<sup>a</sup> | 65<sup>a</sup> | 410 | 21 |
| Input on BD-cellulose II                        | 1,450     | 600                 | 3.1<sup>a</sup> | 65<sup>a</sup> | 410 | 21 |
| Total eluate from ethanol gradient             | 1,300     | 600                 | 2.8<sup>a</sup> | 65<sup>a</sup> | 410 | 21 |
| Pooled fractions from ethanol gradient (BD-cellulose II fraction) | 320 | 470 | 0.70<sup>a</sup> | 51<sup>a</sup> | 1460 | 73 |
| Input on hydroxylapitate                          | 7.5       | 10.8                | 0.70<sup>a</sup> | 51<sup>a</sup> | 1460 | 73 |
| Total eluate from gradient                       | 0.8       | 10.9                | 0.69<sup>a</sup> | 51<sup>a</sup> | 1460 | 73 |
| Pooled fractions from gradient                  | 5.0       | 9.0                 | 0.47<sup>a</sup> | 41<sup>a</sup> | 1800 | 90 |

<sup>a</sup> Adjusted for use of entire preceding fraction.

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The purification procedure is described in the text and Figs. 5, 6, and 7.
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