Evidence That the Nucleic Acid Base Queuine Is Incorporated Intact into tRNA by Animal Cells†

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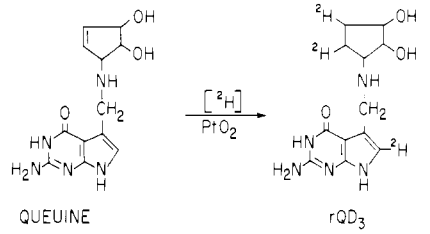
ABSTRACT: Queuine (the base of queuosine, Q) catalytically reduced with tritium or deuterium yields a derivative in which the proton at C-8 (purine numbering system) has been exchanged and the cyclopentene ring has been reduced to a cyclopentane ring. Mouse fibroblast tRNA has been labeled by culturing the cells in medium supplemented with [^{3}H]- and [^{2}H]dihydroqueuine. Such tRNA yields, upon hydrolysis, the nucleoside dihydroqueuosine and a saccharide derivative of dihydroqueuosine. Each product has been identified unambiguously by mass spectrometry and chromatography. Both the ^{3}H- and ^{2}H-labeled material coeluted, and no unlabeled Q nucleoside was found. Therefore, dihydroqueuine is incorporated intact into tRNA in mammalian cells. Furthermore, fractionation of the labeled tRNA on concanavalin A–agarose, which specifically binds the mannosyl-Q-containing tRNA^{Asp}, has shown that the dihydroqueuosine-containing tRNA^{Asp} is mannosylated. This is the first direct evidence that queuine is incorporated intact into mammalian tRNA in vivo.

The modified nucleoside Q[1] occurs exclusively in the first position of the anticodon of specific tRNAs (Kasai et al., 1975a). In mammalian cells, Q is found in tRNA^{His} and tRNA^{Asn}, galactosyl-Q is found in tRNA^{Tyr}, and mannosyl-Q is found in tRNA^{Asp} (the hexose moieties are attached to C-4 of the cyclopentenediol substituent of Q) (Kasai et al., 1976). The function of Q is unknown: long-term Q-deficient, germ-free mice appear normal (Farkas, 1980; Reyniers et al., 1981), and the only phenotypic change thus far observed in an *Escherichia coli* mutant that lacks Q in its tRNA is a marked reduction in viability in the stationary phase of growth (Noguchi et al., 1982). On the other hand, in intact *Xenopus* oocytes, (Q–)tRNA^{Tyr} from *Drosophila* is a suppressor of the amber (UAG) stop codon of tobacco mosaic virus RNA, while the otherwise identical *Drosophilia* (Q+)tRNA^{Tyr} is not (Bienz & Kubli, 1981).

The appearance of Q in tRNA is unique in that it results from a base exchange: in eukaryotes, guanine in the unmodified tRNA is excised and replaced with queuine, the base of Q, to form (Q+)tRNA (Katze & Farkas, 1979; Shindo-Okada et al., 1980; Crain et al., 1980). The enzyme for this base exchange, tRNA-guanine ribosyltransferase (EC 2.4.2.29), has been purified from rabbit erythrocytes (Howes & Farkas, 1978), rat liver (Shindo-Okada et al., 1980), and wheat germ (Walden & Farkas, 1981). The *E. coli* enzyme differs from the eukaryotic ones in that 7-(aminomethyl)-7-deazaguanine is inserted into the tRNA (queuine is not a substrate for the bacterial enzyme), with completion of the synthesis of Q occurring at the polynucleotide level (Okada et al., 1979). In mice, however, queuine is not synthesized de novo but must be obtained from the diet or gut flora (Farkas, 1980; Reyniers et al., 1981). Significant levels of free queuine occur in plant and animal products common to the diet (Katze et al., 1982). Moreover, the ability of germ-free mice to use dietary (Q+)tRNA as a source of queuine implies a mechanism for the salvage of free queuine from dietary (Q+)tRNA (Reyniers et al., 1980), and the salvage of Q base subsequent to the degradation of endogenous tRNA has been demonstrated in tissue-cultured cells (Gündüz & Katze, 1982) and in vitro (Gündüz & Katze, 1984).

Because queuine is incorporated intact into tRNA in vitro (Katze & Farkas, 1979; Shindo-Okada et al., 1980), it has been assumed that exogenous queuine also is incorporated intact into tRNA in vivo. However, the lack of isotopically labeled queuine has prevented a direct test of queuine incorporation into tRNA in vivo. In the present study, two labeled queuine analogues have been synthesized and used as substrates for the insertion reaction in vivo. The labeled compound [^{2}H]dihydroqueuine, rQD$_3$, was prepared by catalytic reduction and exchange of queuine (eq 1) to provide a product



(1)

having deuterium labels in both the upper and lower portions of the molecule. The radioactive analogue [^{3}H]dihydroqueuine, rQT$_3$, was prepared similarly. The mass spectrum of the isolated nucleoside from tRNA can therefore be used to indicate whether incorporation of the intact base has taken place by establishing the integrity of both the upper and lower portions of the molecule.

Tissue-cultured mammalian cells readily incorporate exogenous queuine into their tRNAs. The mouse fibroblast cell

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[1] Abbreviations: queuosine or Q, 5-{[((1*S*,4*S*,5*R*)-4,5-dihydroxy-2-cyclopenten-1-yl)amino]methyl}-7-deazaguanosine (Nishimura, 1983), queuine is the corresponding base; rQD$_3$, queuine reduced with deuterium in the cyclopentene ring and with H replaced by ^{2}H at C-8 in the deazaguanine nucleus; rQT$_3$, tritiated derivative of queuine analogous to rQD$_3$, above; Q*, queuosine containing galactose or mannose bound to C-4 of the cyclopentenediol substituent; (Q+)tRNA, tRNA that contains Q in the first position of the anticodon; RPC-5, reversed-phase 5 chromatography; HPLC, high-performance liquid chromatography; AMT, *N*-(aminomethylene)-2,2,2-trifluoroacetamide; Me$_3$Si, trimethylsilyl (TMS in structures); M, molecular ion; A_{260} unit, amount of material that has an absorbance of 1.0 at 260 nm when dissolved in 1 mL of water and measured with a 1-cm light path.

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line L-M, when cultured in serum-free medium, contains only traces of (Q+)tRNA, but the addition of saturating amounts of queuine to the cultures brings about nearly complete conversion to (Q+)tRNA within 24 h (Katze & Farkas, 1979). L-M cell tRNA has been labeled by culturing the cells in medium supplemented with rQD_3 and/or rQT_3, and the labeled nucleosides isolated from this tRNA have been analyzed by mass spectrometry.

Materials and Methods

Queuine Isolation. Queuine was isolated from third trimester bovine amniotic fluid (Irvine Scientific Co., Santa Ana, CA). Amniotic fluid, 12 L, was filtered through glass wool and then stirred with washed and defined Dowex 50 (H^+), about 2000 g dry wt, for 45 min. The Dowex 50 was allowed to settle, the supernatant fluid was decanted, and the Dowex 50 was transferred to a sintered glass Büchner funnel (3-L capacity), followed by successive elutions with H_2O (12 L), 1.5 N HCl (12 L), H_2O (12 L), and 3 N NH_4OH (3 L). Queuine was found in the dark-colored material eluted by the NH_4OH; this material was pooled, NH_3 was removed by rotary evaporation (room temperature), and then the material was lyophilized. The resultant product was suspended in 300–400 mL of H_2O, clarified by centrifugation, applied to a column of Sephadex G-10 (100 × 5 cm) in H_2O, eluted with H_2O until the effluent shows negligible absorbance at 280 nm, and then eluted with 0.02 M HCl. The material absorbing at 280 nm in the 0.02 M HCl eluate was pooled and lyophilized. Typically, the yield at this point was about 1200 A_{260} units of 40% pure queuine. This crude queuine was dissolved in 1.0 mL of H_2O and subjected in two 0.5-mL portions to preparative HPLC (Altex Ultrasphere-ODS, 1 × 25 cm), with mobile phase A (see Other Methods). The queuine-containing effluent, eluting between 15 and 24 min, was pooled and lyophilized. Queuine is essentially homogeneous at this point. The yield varies between 50 and 500 A_{260} units (2–19 mg), the result of variation in the amniotic fluid lots.

Synthesis of rQD_3 and rQT_3. Queuine was catalytically reduced with deuterium gas and PtO_2 following a procedure suitable for reduction of the double bond and for deuterium exchange at C-8 (Kinoshita et al., 1981). Platinum(IV) oxide, PtO_2 (8 mg, Kawaken Fine Chemicals Co., Ltd., Tokyo, Japan), was added to a 25-mL three-neck round-bottom flask containing 99.8% D_2O (8 mL, Aldrich Chemical Co., Milwaukee, WI). A needle, connected to a nitrogen gas line, was inserted through the rubber stopper in a side neck. A second opening was connected to a deuterium gas line in a similar manner; the third was connected to a gas bubbler, for introduction of queuine. The reaction flask was purged with nitrogen gas, which then was replaced by deuterium gas (Matheson Coleman and Bell, East Rutherford, NJ). The suspension was magnetically stirred while slowly passing deuterium gas through the reaction mixture for 1 h at room temperature. Deuterium gas in the reaction vessel was flushed with nitrogen gas before the addition of queuine (8 mg). Nitrogen atmosphere inside the vessel was replaced with deuterium gas again. The reaction mixture was continuously stirred while deuterium gas was bubbled slowly through the mixture for 6 h at room temperature. After centrifugation, the aqueous phase was removed and dried in a rotary evaporator. The reaction was essentially complete, on the basis of mass spectrometry (see following section) and HPLC analysis using a C_{18} column (Water Associates μBondapak C_{18}, 0.4 × 30 cm) and mobile phase B, 1 mL/min; the detector wavelength was 254 nm. The retention time of rQD_3 was 15.2 min, while that of queuine was 17.2 min.

rQT_3 (0.12 Ci/mmol) was prepared from queuine (by Amersham, Arlington Heights, IL), under the catalytic reduction conditions described for rQD_3. Before use, both rQD_3 and rQT_3 were repurified by HPLC, as described for queuine (above).

Preparation of AMT-Me_3Si Derivatives for Mass Spectrometry. The samples, after being dried under vacuum over P_2O_5, were converted to Me_3Si and AMT-Me_3Si derivatives by adding *N,O*-bis(trimethylsilyl)-2,2,2-trifluoroacetamide plus 1% trimethylchlorosilane, dimethylformamide, and pyridine (20:1:1) and heating at 95 °C for 45 min (Crain et al., 1980; Sethi et al., 1983a).

Preparation of rQD_3- and rQT_3-Labeled L-M Cell tRNA. The L-M cell line (American Type Culture Collection, Rockville, MD) was maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 0.5% Bactopeptone (DIFCO). For preparative cell growth, the medium was further supplemented with penicillin (100 units/mL) and streptomycin (100 μg/mL). To label L-M cell tRNA with rQD_3 or rQT_3, confluent sheets of L-M cells, cultivated either in flasks (Corning, 150-cm^2 growing surface) plus 25 mL of medium or in roller bottles (Bellco, 690-cm^2 growing surface) with 75 mL of medium, were exposed for 24 h to medium supplemented with the derivative (0.01 A_{260} unit/mL), followed by cell harvest and tRNA isolation (Katze, 1978). In some instances, in order to increase the yield of labeled tRNA, the rQD_3- or rQT_3-supplemented medium, which had already been in contact with L-M cells for 24 h, was filter sterilized and used a second time.

Isolation of Nucleosides from tRNA. The rQD_3- and rQT_3-labeled nucleosides were isolated from alkaline hydrolysates of the labeled tRNA. Alkaline hydrolysis (0.3 M KOH, 16 h, 37 °C, in the dark) and isolation of the Q-like mononucleotides by Dowex 1 (formate form) chromatography were as described by Kasai et al. (1975b). The mononucleotide fraction, after evaporation to dryness, was dissolved in 0.1 mL of 0.01 M Tris-HCl (pH 8.3) and incubated (37 °C) with 2 μg of *E. coli* alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) for 1 h, following which an additional 2 μg of enzyme was added and incubation was continued for 1 h. Finally, the entire incubation mixture was subjected to HPLC (Altex Ultrasphere-ODS, 0.46 × 25 cm; mobile phase C, 1.0 mL/min) to resolve the products. This procedure also was used to isolate Q from *E. coli* tRNA (Sigma), as well as Q and Q* from mouse liver tRNA.

Mass Spectrometry. AMT-Me_3Si derivatives were analyzed on a Varian MAT 731 mass spectrometer, under the following conditions: ionizing energy 70 eV; ion-source temperature 250 °C; resolution 1300; sample introduction by direct probe following removal of reagent and solvent in the probe vacuum lock. Mass spectra were recorded by conventional magnetic scanning over a wide mass range as well as by electric scanning at a fixed magnetic field over a limited range. In the case of electric scanning, a Nicolet 1170 signal averager and pulse-counting electronics were used to enhance the signal-to-noise ratio. Exact mass measurements were made at a resolving power of 10 000 with the same signal averager. Mass assignments were made in Ultramark 1621 (L), from PCR, Inc., Gainesville, FL.

Other Methods. tRNA isolation, aminoacylation by a crude mouse liver aminoacyl-tRNA synthetase preparation, recovery of aminoacyl-tRNAs by DEAE-cellulose chromatography, and RPC-5 chromatography were as reported earlier (Katze, 1978; Katze & Farkas, 1979). The use of concanavalin A–Sepharose (Sigma) to isolate the mannosyl-Q containing $tRNA^{Asp}$ was

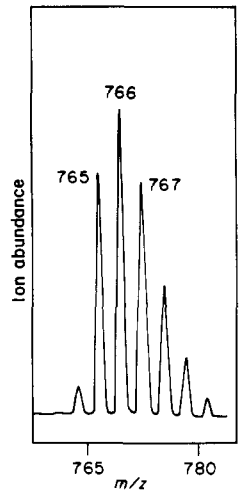


FIGURE 1: Molecular ion region from the mass spectrum of AMT-Me_3Si derivative of the deuterated queuine analogue rQD_3.

as described by Okada et al. (1977). The following mobile phases were employed for HPLC: (A) 0.05 M ammonium formate, pH 4.0–methanol (97:3 v/v) (D. Horton et al., unpublished data); (B) 0.01 M ammonium formate, pH 5.1–methanol (90:10 v/v); (C) 0.05 M ammonium formate, pH 4.0–methanol (95.5:4.5 v/v); (D) 0.005 M heptanesulfonate–0.02 M ammonium acetate, pH 3.2–methanol–tetrahydrofuran (85:14.5:0.5 v/v). All HPLC was performed at room temperature.

Results

Characterization of rQD_3 and rQT_3. The mass spectrum of the AMT-Me_3Si derivative of rQD_3 was compared with the spectrum of the AMT-Me_3Si derivative of queuine (Sethi et al., 1983b) and shown to contain one deuterium atom in the deazaguanine nucleus, as well as a net addition of two deuteriums to the cyclopentenediol side chain, by reduction of the double bond. From the molecular weight of 765 and expected course of the exchange reactions (Kinoshita et al., 1981), these data confirmed the expected structure of rQD_3 shown in eq 1. The resulting molecular ion pattern, shown in Figure 1, is accounted for mostly by natural heavy isotopes of carbon and silicon and reveals incorporation of approximately 47% of a fourth deuterium atom, as shown from the abundance ratio of m/z 765/766. We have demonstrated elsewhere that rQD_3 and rQT_3 are substrates for tRNA-guanine ribosyltransferase in intact cells; however, the reduced queuine derivatives are only about 10% as active as queuine (Gündüz & Katze, 1982; Katze et al., 1983).

Isolation of rQD_3- and rQT_3-Labeled Nucleosides from L-M Cell tRNA. In order to provide sufficient amounts of the isolated nucleosides for mass spectrometric analysis, as well as to provide an internal radioactive label, three preparations of dihydroqueuine-labeled tRNA were studied (Table I). A mixture of these preparations (226 A_{260} units, preparation 1; 157 A_{260} units, preparation 2; 10 A_{260} units, preparation 3) was subjected to alkaline hydrolysis and Dowex 1 chromatography. The resultant chromatogram (not shown) contained an absorbance peak in the position characteristic for Q and Q* monophosphates (Kasai et al., 1975b), which coincided with a radioactive peak that contained greater than 90% of the recovered radioactivity. This material was pooled, evaporated to dryness, and treated with alkaline phosphatase, and

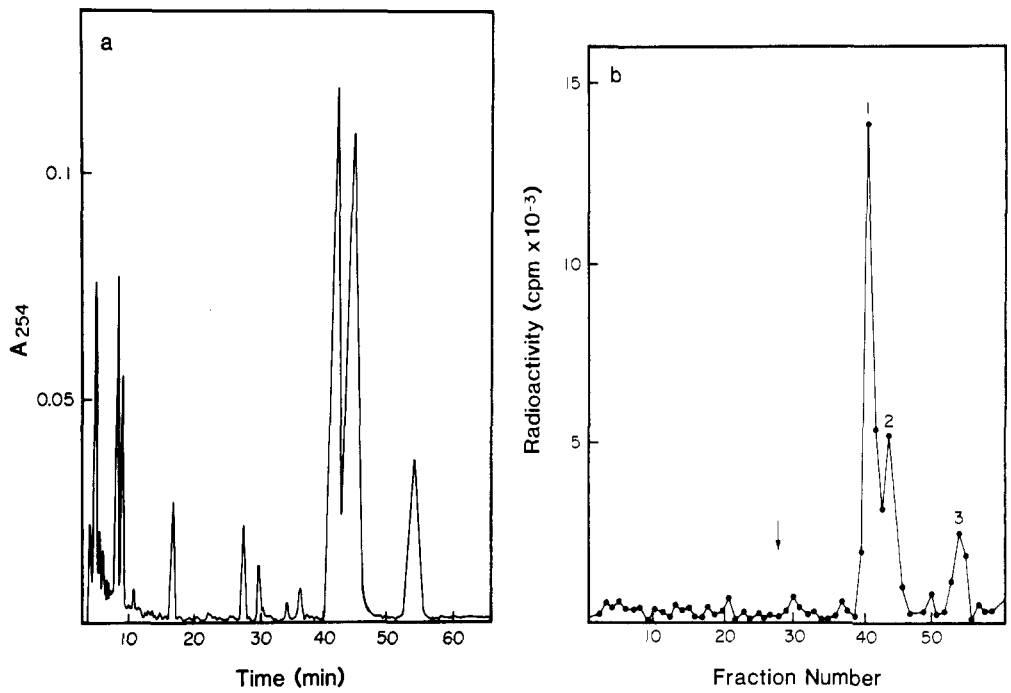


FIGURE 2: HPLC chromatogram of the labeled nucleosides derived from L-M cell tRNA. The labeled L-M cell tRNA preparations described in Table I were mixed (226 A_{260} units, preparation 1; 157 A_{260} units, preparation 2; 10 A_{260} units (273 000 cpm), preparation 3) and subjected to alkaline hydrolysis, followed by Dowex 1 chromatography and then alkaline phosphatase digestion, to yield a mixture of the rQD_3- and rQT_3-labeled nucleosides. These nucleosides were resolved on a C_{18} column (Altex Ultrasphere-ODS, 0.46 × 25 cm) with mobile phase C, 1.0 mL/min. Sample injection (50 μL) was at zero time. The effluent was monitored at 254 nm (a). All fractions (b) were 1 min (1 mL), except for fraction number 42 (its collection was terminated and advanced to fraction 43 just beyond the A_{254} inflexation point following peak 1), which was approximately 0.5 min (0.5 mL). Radioactivity was determined on 10-μL portions of the fractions and is presented as total radioactivity per fraction. We attribute the quantitative difference between the elution profiles for A_{254} (a) and radioactivity (b) to result from different product ratios in the rQD_3- and rQT_3-labeled tRNA preparations. Appropriate fractions were pooled and evaporated to dryness: fractions 40–42 for component 1 (yield 9 μg, 21 000 cpm); fractions 43–45 for component 2 (yield 9 μg, 12 000 cpm); fractions 53–55 for component 3 (yield 2 μg, 5300 cpm). In (b) the arrow indicates the expected elution position of Q nucleoside (determined in a separate experiment, not shown).

Table I: Preparation of rQD_3- and rQT_3-Labeled tRNA[a]

prepn	label	roller bottles (N)	yield of packed cells (mL)	yield of tRNA (A_{260} units)	(Q+)-$tRNA^{Asp}$ (%)[b]
1	rQD_3	62	26	230	78
2	rQD_3[c]	64	25	177	78
3	rQT_3	4	4	20	79

[a] L-M cells were exposed to rQD_3 or rQT_3 (0.01 A_{260} unit/mL) for 24 h, followed by cell harvest and tRNA isolation. [b] The percent (Q+)$tRNA^{Asp}$ was determined by aminoacylation with [^{14}C]aspartic acid, followed by RPC-5 resolution of (Q+)$tRNA^{Asp}$ and (Q–)$tRNA^{Asp}$, as described by Katze & Farkas (1979). [c] The rQD_3-containing medium that had already been in contact with L-M cells for 24 h (preparation 1) was filter sterilized and used for preparation 2.

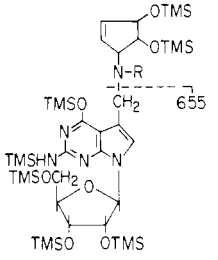
Table II: Isotopic Abundance Pattern from the Mass 655 Region Representing the Deazaguanosine Moiety

m/z	relative intensity Q[a]	$[^2H_3]Q$[b]	$[^2H_3]Q^*$[c]
655	10		
656	43	17	17
657	26	31	32
658	15	29	28
659	5	17	16
660		7	8

[a] Unlabeled Q nucleoside. [b] Component 2 from HPLC separation, Figure 2. [c] Component 1 from HPLC separation, Figure 2.

the resultant rQD_3- and rQT_3-labeled nucleosides were isolated by HPLC (Figure 2). The radioactive fractions were pooled separately, evaporated to dryness, and then analyzed by mass spectrometry. It is notable that both the rQD_3- and rQT_3-labeled material coeluted. Furthermore, no unlabeled Q nucleoside was found. These results are consistent with dihydroqueuine incorporation as an intact unit into mammalian tRNA.

Characterization of rQD_3- and rQT_3-Labeled Nucleosides from L-M Cell tRNA. Trimethylsilylation of Q nucleoside by *N,O*-bis(trimethylsilyl)-2,2,2-trifluoroacetamide in dimethylformamide characteristically produces a mixture of derivatives having structures **1** and **2** (Sethi et al., 1983a). The



1, R = –CH=NCOCF$_3$
2, R = –TMS

molecular ion (m/z 1036 for **1**) and m/z 655 ion (Kasai et al. 1975a) may be used to indicate structural or isotopic changes in the cyclopentyl and deazaguanine moieties.

Components 1 and 2 (Figure 2b) were derivatized, and then mass spectra were recorded first over the mass range from 500 to 1700 daltons to provide an overview of sample integrity. High-sensitivity mass spectra were measured for narrow mass ranges, which included the molecular ion region of Q and Q*. It was established that those derivatives most suitable for analysis corresponded to the AMT-Me_3Si derivative of Q nucleoside in component 2 (analogous to structure **1**) and the

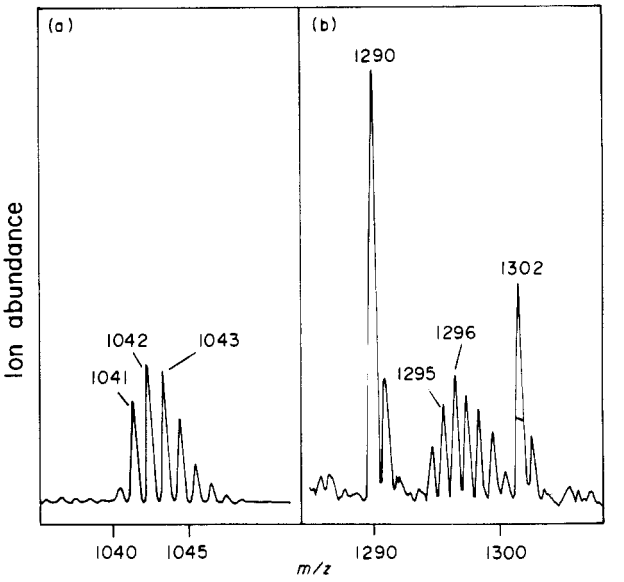
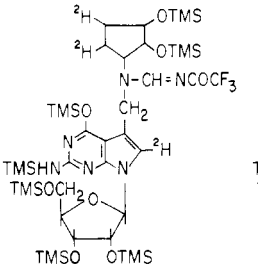


FIGURE 3: Mass spectrum of the molecular ion region from (a) the AMT-Me_3Si derivative of component 2 and (b) the trimethylsilyl derivative of component 1, from L-M cell tRNA. Reference ions m/z 1290.0 and 1302.0 in (b) are derived from a fluorocarbon internal mass standard, used for mass assignment.

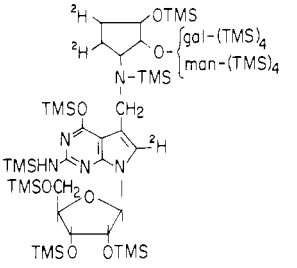
Table III: Exact Mass Values

sample	$C_{12}H_9{}^2HN_4O_5(Me_3Si)_3$ found	theoretical	error (ppm)
component 2	656.3084	656.3080	0.8
component 1	656.3083	656.3080	0.8

trimethylsilyl derivative of Q* in component 1 (analogous to structure **2**). The molecular ion pattern (Figure 3a) from component 2 is 5 mass units greater than that of **1** and, when correlated for differences in the naturally occurring isotopes, is very similar to that exhibited by rQD_3 (Figure 1). A 1 mass unit shift is observed for m/z 655 and its companion ion resulting from hydrogen rearrangement from the cyclopentyl moiety (Kasai et al., 1975a), shown in Table II. That this component is the Q nucleoside analogue of rQD_3 was established unambiguously by measurement of the exact mass of the characteristic (Sethi et al., 1983b) m/z 656 fragment ion, Table III. These results correspond to structure **3**, in which



3, M = 1041



4, M = 1296

one deuterium resides in the deazaguanine moiety (shift of 1 mass unit) and the cyclopentene double bond has been saturated by deuterium (shift of 4 mass units), giving a molecular weight increase of 5 mass units when compared to that of **1**. The results show component 2 to be the Q nucleoside analogue derived by incorporation of the intact rQD_3 base into the tRNA. The constituent of component 1 shows a molecular weight of 1296 (Figure 3b), corresponding of the Me_3Si derivative (as in structure **2**) of the rQD_3 analogue of the hy-

Table IV: Concanavalin A-Sepharose Fractionation of a Mixture of rQT_3-Labeled L-M Cell and Mouse Liver tRNAs[a]

source	unfractionated tRNA				unbound fraction				bound fraction			
	A_{260} units	cpm	acceptance (pmol/A_{260} unit) Asp	His	A_{260} units	cpm	acceptance (pmol/A_{260} unit) Asp	His	A_{260} units	cpm	acceptance (pmol/A_{260} unit) Asp	His
mouse liver	17.2		24	19								
rQT_3-labeled L-M cell	1.3	110 000	ND	ND								
mouse liver plus rQT_3-labeled L-M cell					16.8	53 000	3.5	23	0.5	58 000	720	1.9

[a] Fractionation was performed as described by Okada et al. (1977). The tRNA was dissolved in 0.1 mL of 0.05 M sodium acetate buffer (pH 6.0), 0.9% NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ and loaded on a column of concanavalin A-Sepharose (0.6 × 12 cm) that had been equilibrated with the same buffer. Elution was continued with the same buffer until, beginning at fraction 82, the tRNA bound to the column was eluted with the above buffer containing 50 mM methyl α-D-glucopyranoside. The flow rate was 4.3 mL/h; 0.86-mL fractions were collected. Chromatography was carried out at 4 °C. Fractions 5-12 were pooled for *unbound fraction.* Fractions 84-89 were pooled for *bound fraction.* ND indicates not determined.

permodified nucleoside Q*, structure **4**. This assignment is confirmed by the exact mass of the m/z 656 fragment ion shown in Table III and the isotopic pattern shown in Table II.

The lower isotope peaks observed at m/z 1294 and 1295 evidently result from partial loss of deuterium in the cyclopentyl ring, due to unknown causes. In view of the isotopic distribution shown in the molecular ion pattern, the similarity of m/z 656 region patterns from components 1 and 2 is also unexplained because H (or ^2H) from the cyclopentyl moiety is present in this ion (Kasai et al., 1975a). However, the positional source of rearranged H is unknown, thus leading to uncertainty as to how the m/z 656 region pattern should be affected. In any event, the principal product, M_r 1296, corresponds to the Q* analogue derived from insertion of the intact rQD_3 base, prior to glycosylation that occurs as a separate step (Okada & Nishimura, 1977).

Figure 2b indicates the presence of a third component that contained the radiolabel. A volatile derivative was not formed on trimethylsilylation of component 3; therefore, attempts to characterize it by mass spectrometry failed.

Evidence That rQD_3- and rQT_3-Labeled L-M Cell tRNA Is Mannosylated in Vivo. The preceding having established that component 1, Figure 2, is the rQD_3 analogue of nucleoside Q*; we sought to further characterize the hexose moiety (moieties) attached to the cyclopentane substituent of this Q* analogue. Concanavalin A–Sepharose specifically binds the mannosyl-Q-containing $tRNA^{Asp}$ species (Okada et al., 1977). Therefore, rQT_3-labeled L-M cell tRNA was mixed with mouse liver tRNA and subjected to concanavalin A–Sepharose fractionation. The results (Table IV) indicated that the following. (1) Mouse liver $tRNA^{Asp}$ is significantly enriched in the bound fraction and is, therefore, almost completely in the mannosyl-Q form, as expected from previous results with rabbit and rat liver tRNAs (Okada et al., 1977). (2) Approximately 50% of the rQT_3-labeled L-M cell tRNA cofractionated with the mannosyl-Q $tRNA^{Asp}$. Moreover, when the bound fraction was aminoacylated with [^{14}C]aspartate and subjected to RPC-5 separation, the radioactive elution profile (not shown) corresponded to that expected for (Q+)$tRNA^{Asp}$ alone, and the [^{3}H]rQT_3-derived label coeluted with the [^{14}C]aspartate-derived label.

In a separate experiment, rQT_3-labeled nucleosides were isolated (as above) from the concanavalin A–Sepharose bound and unbound fractions of the rQT_3-labeled L-M cell tRNA. When subjected to HPLC analysis (Altex Ultrasphere-ODS, 0.46 × 25 cm; mobile phase D, 1 mL/min), the labeled nucleoside isolated from the bound rQT_3-labeled tRNA corresponded to component 1, Figure 2 (elution time 11 min); that from the unbound tRNA corresponded to components 2 and 3 (elution time 14 min; components 2 and 3 are not resolved by mobile phase D). These results indicate that component 1 is the mannosyldihydro-Q nucleoside and are consistent with our conclusion (above) that component 2 is the dihydro-Q nucleoside.

Discussion

The present study provides the first direct evidence that the Q base is incorporated intact into mammalian tRNA, in vivo, to yield (Q+)tRNA. By contrast, in *E. coli*, only the lower portion of the base is inserted as a unit following transcription, while the cyclopentenediol moiety is added in one or more subsequent steps (Okada et al., 1979).

Our data from rQD_3 and rQT_3 labeling indicate not only that the 7-deazaguanine and cyclopentanediol units are incorporated intact but also that L-M cells do not oxidize the cyclopentanediol moiety of the reduced analogue to the natural cyclopentenediol state. Furthermore, mannosyldihydro-Q-containing $tRNA^{Asp}$ is formed. Whether the galactosyldihydro-Q nucleoside is formed as well remains to be determined. The present results are in agreement with the recent report (Singhal & Vakharia, 1983) that the (Q+)$tRNA^{Asp}$ of L-M cells is mannosylated.

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Registry No. Queuine, 72496-59-4; dihydroqueuine, 88510-79-6.

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Affinity Labeling of *Escherichia coli* DNA Polymerase I with Thymidine 5′-Triphosphate and 8-Azidoadenosine 5′-Triphosphate: Conditions for Optimum Labeling, Specificity, and Identification of the Labeling Site†

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ABSTRACT: We find that *Escherichia coli* DNA polymerase I, unlike other template-dependent DNA polymerases, has a unique ability to covalently link the bound deoxynucleoside triphosphate as well as 8-azido-ATP upon exposure to ultraviolet light energy. The conditions for the optimum cross-linking of deoxynucleoside triphosphate as well as 8-azido-ATP, a photoaffinity analogue of ATP, to *Escherichia coli* DNA polymerase I have been established. The cross-linking produced via the photoaffinity probe is at least 1 order of magnitude higher than those produced with unsubstituted deoxynucleoside triphosphates. However, in spite of these quantitative differences in the efficiency of cross-linking, the requirements for and characteristics of the cross-linking reactions with both probes are quite similar and corroborate results obtained from the equilibrium dialysis studies of Kornberg and colleagues [Englund, P. T., Huberman, J. A., Jovin, T. M., & Kornberg, A. (1969) *J. Biol. Chem. 244*, 3038–3044]. Thus, the cross-linking of thymidine 5′-triphosphate (dTTP) and 8-azido-ATP to *Escherichia coli* DNA polymerase I is (1) strictly dependent on the presence of a divalent cation, (2) competitively inhibited in the presence of deoxynucleoside and nucleoside triphosphates, (3) only slightly inhibited in the presence of deoxynucleoside monophosphate or pyrophosphate, and (4) sensitive to pyridoxal 5′-phosphate, a triphosphate binding site directed inhibitor of DNA polymerases. Tryptic peptide analysis of *Escherichia coli* DNA polymerase I, labeled with [^{32}P]dTTP and 8-azido[^{32}P]ATP, revealed that the major site of cross-linking of both dTTP and 8-azido-ATP resides in a neutral peptide.

The enzymatic synthesis of DNA is a complex process that involves multiple components (Kornberg, 1980). Although the general features of this catalytic process have been clarified, the mechanism of template-directed base selection is not yet understood. *Escherichia coli* DNA polymerase I (pol I)[1] is the prototype DNA polymerase, and extensive characterization and physical analysis of this enzyme have been carried out (Kornberg, 1980; Lehman & Uyemura, 1976). Recently, through genetic manipulation, the gene coding for pol I has been cloned and sequenced (Joyce et al., 1982), and producer lysogen has been constructed (Kelley et al., 1977; Kelley & Stump, 1979). This development resulted, in turn, in the elucidation of the entire primary amino acid sequence of pol I (Joyce et al., 1982; Brown et al., 1982). These advances, together with the fact that homogeneous preparations of pol I may now be obtained in relatively large quantities, made this enzyme attractive for structure–function studies of substrate binding sites of template-dependent DNA polymerases. Earlier, we had initiated such studies for the development of site-specific reagents using reverse transcriptase from avian

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[1] Abbreviations: pol I, *Escherichia coli* DNA polymerase I; AMV, avian myeloblastosis virus; RLV, Rauscher leukemia virus; dNTPs, deoxynucleoside triphosphates; UV, ultraviolet light; PyP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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