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Biosynthesis of *N*-(Purin-6-ylcarbamoyl)-L-threonine Riboside

INCORPORATION OF L-THREONINE *IN VIVO* INTO MODIFIED NUCLEOSIDE OF TRANSFER RIBONUCLEIC ACID

By GIRISH B. CHHEDA, CHUNG IL HONG, CONRAD F. PISKORZ and G. A. HARMON
General Clinical Research Center, Department of General Surgery, Roswell Park Memorial Institute, Buffalo, N.Y. 14203, *U.S.A.*

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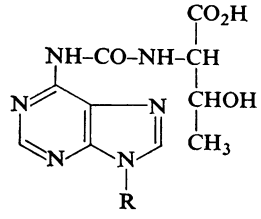
L-[U-^{14}C]Threonine is incorporated into *N*-(purin-6-ylcarbamoyl)-L-threonine riboside of rat liver and *Escherichia coli* tRNA. A pathway is suggested for the biosynthesis of this nucleoside.

N-(Purin-6-ylcarbamoyl)-L-threonine (compound I) and its riboside (compound II) have been isolated and characterized from tRNA (Chheda *et al.*, 1969*a*; Schweizer *et al.*, 1969; Chheda & Hong, 1971) and from human and rat urine respectively (Chheda, 1969). Sequence determination of yeast tRNA^{Ile} revealed that *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II) was a nucleoside adjacent to the anticodon at the 3′-end (Takemura *et al.*, 1969). This modified nucleoside has also been found in several other tRNA species (methionine, lysine and serine tRNA) that respond to the codons beginning with adenosine (Ishikura *et al.*, 1969). In the present paper we describe the incorporation of L-threonine *in vivo* into *N*-(purin-6-ylcarbamoyl)-L-threonine riboside of mammalian and bacterial tRNA and suggest a pathway for its biosynthesis (Chheda & Hong, 1970).

Materials and Methods

Materials

Crude phosphodiesterase (snake venom, *Crotalus adamanteus*) was purchased in freeze-dried form from the Ross Allen Reptile Institute (Silver Springs, Fla., U.S.A.), and was purified in this laboratory by the method of Williams *et al.* (1961). Bacterial alkaline phosphatase was acquired from the Worthington Biochemical Corp., Freehold, N. J., U.S.A.



I, R=H
II, R=1′-β-D-Ribosyl

General methods

Paper chromatograms were run in a descending manner on Whatman no. 1 paper in the following systems (nucleoside spots and bands on paper chromatograms were detected under short-wave u.v. light; amino acids were detected by spraying the chromatograms with 0.1% ninhydrin solution in butanol): A, propan-2-ol–water–conc. NH_3 (7:2:1, by vol.); B, ethyl acetate–2-ethoxyethanol–16% (w/v) formic acid (4:1:2, by vol.); C, butan-1-ol–water–conc. NH_3 (86:14:5, by vol.); D, propan-1-ol–conc. NH_3–water (11:2:7, by vol.); and E, ethyl acetate–propan-1-ol–water (4:1:2, by vol.).

Radioactivity was measured with a Packard Tri-Carb model 3375 liquid-scintillation spectrometer with a counting efficiency of 85% for ^{14}C. For strip scanning of a chromatogram, a strip (2.5cm) covering the desired path was numbered and cut into successive squares (2.5cm × 2.5cm), which were placed into vials for radioactivity counting. All u.v. spectra of the nucleosides were recorded on a Cary model 14 spectrophotometer.

Incorporation studies

Incorporation of L-[*U*-^{14}*C*]*threonine into rat liver tRNA: isolation of* ^{14}*C-labelled N-(purin-6-ylcarbamoyl)-*L*-threonine riboside (compound II) and other nucleosides.* Four Sprague–Dawley rats (approx. 200g body weight) were maintained on a protein-free diet for 12 days; then they were given a total of 250μCi (62.5μCi each in 1.25ml of 0.85% NaCl, sp. radioactivity 201mCi/mmol) of L-[U-^{14}C]threonine through the portal vein. After 5h, the rats were killed, their livers (26g) were removed and the tRNA was extracted at 5°C by the method of Brunngraber (1962) with minor alterations. [The homogenate was

centrifuged at 15000rev./min for 20min in a Sorvall refrigerated centrifuge; before the initial precipitate was dissolved for column purification, a propan-2-ol precipitation procedure described by Zubay (1962) was performed.] The eluate from the column was added to 3vol. of 95% (v/v) ethanol and left overnight at 4°C. The resulting precipitate of tRNA was isolated by centrifugation, then was dissolved in 1.5ml of water, and determined by its u.v. absorption (16.2mg of tRNA with 12360c.p.m.). The terminal amino acid of this tRNA was removed (pH9 for 1h at 37°C) and the tRNA was reprecipitated with ethanol.

A portion of this tRNA (10mg, 7100c.p.m.) was hydrolysed with purified snake venom phosphodiesterase (Williams *et al.*, 1961) and bacterial alkaline phosphatase by the general procedure of Hall (1965). To the hydrolysate was added 2.5mg of pure synthetic *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II) and the solution was separated into its major u.v.-absorbing components by paper chromatography (solvent C). The u.v.-absorbing areas corresponding to the above nucleoside (compound II) marker, as well as to the other major nucleosides, were eluted and purified by paper chromatography, consecutively, in solvents A, B, C and D until pure homogeneous materials of constant specific radioactivity were obtained as shown in Table 1.

Incorporation of L-[*U*-^{14}C]*threonine into Escherichia coli tRNA: isolation of* ^{14}C-*labelled N-(purin-6-ylcarbamoyl)-*L-*threonine riboside (compound II) and other nucleosides. E. coli* mutant S-34-2, which required threonine, lysine and methionine and which had been cultured in beef infusion broth at 37°C for 24h, was added to a 16-litre carboy culture containing the *E. coli* minimal medium (Tatum & Lederberg, 1947) supplemented with L-threonine (30mg/ml), L-lysine (50mg/ml) and L-methionine (50mg/ml). [This mutant (S-34-2) was obtained from Dr. Paul Sypherd, University of California, College of Medicine, Irvine, Calif., U.S.A.] The culture was incubated with vigorous aeration at 27±1°C for 28h. When the growth plateau was established, 250μCi of L-[U-^{14}C]-threonine (sp. radioactivity 201mCi/mmol) was added and the culture was incubated with aeration for 15min and then chilled with solid CO_2. The cells (21.9g; 3.79×10^5c.p.m.) were harvested by centrifugation at 5000rev./min for 10min, then they were washed with water. tRNA (27.3mg) was isolated from the cells by the method of Fleissner & Borek (1961), and then its terminal amino acids were removed.

This tRNA (22mg, 120000c.p.m.) was hydrolysed by snake venom phosphodiesterase and bacterial alkaline phosphatase (Hall, 1965). To the hydrolysate was added 2.0mg of the synthetic *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II) in 1ml of water and then the mixture was separated by paper chromatography in solvent system C. The bands corresponding to the authentic markers were cut out, eluted, freeze-dried and purified by paper chromatography, consecutively, in solvents A, B, C and D until homogeneous materials of constant specific radioactivity were obtained (Table 2).

*Hydrolysis of labelled N-(purin-6-ylcarbamoyl)-*L-*threonine riboside (compound II) isolated from tRNA*

To hydrolyse *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II), 0.5mg of the isolated materials from both rat liver tRNA and *E. coli* tRNA was dissolved in 1ml of 0.5M-NaOH and the solution was heated at 100°C for 30min. Purification of the

Table 1. *Radioactivity in nucleosides of rat liver tRNA*

Rat liver tRNA was labelled *in vivo* with L-[U-^{14}C]threonine as described in the text, and the yield and radioactivity of the nucleosides were determined after hydrolysis of a sample (10mg; 7100c.p.m.).

Nucleoside	Amount [μmol (E_{260} units)]	Total radioactivity (c.p.m.)	Sp. radioactivity (c.p.m./μmol)
Adenosine	3.83 (57)	0	0
Guanosine	4.13 (56)	154	37
Uridine	2.62 (39)	0	0
Cytidine	1.52 (13)	60	39
N-(Purin-6-ylcarbamoyl)-L-threonine riboside (compound II)			
Isolated after dilution with carrier	3.71 (85)	3580	965.0
Actual (calculated)	0.034* (0.78)	3580	105000

* Rat liver tRNA contains 18mol% of adenosine and only 0.16mol% of *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II). On the basis of the 3.83μmol of isolatable adenosine, the calculated quantity of isolatable modified compound II, would be 0.034μmol.

Table 2. *Radioactivity in nucleosides of E. coli tRNA*

E. coli tRNA was labelled with L-[U-^{14}C]threonine as described in the text and the yield and radioactivity of tRNA nucleosides were determined after hydrolysis of a sample (22mg; 120000c.p.m.).

Nucleoside	Amount [μmol (E_{260} units)]	Total radioactivity (c.p.m.)	Sp. radioactivity (c.p.m./μmol)
Adenosine	10.29 (153)	2938	285
Guanosine	21.18 (287)	3367	159
Uridine	5.28 (79)	4452	843
Cytidine	9.54 (82)	5534	580
N-(Purin-6-ylcarbamoyl)-L-threonine riboside (compound II)			
Isolated after dilution with carrier	3.47 (80)	28544	8226
Actual (calculated)	0.036* (0.82)	28544	792900

* *E. coli* tRNA contains 20mol% of adenosine and only 0.07mol% of *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II). On the basis of the 10.29μmol of isolatable adenosine, the calculated quantity of isolatable compound II will be 0.036μmol.

Table 3. *R_F of hydrolysis products of labelled N-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II) by paper chromatography*

Product Solvent ...	R_F A	B	C	D	E
Adenosine (from rat liver tRNA, hydrolysis of compound II)	0.53		0.15	0.61	0.31
Adenosine (hydrolysis of compound II from *E. coli* tRNA)	0.54		0.16	0.60	0.31
Adenosine (authentic)	0.54		0.16	0.60	0.31
Threonine (hydrolysis of compound II from rat liver tRNA)	0.38	0.15			0.02
Threonine (hydrolysis of compound II from *E. coli* tRNA)	0.38	0.15			0.02
Threonine (authentic)	0.37	0.14			0.02

reaction mixture by paper chromatography (solvent B) gave a single u.v.-absorbing product whose spectra and paper-chromatographic properties were identical with those of authentic adenosine (Table 3). The ninhydrin-positive non-u.v.-absorbing material was identical, with respect to its R_F, with authentic L-threonine (Table 3). The band corresponding to marker L-threonine was eluted from the chromatograms of both experiments and samples were used for radioactivity determination and amino acid quantitation (Spackman *et al.*, 1958). The results are recorded in Table 4. In another hydrolysis experiment, the liberated CO_2 was trapped in $Ba(OH)_2$ solution and the resulting $BaCO_3$ was checked for the radioactivity (Table 4).

Results and Discussion

The amount of radioactivity derived from L-[U-^{14}C]threonine in the nucleosides of rat liver tRNA and *E. coli* tRNA (threonineless mutant S-34-2) is shown in Tables 1 and 2 respectively. In each experiment unlabelled synthetic *N*-(purin-6-ylcarbamoyl)-L-threonine (compound II) was added to the tRNA hydrolysate to facilitate the detection of the labelled nucleoside (compound II) by u.v. light. In spite of the dilution with the carrier compound, *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II) had the highest amount of radioactive label, with significantly high specific radioactivity in both the experiments. As the adenosine amounts are 18 and 20mol% in rat liver and *E. coli* tRNA respectively, as compared with 0.16 and 0.07mol% of the modified nucleoside, compound II, the specific radioactivity of *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II), calculated on the basis of the isolatable amounts of adenosine and compound II, is highly significant (see Tables 1 and 2).

On alkaline hydrolysis, the purified *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II)

Table 4. *Radioactivity in hydrolysis products of N-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II)*

	From rat liver tRNA		From *E. coli* tRNA	
Hydrolysis	Total radioactivity (c.p.m.)	(c.p.m./μmol)	Total radioactivity (c.p.m.)	(c.p.m./μmol)
Compound II	1190	965	8105	8226*
Products: Adenosine	0	0	0†	0
CO_2	0	0	0	0
Threonine	770	920	4943	5290*

* These appear to be some impurities which could not be removed from the nucleoside II.

† On the basis of the radioactivity of 2938c.p.m. in 10.29μmol of adenosine isolated from *E. coli* tRNA (Table 2), the radioactivity in the adenosine (0.01μmol) derived from the hydrolysis of *N*-(purin-6-ylcarbamoyl)-L-threonine riboside compound II (8105c.p.m. used for hydrolysis) would be less than 3c.p.m. Considering the limitations of the instrument, this is in good agreement with the finding that the adenosine had no counts.

derived both from rat liver tRNA and from *E. coli* tRNA, gave adenosine, threonine and CO_2. Liquid-scintillation counting of radiochromatograms showed adenosine to be free of radioactive label (Table 4). Only the non-u.v.-absorbing ninhydrin-positive material, which corresponded to authentic threonine, was radioactive. The authenticity of threonine and adenosine was further confirmed by paper-chromatographic comparison in the several solvent systems (Table 3). The liberated CO_2, collected as $BaCO_3$, was free of radioactivity in both cases (Table 4).

The radioactivity in guanosine and cytidine of rat liver tRNA and in all of the major nucleosides of *E. coli* tRNA indicates that the fragments derived from the degradation of L-threonine do get incorporated, but only to a small extent, into purines and pyrimidines of tRNA. The specific radioactivity of these nucleosides is quite low as compared with that of the modified compound II (Tables 1 and 2).

The above results suggest that L-threonine is a precursor for the threonine portion of *N*-(purin-6-ylcarbamoyl)-L-threonine riboside present in rat liver and *E. coli* tRNA. It is also clear from the results in Tables 1 and 2 that none of the carbon atoms of the ureido group, and the purine or ribose moieties of this modified nucleoside (compound II), is derived to any significant extent from the L-threonine (Table 4). As L-threonine is an essential amino acid, it is not unreasonable to suggest that it is incorporated as an intact molecule into *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II) and not as a degradation fragment. The lower incorporation of L-threonine into rat liver tRNA is mainly due to the extensive dilution of the injected label by the large pools of L-threonine present in the animal.

It is reasonable to assume that the biosynthesis of *N*-(purin-6-ylcarbamoyl)-L-threonine riboside, like that of all other modified nucleosides of tRNA, occurs at the macromolecular level (Chheda *et al.*, 1969*b*; Kline *et al.*, 1969; Peterkofsky, 1968), i.e. the L-threonine side chain is attached to inosine or adenosine nucleoside that is adjacent to the anticodon of the precursor tRNA polymer. A possible pathway for the biosynthesis of *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II) in tRNA is as follows. L-Threonine may react with carbamoyl phosphate, or with another C_1 donor, and be converted into *N*-carbamoyl-L-threonine [CH_3–$CH(OH)$–$CH(CO_2H)$–NH–CO–NH_2, compound III] or another activated *N*-carbonylthreonine derivative [CH_3–$CH(OH)$–$CH(CO_2H)$–NH–CO_2A, compound IV, where A is an activating group]. Compound III, or the activated derivative compound IV, could then react with inosine or adenosine respectively in precursor tRNA to give the desired tRNA containing *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (compound II).

To determine the possible C_1 donor for the ureido group, and to see whether the adenosine or inosine adjacent to the anticodon acts as the acceptor of the threonine side chain, preparation of tRNA without this side chain will be necessary.

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