Aminoacyl Nucleosides. VII. *N*-(Purin-6-ylcarbamoyl)threonine. A New Component of Transfer Ribonucleic Acid*

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ABSTRACT: A component of the transfer ribonucleic acid of yeast, *Escherichia coli*, and mammalian tissue has been identified as *N*-(purin-6-ylcarbamoyl)threonine (**1**). The corresponding nucleoside, *N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine, has also been isolated from yeast transfer ribonucleic acid.

Compound **1** has been identified by means of its high-resolution mass spectrum, nuclear magnetic resonance spectrum, and its ultraviolet absorption spectra and by comparison with a synthetic sample.

In the preceding paper (Chheda *et al.*, 1969) we described the isolation and preliminary characterization of three threonine–adenine derivatives that had been isolated from the tRNA of various organisms. The present paper deals with the complete elucidation of the structure of one of these tRNA components, *N*-(purin-6-ylcarbamoyl)threonine (**1**).

Experimental Section

General. *N*-(PURIN-6-YLCARBAMOYL)THREONINE. A sample of this compound was isolated from yeast tRNA (Chheda *et al.*, 1969).

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY. Measurements were made using a Varian Associates HA-100 spectrometer at 32–34° operating in field sweep mode, except during the decoupling experiments when frequency mode was utilized.[1]

HIGH-RESOLUTION MASS SPECTROMETRY. Spectra were obtained with a CEC Model 21-110B spectrometer; a photoplate was used to record the spectra. The sample was introduced directly into the ion source through a vacuum lock. Mass measurements were obtained with an automatic comparator–densitometer operated on-line with an IBM 1800 computer (Biemann, 1968).[2]

CHROMATOGRAPHY. Solvent systems for paper and thin-layer chromatography are described in the preceding paper (Chheda *et al.*, 1969).

Synthesis of Model Compounds. *N*-(PURIN-6-YL)-*N*-METHYLUREA (**11**) was prepared using the general method of the condensation of amines with isocyanates. 2′,3′,5′-Tri-*O*-acetyladenosine (**8**) was condensed with methyl isocyanate. The reaction product, **9**, was hydrolyzed in 1 N hydrochloric acid for 15 min at 100° and *N*-(purin-6-yl)-*N*-methylurea (**11**) was purified by paper chromatography in solvent D (R_F 0.71). The ultraviolet absorption spectra of this sample are shown in Figure 1.

N-(PURINE-6-YLCARBAMOYL)GLYCINE (**12**) was prepared in a similar manner, starting with ethyl isocyanatoacetate and 2′,3′,5′-tri-*O*-acetyladenosine (**8**). The crude semisolid product was hydrolyzed first with 0.1 N sodium hydroxide for 30 min at 75° and then with 0.5 N hydrochloric acid for 15 min at 100°. *N*-(Purin-6-ylcarbamoyl)glycine (**12**) was purified by paper chromatography in solvent D (R_F 0.20). The ultraviolet absorption spectra are shown in Figure 1.

N^6-(*N*-CARBOBENZOXY-L-THREONYL)ADENINE (**5**). A solution of *N*-carbobenzoxy-L-threonine (**4**, 1.01 g, 4.0 mmoles), *p*-nitrophenol (0.61 g, 4.4 mmoles), and dicyclohexylcarbodiimide (0.91 g, 4.4 mmoles) in 10 ml of ethyl acetate was stirred for 45 min at 3° and for an additonal 45 min at 25°. The precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness. The crude *N*-carbobenzoxy-L-threonine *p*-nitrophenyl ester was dissolved in 4 ml of dimethylformamide and the solution was mixed with a hot solution (110°) of 270 mg of adenine (2.0 mmoles) in 4 ml of dimethylformamide and 4 ml of dimethyl sulfoxide. The mixture was stirred for 3 hr at 80° and for 1 hr at 25°. The clear yellow solution was evaporated to yield a syrup, which was then triturated with 25 ml of warm toluene. Adenine (157 mg) was filtered and the filtrate was evaporated to dryness. The residue was crystallized from an ethanol–ether mixture; the yield of **5** was 22 mg, mp 115–120°. This product exhibits the characteristic spectrum of N^6-acyladenine derivatives (Chheda and Hall, 1966).

N^6-(L-THREONYL)ADENINE (**6**). The sample of N^6-(*N*-carbobenzoxy-L-threonyl)adenine (**5**) in a solution of 30% hydrogen bromide in acetic acid was stirred for 30 min at 25°. The mixture was diluted with ether and the precipitated product was collected on a filter, washed with ether, and dried over P_2O_5. The ultraviolet absorption spectra of **6** are shown in Figure 2 and are typical of those of N^6-(α-aminoacyl)adenine deriva-

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[2] The authors acknowledge the use of the facilities of Professor K. Biemann, supported by Grant FR-00317 from the National Institutes of Health, U. S. Public Health Service (Mass Spectrometry Facilities for Biomedical Research).

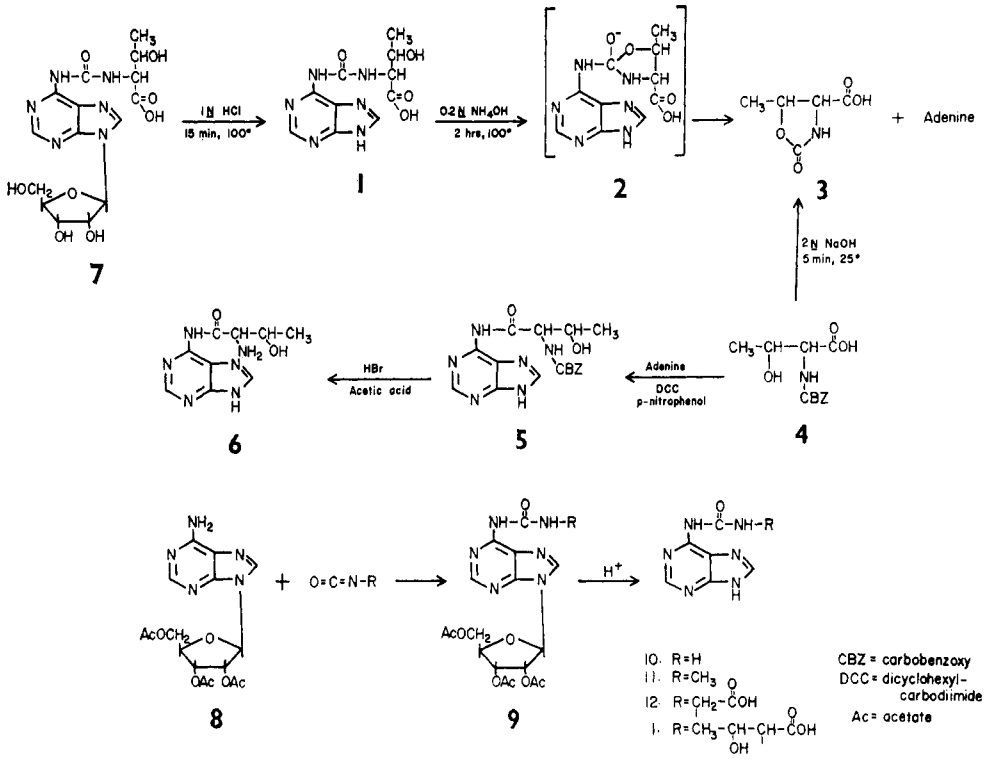

TABLE I: Nuclear Magnetic Resonance Spectral Data on *N*-Formylthreonine and *N*-(Purin-6-ylcarbamoyl)threonine in Pyridine-d_5 (100 Mc) Solvent.

	Chemical shifts (ppm) from Internal Tetramethylsilane Ref						Coupling Constants, J (cps)		
	α-NH	H-2	H-8	α-CH	β-CH	CH_3	α(NH-CH)	α_{CH}-β_{CH}	β_{CH}-CH_3
N-Formylthreonine[a]	9.12 (d)			5.25[c]	4.97[d]	1.53 (d)	9.0	2.5	6.2
N-(Purin-6-ylcarbamoyl)threonine[b]	10.82 (d)	8.74 (s)	8.52 (s)	5.34[c]	5.13[d]	1.70 (d)	8.8	2.5	6.2

[a] 0.04 M. [b] 0.01 M. [c] Quartet. [d] Octet.

tives (Chheda and Hall, 1966). This compound is unstable in aqueous solution and undergoes the general rearrangement for N^6-(α-aminoacyl)adenine derivatives described by Chheda and Hall (1966).

N-(PURIN-6-YL)UREA (**10**). The ultraviolet absorption spectra of this compound (Giner-Sorolla and Bendich, 1958), determined by us, are shown in Figure 1 and are identical with the ultraviolet absorption spectra of the other ureidopurines shown in Figure 1.[3] The nuclear magnetic resonance spectrum of this sample obtained in dimethyl sulfoxide-d_6 showed chemical shifts, parts per million from trimethylsilane capillary: N^6-H (9.86 s), H-2 (8.82 s), H-8 (8.72 s), and NH_2 (7.98 s).

Characterization of N-(Purin-6-ylcarbamoyl)threonine (**1**). GENERAL CHARACTERISTICS OF **1** (ACCORDING TO CHHEDA *et al.*, 1969). The alkaline hydrolysis of **1** yields equimolar amounts of threonine and adenine. According to its electrophoretic mobility, **1** has an acidic dissociation with a pK_a of about 3. The absence of phosphorus- or sulfur-containing groups suggests that this acidic group is due to a carboxyl function.

Acid-Catalyzed Reaction of **1** *with Ethanol.* Anhydrous hydrogen chloride was bubbled into a solution at 25° of 2 mg of **1** dissolved in 100 ml of absolute ethanol. The solution was stirred for 20 min and the ethanol was evaporated *in vacuo*. Residual hydrogen chloride was removed by repeated evaporation of the residue with water. Analysis of the residue by paper chromatography showed the presence of two ultraviolet-absorbing compounds; one corresponded to the starting material and the second moved at a faster rate. The R_F values of the new product compared to those of **1** (Table II of Chheda *et al.*, 1969) are: solvents B, 0.82; D, 0.82; and E, 0,61. On electrophoresis in 0.035 M citrate buffer (pH 3.5) at 40 V/cm

[3] We thank Dr. Giner-Sorolla for providing us with a sample of *N*-(purin-6-yl)urea.

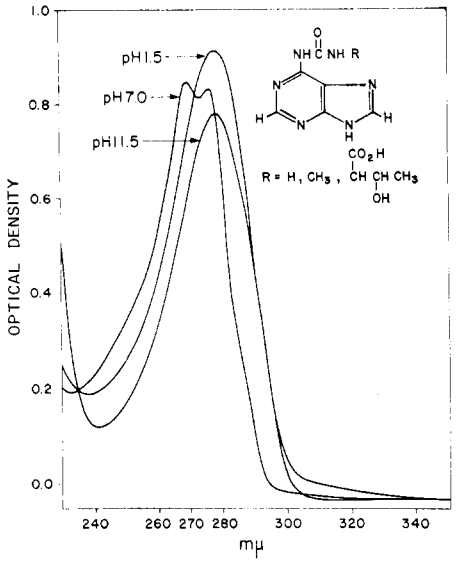

FIGURE 1: The ultraviolet absorption spectra of derivatives of *N*-(purin-6-yl)urea.

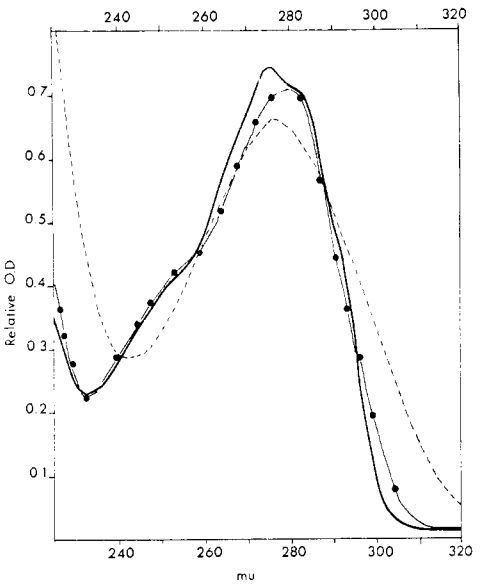

FIGURE 2: The ultraviolet absorption spectra of synthetic N^6-(threonyl)adenine; pH 1.4, ———; pH 7.1, ——·——·——; pH 11.6, – – – – –.

for 1 hr the new product migrated −1.3 cm (**1** migrated +7.0 cm under the same conditions). The ultraviolet absorption spectra of the new product are identical with those of **1**.

The fact that this facile reaction yields a derivative that has greater mobility than **1** and lacks an acidic function represents additional evidence that **1** contains a free carboxyl group.

PRODUCTION OF THE INTERNAL URETHAN OF THREONINE FROM **1**. A sample of **1** (1.0 mg) was hydrolyzed in 0.2 M ammonium hydroxide solution for 2 hr at 100° in a sealed ampule. A quantitative yield of adenine was obtained. The hydrolysate was lyophilized, the residue was dissolved in 5 ml of water, and the solution was passed through a column (1 × 5 cm diameter) of Dowex 50 (H^+) to remove the adenine. The column was washed with 10 ml of water. The combined eluate and washing were evaporated to a volume of 1 ml. This solution gave a negative ninhydrin reaction. However, after an aliquot of the solution was made 0.1 N with respect to sodium hydroxide and heated for 4 hr at 100°, the solution gave a positive ninhydrin reaction corresponding to 1 mole equiv of the amino acid/mole of the starting amount of **1**.

The blocked threonine derivative, obtained by mild alkaline hydrolysis of **1**, was identified as the internal urethan (**3**). This compound was identified by means of high-resolution mass spectrometry.[2] Significant ions in the fragmentation pattern of compound **3** occur at *m/e* 145.0366, 100.0394, 85.0147, and 43.9898. The elemental compositions which fit the exact masses of these ions are $C_5H_7NO_4$ (calcd 145.0374), $C_4H_6NO_2$ (calcd 100.0398), $C_3H_3NO_2$ (calcd 85.0163), and CO_2 (calcd 43.9898). The presumed molecular ion at *m/e* 145 is weak. The loss of CO_2H from this ion yields the strongest ion in the spectrum at *m/e* 100. Further loss of the methyl group leads to the ion at *m/e* 85. The elemental composition of the molecular ion and its fragmentation pattern correspond to that of 5-methyl-2-oxooxazolidine-4-carboxylic acid (**3**, internal urethan of threonine).

Proof of the identity of **3** was obtained by comparing its properties with those of a synthetic sample that had been prepared from *N*-carbobenzoxy-L-threonine (**4**) according to the method of Bergel and Wade (1959). The high-resolution mass spectrum of the synthetic sample is identical with that of the product obtained from **1**. In addition, the chromatographic mobilities of the natural and synthetic samples in solvent systems D and E are identical.

These results show that the ureido bond at the N^6 position of **1** is the most susceptible to cleavage by hydroxyl ions. The hydroxyl group of the threonine moiety probably participates in the hydrolytic reaction leading to the intermediate, **2**, which collapses to form adenine and the oxazolidine **3**. An alternative, although less favored, mechanism for the formation of the

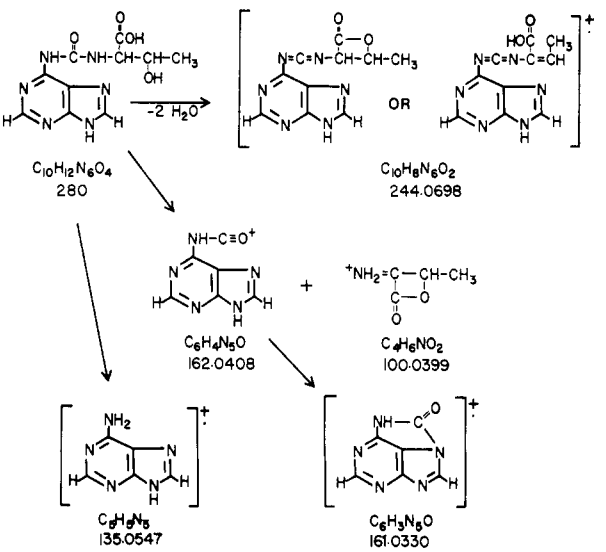

FIGURE 3: The fragmentation pattern of *N*-(purin-6-ylcarbamoyl)-threonine (**1**) obtained in the high-resolution mass spectrometer.

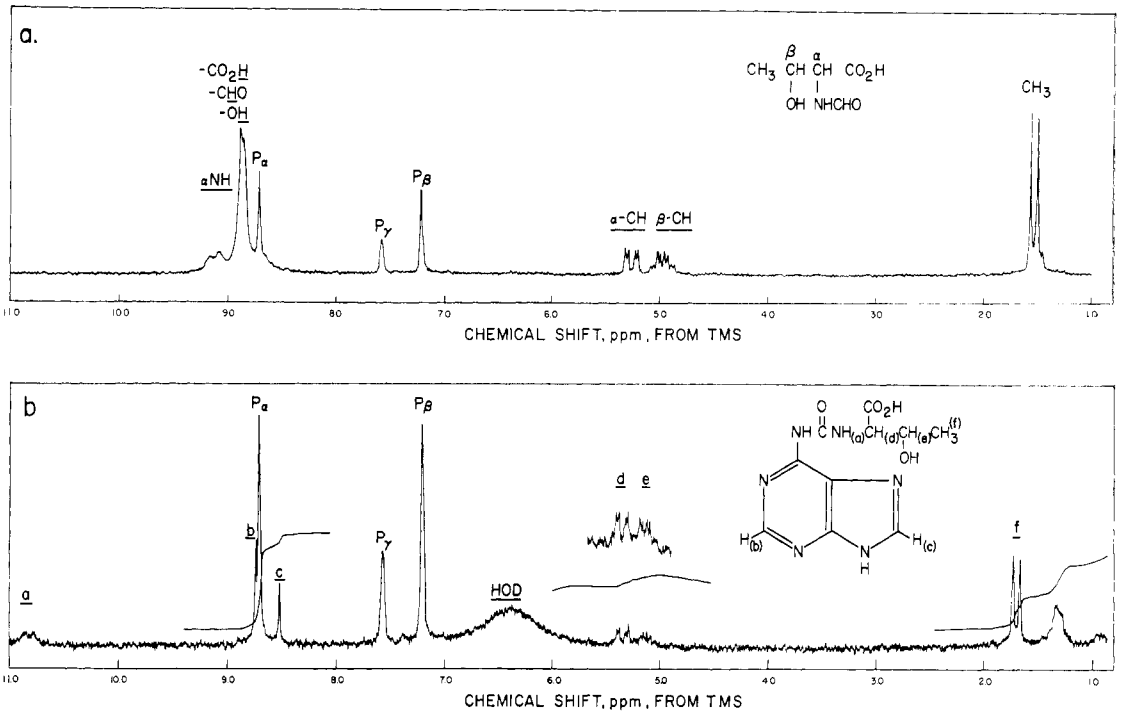

FIGURE 4: Nuclear magnetic resonance spectra taken at 100 MHz; chemical shifts are measured from an internal tetramethylsilane signal. (a) *N*-Formylthreonine, 0.1 M in pyridine-d_5. (b) *N*-(Purin-6-ylcarbamoyl)threonine (**1**) 0.04 M in pyridine-d_5.

oxazolidine would be an immediate cyclization after cleavage of the N^6-ureido bond. In this case the cyclization would have to occur rapidly or the threonine–carbamic acid intermediate would decarboxylate to yield free threonine.

HIGH-RESOLUTION MASS SPECTRUM OF **1**. A possible fragmentation pattern of **1** based on the observed ions is shown in Figure 3. The observed masses of most of the ions agree with the theoretical values within one millimass unit. The fragment with the greatest *m/e* value of 244.0698 indicates an ion with composition $C_{10}H_8N_6O_2$, corresponding to **1** after loss, that occurs either thermally or by electron impact, of two molecules of water. Significant ions are found at 162.0408 and 100.0399. These fragments are probably the result of cleavage α to the carbonyl group, which results in the acyladenine ion of composition $C_6H_4N_5O$ and the dehydrated threonine moiety, $C_4H_6NO_2$. The acyladenine further fragments by the loss of a hydrogen to yield the ion of *m/e* 161.0330, composition $C_6H_3N_5O$. The strongest line in the spectrum corresponds to adenine itself, $C_5H_5N_5$, *m/e* 135.0547. Other ions occurring at 108 and 81 are typical of the loss of hydrogen cyanide from adenine. A doublet differing by 0.0395 mass unit centered at *m/e* 218 also occurs. One line corresponds to an elemental composition of $C_8H_6N_6O_2$ which represents the molecular ion minus water and acetaldehyde; elimination of acetaldehyde is characteristic of threonine fragmentation (Biemann *et al.*, 1961). The other member of the doublet, $C_9H_{10}N_6O$, is generated by loss of water and carbon dioxide from the molecular ion.

NUCLEAR MAGNETIC RESONANCE SPECTRA OF **1** AND *N*-FORMYLTHREONINE. Proton magnetic resonance measurements at 100 MHz were made on **1** in order to obtain additional information about the nature of the side chain. For purposes of comparison, *N*-formylthreonine reference was used as a model *N*-substituted threonine.

The spectrum of *N*-formylthreonine in 0.1 M pyridine-d_5 solution is shown in Figure 4a. The chemical shifts are measured from an internal tetramethylsilane standard. The doublet at δ 1.53 ppm with a J_{H_3} value of 6.2 cps is readily assigned to the methyl group. At 4.97 ppm an octet is observed. In terms of a single first-order analysis of this pattern, the 6.2-cps spacing is found between centers of any two adjacent doublets, indicating that the proton giving rise to this resonance is the β-methine proton. The small coupling of 2.5 cps between doublet members also appears in the quartet centered at 5.25 ppm; this coupling is due to the spin interaction of the vicinal methine protons. The quartet is then due to the α-CH; the other coupling of 9 cps is due to interaction with the NH proton. The 9.0-cps splitting is observed in the broad doublet at 9.12 ppm; this peak is assigned to NH. Aside from the three singlets from the solvent, the split peak at 8.86 ppm could be caused by the formyl and hydroxyl protons, and perhaps by residual formic acid used in preparation of the compound. The carboxyl proton would not be seen because of ionization in the basic pyridine solvent.

The spectrum of **1** in 0.04 M pyridine-d_5 is shown in Figure 4b. With respect to the threonine moiety, the spectral patterns here are essentially identical in terms of the coupling constants with those shown previously for *N*-formylthreonine. The doublet at 1.70 ppm is due to the methyl group. If this peak is irradiated in spin decoupling experiments, the octet at 5.13 ppm collapses, which proves that this latter multiplet is due to the β-CH. Spin decoupling also shows that the broad doublet at 10.82, assigned to the α-NH, is coupled to the proton which gives rise to the quartet at 5.34 ppm, which in

turn must be caused by the α-CH. The singlet resonances at 8.74 and 8.52 ppm are assigned to the H-2 and H-8 protons, respectively, of the adenine ring, based upon the deuterium substitution work of Bullock and Jardetzky (1964). The broad peak at 1.31 ppm may be due to traces of 2-ethoxyethanol left from the partition column purification of **1**.

The only significant chemical shift difference between the spectra of **1** and those of *N*-formylthreonine is the α-*N*-H, which is 1.7 ppm lower field in **1**. Molecular models of **1** indicate that the α-*N*-H can readily undergo hydrogen bond interaction with either *N*-1 or *N*-7 of the purine ring. In addition, a deshielding occurs that is caused by incipient enolization of the amide group.

Given the hygroscopic nature of **1**, the broad resonance at 6.7 ppm is undoubtedly water, and the *N*-9 and hydroxyl protons are not seen because of the exchange. The *N*-6 proton is not observed, possibly because of the exchange with the basic solvent or because of the water present. This proton is quite acidic because of the adjacent electron-withdrawing power of the purine ring and ureido carbonyl. In this regard, it has been possible to observe the N^6 proton using anhydrous samples of some of the synthetic ureido compounds in dimethyl sulfoxide-d_6 solvent. For example, in a 0.18 *M* dimethyl sulfoxide-d_6 solution of *N*-(purin-6-yl) urea, the N^6-H resonated at $\delta = 9.86$ ppm; similarly in 0.094 M *N*-(nebularin-6-yl)-N^1-methylurea, this same proton appeared at $\delta = 9.84$ ppm. Using a synthetic sample of *N*-(purin-6-ylcarbamoyl)threonine (0.12 M), a broad peak at $\delta = 10.07$ ppm appears, which is also assigned to *N*-6. This peak is difficult to assign, possibly because of the presence of 0.5 mole of water of crystallization in the sample. Similarly, the *N*-9 proton is not observed; this is a characteristic feature of the spectrum of adenine and its derivatives because of exchange with the solvent.

ULTRAVIOLET SPECTRA OF **1** AND OTHER UREIDO-N^6-PURINE DERIVATIVES. The ultraviolet absorption spectra of *N*-(purin-6-yl)urea (**10**) are shown in Figure 1. Substitution of the *N* of the urea derivative with CH_3 [*N*-(purin-6-yl)-*N*-methylurea] (**11**) or CH_2COOH [*N*-(purin-6-ylcarbamoyl)glycine] (**12**) does not change the spectra. The spectra of these compounds are identical with those of **1** shown in Figure 4 of the preceding paper (Chheda *et al.*, 1969).

N^6-Acyladenine derivatives have an ultraviolet absorption spectra similar to those shown in Figure 1. For example, the ultraviolet absorption spectra of N^6-threonyladenine (**6**) are shown in Figure 2 (see also Chheda and Hall, 1966).

SYNTHESIS OF N^6-(PURIN-6-YLCARBAMOYL)-L-THREONINE (**1**). Final proof of the structure of **1** was obtained by its synthesis *via* two different routes. (A) 2′,3′,5′-Tri-*O*-acetyladenosine (**8**) was condensed with the isocyanate derivative of *O*-benzylthreonine benzyl ester. The fully protected reaction product was purified by silica gel column chromatography and was then treated with hydrogen bromide in trifluoroacetic acid in order to remove the two benzyl groups and the sugar moiety. *N*-(Purin-6-ylcarbamoyl)threonine (**1**) was crystallized as the hemihydrate from aqueous methanol, mp 219–221°.[4] This product had the correct elemental analysis. The melting point of the natural product is 211–213°. (B) Ethyl *N*-(purin-6-yl)-carbamate (prepared by the procedure of Giner-Sorolla and Bendich (1958)) was condensed with L-threonine in the presence of triethylamine. The product, purified by paper chromatography, has the same properties as the product prepared by method A.[5]

The following properties of the synthetic sample of *N*-(purin-6-ylcarbamoyl)threonine and those of the sample isolated from yeast tRNA were compared and found to be identical: high-resolution mass spectrum, ultraviolet absorption spectra, chromatographic mobility in solvents A–E, and electrophoretic mobility at pH 4.0 and 2.0. The specific rotations were similar.[6]

Discussion

These data support the structure assignment of the threonine–adenine derivative isolated from tRNA as *N*-(purin-6-ylcarbamoyl)threonine (**1**). This compound appears to be well distributed in nature. It has been found in the tRNA of yeast, *E. coli*, and mammalian tissue.

N-(Purin-6-ylcarbamoyl)threonine and its 9-β-D-ribofuranosyl derivative have also been found in human urine (Chheda and Mittelman, 1967; Chheda *et al.*, 1968).

Takemura *et al.* (1969) have isolated a compound from a specific sequence of yeast $tRNA^{Ile}$ which, on acid hydrolysis, yields a product corresponding to **1**. A sample of **1** and that of Dr. Takemura have been compared directly by cochromatography in several solvent systems and by ultraviolet absorption spectra. Dr. Takemura's sample yields threonine on alkaline hydrolysis. This compound occurs adjacent to the 3′ end of the anticodon of $tRNA^{Ile}$.

In light of the results leading to the identification of **1** as *N*-(purin-6-ylcarbamoyl)threonine, our original results on "aminoacyl nucleosides" isolated from tRNA were reexamined. We had reported that a nucleoside had been obtained from yeast tRNA that was an N^6-(α-aminoacyl)adenosine derivative (Hall, 1964; Hall and Chheda, 1965). The original samples of the nucleoside used in the work described in these two publications were no longer available. We isolated another sample of this nucleoside following the procedure of Hall (1965). This sample of "N^6-(aminoacyl)adenosine" was hydrolyzed with 1 N hydrochloric acid under conditions sufficient to remove the sugar moiety; a product identical with *N*-(purin-6-ylcarbamoyl)-threonine (**1**) was obtained. Analysis of the amino acid content of this nucleoside by quantitative ion-exchange chromatography showed that the nucleoside contained only one molecule equivalent of a single amino acid, threonine. This nucleoside sample gave adenosine on alkaline hydrolysis; therefore, it has the structure *N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine [*N*-(nebularin-6-ylcarbamoyl)threonine] (**7**). The original isolated sample (Hall, 1964) on acid hydrolysis in 6 N hydrochloric acid yielded several amino acids, of which glycine, threonine, and valine predominated.

On the basis of the ultraviolet absorption spectral data and chromatographic properties, it is certain that the original

[4] *N*-(Purin-6-ylcarbamoyl)threonine is hygroscopic and unless it is dried at 100° *in vacuo* for several hours, the melting point is about 212–214°.

[5] Full details of the synthesis of *N*-(purin-6-ylcarbamoyl)threonine and related compounds will be reported by G. B. Chheda.

[6] The specific rotation of a sample of the naturally occurring product, **1**, purified by paper chromatography was $[\alpha]_D^{25}$ +42° (*c* 0.12, H_2O). The rotation of a sample of the synthetic material prepared by method A was $[\alpha]_D^{25}$ +30° (*c* 0.2, H_2O). Because the possibility of racemization during synthesis of **1** cannot be excluded and because the sample of the natural product was not crystalline, these data can only be taken as suggestive that the natural material contains L-threonine.

sample possessed the general structure *N*-(nebularin-6-ylcarbamoyl)amino acid. It is not certain whether the original isolated sample was a single amino acid analog of **1** or a mixture. Some of the other amino acids obtained on acid hydrolysis may have been contaminants or were possibly attached to the isolated sample in some way. Thus, at the present time, we cannot exclude the possibility that other amino acid adenosine analogs of **1** occur in tRNA.

Other questions remain unanswered. A small percentage of *N*-(purin-6-ylcarbamoyl)threonine (**1**), isolated as described by Chheda *et al.* (1969), did not have a free carboxylic group. The nature of the blocking group was not determined, although the blocked derivative behaved on paper chromatography like a simple ester (*i.e.*, methyl ester). A precedent exists for the esterification of other components of tRNA that contain carboxyl groups. 2-Thio-5-carboxymethyluridine is isolated as the methyl ester (Baczynskyj *et al.*, 1968) and 5-carboxymethyluridine isolated by Gray and Lane (1968) appears to exist in the tRNA in an esterified form.

The significance of the modified nucleosides to the structure and function of tRNA is the subject of considerable interest. Particularly important is the fact that a modified nucleoside occurs adjacent to the 3′ end of the anticodon. These particular modified nucleosides might be referred to as "hyper" modified nucleosides, in contrast to the simpler modified nucleosides such as methylated, thio, dihydro, etc. They have not been found in any place in the primary sequence of the tRNA molecules other than adjacent to the anticodon. The first of these "hyper" modified nucleosides to be identified, N^6-(Δ^2-isopentenyl)adenosine (Biemann *et al.*, 1966; Hall *et al.*, 1966), occurs in yeast $tRNA^{Tyr}$ and $tRNA^{Ser}$ (Madison and Kung, 1967; Zachau *et al.*, 1966) and in rat liver $tRNA^{Tyr}$ (Staehelin *et al.*, 1968), and a 2-methylthio derivative of this nucleoside occurs in the *E. coli* suppressor $tRNA^{Tyr}$ adjacent to the 3′ end of the anticodon (Burrows *et al.*, 1968; Harada *et al.*, 1968). This particular tRNA component appears to be essential to the codon–anticodon interaction (Fittler and Hall, 1966; Gefter and Russell, 1969). An unidentified nucleoside, compound Y, occurs in the analogous position of the primary sequence of yeast $tRNA^{Phe}$ (RajBhandary *et al.*, 1967), and its presence is essential to the proper functioning of the anticodon region of $tRNA^{Phe}$ (Thiebe and Zachau, 1968). The presence of the "hyper" modified nucleosides in this key position in the primary sequence (adjacent to the 3′ end of the anticodon) of most tRNA molecules may be required for efficient binding of the tRNA to the ribosome.

Although details of the mechanism of the anticodon–codon–ribosome interaction are lacking, the considerable knowledge about the chemical reactivities of *N*-(purin-6-ylcarbamoyl)threonine and N^6-(Δ^2-isopentenyl)adenosine (see Robins *et al.*, 1967) permits some predictions as to how they might participate in this interaction. One of the notable characteristics is that these "hyper" modified nucleosides contain functional groups (organic chemistry definition) such as carboxyl, hydroxyl, and allylic double bond. This fact suggests that they could participate in a variety of chemical reactions such as esterification, peptide-bond formation, etc. The carboxyl group, readily ionizable, could provide increased stability for the tRNA–messenger–ribosome complex by means of electrostatic interaction with the basic ribosomal proteins. Regardless of their exact function the attachment of these reactive and structurally unique groups at a specific location in the primary sequence creates a highly specific active site in the tRNA molecule.

In summary, the tRNA of several organisms contains a component identified as *N*-(purin-6-ylcarbamoyl)threonine. With respect to yeast, about one in five tRNA molecules (assuming there is only one such component per molecule) contains this component. There is a possibility that other such amino acid containing analogs exist in the tRNA.

References

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

Bergel, F., and Wade, R. (1959), *J. Chem. Soc.*, 941.

Biemann, K. (1968), *Advan. Mass Spectr. 4*, 139.

Biemann, K., Seibl, J., and Gapp, F. (1961), *J. Am. Chem. Soc. 83*, 3795.

Biemann, K., Tsunakawa, S., Sonnenbichler, K., Feldmann, H., Dütting, D., and Zachau, H. G. (1966), *Angew. Chem. 78*, 600.

Bullock, F. J., and Jardetzky, O. (1964), *J. Org. Chem. 29*, 1988.

Burrows, W. J., Armstrong, D. J., Skoog, F., Hecht, S. M., Boyle, J. T. A., Leonard, N. J., and Occolowitz, J. (1968), *Science 161*, 691.

Chheda, G. B., and Hall, R. H. (1966), *Biochemistry 5*, 2082.

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

Chheda, G. B., and Mittelman, A. (1967), 154th Meeting of the American Chemical Society, Chicago, Ill., Sept., Abstract C-36.

Chheda, G. B., Mittelman, A., and Grace, J. T. (1968), 156th Meeting of the American Chemical Society, Atlantic City, N. J., Abstract BIOL-212.

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

Gefter, M. L., and Russell, R. L. (1969), *J. Mol. Biol. 39*,145.

Giner-Sorolla, A., and Bendich, A. (1958), *J. Am. Chem. Soc. 80*, 3932.

Gray, M. W., and Lane, B. G. (1968), *Biochemistry 7*, 3441.

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.

Hall, R. H., Robins, M. J., Stasiuk, L., and Thedford, R. (1966), *J. Am. Chem. Soc. 88*, 2614.

Harada, F., Gross, H. J., Kimura, F., Chang, S. H., Nishimura, S., and RajBhandary, U. L. (1968), *Biochem. Biophys. Res. Commun. 33*, 299.

Madison, J. T., and Kung, H. (1967), *J. Biol. Chem. 242*, 1324.

RajBhandary, U. L., Chang, S. H., Stuart, A., Faulkner, R. D., Hoskinson, R. M., and Khorana, H. G. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 751.

Robins, M. J., Hall, R. H., and Thedford, T. (1967), *Biochemistry 6*, 1837.

Schweizer, M. P., Chheda, G. B., Hall, R. H., Baczynskyj, L., and Biemann, K. (1968), 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept, Abstract BIOL-242.

Staehelin, M., Rogg, H., Baguley, B. C., Ginsberg, T., and Wehrli, W. (1968), *Nature 219*, 1363.

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

Thiebe, R., and Zachau, H. G. (1968), *European J. Biochem. 5*, 546.

Zachau, H. G., Dütting, D., and Feldmann, H. (1966), *Z. Physiol. Chem. 347*, 212.

Nucleic Acid Reassociation in Formamide*

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ABSTRACT: Conditions are described for effecting deoxyribonucleic acid renaturation and deoxyribonucleic acid–ribonucleic acid hybridization in formamide solutions at low temperatures.

High specificity and rates of reaction can be achieved by appropriate choice of the formamide and salt concentrations and the temperature. Reaction conditions can be deduced from the relationship between formamide concentration and reduction in the thermal stability: 1% formamide reduces the T_m by 0.72°. These methods are valuable for studies of the prolonged reactions occurring between mammalian nucleic acids.

Renaturation of DNA and hybridization of RNA with DNA are normally studied under reaction conditions involving elevated temperatures. The maximum rate of reaction occurs some 25° below the T_m, or mean thermal denaturation temperature (Marmur and Doty, 1961). However, in studies of nucleic acids of complex organisms where reaction times are prolonged, or where biological activity must be preserved, exposure of nucleic acids to high temperatures presents obvious disadvantages such as chain scission and depurination. These difficulties may be avoided by the use of high concentrations of certain salts, such as 6.2 M $NaClO_4$ (Thomas, 1966) or of aqueous solutions of various organic solvents in which the thermal stability of double-stranded polynucleotides is greatly reduced (Herskovits, 1962; Geiduschek, 1962; Levine *et al.*, 1963). A particularly useful organic solvent is formamide in which DNA can be denatured and renatured at room temperature (Helmkamp and Ts'o, 1961; Marmur and Ts'o, 1961; Subirana and Doty, 1966). This principle has been exploited by Bonner *et al.* (1967) to allow the formation of DNA–RNA hybrids with filter-bound DNA at 25° in the presence of formamide.

The present study explores in detail the relationships among the rate and specificity of reaction, the temperature, and the concentrations of formamide and salt. As a result of the incidence of partially related base sequences (Britten and Kohne, 1968), DNA renaturation or DNA–RNA hybridization reactions involving nucleic acids of mammals or other higher organisms may not display locus specificity. In fact, the extent of cross-reaction among related base sequences is dependent upon the reaction conditions such as temperature and ionic strength (McCarthy and McConaughy, 1968; Church and McCarthy, 1968). Thus it is important not only to establish conditions for high rates of reaction and species specificity but also to characterize the nature of the duplexes being formed. Although in aqueous solution cross-reaction among related base sequences is reduced at higher temperatures or lower ionic strengths (McCarthy and McConaughy, 1968), specificity conditions are much more easily adjusted by variation of ionic strength and formamide concentration. High specificity may be obtained at low temperatures, with rates of reaction several times higher than those obtained at 60 or 70° in aqueous solution.

Materials and Methods

Formamide. The formamide used in these studies was purchased from the Eastman Kodak Co. Optical studies in formamide solution require high solvent purity. The criterion applied was that the optical density of 100% formamide at 270 mμ in 1-cm path-length cells not exceed 0.15. All of the experiments described here were carried out with formamide which meets these specifications.

Isolation of Nucleic Acids. Bacillus subtilis DNA was prepared as described by Marmur (1961) and ^{3}H-labeled DNA was extracted from cells grown in minimal medium to which 0.5 μCi/ml of thymidine-methyl-H^3 was added per hour for 4 hr.

DNA was isolated from *Drosophila melanogaster* pupae by a procedure described elsewhere (Laird and McCarthy, 1968).

Mouse and hamster DNA were extracted from liver as previously described by Church and McCarthy (1968). Labeled mouse DNA was prepared from mouse L cells grown in monolayers with 2 μCi/ml of thymidine-methyl-^{3}H added to the medium (McCarthy and McConaughy, 1968).

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