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SALVAGE OF THE NUCLEIC ACID BASE QUEUINE FROM QUEUINE-CONTAINING TRNA BY ANIMAL CELLS

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SUMMARY: The incorporation of queuine into tRNA and its fate upon tRNA turnover has been studied in the Vero and L-M cell lines. An assay was developed using [^{3}H]dihydroqueuine to detect the queuine acceptance and, thus, the queuine content of tRNA in intact cells. While L-M cells can use only queuine, Vero cells can use either queuine or its nucleoside, queuosine, to form queuine-containing tRNA. Since queuosine is not a substrate for the enzyme which incorporates queuine into tRNA, Vero cells must generate queuine from its nucleoside. When Vero cells are labelled with [^{3}H]dihydroqueuine, the half life of acid insoluble radioactivity is 52 days in queuine-free medium and 3.1 days in queuine-containing medium, indicating that [^{3}H]dihydroqueuine is salvaged from tRNA and reused by Vero cells, but that exogenous queuine can compete with the salvaged [^{3}H]dihydroqueuine. When L-M cells are labelled with [^{3}H]dihydroqueuine, the half life of the acid insoluble radioactivity is 1.2 days in the presence or absence of queuine, indicating the absence of queuine salvage in L-M cells.

INTRODUCTION: The modified nucleoside queuosine (Q) is found exclusively in the first position of the anticodon in tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn} and tRNA^{Asp} (1-3). Its function is unknown: long term Q-deficient, germ-free mice appear normal (4, 5) and the only phenotypic change thus far observed in an Escherichia coli mutant which lacks Q in its tRNA is a

ABBREVIATIONS: Q or nucleoside Q or queuosine 7-{5-[((1S,4S,5R)-4,5-dihydroxy-2-cyclopenten-1-yl)amino]methyl}-7-deazaguanosine; Q base or queuine, the aglycone of queuosine; (Q+)tRNA, tRNA that contains Q in the first position of the anticodon; (Q-)tRNA, the unmodified or precursor form of (Q+)tRNA, which contains guanosine instead of Q in the first position of the anticodon. In this study, Q is used generically to designate Q and its saccharide derivatives containing galactose or mannose bound to carbon 4 of the cyclopentenediol substituent; AGMK, African Green monkey kidney primary or early passage cells; FBS, fetal bovine serum; HPLC, high performance liquid chromatography.

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marked reduction in viability in the stationary phase of growth (6). Unlike other tRNA modifications, Q is synthesized first as a base and then is incorporated irreversibly (in an exchange reaction in which guanine is removed) into preformed tRNA by the enzyme guanine, queuine-tRNA transglycosylase (7, 8). Queuine, the base of Q, appears to be the immediate precursor of Q in mammalian tRNA (7-9). However, animals apparently cannot synthesize queuine de novo, but must obtain it from the diet or from gut flora (4, 5). Significant amounts of free queuine occur in common foods of both plant and animal origin (10) and dietary queuosine-containing tRNA can also serve as a source of queuine (5), implying a mechanism for the salvage of queuine from degraded tRNA.

Tissue cultured mammalian cells readily incorporate exogenous queuine into their tRNAs. The mouse fibroblast cell 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 hours (7). In a reverse experiment, when L-M cells containing (Q+)tRNA are transferred to queuine-free medium, the proportion of (Q+)$tRNA^{Asp}$ declines from 99% to 48% within 24 hr and this decline requires RNA synthesis, i.e., it is eliminated by actinomycin D (11). This rapid loss of (Q+)tRNA from L-M cells cultured in queuine-free medium is consistent with an absence of queuine salvage (i.e., the retrieval of queuine from degraded tRNA) in these cells. Unlike L-M cells, AGMK cells apparently can salvage queuine as deduced from their ability to utilize queuosine as a source of queuine and the very slow loss of (Q+)tRNA from AGMK cells cultured in Q-free medium (11). In the present work, the Vero cell line, a derivative of AGMK cells, is used to further characterize queuine salvage.

MATERIALS AND METHODS: The L-M and Vero cell lines (Am. Type Culture Collection) were cultivated in Dulbecco's modified Eagles' medium (GIBCO) supplemented with: 0.5% Bacto-peptone (DIFCO), L-M; or 5% FBS treated with charcoal plus dextran to remove queuine (12), Vero. Queuine was isolated from third trimester bovine amniotic fluid (Irvine Scientific) by adsorption and elution, first from Dowex-50 (H^+) and then from Sephadex G-10 (7, 10), followed by preparative HPLC (11). [^{3}H]Dihydroqueuine was prepared

from queuine by Amersham, using catalytic reduction followed by HPLC repurification (11). Queuosine was isolated from E. coli tRNA (Sigma) as described (13), alkaline hydrolysis, Dowex-1 (formate) chromatography, alkaline phosphatase treatment (E. coli, Sigma); followed by HPLC (11). (It should be noted that recently purchased lots of tRNA from both Sigma and Boehringer Mannheim contains less than 10% of the expected quantity of queuosine.) [^{3}H]Dihydroqueuosine was isolated as described for queuosine from [^{3}H]dihydroqueuine-containing L-M cell tRNA (Katze, J.R., Cheng, C.S., McCloskey, J.A., unpublished). tRNA isolation, aminoacylation using a crude mouse liver aminoacyl-tRNA synthetase, recovery of aminoacyl-tRNAs by DEAE-cellulose chromatography, and RPC-5 chromatography were as described (7, 12). In order to estimate tRNA degredation rates (14-16), cells were pulsed with [^{3}H]methyl-methionine (1.5 ug/ml for 15 hr), fluid changed into medium containing no [^{3}H]methyl-methionine (defined 0 Time), a portion of the cells were harvested at 0 Time and appropriate later times, tRNA was isolated (12) and the radioactivity determined.

RESULTS AND DISCUSSION: Vero cells cultured in medium supplemented with 10% FBS contain $tRNA^{Asp}$ which is greater than 98% in the Q-containing form (data not shown). In order to make Vero cells Q-deficient, they were cultivated in medium supplemented with 5% FBS which had been treated with dextran and charcoal to remove queuine (12). After five weeks (four passages) cultivation of Vero cells in such medium, (Q+)$tRNA^{Asp}$ comprises less than 6% of the total $tRNA^{Asp}$; however, the relative concentration of (Q+)$tRNA^{Asp}$ can be increased by the addition of pure queuine to the medium (Table 1). Therefore, Vero cells, like L-M cells and the parent AGMK cells, require exogenous queuine to synthesize (Q+)tRNA.

The data presented in Table 1 also demonstrate that queuosine can serve as a source of queuine for Vero cells, the proportion of (Q+)$tRNA^{Asp}$ increasing almost linearly with the amount of added queuosine. However, since queuosine is not a substrate for guanine, queuine-tRNA transglycosylase (Farkas, W. R., Jacobson, K. B. and Katze, J. R., unpublished), Vero cells must be able to degrade queuosine to queuine. Judging from the concentrations required to elicit 50% (Q+)$tRNA^{Asp}$ (Fig. 1), queuine is approximately three fold more efficient than is queuosine. Therefore, the conversion of queuosine to queuine appears to be slow relative to the insertion of queuine into tRNA.

In order to facilitate further examination of queuine metabolism, we have developed a simple assay using [^{3}H]dihydroqueuine. This derivative of

Table 1

Effect of queuine and queuosine on $tRNA^{Asp}$ isocceptors in Vero cells [a]

Addition	Concentration (nM)	% total $tRNA^{Asp}$ in peaks 1(Q)	2(G)	3(Q)	4(G)	%(Q+)$tRNA^{Asp}$ (1+3)
None		1.3	8.2	4.2	86.3	5.5
Queuine	1	1.4	8.1	8.6	82.0	10.0
	4	2.3	5.2	27.8	64.6	30.1
	10	6.2	2.7	76.9	14.2	83.1
Queuosine	12	2.4	6.6	30.0	61.0	32.4
	24	4.8	4.0	64.6	26.6	69.4

[a] Parallel cultures of cells grown to confluency in 150 cm^2 growing surface area flasks (Corning) were exposed (in duplicate) to 25 ml of medium containing the designated concentrations of queuine or queuosine, followed by cell harvest and estimation of $tRNA^{Asp}$ isoaccepting distribution by RPC-5 chromatography (see Methods). Peaks 1 and 3 are (Q+)$tRNA^{Asp}$, peaks 2 and 4 are (Q-)$tRNA^{Asp}$ (7, 12).

queuine, in which the proton at the C-8 (purine numbering system) position has been exchanged with tritium and the cyclopentene ring has been reduced with tritium to yield a cyclopentane ring, remains a substrate which is specifically and irreversibly incorporated into tRNA by guanine, queuine-tRNA transglycosylase; however, it is incorporated approximately ten fold less efficiently than is queuine (11). The incorporation of [^{3}H]dihydroqueuine at a concentration of 1.0 μM, into tRNA by intact Q-deficient Vero cells is complete within 3 hours (actinomycin D is added to prevent new RNA synthesis during this period). Moreover, virtually all of the acid-insoluble radioactivity present in cells exposed to [^{3}H]dihydroqueuine can be accounted for in the tRNA fraction (data not shown).

The ability of Vero cells to utilize queuosine for (Q+)tRNA synthesis was verified with the [^{3}H]dihydroqueuine assay. One set of cells was exposed to queuosine, while a second, untreated set served as control. After 24 hours, the culture fluids were removed, the cell layers were rinsed with queuine-free medium, and then the kinetics of [^{3}H]dihydroqueuine

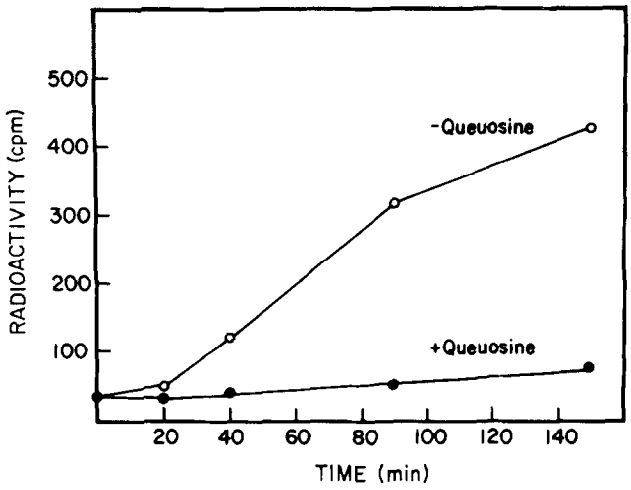


Fig. 1 Effect of queuine and queuosine on the relative (Q+)tRNA^{Asp} in Vero cells. These data were derived from Table 1. (Q+)tRNA^{Asp} represents the sum of peaks 1 and 3.

uptake were determined. The results (Fig. 2) show that the set of cells which had been exposed to queuosine exhibit negligible incorporation of [^{3}H]dihydroqueuine, as would be expected for cells which contain tRNA already saturated with Q. These results support the existence of an acti-

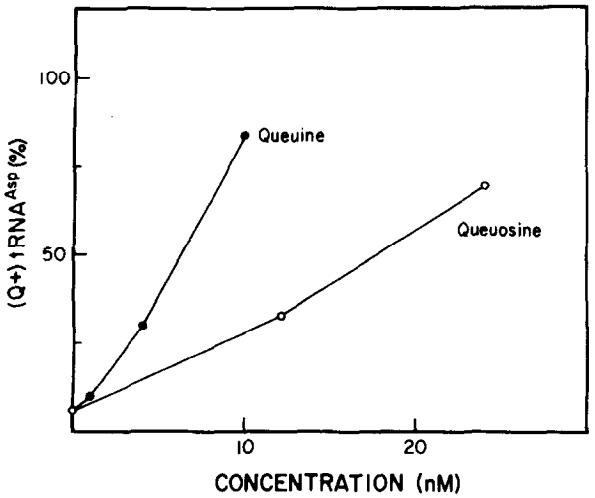
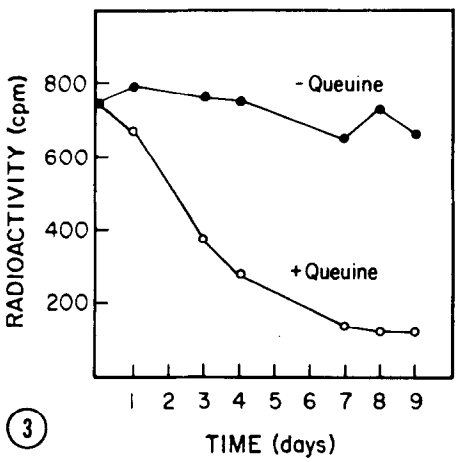


Fig. 2 [^{3}H]Dihydroqueuine uptake into tRNA by Vero cells: effect of pretreatment with queuosine. Parallel cultures (60 mm plates) were exposed to 5 ml control or queuosine (36 nM) containing medium for 24 hours; the medium was removed, the cell sheets were rinsed with control medium and then were incubated with 2 ml medium containing [^{3}H]dihydroqueuine (1 µM, 1.3 x 10^{8} cpm/nmole) and actinomycin D (4 µg/ml). At the designated times the medium was removed, the cell sheets were rinsed with control medium, the cells were transferred with ice cold trichloroacetic acid (5%) to glass fibre filters (Whatman GF/A), washed with cold ethanol (95%), dried and counted in a scintillation counter. The mean of duplicate determinations is presented.

vity which degrades queuosine to queuine. Such an activity also might enable the salvage of queuine after normal tRNA turnover.

We have sought more direct evidence for Q salvage by investigating the fate of [^{3}H]dihydroqueuine-containing tRNA in both Vero cells and L-M cells. Figure 3 summarizes the decay of acid-insoluble radioactivity from Vero cells which had been exposed to [^{3}H]dihydroqueuine for 5 hr, and then "chased" by culturing either in Q-depleted medium (control) or Q-depleted medium plus queuine, changing the medium every 24 hr. The radioactivity incorporated into tRNA remained almost constant (apparent half life = 52 days) over a nine day period in the control cells, but decreased (apparent half life = 3.1 days) at a rate expected for tRNA turnover (14-16) in the cells exposed to queuine. Queuine itself has little effect on tRNA turnover. In a control experiment (not shown), Vero cells were pulse labeled with [^{3}H]methyl-methionine and the decay of radioactivity in the whole tRNA fraction was followed. The results showed that the apparent half life of Vero cell tRNA was the same (4 days) in cells cultured in either the presence or absence of queuine (15nM). We interpret these data to indicate the Vero cells degrade (Q+)tRNA to yield free queuine, which then is inserted into newly synthesized tRNA. When queuine was added to the medium, the free [^{3}H]dihydroqueuine resulting from tRNA degradation was inefficiently reutilized because of dilution by queuine, a much better substrate for the transglycosylase (11).

The same type of experiment was performed with L-M cells, but very different results were obtained (Fig. 4). The decay of acid-insoluble radioactivity from [^{3}H]dihydroqueuine is the same (apparent half life = 1.2 days) in the presence or absence of exogenous queuine. We have investigated further the fate of [^{3}H]dihydroqueuine-tRNA in those cells (Fig. 4) subsequently cultured in the absence of queuine. Each time the cells were fluid changed the spent medium was collected for analysis by HPLC (Fig. 5). The results for the Day 1 sample indicate that almost all of the radioactivity eluted in a single position, Peak 4. Since [^{3}H]dihydroqueuine elu-



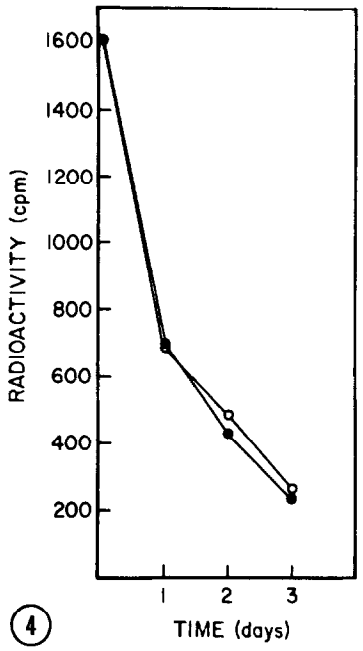


Fig. 3 The decay of [^{3}H]dihydroqueuine-tRNA in Vero cells. Vero cells (60 mm plates) were exposed to 2 ml medium containing [^{3}H]dihydroqueuine (1 μM) for 5 hours, the radioactive medium was removed, the cell sheets were rinsed with control medium, and further culture was performed in 5 ml control medium or control medium plus queuine (15 nM). At the designated times the cells were harvested and the acid insoluble radioactivity determined as in Fig. 2.

Fig. 4 The decay of [^{3}H]dihydroqueuine-tRNA in L-M cells. L-M cells (60 mm plates) were exposed to 2 ml medium containing [^{3}H]dihydroqueuine (5 μM) for 24 hours, then cultured in 5 ml control medium or control medium plus queuine (0.5 μM), changing the medium every 24 hours. At the designated times the cells were harvested and the acid insoluble radioactivity determined as in Fig. 2.

tes in the position of Peak 4, we conclude that most of the radioactivity in the Day 1 sample is [^{3}H]dihydroqueuine remaining from the labeling procedure. Four additional peaks of radioactivity appeared in the Day 2 sample. Of these, Peak 3 cochromatographs with authentic [^{3}H]dihydroqueuosine and, therefore, is assumed to be that compound. The remaining three peaks have not been identified. Our view that Peak 4 is [^{3}H]dihydroqueuine and Peak 3 is the corresponding nucleoside is supported by the progressive increase in Peak 3 and decrease in Peak 4, such that by Day 6 virtually no Peak 4 material remains. We also have examined the medium of Vero cells pulsed with [^{3}H]dihydroqueuine, then cultured in the presence of queuine (15nM) to compete with the reincorporation into tRNA of salvaged

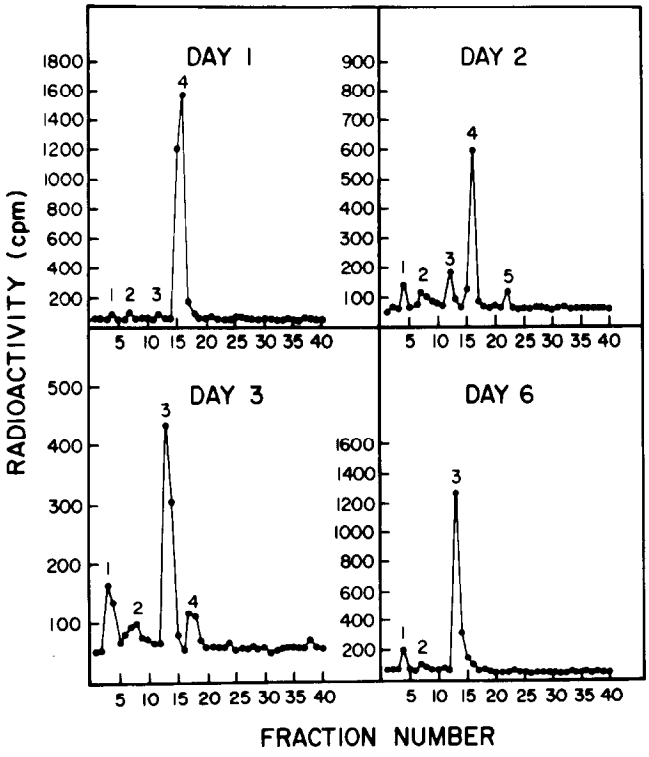


Fig. 5 HPLC analysis of culture fluids obtained from L-M cells labeled with [^{3}H]dihydroqueuine. The cell culture fluids (control medium, no queuine) resulting from the experiment described in Fig. 4 were collected on the days indicated, adsorbed to a column (0.5 ml) of Dowex-50 (H+), eluted as described (footnote 19, ref. 10), and evaporated to dryness. The samples were taken up in 50 µl H_2O and 25 µl of this was subjected to HPLC analysis on a C_{18} column (Altex Ultrasphere ODS, 4.6 x 250 mm). Mobile phase: 0.005 M heptane sulfonate, 0.02 M ammonium acetate, pH 3.20; methanol; tetrahydrofuran (85: 14.5: 0.5, v/v); 1.0 ml/ min, room temp. Fractions of 1 ml were collected; 0.8 ml of each fraction was mixed with 0.7 ml H_2O plus 10 ml 33% Triton X-100 scintillation mixture (17), and the radioactivity determined. Culture fluids for Days 1, 2 and 3 had been in the presence of cells for 24 hours; the culture fluid for Day 6 had been in the presence of cells for 72 hours.

[^{3}H]dihydroqueuine. In contrast to the results with the L-M cells, most of the radioactive material in the Vero cell medium eluted in the position of [^{3}H]dihydroqueuine, even on Day 6, and no material eluted in the position of [^{3}H]dihydroqueuosine (data not shown).

In summary, the present data indicate that Vero cells can salvage and reutilize queuine from degraded (Q+)tRNA, but that L-M cells can degrade (Q+)tRNA no farther than Q nucleoside.

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