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Journal of Chromatography B, 801 (2004) 237–247
JOURNAL OF CHROMATOGRAPHY B
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# Determination of queuosine derivatives by reverse-phase liquid chromatography for the hypomodification study of Q-bearing tRNAs from various mammal liver cells

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Received 21 July 2003; received in revised form 7 November 2003; accepted 17 November 2003

**Abstract**

Three queuosine derivatives (Q-derivatives) have been found at position 34 of four mammalian so-called Q-tRNAs: queuosine (Q) in $tRNA^{Asn}$ and $tRNA^{His}$, mannosyl-queuosine (manQ) in $tRNA^{Asp}$, and galactosyl-queuosine (galQ) in $tRNA^{Tyr}$. An analytical procedure based on the combined means of purified tRNA isolation from liver cells and ribonucleoside analysis by reverse-phase high performance liquid chromatography coupled with real-time UV-spectrometry (RPLC-UV) was developed for the quantitative analysis of the three Q-derivatives present in total tRNA from liver tissues and liver cell cultures. Using this analytical procedure, the rates of Q-tRNA modification were studied in total tRNAs from various mammalian hepatic cells. Our results show that the four Q-tRNAs are fully modified in liver tissues from adult mammals, regardless of the mammal species. However, a lack in the Q-modification level was observed in Q-tRNAs from newborn rat liver, as well in Q-tRNAs from normal rat liver cell cultures growing in a low queuine content medium, and from a rat hepatoma cell line. It is noteworthy that in all cases of Q-tRNA hypomodification, our analytical procedure showed that $tRNA^{Asp}$ is always the least affected by the hypomodification. The biological significance of this phenomenon is discussed.


*Keywords:* Queuosine; tRNA

## 1. Introduction

The hypermodified nucleosides designated as queuosine derivatives (Q-derivatives) are commonly present in transfer ribonucleic acid (tRNA) throughout all kingdoms of life from procaryotes and eucaryotes, including plants, with the exception of archeabacteria, mycoplasma, and yeast species [1,2]. These Q-derivatives are located at the first position of the anticodons of the tRNAs that recognize NAU and NAC codons (His, Asn, Asp, and Tyr) [3].

Four different Q-derivatives have been characterized so far. Among these Q-derivatives, the so-called queuosine (Q) was first described in the "wobble" position of the anticodon (nucleotide 34) of $tRNA^{His}$, $tRNA^{Asn}$, $tRNA^{Asp}$, and $tRNA^{Tyr}$ from *Escherichia coli* B strain [3]. The structure of Q was determined as 7-(4,5-cis-dihydroxy-1-cyclopenten-3-yl-aminomethyl)-7-deazaguanosine [4]. A 2,3-epoxy derivative of Q, called oQ, has been later characterized in $tRNA^{Tyr}$ from *E. coli* MRE 600 strain [5]. Two other Q-derivatives were found in animal species. These latter derivatives beared either a D-mannosyl or a D-galactosyl residue bound by a β-glycoside link to the

*Abbreviations:* Q, queuosine; manQ, mannosyl-queuosine; galQ, galactosyl-queuosine; Q-derivative, queuosine (Q) or its glycosylated derivatives (manQ or galQ); $[Q^+]$tRNA, tRNA that contains Q (or manQ or galQ) in the first position of the anticodon; $[Q^-]$tRNA, the precursor of $[Q^+]$tRNA which contains guanosine (G) instead of Q (or manQ or galQ) in the first position of the anticodon; RPLC-UV, reverse-phase high performance liquid chromatography coupled with real-time UV-spectrometry

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doi:10.1016/j.jchromb.2003.11.022

oxygen atom of carbon-4 of the cyclopentenediol ring of Q [6]. These two glycosylated Q-derivatives were called manQ for mannosyl-queuosine, and galQ for galactosyl-queuosine, and have been shown to be specific to animal $tRNA^{Asp}$ and $tRNA^{Tyr}$, respectively [7].

In eubacteria, synthesis of Q occurs de novo by a complex biosynthetic pathway going first through the formation of an intermediate base 7-(aminomethyl)-7-deazaguanine (preQ1) derived from guanosine triphosphate (GTP) [8]. The preQ1 base is further inserted into the first position of the anticodon (wobble position) of the four tRNAs mentioned above by a base to base exchange reaction with the guanine located at position 34 of the unmodified tRNA. This latter reaction is catalyzed by a procaryotic host tRNA-guanine transglycosylase (Tgt) [9]. The preQ1 contained in these tRNAs is further converted to epoxyqueuosine (oQ) [10,11], and finally to queuosine (Q) [12]. As already mentioned above, only the four tRNAs specific for His, Asn, Asp, and Tyr undergo that insertion. Both oQ and Q occur in procaryotic tRNAs (see in *E. coli* strains, further in the paper).

In contrast, eucaryotes are unable to synthesize neither the precursor nor the queuosine (Q) itself. They must uptake the base counterpart of queuosine, i.e. queuine, as a nutrient compound or from their gut flora [2,13]. Queuine is thus directly inserted into the eucaryotic tRNAs specific for His, Asn, Asp, and Tyr by a guanine exchange reaction involving an eucaryotic host tRNA-guanine transglycosylase [14]. In mammals, three different Q-derivatives, i.e. Q, manQ, and galQ, have been found to date in the so-called Q-tRNAs. All four Q-tRNAs are first modified at the wobble position by the insertion of queuine. Among these Q-tRNAs, the queuosine residues of $tRNA^{Asp}$ and $tRNA^{Tyr}$ are further glycosylated by mean of a mannose- and a galactose-tRNA-queuine glycosyltransferase, respectively [15].

In mammals, the overall amounts of Q-tRNAs bearing a Q-derivative in position 34, i.e. $[Q^+]$tRNAs, versus guanosine-bearing Q-tRNAs in the same position, i.e. $[Q^-]$tRNAs, have been shown to be dependent on the developmental and metabolic state of the considered tissues. Thus, undifferentiated cells [16–18], regenerating rat liver [19], and cultured cell lines [20] contain a large amount of $[Q^-]$tRNAs, whereas adult tissues [19] and differentiated cells [16–18] contain usually the four fully modified ones, i.e. $[Q^+]$tRNAs. Q hypomodification of the Q-tRNAs was also observed in various mammalian tumors [20–22], and its extent was described as correlated with the morphological grading of solid tumors, and as being for value for the prognosis of neoplastic diseases [23,24].

Up to date, the Q-hypomodification in mammalian undifferentiated or tumor cells was mostly determined enzymatically by measuring the incorporation of radioactive guanine into the unfractionated total tRNA obtained from the studied tissues [19–24]. This incorporation of radioactive guanine was performed in vitro using the *E. coli* tRNA-guanine transglycosylase which catalyses the exchange of guanine by queuine at position 34 of $[Q^-]$tRNAs, but not in $[Q^+]$tRNAs that bear already a queuine, whatever the origin of the tRNA molecules [19]. However, the reaction mixture of this in vitro enzymatic method must be free of any enzymatic inhibitors, and requires high amounts of radioactive guanine in order to obtain a full incorporation in $[Q^-]$tRNAs. In addition, such an enzymatic method allows only to determine the level of Q-hypomodification of Q-tRNAs, without being able to distinguish and determine the respective deficiency rate of each Q-tRNA species.

In our studies dealing with the Q-hypomodification in tRNAs from various animal liver cells (undifferentiated cells, replicative cell cultures, tumor cells, etc.), the question arose often whether or not the fourdifferent species of Q-tRNAs were either identically or specifically hypomodified. For this purpose, we developed an analytical procedure for the accurate analysis of the three different Q-derivatives and the corresponding $[Q^+]$tRNAs, to determine and quantify which Q-tRNA species could be hypomodified among the mixture of the unfractionated tRNAs.

The present work reports on the development of this analytical procedure based on the combined means of total tRNA isolation from mammalian cells, Q-derivative purification from enzymatic digests of tRNAs, and nucleoside analysis by reverse-phase high performance liquid chromatography coupled with real-time UV-spectrometry (RPLC-UV). We especially describe the analytical steps developed in our RPLC-UV system for the complete separation, the determination of the elution order, and the accurate quantitation of the three Q-derivatives present in total tRNAs from mammals. The analytical procedure was applied to the quantitative measurement of Q-derivatives and corresponding $[Q^+]$tRNAs in the total tRNAs isolated from liver tissues of various animal species, and from normal or neoplastic rat liver cell cultures. We report also in this paper on our preliminary results that show either a quantitative homogeneity or a significant difference in the levels of Q-modification, as compared to the type of the studied cell material and the species of Q-tRNAs.

## 2. Experimental

### 2.1. Normal rat liver epithelial cell cultures

The primary cultures of rat liver epithelial cells were obtained according to the method developed in our laboratory [25]. Newborn animals from the Wistar US/Commentry strain exclusively were used for these cell cultures. Livers were aseptically removed from newborn rats at 6–10 days post-partum without sexual distinction. The cell culture method involved fractional time dissociation of the tissue by trypsin under mechanical agitation. Each successive dissociation, up to 9, was carried out in Ham F10 culture medium [26] free of calcium and magnesium (Sigma, St Louis, USA), and containing 0.05–0.2% trypsin (Gibco Invitrogen SARL, Cergy-Pontoise, France). The dissociated

liver cells were seeded in culture dishes (60 mm × 15 mm) (Falcon no. 3009, Polylabo, Strasbourg, France) placed in an incubator at 37 °C under a water-saturated atmosphere of air–$CO_2$ (95–5%).

For these primary cell cultures, the growth medium was Ham F10 supplemented with 10% fetal calf serum (Gibco Invitrogen SARL, Cergy-Pontoise, France), and 10% human serum. The first subculture with the same growth medium was performed when the primary culture was near confluency. This occurred generally six to eight days after plating. After three to five passages in the same conditions, the resulting monolayer culture plates contained at least 85% rat liver epithelial cells. The subsequent subcultures were performed in 75 $cm^2$-area cell culture flasks (Falcon no. 3084, Polylabo, Strasbourg, France), using Ham F10 culture medium (Sigma, St Louis, USA) supplemented only with 10% fetal calf serum as growth medium. For specific experiments, some subcultures were grown on Ham F10 culture medium supplemented with 10% horse serum instead of 10% fetal calf serum.

### 2.2. Rat hepatoma cell line

The rat hepatoma cell cultures were obtained from the commercially available 1548 Morris cell line (American Type Culture Collection, CRL-1548). These neoplastic rat liver cells were grown with Ham F10 culture medium supplemented with 10% fetal calf serum.

### 2.3. Preparation of total RNA from animal liver tissues and liver cell cultures

Preparation of total RNA from both liver tissues and cultured liver cells was adapted from Chomczynski and Sacchi [27]. The following protocol describes the technical steps for RNA isolation from 1 g of rat liver tissue. Most of the biochemical reagents used for this protocol were provided by Sigma (St. Louis, USA).

Immediately after removal from the animal, the tissue was minced on ice and homogenized at room temperature in 10 ml of a denaturing solution containing 4 M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol, using a Ultra-Turax homogenizer (Polylabo, Strasbourg, France). To the homogenate were added successively 1 ml of 2 M sodium acetate pH 4.0, 10 ml of water-saturated phenol (Appligène, Illkirch, France), and 2 ml of chloroform-isoamyl alcohol (49:1, v/v), and thorough mixing by inversion was performed after the addition of each reagent. The final suspension was shaked vigorously for 10 s, cooled on ice for 15 min, and centrifuged at 10,000 × *g* for 20 min at 4 C. The aqueous phase supernatant was transferred to a fresh tube, and 10 ml of isopropanol were added to precipitate total RNA. The mixture was placed at −20 °C for at least one hour, and centrifuged at 10,000 × *g* for 20 min at 4 °C. The resulting RNA pellet was dissolved in 3 ml of the denaturing solution, and precipitated again with 3 ml of isopropanol at −20 °C for one hour. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the crude RNA pellet was washed with 3 ml of 75% ethanol; it was further collected by centrifugation, and was dried under vacuum.

This protocol of total RNA preparation was used for either small scale (3 mg liver tissue) or large scale (up to 30 g liver tissue) tRNA preparations from various mammals (newborn rats, beef, sheep, rabbit), and from other animal species such as chickens. The method was also used to isolate tRNA from rat liver cell lines grown as monolayer cultures. In this case, normal or neoplastic cultured cells were lysed directly in the tissue culture flasks by the addition of the above denaturing solution (1 ml of denaturing solution for 75 $cm^2$-area cell culture flask, i.e. for about 10 to 12 × $10^6$ total cells).

### 2.4. Isolation and purification of tRNA from total RNA

Until the last step of RNA preparation depicted above, ribonucleases were inhibited because of the presence of 4 M guanidinium thiocyanate. Therefore, no additional precautions were required to protect RNA from degradation. For the following steps devoted to the isolation and purification of total tRNA from crude RNA preparation, the saline solutions were prepared using distilled water that was treated with diethyl pyrocarbonate (DEPC), and sterilized.

The dried residue of crude RNA was dissolved in 1 ml of 2 M sodium acetate pH 4.5. After addition of 1 ml of 2 M sodium chloride, the mixture was maintained for 3 h at 4 °C to precipitate the high molecular weight RNAs (28S, 18S, and 5.8S rRNAs). The latter were eliminated by centrifugation at 10000 g for 30 min at 4 °C, and the tRNA-containing supernatant was submitted to precipitation with 3 volumes of ethanol at −20 °C for one hour. The precipitate was collected by centrifugation at 10000 g for 30 min at 4;°C, and the resulting pellet of tRNA was dissolved in 1 ml of 0.1 M sodium acetate buffer, pH 4.5. The solution was then applied to a 7 cm × 1 cm i.d. DEAE cellulose (DE 32 Whatman) column previously equilibrated with the same buffer. After washing the column with the sodium acetate buffer containing 0.3 M NaCl, the tRNA was eluted with the sodium acetate buffer containing 0.5 M NaCl. The total tRNA was finally recovered by precipitation with 3 volumes of ethanol at −20 °C and centrifugation, and was dried under vacuum.

### 2.5. Counter-current distribution and aminoacylation test of tRNAs from chicken liver

The partial purification of $tRNA^{Asp}$ and $tRNA^{Tyr}$ isoacceptors from chicken liver tRNA, was performed by counter-current distribution (CCD) adapted from Holley and Merrill [28] and Dirheimer and Ebel [29]. This technique permits the separation of 1–5 g of tRNA. From the CCD distribution pattern obtained for 2 g of chicken liver tRNA

[30], 28 fractions were collected and further submitted to aminoacylation tests, using crude aminoacyl-tRNA synthetases from chicken liver, as previously described [31], in order to localize precisely all tRNAs and especially the four [$Q^+$]tRNAs.

### 2.6. Isolation of $tRNA^{Asp}$ isoacceptors from chicken liver tRNA with Concanavalin A

The Concanavalin A lectin links to any organic molecules bearing an α,D-mannosyl residue. That is the case for manQ-bearing $tRNA^{Asp}$. We adapted from Wosnick and White [32] the technique using Concanavalin A for the isolation of $tRNA^{Asp}$ isoacceptors from chicken liver tRNA. The Concanavalin A coated to Sepharose 4B (Sepharose-Con A gel) was purchased from Sigma (St Louis, USA). The $tRNA^{Asp}$-enriched CCD fractions were pooled and submitted to drying under vacuum. An aliquot of the dry tRNA residue was dissolved in 200 μl of charging buffer containing 50 mM sodium acetate pH 6.0, 150 mM NaCl, 1 mM $MnSO_4$, 1 mM $CaCl_2$, and 5 mM $MgCl_2$. The tRNA solution was added to 400 μg of the Sepharose-Con A gel that was previously washed with the same buffer, and the suspension was slowly stirred for 30 min at 4 °C. After centrifugation, the gel was washed three times with 200 μl of charging buffer to discard all non-bound tRNAs. The bound tRNAs, i.e. manQ-bearing $tRNA^{Asp}$ isoacceptors, were removed from the Con A-Sepharose gel with twice 200 μl of a 0.2 M D-mannose solution. The collected fraction was desalted by centrifugation on Centricon 3 (Amicon, Beverly, USA), and dried under vacuum before further analysis.

### 2.7. Enzymatic hydrolysis of tRNA

The method used for enzymatic hydrolysis of tRNA was adapted from procedures described elsewhere [33,34], and can be summarized as follows: (i) addition to the tRNA sample (100–500 μg of tRNA material) of a known amount of 8-bromoguanosine ($Br^8G$) as an internal nucleoside standard (I.S.) for the further quantitative analysis of nucleosides by RPLC-UV, (ii) digestion overnight of the tRNA sample with nuclease P1 (EC. 3.1.30.1) (Boehringer, Mannheim, Germany), and (iii) combined hydrolysis with snake venom phosphodiesterase (SV-PDE, EC. 3.1.4.1) (Sigma, St Louis, USA) and bacterial alkaline phosphatase (BAP, EC. 3.1.3.1) (Sigma, St Louis, USA) for three hours. The resulting mixture of all ribonucleosides, including $Br^8G$ as I.S., was used in further liquid chromatographic analysis.

### 2.8. Boronate affinity chromatography of the ribonucleosides

Boronate affinity chromatography of enzymatic tRNA hydrolysates was adapted from Gehrke's group [35,36]. We used Affigel 601 (Biorad Laboratories, Richmond, USA), an immobilized boronic acid gel. After swelling for five minutes in water, the boronate gel was sequentially washed with methanol, water, 0.1 M NaCl, 0.1 N formic acid, 0.25 M ammonium acetate, pH 8.8, 50 % methanol/water (v/v), 0.1 N formic acid in 50% methanol/water (v/v), 0.1 M NaCl, and finally resuspended in 0.1 M NaCl. The above treated gel was further packed to a height of 3 cm in a 7 cm × 0.5 cm i.d. glass column which was fitted with a 10 ml reservoir and fine tip plugged with glass wool. Just prior to the chromatography, the gel was equilibrated by passing through the column 15 ml of 0.25 M ammonium acetate, pH 8.8.

Each studied tRNA hydrolysate was adjusted to pH 8.8 by the addition of 250 μl of 2 M ammonium acetate, pH 8.8, and applied to the above column. The column was further washed with 3 ml of 0.25 M ammonium acetate, pH 8.8, and 300 μl of 50% methanol/water (v/v) to discard unbound nucleosides. The bound nucleosides were finally eluted with 4 ml of 0.1 N formic acid in 50% methanol/water (v/v). The resulting eluate was lyophilized to dryness, and redissolved in 200 μl of water for further RPLC-UV analysis.

Prior to re-use, the above column was sequentially washed with 10 ml 0.1 M formic acid in 50% methanol/water (v/v), 10 ml 50% methanol/water (v/v), 10 ml 0.1 N formic acid, and finally re-equilibrated with 15 ml of 0.25 M ammonium acetate, pH 8.8.

### 2.9. Quantitative nucleoside analysis by RPLC-UV

Nucleoside analyses by RPLC-UV of enzymatic tRNA hydrolysates were performed on a Spectra-Physics liquid chromatograph (Thermo Quest France, Les Ulis, France) equipped with (i) a Supelcosil LC 18S column (250 mm × 4.6 mm i.d., Supelco France, St Germain-en-Laye, France) maintained at 26 °C in a controlled temperature oven, (ii) a ternary solvent delivery system at a flow rate of 1 ml/min, and (iii) a forward Spectra Focus scanning detector (Thermo Quest France, Les Ulis, France). The liquid chromatography was carried out using the experimental conditions for the separation of ribonucleosides developed by Gehrke and coworkers [35–38].

The ribonucleosides were quantified using $Br^8G$ added to each sample as an internal standard (I.S.). We used the 255 nm wavelength absorption to obtain optimal sensitivity and accuracy for major and modified nucleosides. Over the years, we have established molecular response factors relative to $Br^8G$ (RMR-$Br^8G$) for 34 ribonucleosides. These RMR-$Br^8G$ were determined from reference nucleosides, or from many pure tRNA species obtained from research collaborators. The RMR-$Br^8G$ for Q-derivative family was established in the present paper from the pure $tRNA^{Tyr}$ of *E. coli* MRE600 strain, as described below.

## 3. Results

### 3.1. Recovery and purity of the tRNAs from liver tissues and liver cell cultures

The amount of recovered RNA material from liver tissues or cell cultures was determined at each step of the tRNA isolation method. The quantitation was performed by absorption spectrometry measurement at 255 nm of the corresponding RNA solutions. From 17 different RNA extractions of 1 g liver tissue from various animal species, 3.1–6.5 mg of crude RNA were recovered, leading to 0.5–0.9 mg of final tRNA amounts. When the tRNA preparations were conducted on 10 cell culture flasks of the liver cell lines, i.e. on about 1 to 1.2 × $10^8$ cultured cells, the recoveries were 0.8–1.2 mg of crude RNA, and 0.10–0.20 mg of total tRNA.

The different tRNA samples were shown to contain very low levels of contaminating proteins, as judged by their 255/280 absorption ratios that ranged between 1.7 and 1.9, whatever the origin of the material. The purity level of the tRNA preparations was also evaluated in term of contaminations by DNA and/or rRNA in the final tRNA samples. This evaluation was done by RPLC-UV nucleoside analysis of tRNA sample aliquots (ca. 50–200 μg) previously submitted to enzymatic digestion, as described above. In our chromatographic conditions, the limit of detection for most ribo- or deoxyribonucleosides was 0.001 absorbance unit at 255 nm, corresponding to about 15 ng of compound. Whatever the tRNA sample analyzed by RPLC-UV, the DNA contamination level was less than 1% since deoxyribonucleosides were under this detection limit, while the presence of a small amount of 2′-*O*-methyladenosine (Am) in the tRNA hydrolysates revealed only a low level (5–8%) of rRNA contamination (results not shown).

### 3.2. HPLC separation of Q-derivatives from enzymatic tRNA digest

Reverse-phase high performance liquid chromatography coupled with a diode-array detector (RPLC-UV) was used for the chromatographic analysis of queuosine molecules (Q-derivatives) present in the tRNAs from animal tissues. To overcome the absence of reference Q-derivatives commercially available, we selected unfractionated tRNAs isolated and purified from beef liver as reference sources of the three queuosines found in mammals and other animal species, i.e. Q, manQ, and galQ.

A typical RPLC-UV nucleoside analysis of total beef liver tRNA after exhaustive enzymatic digestion by nuclease P1, followed by combined snake venom phosphodiesterase (SV-PDE) and bacterial alkaline phosphatase (BAP) hydrolysis is shown in Fig. 1a. Two peaks that eluted at 32.1 min and 32.5 min exhibited UV-absorption spectra whose the profiles were a typical feature for Q-derivatives. However, their identification as Q, manQ or galQ could not be determined directly from their UV-spectra: they

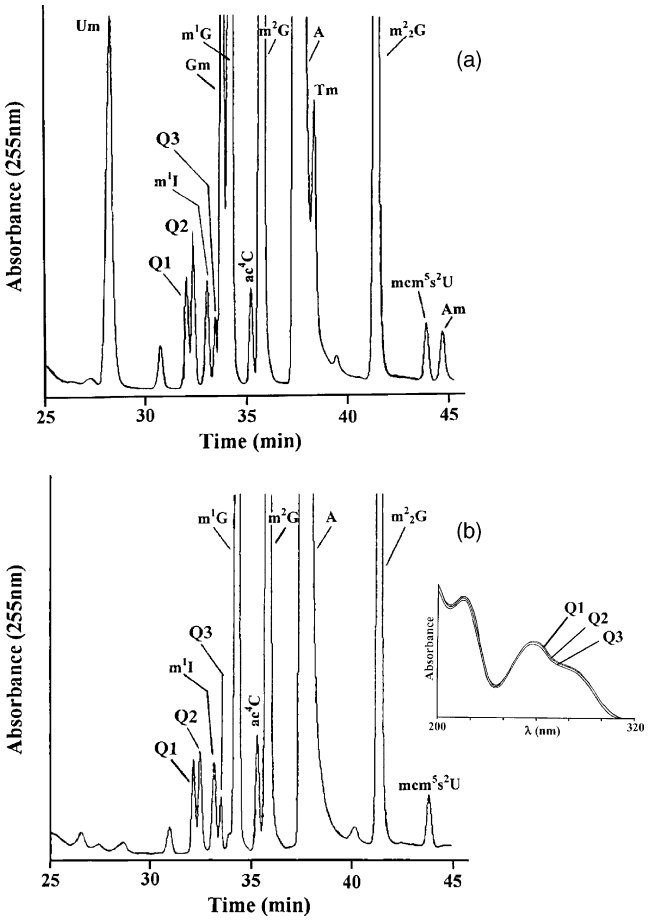


Fig. 1. HPLC chromatograms of nucleosides resulting from combined nuclease P1 plus snake venom phosphodiesterase (SV-PDE) and bacterial alkaline phosphatase (BAP) digestion of total beef liver tRNA: before (a) and after (b) boronate gel (Affigel 601) chromatography of the enzymatic tRNA hydrolysate. The experimental conditions are described in Experimental. The peaks Q1, Q2, and Q3 correspond to the three Q-derivatives found in mammalian liver tRNAs, and their respective UV-spectra are presented as insert in (b) chromatogram. Other peaks on the chromatograms: 2′-*O*-methyluridine (Um); 1-methylinosine ($m^1I$); 2′-*O*-methylguanosine (Gm); 1-methylguanosine ($m^1G$); $N^4$-acetylcytidine ($ac^4C$); $N^2$-methylguanosine ($m^2G$); adenosine (A); 2′-*O*-methylthymidine (Tm); $N^2$,$N^2$-dimethylguanosine ($m^2{}_2G$); 5-methoxycarbonylmethyl-2-thiouridine ($mcm^5s^2U$); 2′-*O*-methyladenosine (Am).

are quasi-identical. We named therefore these peaks Q1 and Q2. A third peak eluting at about 33.5 min had also a UV-spectrum strongly similar to those of Q1 and Q2: this peak was therefore named Q3. However, because the retention time of this third peak in our RPLC-UV system was very close to that of 2′-O-methyl guanosine (Gm) (Fig. 1a), the quantitative measurement of Q3 by RPLC-UV was not possible. The removal of Gm from the enzymatic digest of beef liver tRNAs was necessary.

In order to remove Gm and other 2′-*O*-methylated nucleosides from the nucleoside mixture, the enzymatic digest of total beef liver tRNA was submitted to chromatography on Affigel 601 prior to RPLC-UV analysis. This boronate ma-

trix retains specifically the *cis*-diol nucleosides, whereas the ribonucleosides bearing a methyl group at the position 2′ of the ribose moiety, as well as the eventual deoxyribonucleoside contaminants, will not bind to the gel, and will therefore be eliminated in the washing fraction. As shown in Fig. 1b, Gm nucleoside has been eliminated from RPLC-UV chromatogram when enzymatic digest of beef liver tRNA was previously submitted to boronate gel chromatography. An accurate quantitation of the three Q1, Q2 and Q3 compounds by RPLC-UV was thus possible.

### 3.3. Validation of $Br^8G$ as I.S. for the quantification of Q-derivatives

Because of the multiple sample preparation steps, an internal standard (I.S.) was essential to get the best accuracy of quantitation. The 8-bromo-guanosine ($Br^8G$) was chosen as I.S., because this compound clearly meets the essential criteria required for the most desirable I.S., as previously described by Gehrke's group [36]. $Br^8G$ was therefore added to the tRNA sample prior to any sample manipulation. Finally, the Q-derivatives from mammalian tRNAs were quantified at 255 nm, since that wavelength corresponded to the most sensitive UV-absorption response of both $Br^8G$ and Q-derivative family.

Because none of the Q-derivatives was available as commercial reference standard nucleoside, and synthesis of such nucleosides could have been too expensive, we determined the relative molar ratio to $Br^8G$ (RMR-$Br^8G$) of Q-derivatives using a hydrolysate of the $tRNA^{Tyr}$ from *E. coli* MRE600 strain that was commercially available in pure form and in milligrams amount. The sequence of this *E. coli* $tRNA^{Tyr}$ was shown to contain one "queuosine" residue among a total of 85 nucleotides [39]. The quantitative RPLC-UV analysis was carried out on a 16 μg aliquot of *E. coli* $tRNA^{Tyr}$ enzymatic digest. With the assumption that all the nucleoside modifications in the tRNA were close to 100%, we estimated at about 0.62 nanomole the amount of each nucleoside residue in the analyzed tRNA aliquot, whatever major or modified nucleosides, including the unique queuosine residue. The value of the peak area obtained by RPLC-UV for 0.62 nmol queuosine was then adjusted to 1.0 nmol amount. Finally, the RMR-$Br^8G$ of queuosine was determined by the ratio of this corrected RPLC-UV peak area of queuosine on the RPLC-UV peak area of 1.0 nmol $Br^8G$. A value of 0.47 was obtained by this method for the RMR-$Br^8G$ of queuosine from *E. coli* $tRNA^{Tyr}$.

The structural differences between the Q-derivatives do not alter the electronic structure of their chromophores, and therefore lead to identical UV spectra (see insert in Fig. 1b). This suggests that the queuosine molecules should also have very similar UV-absorption factors and therefore the same RMR-$Br^8G$. The RMR-$Br^8G$ value of 0.47 determined above for the queuosine of *E. coli* $tRNA^{Tyr}$ was thus used for the quantitation of all the Q-derivatives studied in this paper.

### 3.4. Identification of RPLC-UV Q1 peak as mannosyl-queuosine (manQ)

The three queuosines Q1, Q2 and Q3 found in beef liver tRNA did not have fine detailed features in their UV spectra allowing their characterization as either queuosine (Q), or mannosyl-queuosine (manQ), or galactosyl-queuosine (galQ). Their identifications had therefore to be determined by comparison of their RPLC-UV retention times with those of well-known Q-derivatives present in some known procaryotic or eucaryotic specific tRNAs.

Mannosyl-queuosine (manQ) has been shown to be located in the wobble position of aspartic acid tRNA isoacceptors ($tRNAs^{Asp}$) in all animal species so far studied. We isolated the $tRNAs^{Asp}$ from a mixture of tRNAs by linking it through its mannosyl group to a lectin protein, the Concanavalin A coated to Sepharose 4B beads (Sepharose-ConA). To get an enriched manQ-bearing $tRNA^{Asp}$ fraction, we used total chicken tRNA that was first fractionated by counter-current distribution (CCD) into 28 fractions [30]. The $tRNA^{Asp}$-containing fractions were determined by aminoacylation tests for all 20 amino acids on each of the 28 CCD fractions, using crude aminoacyl-tRNA synthetases from chicken or yeast (results not shown). The aminoacylation-charging activity concerning aspartic acid was revealed in the 22–26th CCD fractions.

These $tRNA^{Asp}$-enriched CCD fractions were pooled, and finally submitted to affinity fractionation on Sepharose-ConA gel. The tRNA fraction eluting with 0.2 M mannose solution from the Sepharose-ConA gel represented about 24% of the tRNAs in that pool CCD fractions. Nucleoside analysis by RPLC-UV was performed on the enzymatic hydrolysate of this Sepharose-ConA fraction. The resulting RPLC-UV chromatogram (not shown) confirmed the expected presence of manQ by showing one unique Q-derivative peak eluting at 32.1 min retention time. This manQ peak was collected, and added to an enzymatic hydrolysate of beef liver tRNAs. The RPLC-UV analysis of the mixture showed an increased area of the Q1 peak as compared with that of the original beef liver tRNA digest (not shown).

According to the retention time and the co-elution with Q1 of manQ from chicken tRNAs in our RPLC-UV conditions, the identity of manQ was attributed to the compound Q1 obtained from RPLC-UV nucleoside analysis of unfractionated beef liver tRNAs.

### 3.5. Identification of RPLC-UV Q2 peak as queuosine (Q)

The enzymatic hydrolysates of unfractionated tRNAs isolated from two *E. coli* strains (*E. coli* MRE600 strain and *E. coli* B strain) were submitted to RPLC-UV nucleoside analysis. The RPLC-UV chromatogram of *E. coli* MRE600 strain tRNAs revealed the presence of two peaks of Q-derivatives eluting at 30.9 and 32.6 min retention time (Fig. 2a), while that of *E. coli* B strain tRNAs showed only one Q-derivative peak eluting at 32.6 min (Fig. 2b). Because

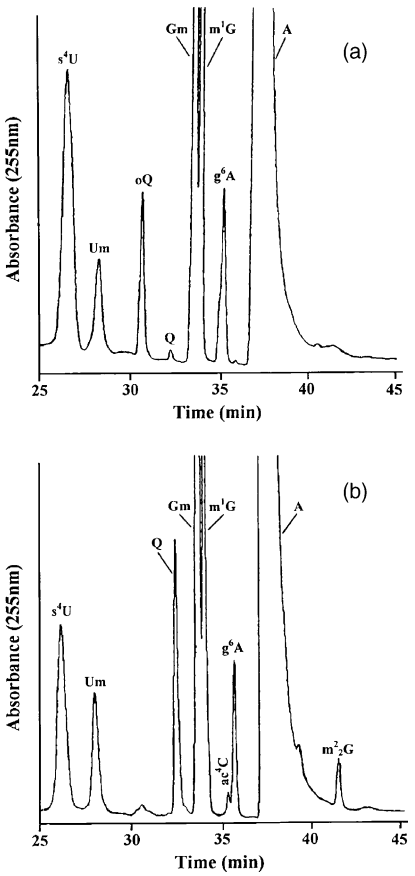


Fig. 2. HPLC chromatograms of nucleosides resulting from enzymatic digestions of total *E. coli* MRE600 strain tRNA (a), and total *E. coli* B strain tRNA (b). The retention times of epoxy-queuosine (oQ) and queuosine (Q) in our RPLC-UV system were determined from these chromatographic analysis. Other peaks on the chromatograms: 4-thiouridine ($s^4U$); 2′-*O*-methyluridine (Um); 2′-*O*-methylguanosine (Gm); 1-methylguanosine ($m^1G$); $N^4$-acetylcytidine ($ac^4C$); $N^6$-glycinocarbonyladenosine ($g^6A$); adenosine (A); $N^2$,$N^2$-dimethylguanosine ($m^2{}_2G$).

these Q-derivatives have already been characterized as epoxyqueuosine (oQ) and queuosine (Q) in MRE600 strain tRNAs [5], and as queuosine (Q) in B strain tRNAs [1], we attributed the retention times of 30.9 min and 32.6 min to the RPLC-UV peaks of oQ and Q, respectively.

When added to an enzymatic beef liver tRNA digest, the Q compound collected from *E. coli* B strain tRNAs co-eluted with the Q2 peak of beef liver tRNAs in our RPLC-UV conditions (not shown). The Q2 peak from beef liver tRNAs eluting at 32.5 min retention time (Fig. 1b) was thus identified as queuosine (Q).

### 3.6. Identification of RPLC-UV Q3 peak as galactosyl-queuosine (galQ)

Since Q1 and Q2 compounds from mammalian tRNAs were characterized as manQ and Q, respectively, the remaining possibility for the chemical structure of Q3 was the third Q-derivative generally found in mammals, i.e. galQ. Confirmation of the identification of Q3 as galQ was obtained using again an enriched chicken tRNA fraction.

Galactosyl-queuosine (galQ) was previously described to be specific for $tRNAs^{Tyr}$ in all animal species so far studied. We therefore searched for the tyrosine aminoacylation-charging activity in the 28 CCD fractions of chicken tRNAs: it was found in the 6–11th fractions. The RPLC-UV nucleoside analysis of these CCD fractions revealed the presence in each fraction of only one chromatographic Q-derivative peak (not shown). The retention time of this peak (33.6 min) was identical to that of Q3 compound described in beef liver tRNAs. From these results, the identity of Q3 was definitively confirmed as galQ.

### 3.7. Variation of Q-modification levels in tRNAs from mammalian liver tissues

The above developed procedure was applied to the quantitative analysis of Q, manQ, and galQ in total tRNAs isolated from different mammal livers. These analysis were done in order to determine and compare the Q-modification levels of Q-tRNAs expressed in these liver tissues, according to: (i) the species of the animals, and (ii) the developmental state (adult or newborn) of rat liver. The results were expressed in picomoles of Q-derivatives per 255 nm absorbance unit (pmol/AU), i.e. per about 40 μg, of starting tRNA material. Quantitative nucleoside analysis by RPLC-UV was extended to 1-methyl-adenosine ($m^1A$), 1-methyl-guanosine ($m^1G$), 2-methyl-guanosine ($m^2G$), and 2,2-dimethyl-guanosine ($m^2{}_2G$). These nucleosides being distributed in a wide array of animal tRNA species, and their amounts in total tRNA being mostly found in constant values, they could thus be considered as references to the whole tRNA contents in the studied cell materials.

Total tRNAs from liver tissues of four different species of adult mammals (rat, beef, sheep, and rabbit) were analyzed by our RPLC-UV procedure. The results presented in Table 1 are the means and extreme values obtained from three to six different tRNA analysis per mammal species. As expected, no significant differences between the four mammal liver tissues were observed in the amounts of $m^1A$, $m^1G$, $m^2G$, and $m^2{}_2G$ nucleosides selected as representative of the whole tRNA liver contents. Whatever the considered mammal species, the amount ranges of Q, manQ, and galQ were also very similar in these tRNAs, showing a quantitative homogeneity in the contents of the corresponding $[Q^+]$tRNAs in the adult mammal livers.

In order to check whether there could be an age-depending variation in the $[Q^+]$tRNA contents of mammal livers, we launched the determination of Q-derivatives amounts in total tRNAs from newborn rat liver (1–10 days of life), and compared to those obtained for adult rat liver. As shown in Fig. 3, the amounts of Q-derivatives in newborn rat liver tRNAs were obviously lower than those in adult rat liver tRNAs, while the amounts of the other selected modified nucleosides ($m^1A$, $m^1G$, $m^2G$, and $m^2{}_2G$) stayed

Table 1
Amounts of queuosine (Q), mannosyl-queuosine (manQ), and galactosyl-queuosine (galQ) present in total tRNAs from liver tissues from four adult mammals: rat, beef, sheep and rabbit

| Nucleosides (pmol/AU) | Rat liver ($n = 6$) | | Beef liver ($n = 3$) | | Sheep liver ($n = 3$) | | Rabbit liver ($n = 4$) | |
|---|---|---|---|---|---|---|---|---|
| | Means | Extreme values | Means | Extreme values | Means | Extreme values | Means | Extreme values |
| Q | 127 | 95–146 | 114 | 93–135 | 110 | 102–118 | 103 | 76–128 |
| manQ | 96 | 81–112 | 79 | 76–82 | 94 | 92–96 | 104 | 79–130 |
| galQ | 42 | 36–60 | 30 | 25–35 | 45 | 44–46 | 44 | 43–45 |
| m1A | 1440 | 1303–1612 | 1300 | 1143–1455 | 1608 | 1549–1645 | 1427 | 1150–1705 |
| m1G | 837 | 807–847 | 748 | 694–803 | 803 | 788–812 | 833 | 793–856 |
| m2G | 1388 | 1351–1433 | 1415 | 1292–1536 | 1545 | 1514–1573 | 1498 | 1410–1585 |
| m22G | 738 | 727–760 | 688 | 671–700 | 765 | 762–766 | 655 | 636–680 |

The tRNA amounts of 1-methyl-adenosine ($m^1A$), 1-methyl-guanosine($m^1G$), 2-methyl-guanosine ($m^2G$), and 2,2-dimethyl-guanosine($m^2{}_2G$) are also mentioned in the table. Means and extremes values are expressed in picomoles of nucleoside per 255 nm absorbance unit (pmol/AU) of tRNA.

constant in both rat liver tissue materials. According to these results, the decrease of the Q-derivative amounts in newborn rat liver should result from a hypomodification of Q-tRNAs in liver of young rats, and not from a decrease of all the molecules of liver tRNAs. It is noticeable that the degree of hypomodification varied according to the species of Q-tRNAs, with deficiency rates of only 23% for manQ-bearing tRNA ($tRNA^{Asp}$), but as high as 45% for Q-bearing tRNAs ($tRNA^{His}$ and $tRNA^{Asn}$), and 64% for galQ-bearing tRNA ($tRNA^{Tyr}$).

### 3.8. Variation of Q-modification levels in tRNAs from rat liver epithelial cell cultures

The Q-modification levels of Q-tRNAs were studied in tRNAs from rat liver epithelial cell cultures, according to the nutritive composition of the culture medium. Two normal replicative cultures of rat liver epithelial cells were used for this study. These rat liver epithelial cell cultures were prepared as described in experimental from livers of newborn rats without sexual selection. The cell cultures were grown in a nutritive culture medium which was supplemented with 10% fetal calf serum, and called “queuine-rich medium” because of the high queuine contents in this serum [40].

The Q-derivative contents were determined in total tRNAs isolated from the two replicative cultures of normal rat liver epithelial cells. No significant differences in their respective Q-derivative contents was observed for seven different subcultures in both lines of rat liver epithelial cells growing in queuine-rich medium. As shown in Fig. 3, the mean of these Q-derivative contents was similar to that of adult rat liver tissues, as compared to the mean Q-derivatives contents of the original newborn rat liver cells used for these cultures.

The rat liver epithelial cells were also grown in the same basal nutritive culture medium, but containing 10% horse serum instead of 10% fetal calf serum. This second culture medium was called “queuine-poor medium” because of the low queuine concentration present in horse serum [40]. Such new nutritive conditions did not affect neither the growing kinetics nor the phenotype shape of the epithelial cells in culture, even after six subcultures in the queuine-poor medium. But the Q-derivative contents of the tRNAs isolated from these cell cultures showed an important decrease since the first passage in queuine-poor medium (Fig. 3). Because no significant variations were observed for the amounts of the other selected modified nucleosides ($m^1A$, $m^1G$, $m^2G$, and $m^2{}_2G$), this decrease of Q-contents in total tRNAs should result from a specific hypomodification of the four Q-tRNAs. The Q-hypomodification rates in queuine-poor medium growing cells were much lower for manQ-bearing tRNA (43%) than for Q-bearing and galQ-bearing tRNAs (84 and 91%, respectively).

In addition, the Q-modification lack stayed remarkably constant for six successive subcultures of normal rat liver epithelial cells in queuine-poor medium. But if the latter was replaced by the queuine-rich medium, the amounts of Q-derivatives in cell culture tRNAs increased rapidly back (after only two days of culture) to their original values, i.e. the values without Q-tRNA hypomodification (not shown).

### 3.9. Variation of Q-modification levels in tRNAs from rat liver neoplastic cell cultures

The neoplastic cultured cells used for this study were obtained from the commercially available rat hepatoma 1548 Morris cell line. The tRNA nucleoside composition of this rat hepatoma cell line was compared with that of normal rat liver epithelial cell cultures in term of Q-derivatives and other selected modified nucleosides. For this comparison, both types of cells were grown in the same queuine-rich culture medium. As shown in Fig. 3, the rat hepatoma cells exhibited a very high level of hypomodification of Q-tRNAs, with deficiencies as much as 81% for manQ-bearing tRNA, up to 98% and even 100% for Q-bearing and galQ-bearing tRNAs, respectively. It is noteworthy that the growth of the hepatoma cells was not affected by this high degree of Q-tRNA hypomodification. It must also be pointed out that manQ-bearing $tRNA^{Asp}$ was again less affected by the hypomodification than both Q-bearing tRNAs ($tRNA^{His}$ and $tRNA^{Asn}$) and galQ-bearing tRNA ($tRNA^{Tyr}$).

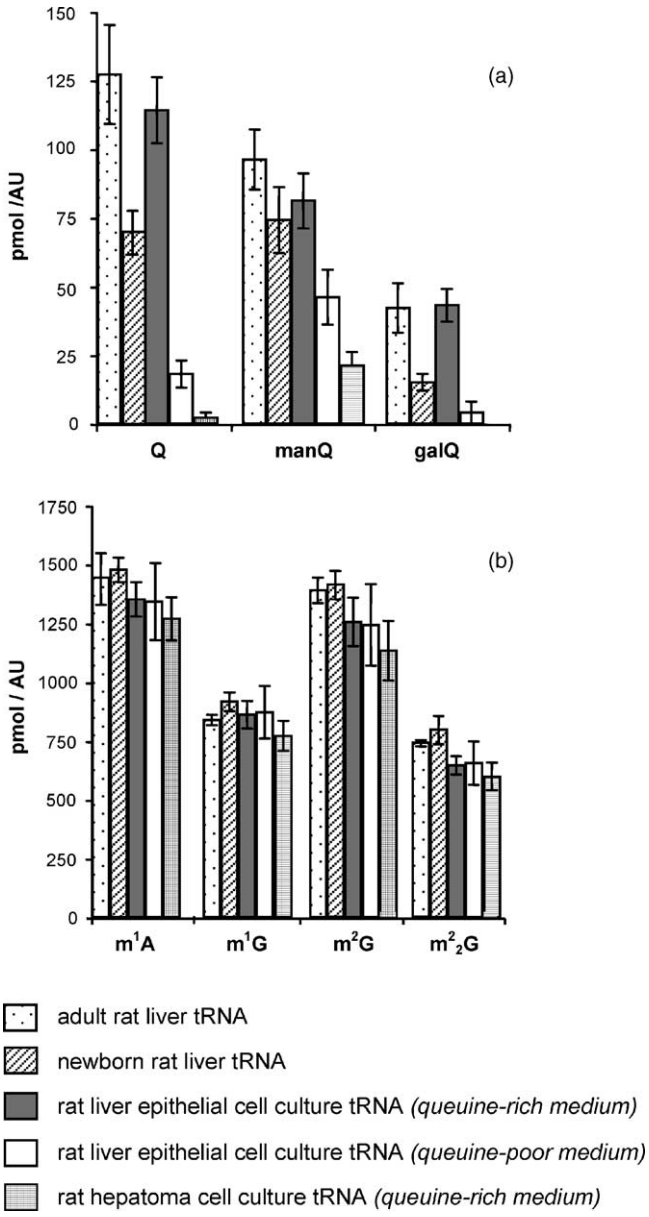


Fig. 3. Variation of the Q, manQ and galQ contents (a) in the total tRNAs isolated from adult rat liver, from newborn rat liver, from rat liver epithelial cell cultures growing either in queuine-rich or in queuine-poor medium, and from rat hepatoma 1548 Morris cell line growing in queuine-rich medium. The results are discussed in the text. For each tRNA preparation, the amounts of 1-methyl-adenosine ($m^1A$), 1-methyl-guanosine($m^1G$), 2-methyl-guanosine ($m^2G$), and 2,2-dimethyl-guanosine ($m^2{}_2G$) are also reported in the figure (b), and can be considered as representative of a constant whole tRNA contents in the studied cell materials.

## 4. Discussion

Reversed-phase high performance liquid chromatography coupled with real-time UV-spectrometry (RPLC-UV) is a powerful combination for the chromatographic analysis of modified nucleosides in tRNA. Using modified nucleosides and isoaccepting tRNAs obtained through commercial chemical suppliers or from many scientists from all parts over the world, the Gehrke's group characterized 67 ribonucleosides by RPLC-UV analysis [37,38]. Among these nucleosides, they described three compounds of the queuosine family isolated from prokaryotic and eukaryotic tRNAs with the following supposed elution times: queuosine (Q), mannosyl-queuosine (manQ), and galactosyl-queuosine (galQ). In addition, the 2,3-epoxy derivative of queuosine (oQ) was not mentioned in the Gehrke's studies on unfractionated tRNAs isolated from *E. coli* MRE600 strain. More recently using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) for the quantitative analysis of eucaryotic Q-derivatives, Marczinke et al. [41] showed that queuosine (Q) was not eluted before but between the two glycosylated Q-derivatives. However, they could not determine which one of the glycosylated Q-derivatives was manQ or galQ.

In the present work, we have characterized precisely on RPLC-UV the four Q-derivatives found in prokaryotic and eukaryotic cells with the following increasing elution order: oQ, manQ, Q, and galQ. The RPLC-UV elution of Q between its two glycosylated derivatives (manQ and galQ) confirmed the Q-derivative elution order partially determined by a HPLC-MS method as mentioned above [41]. It is noteworthy that a slight structure difference, mannose or galactose, could induce an essential difference in the HPLC separation behavior of the two glycosylated queuosines towards the parent nucleoside queuosine. The elution of Q later than manQ in various HPLC system was especially surprising because it did not correspond to the chromatographic retention-structure relationships usually observed between a parent nucleoside and its derivative bearing a neutral modifying group [42].

An internal standard method was also developed to achieve good precision and accuracy of the Q-derivative quantifications. Using 8-bromo-guanosine ($Br^8G$) as an internal standard, the relative molar ratio of Q-derivatives was established from the enzymatic digest of the known sequence *E. coli* MRE600 strain $tRNA^{Tyr}$. Added prior to the enzymatic hydrolysis of the tRNA samples, $Br^8G$ guaranteed the best chemical properties (good stability, elution in boronate gel chromatography quantitatively identical to that of Q-derivatives) for a quantitative recovery of Q-derivatives during the different sample chromatography steps.

Using the combined methods of boronate gel chromatography and RPLC-UV analysis of nucleosides, the best accuracy of quantification could be obtained for each Q-derivative present in the enzymatic hydrolysates of any unfractionated tRNA samples, provided the samples were not contaminated by DNA, rRNA, and proteins. Whatever the origin (liver tissue or liver cell lines) and the amount (a few mg up to 30 g) of starting material, the various tRNA preparations isolated by using the protocol that we have developed revealed to be totally free of DNA, and only very slightly contaminated by proteins and rRNA. In addition, the quantitative recovery of crude RNA from liver cells (means of 4.8 mg per gram of liver tissue), as well as the ratio of

tRNA isolated from crude RNA (about 15%) were in good agreement with the values previously published [43].

Our quantitative RPLC-UV procedure was applied to the unfractionated liver tRNAs isolated from four adult mammal species: rat, beef, sheep, and rabbit. No significant differences between these mammal livers were observed for their Q-derivative contents, and thus for their contents of [$Q^+$]tRNAs. These [$Q^+$]tRNA contents were very similar to those previously determined by others using aminoacylation-charging activity on unfractionated total adult rat liver tRNA [44], and beef liver tRNA [45]. Only slight differences were observed for galQ-bearing $tRNA^{Tyr}$ in adult rat liver, and for manQ-bearing $tRNA^{Asp}$ in beef liver.

Even if these studies were limited to only four mammal species, the constant values of [$Q^+$]tRNA found can be considered to be representative of constant Q-modification levels in adult mammal livers. In contrast, an important decrease of the Q-modification level was observed in Q-tRNAs from newborn rat liver, as compared with those of adult rat liver. Furthermore, our RPLC-UV results on newborn rat liver showed that manQ-bearing tRNA was much less affected by the hypomodification (23% deficiency) than Q-bearing, and galQ-bearing tRNAs (45 and 64% deficiency, respectively). Using the enzymatic method of radioactive guanine incorporation in total tRNA, Singhal et al. [46] have previously described a strong Q-tRNA hypomodification in liver tRNA from one-week old rats, while the Q-tRNAs from adult rat liver were fully modified. However, their analytical procedure did not allow to determine the respective deficiency rate of each individual Q-tRNA species.

The ability of our RPLC-UV procedure for measuring the hypomodification rates of the Q-tRNA family members was also demonstrated in our studies on rat liver epithelial cell cultures. When these replicative cell cultures were grown in queuine-rich culture medium, their [$Q^+$]tRNA contents were almost identical to those of adult rat liver tissue, and not as expected to those of the newborn rat liver cells used for the original cell cultures. However, the [$Q^+$]tRNA contents of these cultured cells decreased markedly if the usual queuine-rich medium of the cell cultures was replaced by a queuine-poor medium. In addition, the decrease in [$Q^+$]tRNA was quantitatively lower for the tRNA bearing manQ ($tRNA^{Asp}$) than for the tRNA bearing galQ ($tRNA^{Tyr}$) and the tRNAs bearing Q ($tRNA^{Asn}$ and $tRNA^{His}$). These Q-tRNA hypomodification rates occurred since the first passage in queuine-poor medium, and stayed constant for the six following subcultures in queuine-poor medium. This hypomodification was however reversal when the queuine-poor medium was replaced by the queuine-rich medium. Thus, Q-deficient tRNAs can be induced in rat liver epithelial cell cultures by decreasing the availability of dietary queuine base in the culture medium.

While the hypomodification of Q-tRNAs from rat liver epithelial cells cultured in queuine-poor medium was provided by a insufficient queuine base supply, the question arose to explain the Q-tRNA hypomodifications of newborn rat liver. In 1973, before the structures of Q-derivatives were known, White et al. [47] showed already that the ratio of [$Q^+$]tRNAs to [$Q^-$]tRNAs varied during the life cycle of *Drosophila*. The tRNAs from the last larval stage contained almost only [$Q^-$]tRNAs, while the tRNAs from adults contained predominantly [$Q^+$]tRNAs. The authors suggested that queuosine in tRNA could play some regulatory roles in cell differentiation. Q-tRNA hypomodification has also been described in undifferentiated cells [16–18], in regenerating rat liver [19], and in cultured cell lines [19,20]. These various types of cells or tissues, including the newborn rat liver, are all characterized by a high rate of cell proliferation involving the turnover of high amounts of tRNAs for the protein synthesis. The queuine supply is an essential element for the full modification of Q-tRNAs, especially in these cases of cell proliferation. The queuine supply by maternal milk or by gut flora could thus be insufficient for a full modification of Q-tRNAs in newborn rat liver in which both cell proliferation and cell differentiation are very active.

We found also a very low modification rate of the Q-tRNAs in rat hepatoma cell line as compared with that of rat liver epithelial cell cultures. Since both types of cell cultures were grown in the same queuine-rich medium, the Q-hypomodification in hepatoma cells could not have been induced by a deficient queuine supply. This seems to be a characteristics of neoplastic cells since Q-tRNA hypomodification could be associated with cancer [20–24]. This led Morris et al. [48] to conclude that Q deficiency in cancer cells could be due to: (i) lack in queuine uptake into cells, (ii) lack in incorporation of queuine into tRNA by tRNA-guanine transglycosylase (Tgt), and (iii) lack in queuine salvage from tRNA degradation. Their demonstration pointed out that the two last statements explain the Q deficiency in cultured cell lines. It is therefore not yet clear if Q deficiency could be used as a malignancy prognosis outcome; all depends probably from the type of tumor and cells, i.e. of the implicated organs.

As already mentioned in tRNAs from newborn rat liver and from epithelial rat liver cells growing in queuine-poor medium, $tRNA^{Asp}$ of rat hepatoma cell line is also less affected by the Q-hypomodification than $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Tyr}$. Whatever the cause of the Q-modification deficiency in tRNAs from eukaryotic cells, there must be a special reason to give a preference to the $tRNA^{Asp}$ to be "better" modified than its three counter-part tRNAs. Functionally, the $tRNA^{Asp}$ integrity, i.e. fully or nearly fully modified, seems to be indispensable to growing of eukaryotic cells. This phenomenom has been already pointed by Farkas [49] who suggested that privileged modification of $tRNA^{Asp}$ could protect the latter from rapid degradation in case of queuine lack. Reyniers et al. [50] supposed that tRNA guanine transglycosylase (Tgt) had higher affinity for $tRNA^{Asp}$ than for the three other tRNAs. None of these hypothesis have been yet confirmed. Nevertheless, one could suppose that a large amount of fully modified $tRNA^{Asp}$ (as far as

manQ is concerned) could be necessary for the synthesis or the regulation of specific proteins having an essential role in the survival of the higher eukaryotic cells. Further work is however necessary to assess such conclusions. Additional work is in progress.

## Acknowledgements

This research had financial support from Ministère des Universités et de la Recherche, and Centre National de la Recherche Scientifique.

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