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METABOLISM OF *N*-(PURIN-6-YLCARBAMOYL)-L-THREONINE RIBOSIDE IN RAT AND MAN

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Abstract—*N*-(purin-6-ylcarbamoyl)-L-threonine riboside-8-^{14}C (Ado-CO-thr-8-^{14}C) and *N*-(purin-6-ylcarbamoyl)-L-[U-^{14}C]threonine riboside (Ado-CO-thr-^{14}C) were synthesized by using adenosine-8-^{14}C and threonine [U-^{14}C], respectively, as labeled starting materials. The Ado-CO-thr-8-^{14}C and Ado-CO-thr-^{14}C were given intravenously and orally to rat and to man. Of the $2{\cdot}4 \times 10^6$ dis/min administered intravenously to rats, 73 per cent was excreted in urine. More than 79 per cent of this excreted activity was in unchanged Ado-CO-thr, 2·2 per cent in the free base, *N*-(purin-6-ylcarbamoyl)-L-threonine (Ade-CO-thr), and less than 2 per cent in adenosine. Radioactivity in adenine, inosine, hypoxanthine and uric acid was less than 0·1 per cent. When the labeled Ado-CO-thr was administered orally, less than 5 per cent of the radioactivity was excreted in urine. Similar results were obtained in man. These data revealed that Ado-CO-thr is quite a stable modified nucleoside *in vivo*. Intravenously administered, Ado-CO-thr was not incorporated into rat t-RNA. *In vitro*, it did not serve as a substrate for adenosine deaminase, xanthine oxidase and adenosine kinase. Incubation of Ado-CO-thr with urease, acylase, protease, peptidases and similar other enzymes did not lead to any structural change in the molecule. It is suggested that naturally occurring urinary Ado-CO-thr originates from t-RNA and is excreted quantitatively in urine.

MODIFIED nucleosides on an average constitute at least 16 per cent of the transfer RNA molecule of bacterial and mammalian origin.[1] It is known that the usual purine nucleosides, adenosine and guanosine, are catabolized to uric acid; however, the modified purine nucleosides of t-RNA are not. It appears that many of the methylated nucleosides are excreted as such without further metabolism.[2] Some hypermodified nucleosides, however, undergo catabolism. For example, the anticodon adjacent nucleoside N^6-(Δ^2-isopentenyl)adenosine (IPA) is, in large part, metabolized to non-ultra-violet absorbing compounds.[3] The present paper deals with the metabolism of another such nucleoside,† the 8-^{14}C labeled *N*-(purin-6-ylcarbamoyl)-L-threonine riboside (Ado-CO-thr).‡ Ado-CO-thr (III, Fig. 1) has been isolated and characterized from various t-RNAs[4,5] and from rat and human urine.[6] It is located adjacent to the anticodon in yeast t-RNA^{Ile}.[7] It appears to be located at a similar position in *Escherichia coli* t-RNA$_3^{Ser}$, t-RNA$_1^{Met}$ and t-RNA^{Lys},[8] and *E. coli* t-RNA^{Asn}.[9] Even

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† Preliminary results of this study were presented at the 62nd Meeting of the American Society of Biological Chemists; *Fedn Proc.* **30**, Part II, 1198 (1971).

‡ The abbreviations Ado-CO-thr for *N*-(purin-6-ylcarbamoyl)-L-threonine riboside and Ade-CO-thr for *N*-(purin-6-ylcarbamoyl)-L-threonine have been suggested by Dr. W. E. Cohn in accord with the IUPAC-IUB Commission on Biochemical Nomenclature.

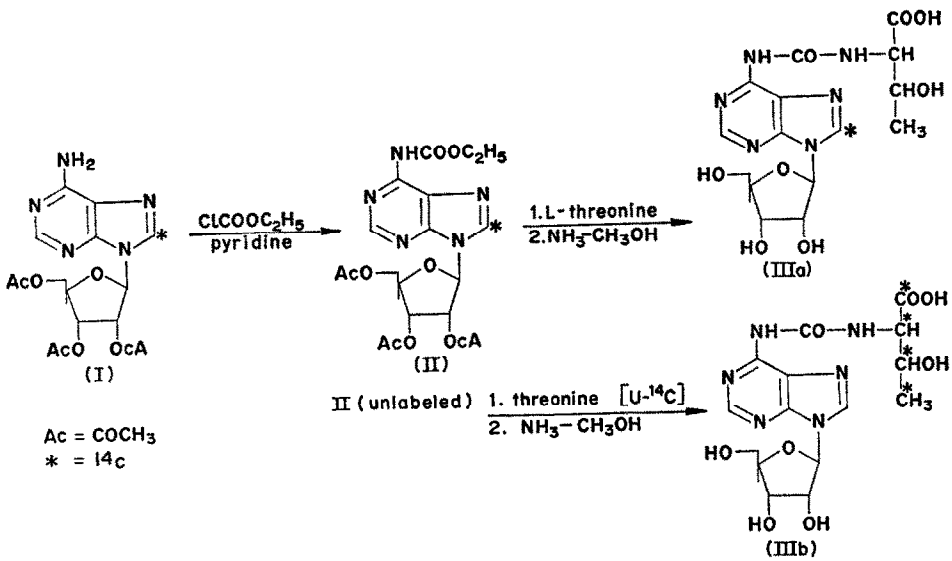

FIG. 1. Synthesis of ^{14}C labeled Ado-CO-thr's.

though Ado-CO-thr itself does not have cytokinin activity, some of its analogs are excellent cytokinins.[10,11] Some analogs have exhibited growth inhibitory properties in cultured mammalian cells[12,13] and some have inhibitory properties toward t-RNA methylase[14] and adenosine kinase.[15] Occurrence of Ado-CO-thr in a key position in the t-RNA molecule and its possible biological significance warranted an investigation of its metabolic fate in rat and man.

MATERIALS AND METHODS

Materials

Adenosine-8-^{14}C (specific radioactivity 47·2 mCi/m-mole) and L-[U-^{14}C]threonine (specific radioactivity 201 mCi/m-mole) were purchased from Schwarz Bio-Research, Orangeburg, N.Y. Celite 545 was purchased from Johns-Manville Company, New York, N.Y. and acid-washed in our laboratory. Preparative thin-layer chromatography (TLC) was carried out on glass plates using Silica gel with PF 254 (W. Merck AG).

Chromatography

The following solvents were used for paper chromatography: (a) isopropanol–conc. ammonium hydroxide–water (7:1:2); (b) ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2); (c) ethyl acetate–*n*-propanol–water (4:1:2) and (d) *n*-propanol–conc. ammonium hydroxide–water (55:10:35).

Whatman No. 3 MM paper was used for the initial preparative paper chromatography of column eluates. Whatman No. 1 was used for comparative identification. All chromatograms were developed in a descending manner. In all instances, appropriate authentic samples were simultaneously chromatographed in parallel lanes with the purified Ado-CO-thr metabolites. Chromatograms were viewed

under short wave ultra-violet lamp. All ultra-violet-absorbing area was eluted and analyzed in a Cary-14 spectrometer. The λ_{max} values are expressed in nanometers (nm).

Determination of radioactivity

Urine samples and paper eluates were assayed for radioactivity in a Packard 3375 liquid scintillation spectrometer. One-hundred μl or 250 μl of sample was absorbed on a 2·5 cm^2 or 2·5 × 5 cm of Whatman No. 3 MM paper. The paper squares were then dropped into scintillation vials containing 20 ml of scintillation liquid and counted. The solid sample (feces) was homogenized and an aliquot corresponding to 0·5 g was placed in a gelatin capsule. The radioactivity was checked after burning the capsule in a Packard Tri-Carb sample oxidizer model 305.

Preparation of the ^{14}C labeled Ado-CO-thr (III*a and* III*b, see Fig.* 1)

2′,3′,5′-*Tri*-O-*acetyl adenosine*-8-^{14}C (I). This compound was prepared by a reaction of 0·545 mg (54 μCi) of adenosine-8-^{14}C (specific radioactivity, 47·2 mCi/m-mole), 266·7 mg of unlabeled adenosine (total, 1 m-mole of adenosine), 5 ml of acetic anhydride and 7 ml of anhydrous pyridine at room temperature for 2 hr.[16] All of this material was used for the next step.

Ethyl 9-(2,3,5-*tri*-O-*acetyl*-β-D-*ribofuranosyl*)-9*H*-*purine*-6-*carbamate*-8-^{14}C (II). This compound was prepared by a reaction of tri-*O*-acetyl adenosine-8-^{14}C (I) (prepared above) with 0·5 ml of ethyl chloroformate in 6 ml of anhydrous pyridine.[16] Total yield of chromatographically pure material was 268 mg (57·5 per cent); λ_{max} pH 6·6 ($\epsilon \times 10^{-3}$), 267 (17·5).

N-(*purin*-6-*ylcarbamoyl*)-L-*threonine riboside*-8-^{14}C (*Ado-CO-thr*-8-^{14}C) (III*a*). This compound was prepared by a reaction of 268 mg (0·575 m-mole) of the (labeled) urethane 8-^{14}C (II), 135 mg (1·13 m-moles) of L-threonine and 25 ml of anhydrous pyridine at 120° for 6 hr;[16] yield, 92·8 mg (39·1 per cent), total radioactivity 11·576 μCi, specific radioactivity 51·45 μCi/m-mole. Ado-CO-thr-8-^{14}C was checked for radiopurity using paper chromatography in solvent A; 99 per cent. Paper chromatography ($R_f \times 100$), solvent A, 23; solvent B, 63; solvent C, 1·6; solvent D, 60. λ_{max} ($\epsilon \times 10^{-3}$), pH 6·5, 269, 277 (22·9, 19·4); pH 1·6, 277 (21·6); pH 12·4, 270, 277, 299 (16·3, 16·0, 11·1).

N-(*purin*-6-*ylcarbamoyl*)-L-[*U*-^{14}C] *threonine riboside* (*Ado-CO-thr*-^{14}C) (III*b*). This compound was prepared by a reaction of 465 mg (1 m-mole) of the unlabeled urethane (II),[16] 61·6 μCi (0·304 μmole) of L-[U-^{14}C]threonine (specific radioactivity, 201 mCi/m-mole), 119 mg (1 m-mole) of cold L-threonine and 30 ml of anhydrous pyridine at 120° for 6 hr; yield 89·3 mg (21·65 per cent) with a total radioactivity of 14·85 μCi (specific radioactivity 68·6 μCi/m-mole). Ado-CO-thr-^{14}C was tested for radiopurity using paper chromatography in solvent A; 88·31 per cent. [Storage over a long period of time (more than 3 months) at room temperature leads to some decomposition.]

Intravenous administration of Ado-CO-thr. To 77·1 mg of unlabeled Ado-CO-thr in sterile saline solution was added 22·9 mg (2·86 μCi) of Ado-CO-thr-8-^{14}C and the solution was filtered through a MF-Millipore filter (pore size, 0·45 μm, Millipore Corp., Bedford, Mass.). The filter was washed with 10 ml of 70% ethanol and the combined filtrate was mixed with 1000 ml of sterile 5% dextrose in isotonic saline, which was infused into a human volunteer over a 3·5-hr period. Before the infusion,

the subject emptied his bladder and immediately after completion of the infusion, the first urine sample was collected.

A solution of 1 ml of sterile saline and Ado-CO-thr-8-^{14}C (8·1 and 16·5 mg) or Ado-CO-thr-^{14}C (10 mg) was injected into two rats (Sprague–Dawley, approx. 200 g body wt) through the tail vein and in another experiment through the portal vein. After completion of the injection, the urines were collected for 48 hr.

Oral administration of Ado-CO-thr. To 174 mg of Ado-CO-thr in 150 ml of orange juice was added 26·24 mg (3·365 μCi) of Ado-CO-thr-8-^{14}C. This mixture was given to a volunteer in one dose. The bladder was emptied immediately before oral administration of the compound.

Ado-CO-thr-8-^{14}C (5·7 and 18·2 mg) or Ado-CO-thr-^{14}C (20 mg) was dissolved in 1 ml of water and given to two 200 g rats. After administration, the urine was collected for 48 hr. Feces for 48 hr was also collected. Precautions were taken to avoid contamination of feces by urine.

Isolation, purification and identification of urinary metabolites

Prior to paper chromatography, urine samples* were desalted by passage through a charcoal–Celite column. Charcoal (5 g) was mixed with 5 g of Celite 545 and 5 ml of water. This was packed into a column (diameter 2·5 cm) on a Celite filter bed of 3 g and another Celite bed of 3 g was packed on the top of the charcoal–Celite column. This was washed with 50 ml of water and urine sample or the aliquot was then applied to the column. The column was washed with water (500 ml) until no chloride ion was found. The charcoal bound metabolites were eluted with 300–400 ml of 2 N NH_4OH in 50% ethanol. The eluate was evaporated to dryness at 25° and the residue was dissolved in 1 ml of water. Total radioactivity was estimated at this point. To the desalted urine sample was added 0·2 mg of each of the authentic samples of *N*-(purin-6-ylcarbamoyl)-L-threonine (Ade-CO-thr),[16] adenosine, adenine, inosine, hypoxanthine and uric acid. The paper chromatographic purification (see Ref. 16 for R_f values) was performed generally in order of solvents A, B, C and D. Each purified metabolite was subjected to ultra-violet quantitation and measurement of radioactivity. Metabolites were identified by comparison of their paper chromatographic mobilities and ultra-violet spectra with those of the authentic samples.

Isolation of t-RNA and Ado-CO-thr from rat liver

Rats receiving Ado-CO-thr-8-^{14}C through the portal and tail vein were sacrificed after 6 and 48 hr of urine collection respectively. The livers were removed and processed by a standard procedure for isolation of t-RNA.[17] Ado-CO-thr was isolated by enzymatic hydrolysis of t-RNA.[18]

Stability of the labeled Ado-CO-thr—model experiment

In aqueous solution, Ado-CO-thr degrades partially to adenosine and threonine when allowed to stand at room temperature for 2 months. Thus it was necessary to

* The volume of rat urine sample used was 10–20 ml. The volume of human urine sample used was 100–150 ml and concentrated to 50 ml *in vacuo*. After cooling at 0–5° overnight, the precipitate (mainly uric acid) was removed on a filter and the filtrate was applied to a charcoal–Celite column (10 g each).

determine the degree of degradation under our experimental conditions. A solution containing Ado-CO-thr-8-^{14}C ($2{\cdot}1 \times 10^5$ cpm) and a generous amount of cold Ado-CO-thr (total optical density 695 at 269 nm) was processed in the same manner as the urine sample. The results are shown in Table 1.

TABLE 1. STABILITY AND RECOVERY STUDIES ON Ado-CO-thr-8-^{14}C*

Compound	Radioactivity of purified compound (10^3 counts/min)	% of Total radioactivity applied	% of Total radioactivity after charcoal column†	% of Total purified radioactivity
Ado-CO-thr	156·52	75·25	88·83	97·1
Ade-CO-thr	0·6	0·28	0·34	0·37
Adenosine	2·89	1·38	1·64	1·8
Adenine	0	0	0	0
Total	160·01	76·91	90·81	99·27

* Ado-CO-thr-8-^{14}C containing $2{\cdot}1 \times 10^5$ counts/min was applied to a charcoal–Celite column and was processed and purified in a manner similar to the normal run.
† $1{\cdot}762 \times 10^5$ counts/min (15·3 per cent loss of radioactivity in column process).

Incubation of Ado-CO-thr and Ade-CO-thr with enzymes

Ado-CO-thr and its base, *N*-(purin-6-ylcarbamoyl)-L-threonine (Ade-CO-thr), were incubated at room temperature and at 37° with various enzymes. To 2·9 ml of Ado-CO-thr (0·225 μmole) in an appropriate buffer was added 0·1 ml of enzyme (1 mg) in the same buffer. Ultra-violet spectra (350–220 nm) were recorded immediately for zero time, and then at various time intervals. Blank cuvettes contained buffer and enzyme, but no substrate. The characteristic peak at 269 nm and the shoulder at 277 nm were the indications that the substrate was intact. The following enzymes and cell extracts were used under proper pH and incubation conditions adapted from the procedures described in Ref. 19: urease (jack bean) (0·75 M phosphate, pH 7·0), protease (0·08 M Tris, pH 7·8), acylase (hog kidney) (0·75 phosphate, pH 7·0), peptidase (0·08 M Tris, pH 7·5), arginase (pH 9·5), snake venom (0·1 M Tris, pH 8·8), rat liver cell extract, yeast cell extract, and *E. coli* cell extract (0·01 M Tris, pH 7·5). Ado-CO-thr was inert to the action of adenosine deaminase (0·05 M phosphate, pH 7·0) and xanthine oxidase (0·05 M phosphate, pH 7·5).

RESULTS

Results in rat. In the case of intravenous administration, 73 per cent of the radioactivity was excreted in urine over a period of 48 hr (Table 2). Table 3 shows the quantitative estimation of the metabolites isolated from rat urine after administration of the labeled Ado-CO-thr. In the case of intravenous experiment, 79 per cent of the excreted radioactivity was found in the unchanged Ado-CO-thr and adenosine had only about 2·2 per cent of radioactivity. Adenosine counts in major part appear to be due to an artifact, since in a model experiment it was established that Ado-CO-thr undergoes a slight degradation to adenosine, during the separation by column and paper chromatography (Table 1). Adenine and inosine contained less than 0·1 per cent, while hypoxanthine and uric acid were completely devoid of radioactivity.

TABLE 2. EXCRETION OF RADIOACTIVITY IN URINE (48 hr) AFTER ADMINISTRATION OF LABELED Ado-CO-thr TO MAN AND RAT

Administered to	Route of administration	Location of label in Ado-CO-thr	Amount administered		Radioactivity excreted	Recovery (%)
			(mg)	(dis/min × 10^6)	(dis/min × 10^6)	
Rat	i.v. (tail vein)	8-^{14}C	8·1	1·85	1·35	73
Rat	i.v. (tail vein)	thr-^{14}C	10·1	2·88	2·07	72
Man	i.v.	8-^{14}C	100·0	6·35	5·14	81
Rat*	Oral	8-^{14}C†	5·7	2·27	0·110	4·8
Rat	Oral	thr-^{14}C	20·0	5·62	0·16	3·00
Man	Oral	8-^{14}C	200·0	7·47	0·054	0·72

* Forty-eight-hr feces gave 0·878 × 10^6 dis/min (38·5 per cent recovery) of the administered radioactivity. Total recovery from the urine, feces and chow (48 hr) was 45·5 per cent (1·038 × 10^6 dis/min).
† Specific radioactivity of this batch of Ado-CO-thr was 88·8 μCi/m-mole.

In the case of oral administration, less than 5 per cent of the administered radioactivity was excreted in the urine over a period of 48 hr. The majority of the counts (40 per cent) were found in a 48-hr collection of the rat feces. The radioactivity excreted in urine was predominantly as Ado-CO-thr and Ade-CO-thr (Table 3). Ade-CO-thr and other metabolites might be formed by degradation of Ado-CO-thr in the gastrointestinal tract before absorption, since Ado-CO-thr in the blood is stable (Table 3).

Results in man. Figure 2 represents the urinary excretion pattern in a normal human subject after intravenous and oral administration of Ado-CO-thr-8-^{14}C. More than 60 per cent of the administered radioactivity was excreted in urine during the first 4 hr. A total of 81 per cent of the intravenously administered radioactivity was excreted over a period of 48 hr (Table 2). Attempts to determine plasma levels of the radioactivity, when half of the dose had been infused, failed due to the low levels of the activity in 10 ml of blood. Table 3 shows the quantitative estimation of the metabolites after purification to constant specific activity. In the case of an intravenous experiment, 69 per cent of the excreted activity was found as unchanged Ado-CO-thr. No other metabolites with significant radioactivity were found. About thirty per cent of radioactivity was lost in column and paper chromatographic purifications. In the case of oral administration of Ado-CO-thr-8-^{14}C (Fig. 2 and Table 2), only 1 per cent of the administered radioactivity was excreted over a period of 48 hr. More than 50 per cent of the recovered counts in urine was excreted during the first 4 hr. Of this excreted activity, 98 per cent was in the unchanged Ado-CO-thr. Again, the losses were heavy in the isolation and purification.

DISCUSSION

From the results obtained, it is clear that Ado-CO-thr is a metabolically stable modified nucleoside. Both in man and rat, most of the intravenously administered Ado-CO-thr is excreted in urine as an unchanged material. When administered orally, less than 5 per cent of the radioactivity is excreted in urine, while 40 per cent of activity was found in rat feces. The poor urinary excretion of radioactivity in the case of oral administration may be due to: (a) poor absorption of Ado-CO-thr by the gastro-

TABLE 3. URINARY METABOLITES ISOLATED FROM RAT AND HUMAN URINE COLLECTIONS (48 hr) AFTER LABELED Ado-CO-thr-8-^{14}C ADMINISTRATION (i.v. AND ORAL)*

		% of Radioactivity in purified products					
		Intravenous			Oral		
Subject	Metabolites	% of Administered	% of Total excreted	% of Total purified	% of Administered	% of Total excreted	% of Total purified
Rat	Ado-CO-thr	57·5	78·8	94·0	1·72	35·84	51·2
	Ade-CO-thr	1·57	2·15	2·57	1·35	28·21	40·3
	Adenosine	1·46	2·00	2·38	0·04	0·77	1·1
	Adenine	0·007	0·01	0·01	0·05	0·98	1·4
	Others†	0·015	0·02	0·03	0·20	4·13	5·9
	Total	60·552	82·98	98·99	3·36	69·93	99·9
Man	Ado-CO-thr	55·9	69	100	0·07	9·25	97·5
	Ade-CO-thr				0·002	0·24	2·5
	Total	55·9	69	100	0·072	9·49	100

* Similar results were obtained from the experiments using Ado-CO-thr-^{14}C.

† In the case of intravenous experiment, this represents inosine only; however, in the case of oral experiment, it represents inosine, hypoxanthine and uric acid.

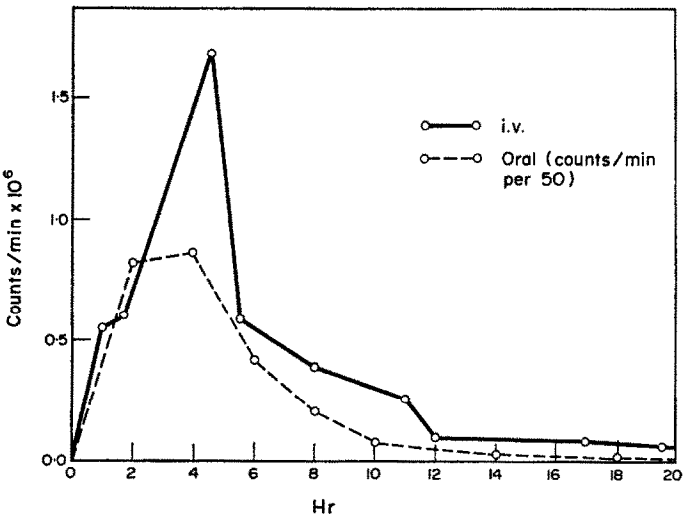

FIG. 2. Pattern of excretion of radioactivity in human urine after intravenous and oral administrations of Ado-CO-thr-8-^{14}C.

intestinal tract or (b) degradation of Ado-CO-thr by various organisms. *In vivo*, Ado-CO-thr does not undergo catabolism to 2,8-dioxo Ade-CO-thr or to uric acid, though to a small extent its glycosidic bond is cleaved. Most of the adenosine and adenine formed is due to an artifact of purification process. *In vitro*, Ado-CO-thr was inert to the common nucleoside enzymes like xanthine oxidase, adenosine deaminase and adenosine kinase.[15] These results suggest that substitution at N^6 of adenosine by carbamoyl threonine alters the steric and electron characteristics of the purine portion of Ado-CO-thr. The ureido side chain of Ado-CO-thr is unaffected by enzymes such as urease, protease, peptidases and esterase. It appears that common drug-metabolizing enzymes present in liver microsomes do not attack the ureido moiety. For example, in the hypoglycemic agents, tolbutamide and acetohexamide (Fig. 3), ureido groups remain intact.[20] The major metabolic alterations occur in the phenyl ring and its substituents.[20] Similarly, in anticonvulsant phenacetylurea (Fig. 3), major metabolic changes occur in phenyl ring while the ureido moiety remains intact.[21] In the latter case, though, some cleavage of the *N*-phenylacyl bond occurs.[21]

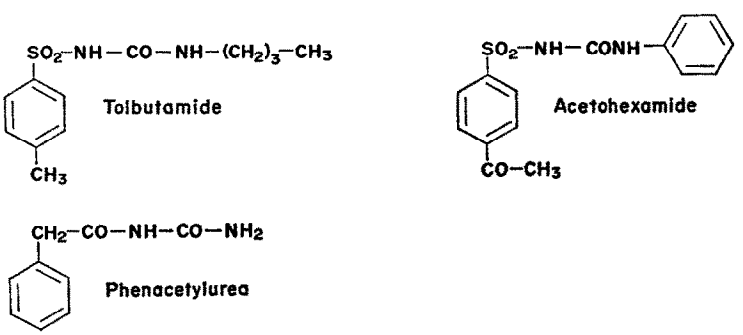

FIG. 3. Chemical structures of tolbutamide, acetohexamide and phenacetylurea.

From the present results on the metabolic fate and the earlier biosynthetic studies on Ado-CO-thr,[18,22] the origin of naturally occurring urinary Ado-CO-thr can be postulated. We suggest that the urinary Ade-CO-thr and Ado-CO-thr[4] are derived from t-RNA.[2] It is now established that free Ado-CO-thr cannot be incorporated into t-RNA,[18,23] and that it is biosynthesized most probably at a polynucleotide level. In a normal turnover process, the Ado-CO-thr containing t-RNA should liberate Ado-CO-thr. The latter, like pseudouridine,[24] is not metabolized further, but is excreted unchanged in urine.[2,6] Since Ado-CO-thr is exclusively present in t-RNA[4] and not absorbed from the diets, urinary Ado-CO-thr or Ade-CO-thr must originate from the metabolism of t-RNA.

The results of the present study can be used to confirm our calculations of t-RNA turnover based on pseudouridine,[24] as well as to compare the calculated and actual amounts of modified nucleosides excreted in urine. Based on 40 mg of urinary pseudouridine excretion and 3·5 mole % content of pseudouridine in human t-RNA, we have determined that approximately 1535 mg of t-RNA is turned over per day (Table 4). The calculated and actually excreted amounts of N^6-methyladenosine and

TABLE 4. COMPARISON OF CALCULATED AND FOUND LEVELS OF MODIFIED NUCLEOSIDES

Modified nucleoside	Isolated levels (mg in 24-hr urine)	Calculated levels (1535 mg t-RNA/day)*	Mole %
N^6-Methyladenosine	11·0	9·8	0·75 (human spleen)
Ado-CO-thr	4–4·2†	3·7	0·19 [liver (calf)]
Uric acid	370–470	330	equivalent to (100–16)/2 = 42%‡

* Amount of t-RNA required to release 40 mg of pseudouridine (ψU)

$$= \frac{\text{urinary } \psi\text{U (in mg)}}{\text{mole \%} \times \text{mol. wt of } \psi\text{U}} \times (\text{av. mol. wt of nucleotide}) \times 100$$

$$= \frac{40}{3{\cdot}5 \times 244{\cdot}2} \times 328 \times 100 = 1535 \text{ mg of t-RNA.}$$

† Four mg; isolated on the basis of isotope dilution technique (G. B. Chheda and C. F. Piskorz, unpublished results).

‡ In order to determine the potential mole % of uric acid that could be derived from adenosine and guanosine released from t-RNA, an average of 16 mole % of the modified nucleosides was subtracted from 100. Then considering that half of the major nucleosides were purines, the resulting figure was divided by 2.

uric acid in urine are in fair agreement. Our recent measurement of Ado-CO-thr by the isotope dilution technique using Ado-CO-thr-8-^{14}C showed that Ado-CO-thr is excreted in the range of 4 to 4·2 mg/day in normal human urine.* This figure is in good agreement with the calculated value.

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* G. B. Chheda and C. F. Piskorz, unpublished results.

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