Richards, E. G. (1968), *European J. Biochem. 6*, 88.
Rushizky, G. W., and Sober, H. A. (1964), *Biochem. Biophys. Res. Commun. 14*, 276.
Schweizer, M. P., Broom, A. D., T'so, P. O. P., and Hollis, D. P. (1968), *J. Am. Chem. Soc. 90*, 1042.
Sevag, M. G., Lackmann, D. B., and Smolens, J. (1938), *J. Biol. Chem. 124*, 425.
Simpkins, H., and Richards, E. G. (1967a), *Biochemistry 6*, 2513.
Simpkins, H., and Richards, E. G. (1967b), *Biopolymers 5*, 551.
Staehelin, M., Rogg, H., Baguley, B. C., Ginsberg, T., and Wehrli, W. (1968), *Nature 219*, 1363.
Tinoco, Jr., I. (1968), *J. Chim. Phys. 65*, 91.
Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.
Van Holde, K. E., Brahms, J., and Michelson, A. M. (1965), *J. Mol. Biol. 12*, 726.
Zachau, H. G., Dütting, D., and Feldman, H. (1966), *Angew. Chem. 78*, 392.
Zavil'gel'skii, G. B., and Li, L. (1967), *Molek. Biol. 1*, 323.

Aminoacyl Nucleosides. VI. Isolation and Preliminary Characterization of Threonyladenine Derivatives from Transfer Ribonucleic Acid*

Girish B. Chheda,† Ross H. Hall,†,‖ David I. Magrath,‡ J. Mozejko,† Martin P. Schweizer,† Lubomyr Stasiuk,† and Peter R. Taylor†,§

ABSTRACT: Transfer ribonucleic acid was hydrolyzed by acid under conditions that released the purine residues. Examination of the hydrolysate by means of ion-exchange chromatography on a sulfonic acid resin revealed the presence of a "new" component. This component contains 1 mole of adenine and 1 mole of threonine. It has been detected in the unfractionated transfer ribonucleic acid of yeast (0.28 mole %), *Escherichia coli* (0.07 mole %), and calf liver (0.19 mole %).

A number of articles reports the presence of amino acids or small polypeptides, apart from the amino acids attached to the acceptor end of tRNA molecules, bound to nucleic acids. Ingram and Sullivan (1962) and Akashi *et al.* (1965), for example, have reported the presence of amino acids bound to RNA which cannot be removed through the use of extensive deproteinizing procedures. Balis *et al.* (1964) and Olenick and Hahn (1964) have reported the presence of amino acids in highly purified preparations of DNA isolated from a variety of sources. The nature of the amino acid–nucleic acid linkage, however, has not been elucidated. Bogdanov *et al.* (1962) have reported that tRNA contains amino acids attached to the phosphate residues. Harris and Wiseman (1962) have also reported the presence of small polypeptides attached to the phosphate residue of yeast nucleic acid; the exact nature of such complexes has not been described.

Hall (1964) and Hall and Chheda (1965) reported that amino acid nucleoside derivatives had been isolated from yeast tRNA in which the aminoacyl group was attached to the N^6 position of adenosine. It appeared that a series of these compounds existed and that they all possessed the same basic structure. We have continued our investigations of these amino acid adenosine derivatives of tRNA in order to clarify their exact nature and to establish their significance to tRNA structure and function.

The isolation procedure used in the original work (Hall, 1964, 1965) was lengthy and the yield of the desired amino acid adenosine derivatives was very low. Therefore, more convenient and efficient methods for isolation were sought. Purines are readily cleaved from RNA by mild acid hydrolysis and this procedure seemed to offer a relatively fast and effective means of obtaining the free base of the amino acid nucleosides. Yeast tRNA was subjected to such a procedure and the released purine derivatives were separated by means of ion-exchange chromatography. The acid hydrolysate of yeast tRNA yielded three amino acid adenine derivatives, each of which contained threonine. This paper describes the method of isolation, the preliminary characterization of these compounds, and the detection of threonine–adenine residues in tRNA of *E. coli* and mammalian tissue. The accompanying

* From the Department of Experimental Therapeutics, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, New York 14203, and Department of Biochemistry, The Australian National University, Canberra, Australia. Work supported in part by grants from the National Cancer Institute, U. S. P. H. S. (CA-04640), and the Division of Metabolic Biology, National Science Foundation (GB-5992).

† Roswell Park Memorial Institute, Buffalo, N. Y.
‡ Australian National University, Canberra, Australia.
§ Present address: Ramsgate, Kent, England.
‖ Present address: Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada. Please send inquiries to this address.

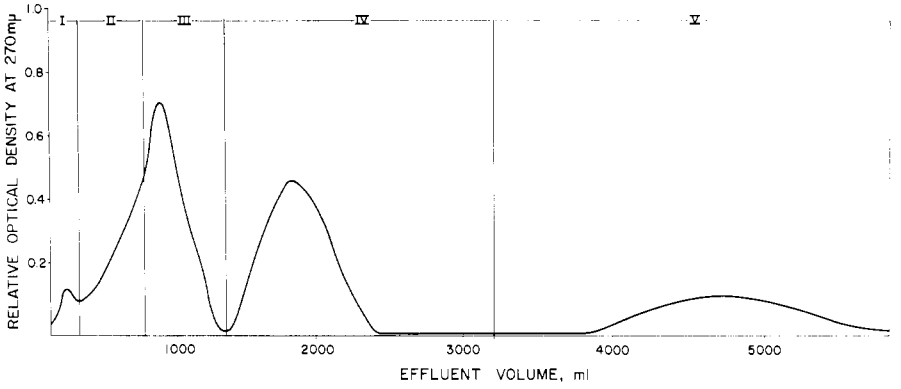

FIGURE 1: Resolution of a hydrochloric acid digest of 30 g of yeast tRNA on a column (2.54 × 100 cm) of Dowex 50W-X8 (H^+) (200–400 mesh). The column was developed with a linear gradient of hydrochloric acid (0.2–0.4 N; total volume 8 l.) at 200 ml/hr. The effluent was divided into fractions as indicated.

paper (Schweizer *et al.*, 1969) describes the complete elucidation of the basic structure of the threonine–adenine derivatives.

Experimental Section

Preparation of RNA Samples. Yeast tRNA was prepared from Baker's yeast by the method of Holley (1963). The sample had a sedimentation value of 3.8 (Spinco Model E ultracentrifuge, Schlieren optics). This RNA sample accepted 34 μμ-moles of serine/A_{260} unit (for details, see Fittler and Hall, 1966). In order to demonstrate that the threonine–adenine derivative did not arise because of protein or amino acid contamination, portions of the main tRNA sample were subjected to the additional purification procedures described below.

E. coli and calf liver tRNA were obtained from the Nutritional Biochemical Corp. Rat liver tRNA was prepared according to Brunngraber (1962). Calf liver rRNA was prepared according to the method of Kirby (1965). rRNA from *E. coli* B and yeast was prepared according to the method of Stanley and Bock (1965).

Treatment of Yeast tRNA with Chloroform–Butanol. A solution of 3.0 g of yeast tRNA in 500 ml of water was mixed with 472 ml of chloroform and 118 ml of 1-butanol. The mixture was shaken vigorously on a reciprocating shaker for 30 min, and was then centrifuged at 13,000*g* for 10 min. The aqueous phase was again treated with the chloroform–1-butanol mixture; 2.25 g of sodium acetate and 1100 ml of ethanol were added. The mixture was kept at 4° for 16 hr, then centrifuged at 13,000*g* for 19 min. The precipitate was washed with two 150-ml portions of ethanol–water (3:1), and then with 95% ethanol. The precipitate was air dried (wt 2.7 g).

"Stripped" Yeast tRNA. A solution of 2 g of the yeast tRNA in 200 ml of water was adjusted to pH 8.85 by the addition of 0.1 N sodium hydroxide and incubated at 37° for 1 hr. The solution was neutralized with 0.1 N hydrochloric acid and dialyzed for 24 hr at 4° against distilled water (two lots, 20 l. each lot). The solution in the dialysis bag was concentrated *in vacuo* to a volume of 45 ml. Sodium acetate (1.0 g) and ethanol (100 ml) were added to the solution which was kept at −10° for 30 min. The precipitated tRNA was collected by centrifugation and washed twice with cold ethanol–water (2:1). The yield of tRNA was 834.77 mg.

Isolation of Compound **1**. Yeast tRNA (30 g) was dissolved in 300 ml of water and the pH of the solution was adjusted to 4.0 by the addition of concentrated hydrochloric acid. The solution was transferred to a 2-l. round-bottom flask warmed to 93°. Hydrochloric acid (5 N, 81 ml) was added dropwise with stirring to this solution and the solution was heated for 15 min at 100°. The reaction mixture was cooled rapidly to 25° and evaporated to dryness *in vacuo* in a rotating flash evaporator (liquid nitrogen in the trap). The residue was washed several times by adding methanol and evaporating the mixture. The residue was left for 24 hr at 25° in a vacuum desiccator over potassium hydroxide pellets and was then dissolved in 185 ml of water. The pH of the solution was adjusted to 6 by the addition of 5 N sodium hydroxide (35 ml was required), and the mixture was centrifuged for 30 min at 15,000*g*. The precipitate was washed with 50 ml of water, and the combined solutions (the solutions contained 565,000 A_{260} units) were run onto the top of a previously prepared column (2.54 × 100 cm) of Dowex 50W-X8 (H^+) (200–400 mesh). (The column had been equilibrated by washing with 5 l. of 0.001 M hydrochloric acid.) The column was developed with a linear gradient of hydrochloric acid (0.2–0.4 N HCl). The elution pattern obtained is shown in Figure 1.

Analyses of the eluted fractions by paper chromatography in solvent system D, followed by ultraviolet absorption spectroscopy of the separated components, showed that compounds with the characteristic spectra of **1** were located in fractions 1, 3, and 5. Rather than working up the fractions individually at this stage, fractions 1, 3, and 5 were combined and evaporated to dryness *in vacuo*. The residue was repeatedly dissolved in methanol and concentrated to dryness to free it of hydrogen chloride. The residue (111 mg) was dissolved in 7.5 ml of the lower phase of solvent F and filtered; this solution was mixed with 15 g of Celite-545 and the mixture was packed on the top of a previously prepared column of Celite-545 and solvent F (2.54 × 40 cm). The column was prepared and developed according to the basic procedure of Hall (1962). The elution profile of the column is shown in Figure 2.

Fraction 2 (Figure 2) contained a compound (115 A_{276} units, pH 7.0) with ultraviolet absorption spectra identical with

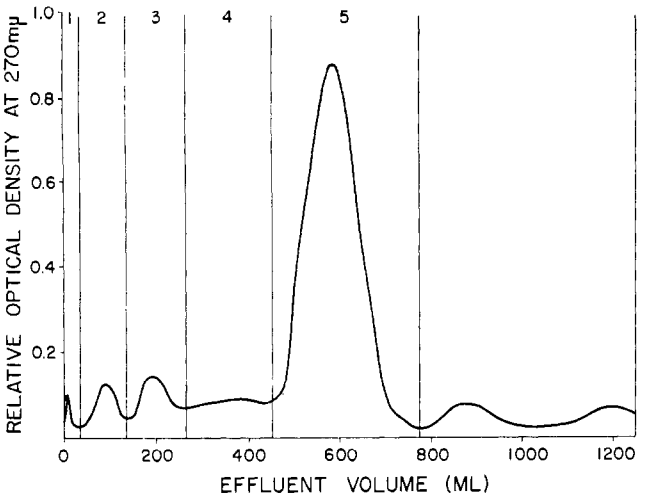

FIGURE 2: Elution profile of the combined material in fractions 1, 3, and 5 (Figure 1) fractionated on a partition column of Celite-545 (2.54 × 40 cm), solvent F. The effluent was divided into fractions as indicated.

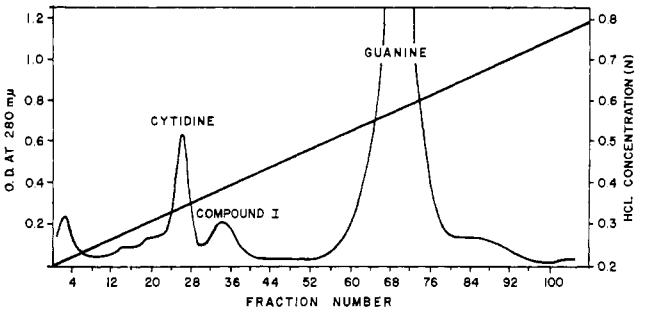

FIGURE 3: Elution profile of an acid hydrolysate of calf liver tRNA fractionated on a column (0.9 × 5 cm) of Dowex 50W-X8 (H^+) (200–400 mesh). The column was developed with a linear gradient of hydrochloric acid (0.2–0.8 N, total volume 500 ml) at 1.2 ml/min.

those of **1**. For convenience, it will be designated **2**. Fraction 3 contained a third compound (**3**; 120 A_{276} units, pH 7.0) with an ultraviolet absorption spectrum identical with compound **1**. Fraction 5 contained **1** as well as N^2,N^2-dimethylguanine and 1-methylhypoxanthine. Hypoxanthine was located in the next small fraction after fraction 5 (Figure 2).

Compound **1** was purified by means of partition chromatography (Hall, 1962) in solvent system E on a column of Celite-545[1] (1.83 × 30 cm). N^2,N^2-Dimethylguanine (100 A_{250} units, pH 7.0) and 1-methylhypoxanthine (110 A_{250} units, pH 7.0) were eluted in a single peak between the second and third holdback volumes. This mixture was separated by paper chromatography in solvent D. Compound **1** was eluted by washing the column with 500 ml of water. The water solution was evaporated to a small volume and filtered. This solution (6.0 ml) was used for the analytical studies. Paper chromatography in solvents A, B, C, D, and E showed that **1** was free of ultraviolet-absorbing contaminants. The yield was 880 A_{275} units, pH 7.0.[2] A lyophilized sample melted at 211–213°.

Routine Assay Procedure for Compound **1**. This procedure is described in terms of a sample of calf liver tRNA. A solution of tRNA (100 mg in 1.3 ml of water) was heated to 100° in a boiling-water bath in a 10-ml erlenmeyer flask. Hot 2 N hydrochloric acid (1.3 ml) was added, the flask was stoppered, and heating was continued for 10 min. The flask was then rapidly cooled in ice water. The solution was evaporated *in vacuo* and the gummy residue was reevaporated twice with small portions of water and triturated with several changes of anhydrous ether. The solid was placed in a vacuum desiccator over potassium hydroxide pellets for 4 hr. The residue was dissolved in 3 ml of water and the solution was clarified by centrifugation. The pH of the solution was adjusted to 7 by the addition of 2 N sodium hydroxide, and the mixture was cooled in an ice bath for 1 hr. Precipitated guanine was removed by centrifugation and washed once with water. The combined supernatant and wash were applied to a column of previously prepared Dowex 50W-X8 (H^+) (200–400 mesh; column size 0.9 × 8 cm). The column was washed with water (approximately 20 ml) until the pH of the effluent was approximately 5. Washing was continued with 0.001 N hydrochloric acid (75 ml) and the column was developed by hydrochloric acid in a linear gradient (0.2–0.8 N hydrochloric acid; total volume, 500 ml) at 1.2 ml/min flow rate. The elution profile is shown in Figure 3. The yield of **1** obtained from the calf liver RNA and from the other samples of tRNA is shown in Table I. The small scale of this procedure did not permit an examination of the hydrolysates for the presence of **2** and **3**.

In a series of experiments, the effect of varying hydrolysis time from 1 to 30 min on the yield of **1** from yeast tRNA was measured. The maximum yield was obtained after 10 min of hydrolysis at 100°.

Preliminary Characterization of Compound **1**. The ultraviolet absorption spectra of **1** are shown in Figure 4. R_F values on paper chromatography of compound **1** are shown in Table II. The compound gave a negative ninhydrin test. A sample of **1** was hydrolyzed in 0.1 N sodium hydroxide solution for 3 hr at 100°. Chromatography of the hydrolysate in solvent systems A–E showed a single ultraviolet-absorbing product with mobilities identical with those of adenine. In addition, the ultraviolet absorption spectra of the product were identical

TABLE I: Amount of Compound **1** Isolated from RNA.

RNA	Moles/100 Moles of Nucleotides[a]
Yeast tRNA	0.28 ± 0.02
Yeast tRNA (stripped)	0.25
Yeast tRNA ($CHCl_3$–1-butanol)	0.20
E. coli tRNA	0.07
Calf liver tRNA	0.19
Rat liver tRNA	0.13
Yeast rRNA	Not detected
E. coli rRNA	Not detected
Calf liver rRNA	Not detected

[a] ϵ_{max} 17,000 for **1**.

[1] Registered trademark of Johns-Manville Corp.

[2] An ϵ_{max} value at $\lambda_{276}^{pH\ 2.0}$ of 17,000 is used and was derived by hydrolyzing a sample of **1** quantitatively to adenine. Using a molecular weight of 280, the yield of **1** can be calculated as 14.6 mg.

TABLE II: Paper Chromatography.

Compound	R_F Values (× 100)					
	Solvent Systems[a]					
	A	B	C	D	E	G
Compound **1**	2	47	43	37	4	
Compound **2**		82		82	61	
Compound **3**				53		
Adenine	33	59	28	46	22	
Base from **1**	32	57	28	46	22	
Base from **2**	30	56		45	20	
Threonine		53		40		43
Serine		46		33		36
Amino acid from **1**		52		39		42
Amino acid from **2**				40		44
Amino acid from **3**				38		45

[a] Solvent systems are: (A) 1-butanol–water–concentrated ammonium hydroxide (86:14:5, v/v), (B) 2-propanol–1% aqueous ammonium sulfate (2:1, v/v), (C) 2-propanol–concentrated hydrochloric acid–water (680:170:144, v/v), (D) 2-propanol–water–concentrated ammonium hydroxide (7:2:1, v/v), (E) ethyl acetate–*n*-propyl alcohol–H_2O (4:1:2, v/v), (F) ethyl acetate–2-ethoxyethanol–0.25% aqueous formic acid (4:1:2, v/v), and (G) 1-butanol–acetic acid–water (5:2:3, v/v).

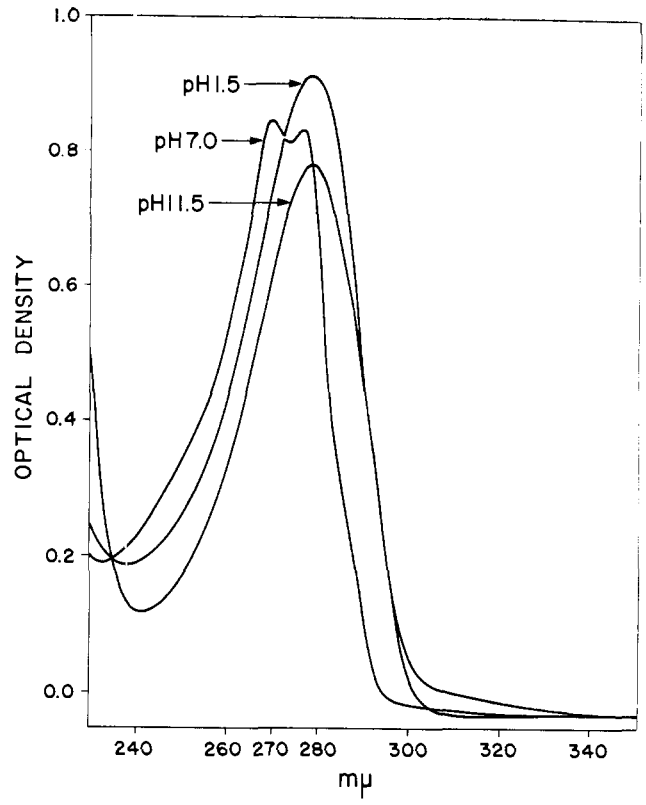

FIGURE 4: Ultraviolet absorption spectra of compound **1**, *N*-(purin-6-ylcarbamoyl)threonine.

with those of adenine. The developed chromatograms also showed a single ninhydrin-reacting spot corresponding to the mobility of DL-threonine. Compound **1** gave a negative sulfur test (sodium nitroprusside and lead acetate tests). Compound **1** remained unchanged after prolonged digestion with bacterial alkaline phosphatase and gave a negative phosphorus test (Lowry and Lopez, 1946).

A stock solution (1 ml) of **1** (0.40 μmole, ε 17,000) was made 0.1 N with respect to sodium hydroxide, and the solution was heated in a sealed tube for 3 hr at 100°. The amount of adenine in the hydrolysate determined by its ultraviolet absorption spectra was 0.41 μmole, and the amount of threonine determined by a quantitative ninhydrin procedure was 0.40 μmole. The amino acid in the alkali hydrolysate was also determined by quantitative ion-exchange chromatography (Spackman *et al.*, 1958);[3] 1 mole equiv (to adenine) of threonine was detected, as well as a trace of glycine and serine (the amount of these two amino acids was too small to integrate).

A sample of **1** was hydrolyzed in 0.2 M ammonium hydroxide for 2 hr at 100°. Chromatographic examination of the hydrolysate showed a quantitative yield of adenine. After the removal of ammonia, the hydrolysate gave a negative ninhydrin test indicating that the amino group of the threonine residue was still blocked. Characterization of the derivative, 5-methyl-2-oxooxazolidine-4-carboxylic acid, is described in the accompanying paper (Schweizer *et al.*, 1969).

pK Values. The electrophoretic mobilities of compound **1** (Table III) show that it has an acidic dissociation between pH 2.5 and 3.0. It is also evident that a basic dissociation (probably ring protonation) exists between pH 2 and 2.5. This dissociation is confirmed by the ultraviolet absorption spectra, which show a chromophoric change between pH 2.0 and 3.0. The chromophoric change at alkaline pH values is probably due to ionization at N-9.

Characterization of Compounds **2** *and* **3**. Compounds **2** and **3** have the following characteristics in common with those of **1**. The ultraviolet absorption spectra of **2** and **3** are identical with those of **1** (Figure 4). Hydrolysis of **2** and **3** by 0.1 N sodium hydroxide for 4 hr at 100° yields adenine (identified by paper chromatography (Table I) and ultraviolet absorption spectra) and threonine (identified by paper chromatography; Table I). Quantitative ion-exchange analysis of **2** showed that the sample contained 0.8 mole equiv of threonine/mole of adenine.

The electrophoretic mobilities of **2** and **3** (Table III) show that the acidic function evident in **1** is not present in these two compounds. This conclusion is also supported by the faster paper chromatographic mobilities of **2** and **3** in basic solvent system (Table II). Compound **2** was hydrolyzed in 0.4 M hydrochloric acid for 20 min at 100°. Chromatographic analysis of the hydrolysate in solvent D showed the presence of two new ultraviolet-absorbing compounds: one corresponded to **1** and the second compound had an intermediate R_F value between those of **1** and **2**. The ultraviolet absorption spectra of the two hydrolytic products remained unchanged from those of **2**.

Experiment to Determine Whether Any of the Terminal

[3] We thank Dr. L. Kress and Dr. W. Chan for performing the amino acid determinations.

TABLE III: Paper Electrophoresis.[a]

Compound	pH	Time (min)	Migration from origin (cm)
Adenine	4.0	60	−11.0
Compound **1**	4.0	60	+12.0
Compound **1**	3.5	60	+7.0
Compound **2**	3.5	60	−2.2
Compound **3**	3.5	60	−2.2
Compound **1**	3.0	45	+1.3
Adenosine 5′-phosphate	3.0	45	+2.2
Compound **1**	2.0	40	−4.1
Adenosine 5′-phosphate	2.0	40	0.0

[a] All experiments were carried out in a Savant flat-bed apparatus at 40 V/cm. Buffers used: pH 4.0, 0.05 M citrate; pH 3.5, 0.035 M citrate; pH 3.0, 0.2 M ammonium formate; pH 2.0, 1 M acetic acid–0.75 M formic acid.

Adenosine Residues of tRNA Are N^6 Substituted. A solution of 30 mg of yeast tRNA that had been charged with all 20 amino acids (Hoskinson and Khorana, 1965) and 1.5 mg of pancreatic RNase in 4 ml of 0.01 M phosphate buffer (pH 7.0) was incubated for 45 min at 25°. The solution was passed through a column (2 × 8 cm) of DEAE-cellulose (Cl^-) and the column was washed with 100 ml of water. Under these conditions, 0.7 mole % of *O*-aminoacyladenosine derivatives was obtained. Acid hydrolysis yielded adenine. No trace of a threonine derivative was detected on paper chromatography. If these derivatives had been located in the terminal position of the tRNA molecules, they would have been detected under the conditions of this experiment.

Discussion

The principal compound, **1**, isolated in this study consists of one residue of adenine and one of threonine. The evidence obtained by electrophoresis and paper chromatography indicates that this product has an acidic dissociation corresponding in strength to that of a carboxylic group, which strongly suggests that the carboxyl group of the threonine residue is free.

Compounds **2** and **3** are closely related to **1**, as shown by their analytical data and the ultraviolet absorption spectra. Compound **2** can be converted into **1** on mild acid hydrolysis. The electrophoretic and paper chromatographic mobilities of **2** and **3** show that a free carboxylic acid group is not present; these facts suggest that these compounds may be esterified derivatives of **1**. Precedence exists for the occurrence of an esterified carboxyl group in a yeast tRNA component (Baczynskyj *et al.*, 1968). In addition, although evidence is lacking, it is conceivable that the hydroxyl group of threonine could also be substituted.

Compound **1** probably is widely distributed in nature; it occurs in the tRNA of yeast, *E. coli*, and mammalian tissue. The values reported in Table I must be taken as minimal values since in addition to factors of sample purity and losses during the assay, it is possible that the level of **1** varies according to the growth stage or condition of cells and tissue at the time of the tRNA extraction. Assuming that only one residue of **1** occurs per molecule of tRNA, according to these data several molecular species of tRNA would contain such a residue. For example, for yeast tRNA at least one tRNA molecule out of five contains a threonine–adenine derivative.

Although **1** was not detected in rRNA the analyses were not sensitive enough to detect one residue of **1** per rRNA molecule.

The ability to isolate **1** in adequate amounts made possible an extensive investigation of its chemical structure. The data presented in the accompanying paper (Schweizer *et al.*, 1969) show that **1** is *N*-(purin-6-ylcarbamoyl)threonine.

References

Akashi, S., Murachi, T., Ishihara, H., and Goto, H. (1965), *J. Biochem. (Tokyo) 58*, 162.

Baczynskyj, L., Biemann, K., and Hall, R. H. (1968), *Science 159*, 1481.

Balis, M. E., Salser, J. S., and Elder, A. (1964), *Nature 203*, 1170.

Bogdanov, A. A., Prokof'ev, M. A., Antonovich, E. G., Terganova, G. V., and Anisimova, V. M. (1962), *Biokhimiya 27*, 266.

Brunngraber, E. F. (1962), *Biochem. Biophys. Res. Commun. 8*, 1.

Fittler, F., and Hall, R. H. (1966), *Biochem. Biophys. Res. Commun. 25*, 441.

Hall, R. H. (1962), *J. Biol. Chem. 237*, 2283.

Hall, R. H. (1964), *Biochemistry 3*, 769.

Hall, R. H. (1965), *Biochemistry 4*, 661.

Hall, R. H., and Chheda, G. B. (1965), *J. Biol. Chem. 240*, PC 2754.

Harris, G., and Wiseman, A. (1962), *Biochim. Biophys. Acta 55*, 374.

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

Hoskinson, R. M., and Khorana, H. G. (1965), *J. Biol. Chem. 240*, 2129.

Ingram, V. M., and Sullivan, E. (1962), *Biochim. Biophys. Acta 61*, 583.

Kirby, K. S. (1965), *Biochem. J. 96*, 266.

Lowry, O. H., and Lopez, J. A. (1946), *J. Biol. Chem. 162*, 421.

Olenick, J. G., and Hahn, F. E. (1964), *Biochim. Biophys. Acta 87*, 535.

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

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Stanley, W. M., and Bock, R. M. (1965), *Biochemistry 4*, 1302.