*Amer. Chem. Soc. 81,* 62.
Danyluk, S. S., and Hruska, F. E. (1968), *Biochemistry 7,* 1038.
Dorman, D. E., and Roberts, J. D. (1970), *Proc. Nat. Acad. Sci. U. S. 65,* 19.
Eigen, M. (1964), *Angew. Chem., Int. Ed. Engl. 3,* 1.
Gellert, M., Lipsett, M. N., and Davies, D. R. (1962), *Proc. Nat. Acad. Sci. U. S. 56,* 2013.
Jardetzky, C. D., and Jardetzky, O. (1960), *J. Amer. Chem. Soc. 82,* 222.
Jardetzky, O., Pappas, P., and Wade, N. G. (1963), *J. Amer. Chem. Soc. 85,* 1657.
Johnson, C. A., Jr. (1965), *Advan. Magn. Resonance 1,* 39.
Jones, J. W., and Robins, R. K. (1963), *J. Amer. Chem. Soc. 85,* 193.
Martin, D. M. G., and Reese, C. B. (1967), *Chem. Commun.,* 1275.
McConnell, B., Raszka, M., and Mandel, M. (1972), *Biochem. Biophys. Res. Commun. 47,* 692.
McConnell, B., and Seawell, P. C. (1972), *Biochemistry 11,* 4382.
McConnell, B., and von Hippel, P. H. (1970), *J. Mol. Biol. 50,* 297.
Molday, R. S., and Kallen, R. G. (1972), *J. Amer. Chem. Soc. 94,* 6739.
Raszka, M. J. (1971), Ph.D. Dissertation, Appendix B, University of Hawaii.
Raszka, M. (1974), *J. Biol. Chem.* (in press).
Raszka, M., and Kaplan, N. O. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2025.
Raszka, M., and Kaplan, N. O. (1974), *Proc. Nat. Acad. Sci. U. S.* (in press).
Schweizer, M. P., Broom, A. D., Ts'o, P. O. P., and Hollis, D. P. (1968), *J. Amer. Chem. Soc. 90,* 1042.
Shimanouchi, T., Tsubor, M., and Kyogoku, Y. (1964), *Advan. Chem. Phys. 7,* 435.
Shoup, R. R., Miles, H. T., and Becker, E. D. (1972), *J. Phys. Chem. 76,* 64.
Suchy, J., Mieyal, J. J., Bantle, G., and Sable, H. Z. (1972), *J. Biol. Chem. 247,* 5905.

# The Enzymatic Synthesis of *N*-(Purin-6-ylcarbamoyl)threonine, an Anticodon-Adjacent Base in Transfer Ribonucleic Acid†

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ABSTRACT: Almost all *Escherichia coli* tRNAs which have codons with a 5′-adenosine have an unusual nucleoside adjacent to the 3′ end of the anticodon the systematic name of which is *N*-[*N*-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine ($t^6A$). An enzyme has been isolated from *E. coli* which can synthesize this nucleoside in tRNA when provided with a tRNA deficient in $t^6A$ and with L-threonine, bicarbonate, ATP, and $Mg^{2+}$. The $t^6A$-deficient tRNA was isolated from a threonine-starved culture of an *E. coli* strain which is a threonine auxotroph and is also a relaxed control mutant (capable of synthesizing RNA in the absence of protein synthesis). The $t^6A$-deficient tRNA was treated with periodate to destroy the 3′-ribose so that the enzyme assay could depend upon the incorporation of labeled L-threonine into the tRNA to form $t^6A$ without interference by the threonine incorporating activity of the *E. coli* threonyl-tRNA synthetase. Using this assay, an enzyme was purified from *E. coli* extracts. It catalyzed the bicarbonate- and ATP-dependent incorporation of L-threonine into $t^6A$-deficient tRNA. The product of the reaction was established as $t^6A$ by comparison with an authentic sample. The same purified enzyme could incorporate glycine in place of threonine into $t^6A$-deficient tRNA. Others had previously found a small amount of the glycine analog of $t^6A$ in tRNA; the present findings indicate that a single enzyme incorporates threonine and glycine into the same tRNA species. Since glycine competes with threonine, the tRNA synthesized in threonine-deficient cells would have more than the normal content of the glycine analog of $t^6A$. This could explain why the $t^6A$-deficient tRNA used here as substrate was found to accept much less threonine than was predicted from the amount of tRNA that was synthesized during the threonine starvation.

Transfer RNAs whose codons have a 5′-adenosine usually bear in the position adjacent to the 3′-uridine of the anticodon an unusual nucleoside containing a threonine residue (Takemura *et al.,* 1969; Ishikura *et al.,* 1969; Kimura-Harada *et al.,* 1972a; Powers and Peterkofsky, 1972b). The structure of this nucleoside was determined by Chheda *et al.* (1969) and Schweizer *et al.* (1969) to be *N*-[*N*-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine ($t^6A$).[1]

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[1] Abbreviations used are: $t^6A$, *N*-[*N*-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine or the simpler and more descriptive name suggested by Dr. W. E. Cohn, Office of Biochemical Nomenclature, $N^6$-(*N*-threonylcarbonyl)adenosine; $t^6Ade$, *N*-(purin-6-ylcarbamoyl)-threonine or $N^6$-(*N*-threonylcarbonyl)adenine; SD enzyme, total cell soluble proteins freed of low molecular weight material and of nucleic acids by passage through Sephadex G-25 and DEAE-cellulose columns; $rel^-$, a mutation in *E. coli* which allows RNA synthesis to occur in the absence of protein synthesis.

The simpler trivial name $N^6$-(*N*-threonylcarbonyl)adenosine which has been suggested for this nucleoside[1] more clearly indicates the derivation of its components from threonine, $CO_2$, and adenosine which is the subject of this paper. The base of this nucleoside has the systematic name *N*-(purin-6-ylcarbamoyl)threonine ($t^6$Ade) or the trivial name $N^6$-(*N*-threonylcarbonyl)adenine.

The importance of this $t^6A$ in preventing codon misreading at the adjacent 3′-uridine of the anticodon was first suggested by Dube *et al.* (1968) even before the structure of $t^6A$ was completely worked out. In the case of the initiating $tRNA^{fMet}$ of *Escherichia coli* there is no $t^6A$ or any other modification of the adenosine adjacent to the anticodon (Dube *et al.*, 1968) and this tRNA responds not only to the normal methionine codon AUG but also to the valine codon GUG (Clark and Marcker, 1966; Ghosh *et al.*, 1967; Volckaert and Fiers, 1973). In this case a wobble response of a U to an A or a G occurs at the 5′ end of the codon like the more common wobble at the 3′ end of codons. All the other tRNAs of *E. coli* which respond to codons with a 5′-adenosine have the $t^6A$ (see above for references) and fail to show this particular kind of wobble. Furthermore, in the case of yeast, the initiating $tRNA^{fMet}$ has the $t^6A$ (Simsek and RajBhandary, 1972) and responds only to AUG, not to GUG (Stewart *et al.*, 1971). Thus the $t^6A$ apparently plays an important role in the correct codon–anticodon interaction of a whole class of tRNAs (see also Högenauer *et al.*, 1972; Miller and Schweizer, 1972).

Little information has been available on the mechanism of biosynthesis of this important modified nucleoside in tRNA. The *in vivo* utilization of L-threonine for the synthesis of $t^6A$ in tRNA has been reported by Powers and Peterkofsky (1972a) and by Chheda *et al.* (1972). Recently Körner and Söll (1974) described an *in vitro* system from *E. coli* which utilized threonine and ATP for the synthesis of $t^6A$ in $t^6A$-deficient tRNA. The present paper describes the purification of an enzyme from *E. coli* which carries out this synthesis on $t^6A$-deficient tRNA utilizing threonine, ATP, and, as the source of the one carbon fragment, bicarbonate. With this system it was possible to show that the same enzyme preparation can utilize glycine in place of threonine to synthesize the glycine analog of $t^6A$ in $t^6A$-deficient tRNA.

## Experimental Section

*Materials.* [$^{14}$C]-L-Threonine, [$^{3}$H]-L-threonine, [$^{14}$C]glycine, and [$^{14}$C]$NaHCO_3$ were obtained from Schwarz/Mann. Bio-Gel A 1.5m (200–400 mesh) and Bio-Gel P 100 (100–200 mesh) came from Bio-Rad Labs. *E. coli* CP 79 ($Thr^-$, $Arg^-$, $His^-$, $Leu^-$, $thi^-$, $rel^-$) was kindly provided by Dr. Joel Weiner.

*Growth of E. coli CP 79 for Synthesis of $t^6A$-Deficient tRNA. E. coli* CP 79 was grown in the medium of Davis and Mingioli (1951). Solutions of the following were autoclaved separately and added to 1 l. of medium before use: 10 g of glucose, 30 mg of L-threonine, 30 mg of L-arginine · HCl, 30 mg of L-histidine · HCl, 30 mg of L-leucine, and 3 mg of thiamine · HCl. An inoculum (2 l.) was grown up in four 2-l. baffle flasks with shaking at 37° overnight. This was used to inoculate 30 l. of medium in two 20-l. carboys. The cells were grown at room temperature with vigorous aeration until they had reached late-log phase. They were then harvested, washed twice with medium without threonine, and reincubated overnight at room temperature in 15 l. of medium without threonine. In the absence of threonine, and therefore of protein synthesis, this relaxed control mutant continues to synthesize tRNA which under these conditions would necessarily be deficient in $t^6A$.

*Preparation of $t^6A$-Deficient Periodate-Treated tRNA.* tRNA was isolated from 40 g of the above threonine-starved cells essentially by the method of Holley (1963). The tRNA was then treated with periodate in order to destroy the 3′-ribose and thus prevent any incorporation of threonine onto threonine tRNA by the threonyl-tRNA synthetase. The tRNA dissolved in 25 ml of 0.01 M sodium acetate (pH 4.5) was treated with 2.5 ml of 0.5 M $NaIO_4$, and the solution was incubated 30 min at room temperature in the dark. Another equal portion of $NaIO_4$ was added and the incubation repeated. Ethylene glycol (1 ml) was then added to destroy $NaIO_4$. Then the solution was dialyzed against 2 l. of 0.01 M sodium acetate (pH 4.5) containing 1 ml of ethylene glycol, then against 2 l. of the buffer without ethylene glycol. The tRNA was precipitated with alcohol, collected, dried, dissolved in 10 ml of water, distributed into 10 tubes, and lyophilized. These dried portions of deficient tRNA were stored in the freezer and reconstituted with 1 ml of water as they were needed. In this paper, $t^6A$-deficient tRNA is understood to be this periodate-treated material.

*Assay for $t^6A$ Synthesis.* The assay contained the following in a final volume of 0.20 ml: 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 25 mM KCl, 5 mM dithioerythritol, 10 mM ATP, 0.05 mM [$^{14}$C]-L-threonine (20 Ci/mol), 25 mM $NaHCO_3$, $t^6A$-deficient tRNA in the amount indicated, and enzyme as indicated. Incubation was for 20 min at 37°. tRNA along with enzyme protein was then precipitated by adding 2 ml of 8% trichloroacetic acid. The precipitate was collected on a glass fiber filter membrane (Type E, Gelman Instrument Co.) and washed well with 5% trichloroacetic acid and then with 95% ethanol. The membrane was dried, placed in a vial with toluene-based scintillation fluid, and counted in a Packard liquid scintillation counter with an efficiency of 75%.

One unit of $t^6A$ synthetase activity catalyzes 1 pmol of bicarbonate-dependent incorporation of labeled L-threonine into $t^6A$-deficient tRNA in 20 min at 37°.

*Assay for Threonyl-tRNA Synthetase.* Column fractions of enzyme were assayed directly (5–20 μl per assay) under essentially the conditions described by Leis and Keller (1971), except that [$^{14}$C]-L-threonine (20 Ci/mol) replaced labeled methionine, and 0.2 mg of bulk *E. coli* tRNA (General Biochemicals) was present in the final volume of 0.2 ml.

*Preparation of E. coli Soluble (SD) Enzyme.* All steps were carried out at 0–2°. Frozen *E. coli* B cells (100 g from Grain Processing Corp.) were suspended in 250 ml of 10 mM Tris-HCl (pH 7.5)–10 mM magnesium acetate. Aliquots of 80 ml at a time were sonicated with a Branson sonifier for 2 min with intervals for cooling. After a low-speed centrifugation, ribosomes were removed by centrifuging for 2.5 hr at 50,000*g*. Total protein was precipitated from the supernatant by 80% saturation with ammonium sulfate, collected by centrifugation, suspended in and then dialyzed against 20 mM Tris · HCl (pH 7.5)–0.1 mM dithioerythritol. The protein was then freed of RNA on a DEAE-cellulose column as described by Leis and Keller (1971). Except for the substitution of dialysis for gel filtration because of the large volumes involved, this enzyme preparation is equivalent to those authors' SD enzyme and therefore it is called SD enzyme in this paper.

TABLE I: Requirements for Synthesis of $t^6A$ by an *E. coli* SD Enzyme Preparation.[a]

| System | [$^{14}$C]Threonine Incorporated (cpm) |
|---|---|
| Complete | 433 |
| − ATP | 60 |
| − $NaHCO_3$ | 152 |
| − $MgCl_2$ | 55 |
| − $t^6A$-deficient tRNA | 40 |
| − Enzyme | 50 |

[a] The assay system (see Experimental Section) contained 0.3 mg of $t^6A$-deficient tRNA and 0.4 mg of SD enzyme protein in a volume of 0.20 ml.

*Ammonium Sulfate Fractionation of E. coli SD Enzyme Preparation.* Ammonium sulfate pellets were obtained at 35, 50, and 80% saturation, and suspended and dialyzed as above. Assay showed that 80% of the $t^6A$ synthetase activity was in the 50–80% cut, so this material was saved and stored frozen in 5-ml aliquots.

*Preparative Synthesis of Labeled $t^6A$ in tRNA.* The preparative incubation contained the following in a volume of 2 ml: 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 25 mM KCl, 5 mM dithioerythritol, 10 mM ATP, 0.025 mM [$^{14}$C]-L-threonine (50 Ci/mol), 25 mM $NaHCO_3$, 1–2 mg of $t^6A$-deficient tRNA, and 4 mg of SD enzyme protein. After incubation for 20 min at 37°, the mixture was extracted in the usual way with phenol and then with ether, and the aqueous phase was applied to a Sephadex G-25 column (20 ml) which had been equilibrated with 0.5 M Tris-HCl (pH 8.0). The tRNA was eluted with the same buffer, precipitated with alcohol, collected, and dried *in vacuo*.

When $t^6A$ labeled with [$^{14}$C]$NaHCO_3$ was prepared, the above was scaled down to 0.2 ml, and 15 mM [$^{14}$C]$NaHCO_3$ (50 Ci/mol) and 1 mM L-threonine were substituted for unlabeled $NaHCO_3$ and labeled L-threonine. After the incubation, water was added to 2 ml, and the mixture was treated as above.

*Acid Hydrolysis of Enzymatically Labeled tRNA.* The labeled tRNA was dissolved in 0.5 ml of water, and 0.5 ml of 2 N HCl was added. The solution was heated for 15 min in a boiling water bath to release purines including the labeled $t^6Ade$ from the tRNA (Chheda *et al.*, 1969). The resulting solution was flash evaporated, water was added, it was flash evaporated again, and this was repeated once more to remove all traces of HCl.

*Paper Electrophoresis of Purines Released from Labeled tRNA.* The solution of the purines freed of HCl was spotted along with standards onto filter paper (S and S 589 Orange) moistened with 1% acetic acid which had been adjusted to pH 7.8 with $NH_4OH$. Electrophoresis was performed in an apparatus with a water-cooled flat bed at 50 V/cm for 45 min. The paper was dried and cut into 1 × 4 cm strips which were counted in a liquid scintillation counter as above.

## Results

*Synthesis of $t^6A$ with an E. coli (SD) Enzyme Preparation.* The assay adopted depended on the incorporation of [$^{14}$C]-L-threonine into acid-insoluble form when incubated under certain conditions with a tRNA preparation deficient in $t^6A$. The deficient tRNA was obtained from a mutant *E. coli* CP 79 which is a threonine auxotroph and a relaxed control mutant capable of synthesizing RNA in the absence of protein synthesis. When cells of this strain are starved for threonine they continue to synthesize tRNA and this tRNA must be deficient in $t^6A$. The tRNA prepared in this way was treated with periodate to completely destroy all amino acid acceptor activity, since obviously threonine incorporation caused by threonyl-tRNA synthetase would interfere with the assay.

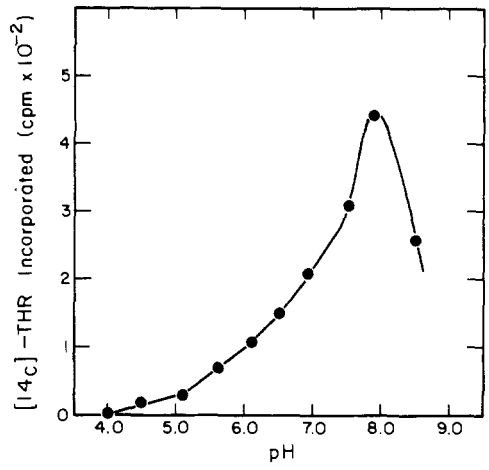


FIGURE 1: Dependence on pH of $t^6A$ synthesis by an *E. coli* SD enzyme preparation. The assay (see Experimental Section) included 0.4 mg of SD enzyme protein and 0.3 mg of $t^6A$-deficient tRNA in a volume of 0.20 ml. Final buffer concentration was 0.20 M. Buffers used were: sodium acetate (pH 4.0, 4.5), sodium maleate (pH 5.1–6.9), and Tris-HCl (pH 7.5–8.5).

Using this system, it was found that an *E. coli* soluble fraction (the SD enzyme), which had been freed of endogenous tRNA as required for this assay, could incorporate labeled threonine into the $t^6A$-deficient tRNA, and this activity depended upon the presence of ATP, $Mg^{2+}$, and bicarbonate (Table I). The requirement for bicarbonate was especially encouraging since this definitely distinguished the $t^6A$ synthetase reaction from incorporation due to the threonyl-tRNA synthetase.

The optimum bicarbonate concentration was found to be quite high, about 8 mM. In the absence of added bicarbonate there was a small but significant reaction which could be due to the presence of bicarbonate in the enzyme or in the buffers or could be due to the incomplete destruction by the periodate of threonine tRNA in the $t^6A$-deficient tRNA. If 1–2% of the original threonine tRNA had survived the periodate treatment, this could account for the observed bicarbonate-independent threonine incorporation. Since a 1–2% survival was a definite possibility, it seemed best to take only the bicarbonate-dependent incorporation as a measure of the $t^6A$ synthetase.

The optimum conditions for the $t^6A$ synthetase activity were then determined. An ATP concentration curve showed a sharp optimum at 10–15 mM; above this the activity dropped sharply even though an equivalent concentration of $Mg^{2+}$ was added when the ATP concentration exceeded 10 mM. With the ATP held at 10 mM, the optimum concentration of $Mg^{2+}$ was determined to be 20 mM. The pH curve showed a sharp optimum at pH 7.7–8.2 (Figure 1). The loss of activity above 8.2 could be due to the instability of $t^6A$ in alkali. Chheda *et al.* (1969) and Schweizer *et al.* (1969) found that $t^6A$ in mild alkali (0.2 M $NH_4OH$) rearranges to

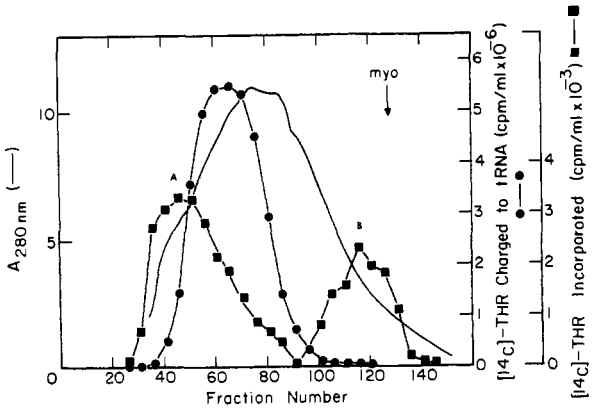


FIGURE 2: Gel filtration chromatography of *E. coli* SD enzyme on an agarose gel column. *E. coli* SD enzyme (500 mg of protein in 5 ml after concentration by dialysis against 50% glycerol) was applied to a Bio-Gel A 1.5m column (1.4 × 80 cm) previously equilibrated with 20 mM Tris-HCl (pH 8.0)-0.1 mM dithioerythritol-0.1 M KCl, and eluted with the same buffer. The column was run at 4°. Fractions of 1.2 ml were collected at a flow rate of 0.1 ml/min. Aliquots were assayed for threonyl-tRNA synthetase (●) using bulk *E. coli* tRNA, and for apparent $t^6A$ synthetase activity (■) using $t^6A$-deficient tRNA; (—) $A_{280\,nm}$. The same results were obtained when the KCl concentration of the buffer solution was raised from 0.1 to 0.5 M and when the KCl was replaced by 10 mM $MgCl_2$.

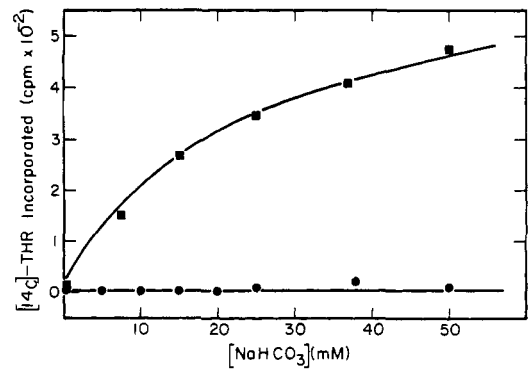


FIGURE 3: Dependence on bicarbonate of threonine incorporation activity of agarose gel column fractions. The two peaks of apparent $t^6A$ synthetase activity from the Bio-Gel A 1.5m column, Figure 2, were pooled separately and then assayed for [$^{14}$C]-L-threonine incorporation as a function of the added bicarbonate concentration. Each assay tube contained 0.2 mg of $t^6A$-deficient tRNA in a volume of 0.2 ml; (●) first peak, 0.14 mg of protein per assay; (■) second peak, 0.04 mg of protein per assay.

release the carbonylthreonine fragment from the adenine in the form of an internal urethane, *i.e.*, 5-methyl-2-oxooxazolidine-4-carboxylic acid. A separate experiment was therefore run to determine the alkali lability of the enzymatically synthesized $t^6A$. After the usual enzymatic synthesis at pH 8, the pH was raised to 10 and incubation continued for 20 min at 37°. At the end of the pH 10 incubation, assay showed that the synthesized $t^6A$ had been completely destroyed.

The time curve for the reaction showed that it had gone to completion by 20 min with 2 mg/ml of SD enzyme protein and 1.5 mg/ml of deficient tRNA. A calculation from the maximum incorporation obtainable with the $t^6A$-deficient tRNA indicated that it was less deficient in $t^6A$ than had been hoped. If the cells which synthesized it were indeed completely starved for threonine, if threonine were the only amino acid which could be added to the adenosines in the newly synthesized tRNA, and if the cells synthesized RNA equivalent to 50% of the initial RNA during the starvation period, then 33% of the isolated tRNA should have been deficient in $t^6A$. Taking the value for the normal $t^6A$ content of *E. coli* tRNA as 0.07 mol/100 mol of nucleotides (Chheda *et al.*, 1969), 33% of this would be 0.023 mol/100 mol of nucleotides theoretical acceptor activity. The actual incorporation gave only 0.003 mol/100 mol of nucleotides or 13% of the theoretical value. The low value obtained is probably due to the fact that glycine can be used in place of threonine for modification of the same tRNAs (see below).

*Purification of the $t^6A$ Synthetase Activity.* The *E. coli* SD enzyme was concentrated by dialysis against 50% glycerol and chromatographed on an agarose gel (Bio-Gel A 1.5m) column (Figure 2). The column fractions were assayed both for $t^6A$ synthetase and for threonyl-tRNA synthetase. A peak of the latter was found in the expected location; the enzyme is known to have a molecular weight of 116,000 (Hirsh, 1968). There appeared to be two peaks of $t^6A$ synthetase activity, a first peak having an apparent molecular weight of about 260,000 and a second peak, 22,000. When the best fractions of the first and second peaks were pooled separately and tested, it was found, however, that only the enzyme in the second peak carried out a [$^{14}$C]threonine incorporation which was dependent upon the addition of bicarbonate (Figure 3). That the second peak was indeed the desired $t^6A$ synthetase was established by showing that the product of the incorporation after acid hydrolysis of the tRNA to release purines had the same electrophoretic mobility as authentic $t^6Ade$ (see below).

When a 50–80% $Am_2SO_4$ cut of the SD enzyme was chromatographed in the same way on the agarose gel column (Bio-Gel A 1.5m), both the bicarbonate-independent first peak and the bicarbonate-dependent second peak appeared as well as the peak of threonyl-tRNA synthetase as in Figure 2. The purification of the $t^6A$ synthetase achieved by these steps is summarized in Table II.

Because the apparent molecular weight of the $t^6A$ synthetase on this particular type of agarose gel column was unexpectedly low, it was decided to check the value by chromatography on a different type of gel column. Figure 4 shows the result of fractionation on a polyacrylamide gel column. In this case the assay for $t^6A$ synthetase was made specific by determining only the bicarbonate-dependent incorporation of labeled threonine into deficient tRNA. This was done by assaying each column fraction without added

TABLE II: Partial Purification of *E. coli* $t^6A$ Synthetase Activity.

| Purification Step[a] | Volume (ml) | Protein (mg) | Activity[b] (units) | Specific Activity[b] (units/mg of protein) |
|---|---|---|---|---|
| SD enzyme | 270 | 6900 | 900 | 0.13 |
| Ammonium sulfate, 50–80% | 34 | 1420 | 475 | 0.33 |
| Bio-Gel A 1.5m chromatography | 25 | 95 | 100 | 1.05 |

[a] The purification steps were performed as described in the Experimental Section and in Figure 2. [b] The activity represents bicarbonate-dependent incorporation of [$^{14}$C]-L-threonine (see Experimental Section and Figure 4).

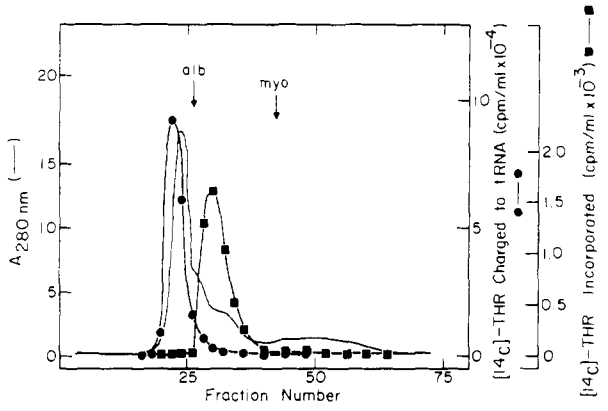


FIGURE 4: Polyacrylamide gel filtration chromatography of the 50–80% $Am_2SO_4$ fraction from the *E. coli* SD enzyme. The 50–80% $Am_2SO_4$ fraction (150 mg of protein in 2.5 ml) was applied to a Bio-Gel P 100 column (1.4 × 22 cm) which was operated under conditions described in Figure 2. Fractions of 0.9 ml were collected at a flow rate of 0.25 ml/min. They were assayed for threonyl-tRNA synthetase activity (●) using bulk *E. coli* tRNA and for $t^6A$ synthetase activity (■) by determining the bicarbonate-dependent incorporation of [$^{14}$C]-L-threonine using $t^6A$-deficient tRNA. To do this, a blank containing no added bicarbonate was run on each column fraction. The blank value was subtracted from the value obtained in the assay of the same fraction with 25 mM $NaHCO_3$; (—) $A_{280\ nm}$. The arrow marked "alb" indicates the peak tube of human serum albumin (70,000) and the one marked "myo" that of horse myoglobin (17,800) run on the same column as above.

bicarbonate and with 25 mM bicarbonate added, and subtracting the value without bicarbonate from the value with bicarbonate. A single peak of $t^6A$ synthetase was found, the molecular weight of which appeared to be about 50,000–60,000. It was a sharp peak suggesting that only a single protein was involved.

The purified $t^6A$ synthetase is quite unstable when kept at 4° even in the presence of 0.1 mM dithioerythritol. This makes the yield of enzyme from the gel columns rather low; during the 36 hr required to run the Bio-Gel A 1.5m column, at least 75% of the activity was lost, and in some experiments up to 90% was lost. The enzyme cannot be stabilized with glycerol or ethylene glycol, as these compounds inhibit the enzyme about 50% at a concentration of 10% (v/v). The column fractions did, however, retain their activity well when stored frozen. The enzyme was stored frozen in small aliquots, used immediately upon thawing, and was not refrozen.

The addition of 100 mM KCl inhibited the enzyme 100%, though 25 mM KCl had very little effect on the activity. Ammonium chloride at 10 mM inhibited the enzyme about 90%.

TABLE III: Affinity of Substrates and Inhibitor for Purified $t^6A$ Synthetase.[a]

| Substrate or Inhibitor | Michaelis Constant $K_M$ (M) | Inhibitory Constant $K_I$ (M) |
|---|---|---|
| ATP | $5.9 \times 10^{-3}$ | |
| $NaHCO_3$ | $6.8 \times 10^{-3}$ | |
| L-Threonine | $7.7 \times 10^{-5}$ | |
| Glycine[b] | $1.3 \times 10^{-4}$ | |
| D-Threonine | | $3 \times 10^{-4}$ |

[a] The methods used are given in Figure 5. [b] The bicarbonate-dependent [$^{14}$C]glycine incorporation was measured as in Figure 5 for [$^{14}$C]-L-threonine.

TABLE IV: Effect of Added Amino Acids on the Synthesis of $t^6A$.[a]

| Addition | [$^{14}$C]-L-Threonine Incorporated (cpm) + 25 mM $NaHCO_3$ | [$^{14}$C]-L-Threonine Incorporated (cpm) − $NaHCO_3$ |
|---|---|---|
| None | 403 | 51 |
| Glycine, 1 mM | 108 | 36 |
| L-Valine, 1 mM | 328 | 44 |
| L-Glutamine, 10 mM | 366 | 54 |
| L-α-Aminobutyric acid, 1 mM | 469 | 45 |
| D-Threonine, 1 mM | 127 | 47 |
| L-*allo*-threonine, 1 mM | 390 | 40 |

[a] The assay (see Experimental Section) included in a volume of 0.20 ml, 0.2 mg of $t^6A$-deficient tRNA, 40 μg of enzyme protein purified on Bio-Gel A 1.5m (see last line of Table II), and 0.05 mM [$^{14}$C]-L-threonine.

The $K_M$ of the purified enzyme for ATP was found to be $6 \times 10^{-3}$ M and the $K_M$ for bicarbonate, $6.7 \times 10^{-3}$ M (Table III).

*Amino Acid Specificity of the Purified $t^6A$ Synthetase.* There have been a number of reports that tRNA also contains $t^6A$-like nucleosides having a glycine or serine instead of a threonine (Chheda *et al.*, 1969; Schweizer *et al.*, 1970; Kimura-Harada *et al.*, 1972a; Cunningham and Gray, 1974). Kimura-Harada *et al.* (1972a) found small amounts of glycine and serine on hydrolyzing the $t^6A$ preparation from certain purified tRNAs. This raised the possibility that the glycine and serine analogs to $t^6A$ may be present as minor variants in place of $t^6A$ in the anticodon adjacent position in individual tRNA species which have $t^6A$. One way to approach this problem is to determine the amino acid specificity of the purified $t^6A$ synthetase. To see if the enzyme can utilize glycine, the effect of unlabeled glycine on the labeled threonine incorporation was first determined (Table IV). Glycine, when present in 20-fold excess over the threonine, inhibited the incorporation of the latter about 80%. Then [$^{14}$C]glycine was tested and found to be incorporated in place of L-threonine into the $t^6A$-deficient tRNA by the purified $t^6A$ synthetase under the conditions for threonine incorporation. The $K_M$ for glycine was found to be only a little higher ($1.3 \times 10^{-4}$ M, Table III) than the $K_M$ for L-threonine ($7.7 \times 10^{-5}$ M, Figure 5 and Table III). D-Threonine was found to inhibit glycine incorporation (Table V) to the same extent as it does L-threonine incorporation (Table IV). (The latter inhibition was shown (Figure 5) to be competitive with a $K_I$ of $3 \times 10^{-4}$ M.) These results support the idea that the glycine and threonine incorporation are brought about by the same $t^6A$ synthetase. This idea is further strengthened by the fact that the two activities decay at the same rate when the purified enzyme loses its activity at 4°.

The fact that L-glutamine even in 200-fold excess over threonine has little or no inhibitory action on $t^6A$ synthetase (Table IV) argues strongly against $t^6A$ synthesis as a secondary activity of carbamyl phosphate synthetase (Anderson

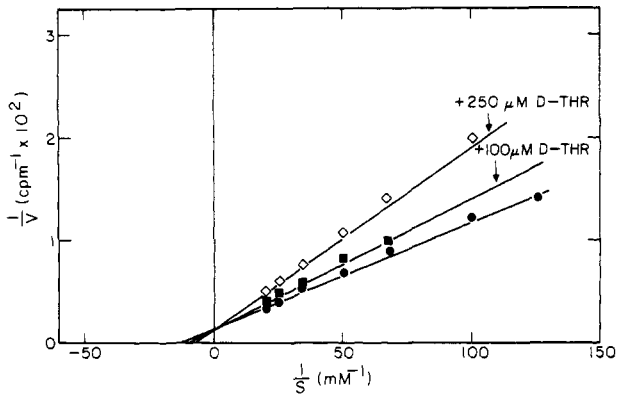


FIGURE 5: Lineweaver-Burk plots showing Michaelis constant ($K_M$) for L-threonine and inhibition by D-threonine with the t⁶A synthetase. Bicarbonate-dependent [¹⁴C]-L-threonine incorporation was measured at the indicated concentrations of L-threonine. To do this, a blank was run without added $NaHCO_3$ at each threonine concentration, and the blank value was subtracted from the value obtained with 25 mM $NaHCO_3$. Assays contained t⁶A-deficient tRNA and enzyme was purified as in the last line of Table II. Incubation time was 10 min. (●) t⁶A synthesis as a function of L-threonine concentration; (■) same with 100 μM D-threonine added; (◇) same with 250 μM D-threonine added.

*et al.*, 1970). Powers and Peterkofsky (1972a) have presented evidence that carbamyl phosphate synthetase is not essential for t⁶A synthesis *in vivo*.

*Identification of the Enzymatically Labeled Product as t⁶A.* An authentic sample of unlabeled t⁶Ade to be used as a marker was first prepared from bulk yeast tRNA essentially by the method of Chheda *et al.* (1969) (Elkins, 1973). The identity of the product was confirmed by comparison of its ultraviolet spectrum in acid, neutral, and alkaline solution with the published spectra of t⁶Ade (Chheda *et al.*, 1969). Then the labeled product of the t⁶A synthetase was acid hydrolyzed and chromatographed on a Dowex 50-X8 column as in Chheda *et al.* (1969). The labeled product eluted at the same position as the authentic sample of t⁶Ade (Figures 3–8 in Elkins, 1973). A smaller labeled peak followed the main peak of t⁶Ade. This peak has not been identified yet; it could be the $N^6$-methyl derivative of t⁶Ade (Kimura-Harada *et al.*, 1972b). As a further check on the enzymatic product, a doubly labeled product was prepared starting with [³H]-L-threonine and [¹⁴C]$NaHCO_3$. The two labels migrated together on electrophoresis and with the same mobility as that of authentic t⁶Ade (Figure 6).

## Discussion

An enzyme system has been developed for the synthesis of t⁶A by the modification of adenosines in t⁶A-deficient tRNA. An enzyme which can carry out this reaction has been purified from the soluble protein of *E. coli* by DEAE-cellulose treatment, ammonium sulfate fractionation, and gel filtration chromatography. The reaction is dependent upon bicarbonate, and labeled bicarbonate appears in the t⁶A product, which establishes that bicarbonate or $CO_2$ is the precursor of the carbonyl group in t⁶A. ATP is required for the synthesis; the number of moles of ATP required per mole of product and the nature of the split products of ATP have not yet been determined. Since two covalent linkages are formed, one would predict that at least 2 mol of ATP would be required.

These experiments have been particularly valuable in elucidating the question of the synthesis of the analog of t⁶A

TABLE V: Effect of D- and L-Threonine on the Incorporation of [¹⁴C]Glycine by the Purified t⁶A Synthetase.[a]

| Addition | [¹⁴C]Glycine Incorporated (cpm) | |
|---|---|---|
| | + 25 mM $NaHCO_3$ | − $NaHCO_3$ |
| None | 640 | 42 |
| D-Threonine, 1 mM | 196 | 34 |
| L-Threonine, 1 mM | 224 | 38 |

[a] The system was as for t⁶A synthesis (see Table IV) except that [¹⁴C]glycine (0.05 mM, 10 Ci/mol) was substituted for [¹⁴C]-L-threonine.

containing glycine which has been found in tRNAs, sometimes present with the major t⁶A in a single purified species of tRNA (Chheda *et al.*, 1969; Schweizer *et al.*, 1970; Kimura-Harada *et al.*, 1972a; Cunningham and Grey, 1974). From the experiments presented here on glycine incorporation into t⁶A-deficient tRNA, it would appear that the same enzyme is involved in the utilization of both glycine and threonine. Each amino acid competes with the incorporation of the other, and they are both inhibited by D-threonine. The activity for both, which resides in the same column fractions, decays at the same rate on storage of the enzyme at 4°. Further work will be needed to determine if serine, which also appears in a t⁶A-like nucleoside (see above references), behaves like glycine in the enzymatic system.

The findings on glycine may help to explain the fact that the tRNA synthesized during threonine starvation in the relaxed control *E. coli* CP 79 was not as deficient in modification as would be predicted. The t⁶A-deficient tRNA may have had more than the normal content of the glycine analog of t⁶A. It may be that the only way to prepare a tRNA in which the particular adenosines are largely unmodified would be to use a relaxed control *E. coli* requiring threonine, glycine, and probably also serine, and to starve for all three of these amino acids.

During the purification of the t⁶A synthetase, the activity was not lost as it could have been if more than one enzyme were required for the reaction. All the evidence so far

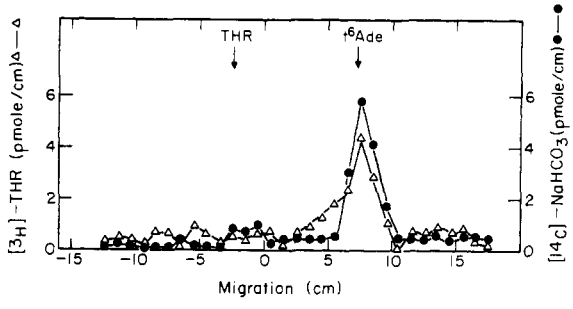


FIGURE 6: Paper electrophoresis of acid-hydrolyzed tRNA which had been doubly labeled enzymatically with [³H]-L-threonine and [¹⁴C]$NaHCO_3$. Doubly labeled tRNA was synthesized and hydrolyzed as described in the Experimental Section. The dried material was dissolved in 25 μl of water, spotted on the moistened filter paper, and electrophoresed (see Experimental Section). The markers of L-threonine and authentic t⁶Ade, which were also applied to the paper, were visualized by ninhydrin reaction and uv absorbance, respectively, and are indicated by the arrows. (△) [³H]Threonine incorporated, pmol/cm of paper; (●) ¹⁴C incorporated from [¹⁴C]$NaHCO_3$, pmol/cm of paper.

suggests that a single enzyme carries out the reaction, though this will need to be confirmed by further purification. If a single enzyme protein carries out the reaction, it would have to bind, though probably not simultaneously, a large number of substrates, L-threonine (or glycine), $HCO_3^-$, unmodified adenosine in certain specific tRNAs, and at least two molecules of ATP. A precedent for such a complex enzyme is found in the case of a very similar reaction, the formation of carbamyl aspartate from ammonia, $CO_2$, aspartate, and ATP by a single enzyme from *Neurospora* (Williams and Davis, 1968). This enzyme has the combined activity of carbamyl phosphate synthetase and aspartate transcarbamylase. There would be a rationale for such a complex enzyme in the case of $t^6A$ synthesis; if all the steps occur on a single enzyme, this would obviate the necessity of a buildup in the cytosol of substrate levels of free intermediates such as a possible *N*-(phosphocarboxy)threonine or $N^6$-(phosphocarboxy)adenosine in tRNA, the two analogs of carbamyl phosphate in this reaction.

## References


Anderson, P. M., Wellner, V. P., Rosenthal, G. A., and Meister, A. (1970), *Methods Enzymol. 17A,* 235.

Chheda, G. B., Hall, R. H., Magrath, D. I., Mozejko, J., Schweizer, M. P., Stasiuk, L., and Taylor, P. R. (1969), *Biochemistry 8,* 3278.

Chheda, G. B., Hong, C. I., Piskorz, C. F., and Harmon, G. A. (1972), *Biochem. J. 127,* 515.

Clark, B. F. C., and Marcker, K. A. (1966), *J. Mol. Biol. 17,* 394.

Cunningham, R. S., and Gray, M. W. (1974), *Biochemistry 13,* 543.

Davis, B. D., and Mingioli, E. S. (1951), *J. Bacteriol. 60,* 17.

Dube, S. K., Marcker, K. A., Clark, B. F. C., and Cory, S. (1968), *Nature (London) 218,* 232.

Elkins, B. N. (1973), Thesis, Cornell University, Ithaca, N.Y.

Ghosh, H. P., Söll, D., and Khorana, H. G. (1967), *J. Mol. Biol. 25,* 275.

Hirsh, D. I. (1968), *J. Biol. Chem. 243,* 5731.

Högenauer, G., Turnowsky, F., and Unger, F. M. (1972), *Biochem. Biophys. Res. Commun. 46,* 2100.

Holley, R. W. (1963), *Biochem. Biophys. Res. Commun. 10,* 186.

Ishikura, H., Yamada, Y., Murao, K., Saneyoshi, M., and Nishimura, S. (1969), *Biochem. Biophys. Res. Commun. 37,* 990.

Kimura-Harada, F., Harada, F., and Nishimura, S. (1972a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 21,* 71.

Kimura-Harada, F., von Minden, D. L., McCloskey, J. A., and Nishimura, S. (1972b), *Biochemistry 11,* 3910.

Körner, A., and Söll, D. (1974), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 39,* 301.

Leis, J. P., and Keller, E. B. (1971), *Biochemistry 10,* 889.

Miller, J. P., and Schweizer, M. P. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 450.

Powers, D. M., and Peterkofsky, A. (1972a), *Biochem. Biophys. Res. Commun. 46,* 831.

Powers, D. M., and Peterkofsky, A. (1972b), *J. Biol. Chem. 247,* 6394.

Schweizer, M. P., Chheda, G. B., Baczynskyj, L., and Hall, R. H. (1969), *Biochemistry 8,* 3283.

Schweizer, M. P., McGrath, K., and Baczynskyj, L. (1970), *Biochem. Biophys. Res. Commun. 40,* 1046.

Simsek, M., and RajBhandary, U. L. (1972), *Biochem. Biophys. Res. Commun. 49,* 508.

Stewart, J. W., Sherman, F., Shipman, N. A., and Jackson, M. (1971), *J. Biol. Chem. 246,* 7429.

Takemura, S., Murakami, M., and Miyazaki, M. (1969), *J. Biochem. (Tokyo) 65,* 489.

Volckaert, G., and Fiers, W. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 35,* 91.

Williams, L. G., and Davis, R. H. (1968), *Genetics 60,* 238.