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Biochemistry

A factor in serum and amniotic fluid is a substrate for the tRNA-modifying enzyme tRNA-guanine transferase

(modified nucleoside Q/tRNA^{Asp}/tRNA^{His}/posttranscriptional modification)

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ABSTRACT **Q factor, a substance found in animal serum that enables cultured mammalian cells (L-M) to produce tRNA containing queuine (the base of "nucleoside Q", queuosine), has been purified to homogeneity from bovine amniotic fluid. Q factor causes the appearance of Q-containing tRNA^{Asp} in the L-M cells cultivated in serum-free medium, and this was used as an assay to monitor the purification of Q factor. Q factor is a competitive inhibitor of guanine for rabbit reticulocyte tRNA-guanine transferase, with a K_I of 4.5×10^{-8} M. Q factor is inactivated in both the L-M cell and tRNA-guanine transferase assays by treatment with periodate or cyanogen bromide, both of which react with queuine. In L-M cells, nearly complete conversion of Q-free to Q-containing tRNA^{Asp} is observed within 24 hr after addition of pure Q factor to the medium; actinomycin D, cycloheximide, and cycloleucine, inhibitors of RNA synthesis, protein synthesis, and nucleic acid methylation, respectively, do not inhibit this conversion. The product of the reaction, catalyzed by pure rabbit reticulocyte tRNA-guanine transferase, between Q factor and rabbit reticulocyte Q-free tRNA^{His} is chromatographically indistinguishable from Q-containing tRNA^{His}.**

The hypermodified guanosine derivatives queuosine (Q) and modified queuosine (Q*) are found in the first position of the anticodons of tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn}, and tRNA^{Asp}, which form a set in that they accommodate the codons NA^U_C (1, 2). Queuosine is 7-{[(*cis*-4,5-dihydroxy-2-cyclopenten-1-yl)-amino]-methyl}-7-deazaguanosine (1); Q* designates derivatives of Q having either a mannose or a galactose residue at the 4 or 5 position of the cyclopentenediol (2). The Q-containing tRNAs from tumors or tissue cultured cells often show differences in chromatographic patterns when compared with the same tRNAs from normal animal tissue such as liver (refs. 3–7 and refs. therein). This phenomenon is explained by variations in the extent of the Q modifications. The tRNA isoaccepting species that contain Q, [Q+]tRNA, are eluted from RPC-5 and Aminex A-28 columns earlier than are their unmodified counterparts, [Q−]tRNA, in which the queuine position is occupied by guanine (3–10). The additional positive charge of Q is sufficient to explain this chromatographic difference.

tRNA-guanine transferase (formerly called guanine insertase), described by Farkas and coworkers in mammalian reticulocytes (11, 12), catalyzes the exchange of guanine into tRNAs of the NA^U_C codon set. In homologous enzyme-tRNA systems, [Q−]tRNAs, but not [Q+]tRNAs, are substrates for the enzyme (9, 10, 13, 14), which excises a specific base by cleaving the *N*-glycosyl bond and replaces that base with guanine. Studies with the pure reticulocyte enzyme show that the base replaced by guanine is the guanine residue in the first position of the anticodon (N. K. Howes and W. R. Farkas, unpublished data) and similar results have been obtained with the *Escherichia coli* and *Drosophila* enzymes (refs. 13 and 14; K. B. Jacobson and W. R. Farkas, unpublished data). These findings suggest that tRNA-guanine transferase participates in the substitution. The enzyme is thought to remove guanine from the first position of the anticodon and to replace it with queuine or a precursor of it (9, 15–18); however, in the absence of this substrate, it catalyzes the previously noted (11, 12) exchange of guanine for guanine.

Katze (19) has described a serum component, designated Q factor, which enables cells in tissue culture to synthesize [Q+]tRNAs from [Q−]tRNA. The properties of Q factor are consistent with it being either a substrate for the guanine-transferring enzyme or a precursor that is converted into a substrate. In the present communication, we describe the purification of Q factor to homogeneity from bovine amniotic fluid and show that it is a substrate for the reticulocyte tRNA-guanine transferase.

MATERIAL AND METHODS

Yeast tRNA was obtained from Miles, [^{3}H]guanine [specific activity, 1 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)] from ICN, [^{3}H]histidine (1 Ci/mmol) and [^{14}C]histidine (0.24 Ci/mmol) from Schwarz/Mann, and protein assay solution and globulin standard from Bio-Rad. Buffers, salts, and L amino acids were reagents grade. Rabbit liver guanine aminohydrolase (1.0 unit/mg) was from Sigma and bovine amniotic fluid was from Pel-Freez. Protein was assayed as described (20), as was the guanylation reaction (9). tRNA-guanine transferase was purified to homogeneity from rabbit erythrocytes as described (21), as was the *in vivo* Q factor activity in L-M cells cultured in the absence of serum (19).

Purification of Q factor. Bovine amniotic fluid (517 ml) was subjected to ultrafiltration (Amicon hollow fiber cartridge, type H1P10); the retentate was washed with two 50-ml portions of water. The entire ultrafiltrate (600 ml) was added to a column (5×88 cm) of Sephadex G-100 in water; elution was with water until the effluent showed negligible absorbance at 280 nm, with 0.02 M HCl until the effluent showed no material absorbing at 280 nm, and finally with 0.45 M NH_4OH. Significant Q-factor activity was found only in the 0.02 M HCl eluate. The active fraction was lyophilized, redissolved in methanol, and subjected

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Abbreviations: Q, 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 derivative Q*.

to preparative thin-layer chromatography on cellulose plates (Eastman Chromogram type 13225), with the following solvent systems in sequence: A, 3% ammonium formate; B, isopropanol/2% ammonium acetate, 1:1 (vol/vol) (22); C, 1-butanol/glacial acetic acid/H_2O, 4:1:1 (vol/vol) (22). After each chromatographic step, the areas of interest (identified under UV light) were scraped from the plates, and the active material was eluted with 0.02 M HCl and lyophilized to dryness. Q-factor activity, determined by the L-M cell $tRNA^{Asp}$ assay (19), was found to be associated solely with a light-blue fluorescing material that exhibited R_F values in solvents A, B, and C of 0.41, 0.43, and 0.17, respectively.

***In vitro* Assay of Q Factor.** The reaction mixture contained 10 μmol of *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes)·HCl buffer (pH 7.4), 1 nmol of [^{3}H]guanine, 53 μmol of KCl, 5 μmol of 2-mercaptoethanol, 1.25 A_{260} units of yeast tRNA, 50 μl of guanylating enzyme, and Q factor in a final volume of 0.60 ml; it was incubated at 37°C for 2 hr. The reaction was terminated by the addition of 3 ml of ice-cold 3 M HCl. After 5 min at 0°C, the precipitated tRNA was collected on 2.4-cm glass-fiber filters; these were washed with cold 3 M HCl and cold 95% ethanol, dried, and transferred to vials for scintillation counting.

Reaction of Q Factor with BrCN. The reaction of Q factor with BrCN was a modification of a described method (9). Q factor (0.125 A_{260} unit dissolved in 0.2 ml of 0.04 M Na_2CO_3 at pH 8.9) and BrCN (2.5 mg dissolved in 1 ml of ethanol) were mixed with gentle stirring for 15 min. The reaction mixture was added to a small column containing 2 g of Dowex 50(H^+). The column was washed serially with 50 ml of water, 20 ml of 1.5 M HCl, 25 ml of H_2O, and then 3M NH_4OH. Preliminary experiments had shown that Q factor treated in this manner was eluted by 3M NH_4OH. NH_3 was removed in a gentle stream of nitrogen at 37°C and the sample was concentrated to 0.25 ml. A control without BrCN (sham BrCN) was treated similarly.

Reactions of Q Factor with Periodate. The reaction of Q factor with IO_4^- was a modification of a described method (10). Q factor (0.13 A_{260} unit dissolved in 0.2 ml of 0.1 M sodium acetate at pH 5.2) was adjusted to 0.02 M in sodium metaperiodate. The reaction was allowed to proceed in the dark for 30 min at 22°C and was stopped by the addition of excess glycerol. Q factor was recovered as described above for the BrCN experiment. A control, with NaI for $NaIO_4$ (sham IO_4^-), was treated similarly.

Reversed-Phase-5 Chromatography. tRNA was L-M cell aminoacylated with [^{3}H]- or [^{14}C]aspartic acid, by using a crude aminoacyl-tRNA synthetase preparation from mouse liver, and was subjected to reversed-phase chromatography as described (19).

Preparation of Rabbit Liver Histidyl-tRNA Synthetase. Rabbit liver (90 g) was homogenized at high speed in a refrigerated Waring blender for 10 min in 250 ml of 0.01 M Tris·HCl, pH 7.4/0.01 M $MgCl_2$/1 mM dithiothreitol/and 10% (vol/vol) glycerol. After centrifugation (18,000 × *g* for 30 min), the supernatant was passed through a DEAE-cellulose column (2 × 25) cm equilibrated at 4°C with the homogenizing buffer. The column was eluted with the same buffer containing 0.2 M KCl. The eluted fractions were assayed for histidyl-tRNA synthetase activity (24), and the active fractions were pooled and stored at −70°C.

Preparation of Reticulocyte tRNA. Reticulocytes (11) were suspended in 1 vol of 0.01 M sodium acetate, pH 5.0/5 mM EDTA/0.1 M NaCl and were extracted (5 min, 22°C) with an equal volume of water-saturated phenol. The phenol phase was reextracted with 0.5 vol of the sodium acetate buffer, the two aqueous phases were pooled, and the RNA was recovered by ethanol precipitation. The precipitate was dissolved in 0.05 M sodium acetate, pH 4.5/5 mM 2-mercaptoethanol/5 mM EDTA/0.01 M $MgCl_2$/0.3 M NaCl and added to a DEAE-cellulose column equilibrated with the same buffer. The column was washed with 10 column volumes of buffer, following which the tRNA was eluted with 1.0 M NaCl dissolved in the same buffer. The tRNA was located in the eluate by its absorbance at 260 nm and was recovered by ethanol precipitation; the precipitate was dried *in vacuo* and stored as a powder until used. In order to isolate [Q−]$tRNA^{His}$ or [Q+]$tRNA^{His}$, reticulocyte tRNA was aminoacylated with [^{3}H]histidine and the major [Q+]$tRNA^{His}$ species [reticulocyte $tRNA_2^{His}$ (9)] and the [Q−]$tRNA^{His}$ species [reticulocyte $tRNA_3^{His}$ (9)] were resolved by reversed-phase chromatography (23). The appropriate tRNA fractions were pooled separately and deacylated (pH 9.0, 37°C, 1 hr), and the tRNA was recovered as above. Reticulocyte tRNA was charged by using rabbit liver histidyl-tRNA synthetase.

RESULTS

Isolation of Q Factor. Q factor was purified to chromatographic homogeneity from bovine amniotic fluid by using ultrafiltration, Sephadex chromatography, and preparative thin-layer chromatography. It was homogeneous after the second thin-layer chromatographic step. Recovery was 27% of the total activity in the starting material. To test the activity of pure Q factor, L-M cells cultivated in serum-free medium were exposed for 24 hr to increasing concentrations of Q factor (Table 1). It is evident that $tRNA^{Asp}$ peaks 1 and 3 [the [Q+]$tRNA^{Asp}$ (19)] were virtually absent in cells cultivated in serum-free medium. Peaks 1 and 3 increased, with a reciprocal decrease in peaks 2 and 4 [the [Q−]$tRNA^{Asp}$ (19)], as a function of the Q-factor content of the growth medium. Similar results have been observed (19) with unfractionated bovine amniotic fluid. These results indicate the absence of inhibiting or potentiating activities in serum or amniotic fluid. Therefore, a direct relationship between the presence of Q factor in the culture medium and the appearance of [Q+]$tRNA^{Asp}$ in cells is established.

Q Factor Inhibits the Insertion of Guanine into tRNA by tRNA-Guanine Transferase. Okada *et al.* (15) have reported that mammalian tRNA-guanine transferase can excise queuine from *E. coli* [Q +]tRNA and insert guanine in its place. Therefore, we tested the possibility that Q factor might act by inhibiting the enzyme. Our data show that Q factor is indeed an inhibitor of the guanine exchange reaction (Fig. 1; Table 2).

Table 1. Effect of increasing amounts of Q factor on $tRNA^{Asp}$ peaks from L-M cells

Q-factor, A_{260} unit/ 50 ml	% of total $tRNA^{Asp}$ in peaks 1 (Q+)	2 (Q−)	3 (Q+)	4 (Q−)	% [Q+]$tRNA^{Asp}$*
0	1.0	16	2.0	81	3.0
0.01	7.0	10	36	48	43
0.02	11	5.0	53	31	64
0.05	15	1.0	78	6.0	93
0.10	17	1.0	79	3.0	96
0.25	16	1.0	83	1.0	99

Parallel cultures of cells were inoculated with serum-free medium on day 0 and the medium was replaced with medium containing the designated concentrations of Q factor on day 3. The cells were harvested 24 hr later. After isolation of tRNA, aminoacylation, and reversed-phase chromatography, percentage values were determined from the radioactivity present in each peak.

* Represents the sum of peaks 1 and 3.

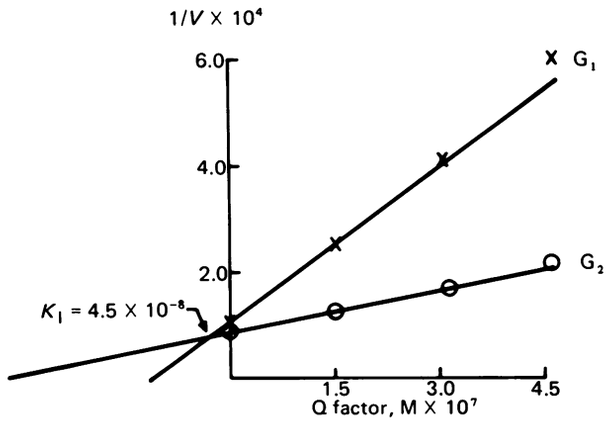

FIG. 1. K_1 of Q factor. The initial rate was determined at the indicated concentrations of Q factor and at two guanine concentrations ($G_1 = 3.8 \times 10^{-7}$ M and $G_2 = 8.8 \times 10^{-7}$ M). The reaction solutions contained 4.0 units of homogeneous tRNA-guanine transferase; the assay conditions were as described in the text. Each point represents the mean of samples run in duplicate, and the experiment was repeated with identical results. For competitive inhibitors, when the reciprocal of the initial velocity ($1/V$) is plotted against the inhibitor concentration at two different substrate concentrations, the point of intersection of the lines lies above the abscissa at a point $-K_1$ distant from the ordinate (25). The Q-factor concentration was estimated from the extinction coefficient at 260 nm for Q (calculated from ref. 1).

The data summarized in Fig. 1 show that the inhibition is competitive and that the K_1 for Q factor is 4.5×10^{-8} M.

Before proceeding further we thought that it was necessary to rule out the possibility that the inhibitor was guanine fortuitously copurified with Q factor. Therefore, we incubated Q factor with the enzyme guanine aminohydrolase to deaminate any guanine present to xanthine, which is neither a substrate nor an inhibitor of the mammalian tRNA-guanine transferase (26). This treatment effected no diminution of the capacity of Q factor to inhibit the guanine exchange reaction (data not shown). That Q factor is not guanine was demonstrated also in another way. [^{14}C]Guanine and Q factor were mixed and added to a column of Dowex 50 (H^+), which was then washed successively with H_2O, 1.5 M HCl, water to remove HCl, and finally 3.0 M NH_4OH. All of the guanine was eluted with 1.5 M HCl; however, Q-factor activity was eluted only in the NH_4OH fraction (data not show). This experiment confirms that Q factor is more positively charged than guanine.

Reaction of Q factor with BrCN and IO_4^-. That Q factor is a low molecular weight compound other than guanine raised the possibility that it is related to Q. Queuine reacts with IO_4^- because of the vicinal hydroxyl groups in the cyclopentenediol ring and with BrCN because of the secondary amine moiety (1). Therefore, Q factor was treated with these two reagents and then tested for its capacity to inhibit tRNA-guanine transferase as well as for its capacity to stimulate the cellular production of [Q+]tRNA. The data in Tables 2 and 3 show that reaction with either BrCN or IO_4^- destroys the capacity of Q factor to inhibit tRNA-guanine transferase and to stimulate tissue-cultured cells to produce [Q+]tRNA.

Table 2. Effect of BrCN and periodate treatments of Q factor, assayed by guanine incorporation assay

Exp.	Addition*	[^{3}H]Guanine incorporated into tRNA, cpm X 10^{-3}	Inhibition of enzymatic incorporation of guanine, %
1	None	43	0
	Q factor	4.3	90
	Q factor (BrCN treated)	39	9
	Q factor (BrCN omitted)	5.1	88
2	None	19	0
	Q factor	2.1	89
	Q factor (IO_4^- treated)	17	11
	Q factor (IO_4^- omitted)	2.8	85

* Where noted 0.5 A_{260} unit of Q factor or treated Q factor was added to the reactions. Assay conditions are described in the text.

Effect of Actinomycin D, Cycloheximide, and Cycloleucine on Q Factor-Induced Appearance of Q-Containing tRNA in L-M Cells. When added simultaneously with Q factor, actinomycin D (4 μg/ml), cycloheximide (10 μg/ml), and cycloleucine (1 mg/ml)—inhibitors of RNA synthesis (28), protein synthesis (29), and nucleic acid methylation (27), respectively—did not inhibit the conversion of [Q−]- to [Q+]tRNA^{Asp} (Table 3).

Q Factor Is Incorporated into tRNA by tRNA-Guanine Transferase. The results show that Q factor inhibited the enzymatic incorporation of guanine into tRNA, that Q-factor has many properties that are identical to those of Q or its base, and that RNA synthesis, nucleic acid methylation, and protein synthesis are not required for the *in vivo* activity of Q factor. These findings suggested, therefore, the possibility that Q factor and queuine are identical and also are the physiological substrate for the guanine transferase. In order to test this possibility, [Q−]tRNA^{His} prepared by reversed-phase chromatography was incubated with homogeneous guanine transferase plus Q factor; a control was incubated identically but without Q factor. The products from the reaction of [Q−]tRNA^{His} with and without Q factor were aminoacylated with [^{3}H]histidine and [^{14}C]histidine, respectively, and analyzed by reversed-phase chromatography. In a similar experiment, the product of the enzymatic reaction was cochromatographed with authentic [^{14}C]histidine-labeled [Q+]tRNA^{His} and the two tRNAs eluted as one peak (results not shown). The results of this experiment, summarized in Fig. 2, show that most of the [Q−]tRNA^{His} incubated in the presence of Q factor coeluted with [Q+]tRNA^{His}. The control, incubated in the absence of Q-factor, eluted from the column in its original position, that of [Q−]tRNA^{His}.

Table 3. Effect of $BrCN^-$ at periodate-treated Q factor and some metabolic inhibitors on the production of [Q+]tRNA^{Asp} by L-M cells

Exp.	Addition	% [Q+]tRNA^{Asp}*
1†	None	3.0
	Q factor	91
	Q factor (BrCN treated)	5.0
	Q factor (BrCN omitted)	94
	Q factor (IO_4^- treated)	3.0
	Q factor (IO_4^- omitted)	97
	Q factor + actinomycin D	98
	Q factor + cycloheximide	96
2‡	Q factor	99
	Q factor + cycloleucine	99

* Determined as in Table 1.

† The culture protocol was as in Table 1; where noted, 0.1 A_{260} unit of Q factor or treated Q factor per 50 ml of medium was added 24 hr prior to harvest; where noted, actinomycin D (4 μg/ml) or cycloheximide (10 μg/ml) was added at the same time as the Q factor.

‡ The culture protocol was as in Table 1, except that 0.075 A_{260} unit of Q factor per 50 ml of medium containing $\frac{1}{10}$th the normal concentration of amino acids [high methionine levels partially counteract the effects of cycloleucine (27)], with or without cycloleucine (1 mg/ml), was added 24 hr prior to harvest.

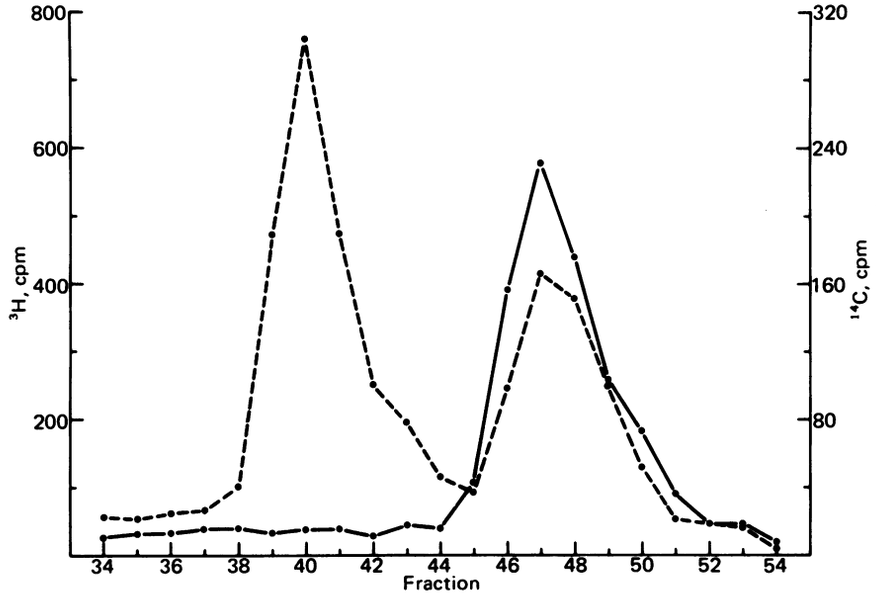

FIG. 2. Q factor is inserted enzymatically into [Q−]tRNA^{His}. [Q−]tRNA^{His} was incubated with 8 units of pure reticulocyte tRNA-guanine transferase and 0.11 A_{260} unit of pure Q factor for 2 hr at 37°C under standard guanylating conditions. A control was treated identically except that Q factor was omitted. The Q factor-treated and control tRNAs were recovered by ethanol precipitation, aminoacylated with [^{3}H]histidine and [^{14}]histidine, respectively, and subjected to cochromatographic analysis by reversed-phase chromatography. ●– –●, Sample incubated with Q factor and charged with [^{3}H]histidine; o——o, control incubated without Q factor and charged with [^{14}C]histidine.

DISCUSSION

Our results suggest that Q factor is queuine. Q factor affects the production of [Q+]tRNA both *in vivo* and *in vitro*. Furthermore, Q factor exhibits an absorption spectrum in H_2O like that of Q nucleoside (1), with maxima near 220 and 260 nm and a shoulder at 280 nm (unpublished data), and a preliminary mass spectroscopic study of Q factor indicates the presence of a 7-deazaguanine moiety and the absence of ribose or other sugar moieties (J. A. McCloskey and P. F. Crain, personal communication). The presence of a secondary amine function in Q factor is compatible with the facts that Q factor binds more tightly to Dowex-50 (H^+) than does guanine and that Q factor reacts with BrCN. The presence of a cyclopentenediol group is compatible with the reaction of Q factor with periodate. Because there is no ribose in Q factor, the diol group of queuine would account for the reaction with periodate. We have not investigated whether the double bond of the cyclopentene ring is present in Q factor.

Reticulocyte tRNA-guanine transferase was discovered because it incorporates guanine into tRNA (12), but it now appears that queuine incorporation and not guanine exchange may be the biological role of this enzyme. The question arises then, can queuine compete with guanine for the enzyme? The K_m of pure reticulocyte guanine transferase for guanine is 1.5×10^{-7} M (21), and we report a K_1 of 4.5×10^{-8} for Q factor, which appears to be queuine. Thus, the affinity of the enzyme would be 3 times greater for queuine than for guanine. Moreover, queuine insertion would be irreversible, because homologous [Q+]tRNA does not appear to be a substrate for the mammalian tRNA-guanine transferase, whereas guanine incorporation is reversible (9, 10).

Okada *et al.* (15, 16, 18) reported that in *E. coli* a cyclopentenediol-lacking precursor of Q, 7-(aminomethyl)-7-deazaguanine, is inserted into tRNA. Further modification must be completed at the polynucleotide level because queuine is not a substrate for the *E. coli* guanine transferase (18). We speculate from the present results that the mammalian enzyme is capable of incorporating queuine into tRNA and that queuine may be the physiological substrate. Presumably, mammalian cells that contain large amounts of [Q−]tRNA have adequate amounts of the enzyme to incorporate queuine into tRNA but lack the queuine. L-M cells cultivated in the presence of fetal bovine serum exhibit substantial quantities of [Q+]tRNA, but only trace quantities of [Q+]tRNA are found in L-M cells cultivated in the absence of serum (19). However, cells cultivated with or without serum exhibit similar levels of guanine transferase activity (unpublished data). We propose that the tRNA-guanine transferase-catalyzed step in Q biosynthesis differs between mammalian cells and *E. coli*, the immediate nonpolynucleotide-attached precursor of Q being queuine in mammalian cells and a precursor of queuine in *E. coli*. It should be noted that mammalian tRNA-guanine transferase may recognize precursors of queuine in addition to queuine as its substrate but this point has not been tested. Therefore, it remains to be determined whether mammalian cells also use the *E. coli* Q biosynthetic route.

The present report also shows that the enzyme guanine aminohydrolase, which is ubiquitous and is responsible for the catabolism of guanine, does not attack Q factor, probably accounting for the fact that Q factor exists in significant quantities in serum and amniotic fluid.

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