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The role of the guanine insertion enzyme in Q-biosynthesis in Drosophila melanogaster

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ABSTRACT

Drosophila tRNA can be guanylated by a crude enzyme from rabbit reticulocytes. Guanylating activity is also present in crude extracts of adult Drosophila. A major product of this reaction as well as several minor ones were resolved by RPC-5 chromatography. The main substrate of both the Drosophila and rabbit reticulocyte enzymes was the non-Q-containing aspartic acid tRNA, $tRNA^{Asp}_{2\gamma}$. The Q-lacking (γ) forms of asparagine, histidine and tyrosine tRNAs were also substrates and gave rise to the minor products of the reaction. In contrast, the Q- or Q*-containing (δ) forms of these tRNAs appear not to be substrates. The evidence strongly suggests that the guanylating enzyme is involved in Q biosynthesis and would be better termed a guanine replacement or pre-Q insertion enzyme.

INTRODUCTION

The hypermodified nucleosides Q and Q*, which are guanosine derivatives, are found in the first position of the anicodons of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$, and $tRNA^{Tyr\ 1-3}$. The structure of Q has been determined as 7-(4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine[4], whereas Q* has either a mannose or galactose residue at the 4th position of the cyclopentene diol[5]. Since Q finds such wide distribution in both prokaryotes and eukaryotes[6], and since it represents the only known example of a purine skeleton being modified to a 7-deaza structure[4], the modification process giving rise to Q is of considerable interest.

In Drosophila, Q is found in the early eluting (δ) forms of $tRNA^{Asn}$, $tRNA^{His}$, and $tRNA^{Tyr\ (2,3)}$. The δ form of $tRNA^{Asp}$ contains the related base Q*[7]. During Drosophila development, the relative proportions of the modified (δ) and unmodified (γ) forms of these tRNAs change coordinately: the δ isoacceptor forms decrease relative to the γ forms until late larval stage, when the trend reverses and the predominant form in the adult becomes the Q-containing δ form[2]. It has been proposed that this trend represents

either a delay in the onset of Q-biosynthesis until pupation, or a demodification of Q-containing isoacceptors up to the pupal stage[2].

A guanine insertion reaction, specific to tRNAs of the Q-family, was first described by Hankins and Farkas[8]. In rabbit reticulocytes, only Q-lacking forms of these tRNAs were substrates [8,9,10]. On the other hand, when purified E. coli tRNAs were used as the substrate for the reticulocyte guanylating enzyme, the Q base was specifically excised and replaced by guanine[11]. The E. coli enzyme will not normally guanylate E. coli tRNA, but will guanylate yeast tRNA (which contains no Q)[11] or tRNA from methyl-deficient E. coli (a Q-lacking form)[12]. These data suggest, that in vivo, the substrates of the guanine insertion enzymes are the Q-lacking tRNAs of this family.

The guanine insertion reaction was investigated in Drosophila in order to clarify the relevance of this mechanism to the changes in Q-family tRNAs during Drosophila development. This study reports that the unmodified (γ) isoacceptor forms of Drosophila tRNA are the major substrates for both the rabbit reticulocyte guanylating enzyme and a corresponding Drosophila enzyme and suggests that the guanylating enzyme is indeed involved in Q-biosynthesis.

MATERIALS AND METHODS

[^{3}H] guanosine (7.6 Ci/mmole) was obtained from Amersham. The [^{3}H] and [^{14}C] amino acids were from New England Nuclear Corporation. Guanosine was from Boehringer Mannheim. Adogen 464 was a gift of the Ashland Chemical Co., and Plaskon CTFE 2300 powder was a gift from Allied Chemical Corp. Napthoxyacetylester of N-hydroxy-succinimide was from Sigma Chemical Co.

a) Growth of Drosophila - Wild type Drosophila were grown at 25°C, at 60-70% relative humidity, in plexiglass boxes with ventilation ports. The medium was composed of 10% brewers yeast, 10% sucrose, 1.5% agar, 1.0% propionic acid, and 0.001% chloramphenicol. Collected organisms were stored at -20°C.

b) Isolation and Aminoacylation of tRNA - Transfer RNA was isolated by the phenol method of Kirby[13] and DEAE-cellulose chromatography procedure of Kelmers et al.[14], except that 0.01 M sodium acetate (pH 4.5) was the buffer used. Crude aminoacyl-tRNA synthethases were prepared by modifications[2] to the method of of Twardzik et al.[15], and the tRNA was aminoacylated as previously described[2].

c) Preparation of Rabbit Reticulocyte Guanylating Enzyme - Reticulocytosis

was induced as described by Hankins and Farkas[8]. Plasma was removed and the cells washed with 4 vol. of 0.01 M Tris-HCl (pH 7.5) containing 0.001 M 2-mercaptoethanol. The cells were homogenized on ice, centrifuged at 12,000 x g for 15 min., and the supernatant further centrifuged at 105,000 x g for 2 h. The supernatant was adjusted to 0.3 M KCl, 10% glycerol and applied to a DEAE-cellulose column equilibrated with the same buffer. Fractions were pooled according to maximum absorbance at 280 nm, then brought to 60% saturation with ammonium sulphate. This was then centrifuged and the pellet redissolved in the homogenizing buffer, dialysed overnight against the same buffer containing 50% glycerol, and stored at -20°. The glycerol was removed immediately before use by Sephadex G-25 chromatography.

d) Assay of Guanylating Activity - The procedure of Farkas et al.[16], was modified as follows: each 0.2 ml reaction volume contained 0.5 mg enzyme, 0.02 M Tris-HCl (pH 7.5), 0.1 M KCl, 3-5 A_{260} units tRNA, and 15 µCi [^{3}H] guanosine (1615 Ci/mole). After incubation at 37°C, the entire reaction mixture was applied to a 0.7 x 4 cm DEAE-cellulose column at 4°C. The column was equilibrated with 0.01 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, 0.001 M 2-mercaptoethanol, and 0.25 M NaCl. The column was washed with the same buffer until the radioactivity in the eluate dropped to background level, and the tRNA was eluted from the column with buffer containing 0.75 M NaCl.

e) Reverse Phase Chromatography - The RPC-5 system of Pearson et al.[17] was used.

f) Isolation of Drosophila tRNAs - Drosophila tRNA $^{Asp}_{2\delta}$ was isolated by Con A-Sepharose chromatography[7]. Other Q-containing (δ) isoaccpetor tRNAs were isolated by periodate modification[18]. Drosophila tRNA $^{Asp}_{2\gamma}$ was purified by the napthoxyacelytation procedure[2].

g) Nucleotide Analysis - ^{3}H-labelled guanylated tRNA was digested with ribonuclease T_2 and the nucleotides separated on cellulose thin-layer plates with isobutyric acid/0.5 N NH_4OH (5/3)[19].

RESULTS

The rabbit reticulocyte lysate was assayed for guanine insertion activity using crude Drosophila tRNA as substrate. The rate of incorporation was similar to that reported for E. coli and reticulocyte tRNA (Fig. 1). The maximum incorporation of 2 pmol/A_{260} unit represents 1-2% guanylation of the Q-family tRNAs. That the incorporation into Drosophila tRNA was at an internal position was demonstrated by RNase T_2 digestion of guanylated tRNA and thin-layer chromatography. Fig. 2 shows that after hydrolysis, the

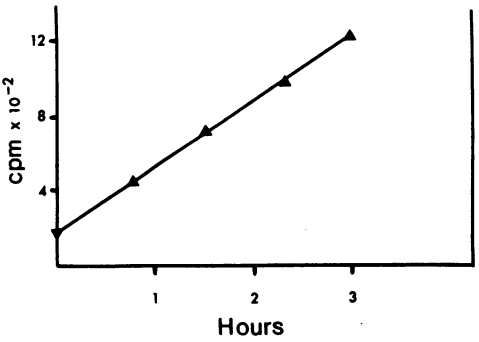

Figure 1. Time course of the guanylation reaction. The incubation mixture was increased proportionately from that described in Methods. Aliquots were removed and the extent of guanylation determined at each time point by DEAE-cellulose chromatography.

labelled nucleotides comigrate with an authentic sample of GMP.

Drosophila tRNA containing 30% δ form of the Q-tRNAs, isolated from adult flies, was guanylated with the rabbit reticulocyte enzyme and fractionated by RPC-5 chromatography. The distribution of incorporated counts in crude tRNA showed one major peak with a predominant shoulder as well as several minor peaks (Fig. 3a). The size of the minor peaks was variable.

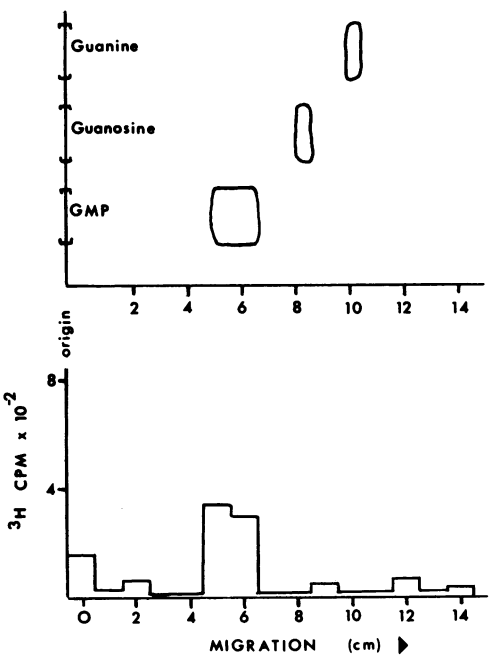

Figure 2. Chromatography of ribonuclease T_2 products from [^{3}H] labelled guanylated tRNA. Drosophila tRNA (4 A_{260} units), guanylated with the rabbit reticulocyte enzyme, was digested with ribonuclease T_2 and the resultant nucleotides chromatographed in one dimension on cellulose thinlayer plates as described in the Methods. The labelled nucleotide was detected by scraping 1 cm bands, eluting with 0.01 N HCl and counting.

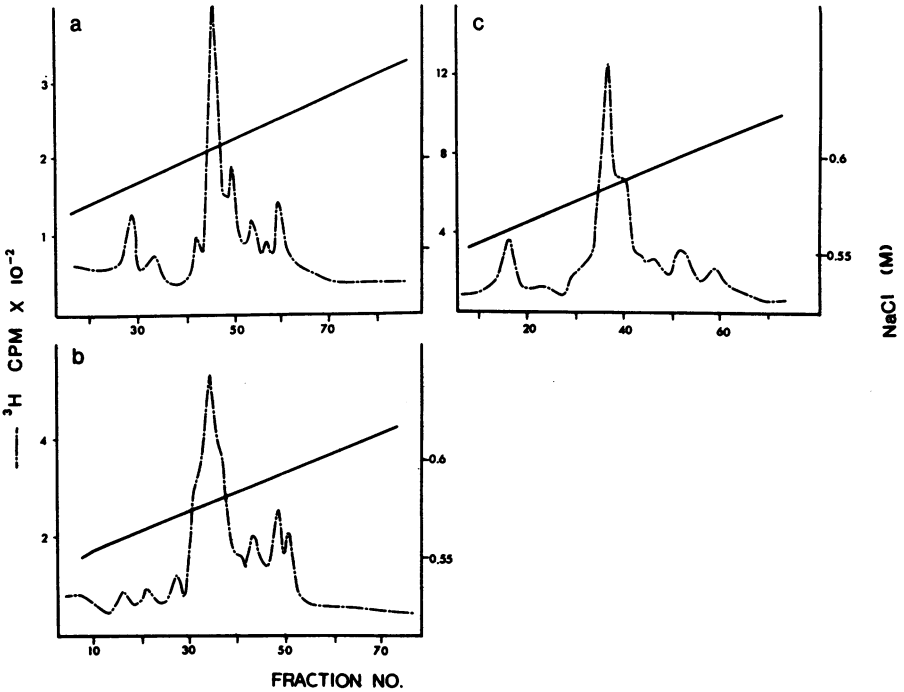

Figure 3. RPC-5 chromatography of crude adult Drosophila tRNA guanylated by rabbit reticulocyte and Drosophila extracts. Crude Drosophila tRNA (containing 30% δ form of $tRNA^{Tyr}$ and $tRNA^{His}$) was guanylated as described in Methods. The tRNA was chromatographed on 0.9 x 20 cm RPC-5 columns at 37°C by 100 ml, 0.55 M - 0.65 M NaCl gradients: (a) tRNA guanylated with rabbit reticulocyte extract; (b) tRNA guanylated with Drosophila extract; (c) mixture of tRNAs guanylated separately with Drosophila and rabbit reticulocyte extracts.

A Drosophila extract prepared as described for the preparation of aminoacyl-tRNA synthetases was analysed for guanylating activity. Such an activity was found and chromatography of the guanylated tRNA revealed a similar pattern as with the rabbit enzyme (Fig. 3b). To verify that both the rabbit reticulocyte and the Drosophila enzymes guanylated the same tRNAs, samples guanylated by both enzyme preparations were co-chromatographed. Figure 3c shows that there was no difference in peak distribution, indicating that the products of the two enzymes are chromatographically indistinguishable. Since the Drosophila enzyme preparation was high in ribonuclease activity, the rabbit reticulocyte preparation was routinely used for the remainder of the work.

Using purified E. coli tRNA and the rabbit reticulocyte enzyme, Okada et al.[11] demonstrated that guanylation of wild type E. coli tRNA specifically involved the replacement of Q with guanine in the first position of the anticodon. To determine if the Q-containing isoacceptor forms of Drosophila tRNA were also the substrates for the rabbit reticulocyte enzyme, these species were isolated from crude Drosophila tRNA.

Using Concanavalin A-Sepharose affinity chromatography, both Drosophila $tRNA_{2\delta}^{Asp}$ and crude Drosophila tRNA minus $tRNA_{2\delta}^{Asp}$ were prepared[7]. the samples were recovered by ethanol precipitation, guanylated and chromatographed on RPC-5 columns. It was found that $tRNA_{2\delta}^{Asp}$ was not a substrate for the enzyme and that the RPC-5 profile of guanylated Drosophila tRNA minus $tRNA_{2\delta}^{Asp}$ (Fig. 4b) was identical to that reported for total tRNA. Thus, unlike E. coli tRNA[11], the Q-containing $tRNA_{2\delta}^{Asp}$ in Drosophila is not the main substrate for this reaction.

To determine if the Q-containing isoacceptors of $tRNA^{Asn}$, $tRNA^{His}$, and $tRNA^{Tyr}$ in Drosophila could serve as substrates for the guanylating enzyme, crude tRNA from adult flies was subjected to sodium periodate oxidation[3,18]. The modification procedure is specific to the Q-containing isoacceptor tRNAs by virtue of the cis-diol function of the cyclopentene side chain ($tRNA_{2\delta}^{Asp}$ is protected due to the mannose substitution in position 4 of this ring in Drosophila). Using this procedure, a purified Q-containing tRNA fraction and crude tRNA minus the Q forms were prepared and guanylated. It was found that the periodate-modified Q-containing tRNAs did not accept [^{3}H] guanine with the rabbit enzyme, and that the RPC-5 profile of total tRNA minus the Q-containing isoacceptors was identical to that of total tRNA (Figure 4c). The non-involvement of the Q-containing forms was also shown by the guanylation of Drosophila tRNA with virtually no δ isoacceptor forms of these tRNAs, isolated from 3rd instar larvae (Figure 4d). The profile is characteristic of tRNA from adult flies, indicating that in Drosophila, the γ or unmodified forms of the Q-base family tRNAs serve as substrates for the reticulocyte guanylating enzyme.

In order to examine the substrates for guanylation more closely, crude Drosophila tRNA, high in γ isoacceptor tRNA, was fractionated by RPC-5 chromatography. The column was assayed for acceptance of aspartic acid, asparagine, histidine and tyrosine (Fig. 5), and peak fractions were guanylated with the reticulocyte lysate. The guanylated fractions were individually chromatographed on RPC-5 columns (Fig. 6) and compared with the profile of guanylated (crude) Drosophila tRNA (Figure 3). By comparing the

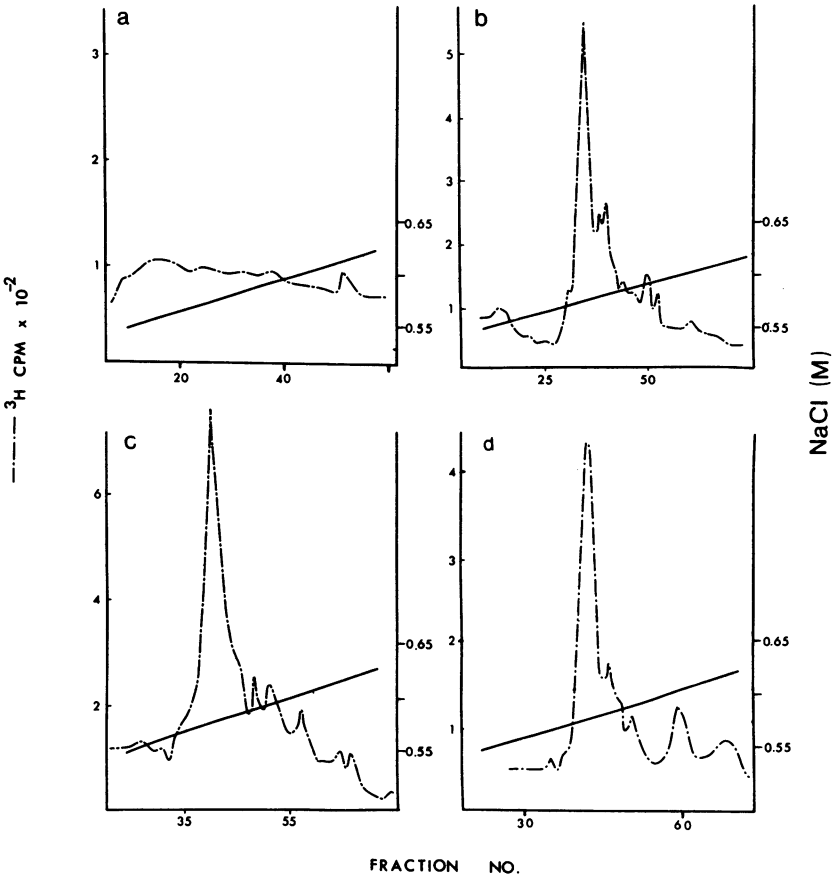

Figure 4. Demonstration that Q- and Q*-containing tRNAs are not substrates for the guanylating enzyme. Various Drosophila tRNAs guanylated and analysed as in Figure : (a) purified $tRNA^{Asp}_{2\delta}$; (b) crude tRNA lacking $tRNA^{Asp}_{2\delta}$; (c) crude tRNA lacking δ forms of Q family; (d) crude tRNA from 3rd instar larvae with 5% δ form of Q-containing tRNAs.

guanylated substrates (Figure 6) with the products in Figure 3, it is concluded that the tRNA species giving rise to the main peak in Figure 3 is $tRNA^{Asp}_{2\gamma}$ (Figure 6-2) and that the prominent shoulder in Figure 3 arises from guanylation of $tRNA^{Tyr}_{1\gamma}$ (Figure 6-5). It is again evident from Figure 6-1 that $tRNA^{Asp}$ (Q*) is not a substrate for guanylation.

To confirm that the unmodified isoacceptor form $tRNA^{Asp}_{2\gamma}$ is the main guanylation substrate in Drosophila, this species was purified. The guanylated product of $tRNA^{Asp}_{2\gamma}$ aligns with the A_{260} and elutes in the same position

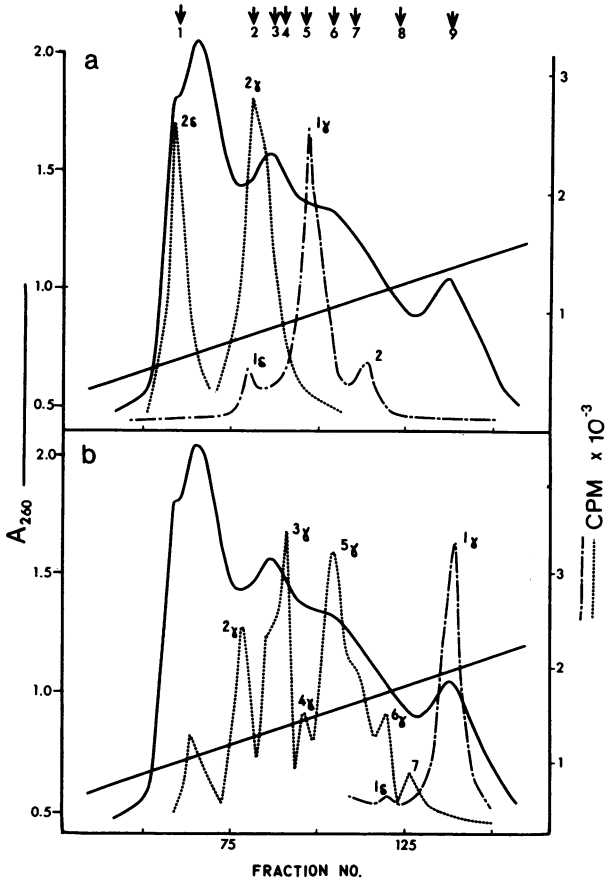

Figure 5. RPC-5 fractionation of Drosophila tRNA. Crude adult tRNA (150 mg with about 95% γ form of $tRNA^{Tyr}$, $tRNA^{His}$ and $tRNA^{Asn}$) was chromatographed on a 2.5 x 100 cm RPC-5 column. Elution was at 37°C with a 3 l 0.55 - 0.65 NaCl gradient. Amino acid acceptance was measured on 25 µl aliquots of each fraction: (a) ....[^{14}C] aspartic acid acceptance; _.-.-_[^{14}C] tyrosine acceptance; (b) ....[^{14}C] asparagine acceptance; _.-.-_[^{14}C] histidine acceptance. The fractions indicated (1-9) were further analysed by guanylation.

as the main guanylated peak from crude Drosophila tRNA (Fig. 7).

DISCUSSION

Drosophila is an ideal organism in which to study the biosynthesis of the hypermodified bases Q and Q* because of the presence of both Q-containing (δ) and Q-lacking (γ) forms of the same $tRNA^2$. The properties of the

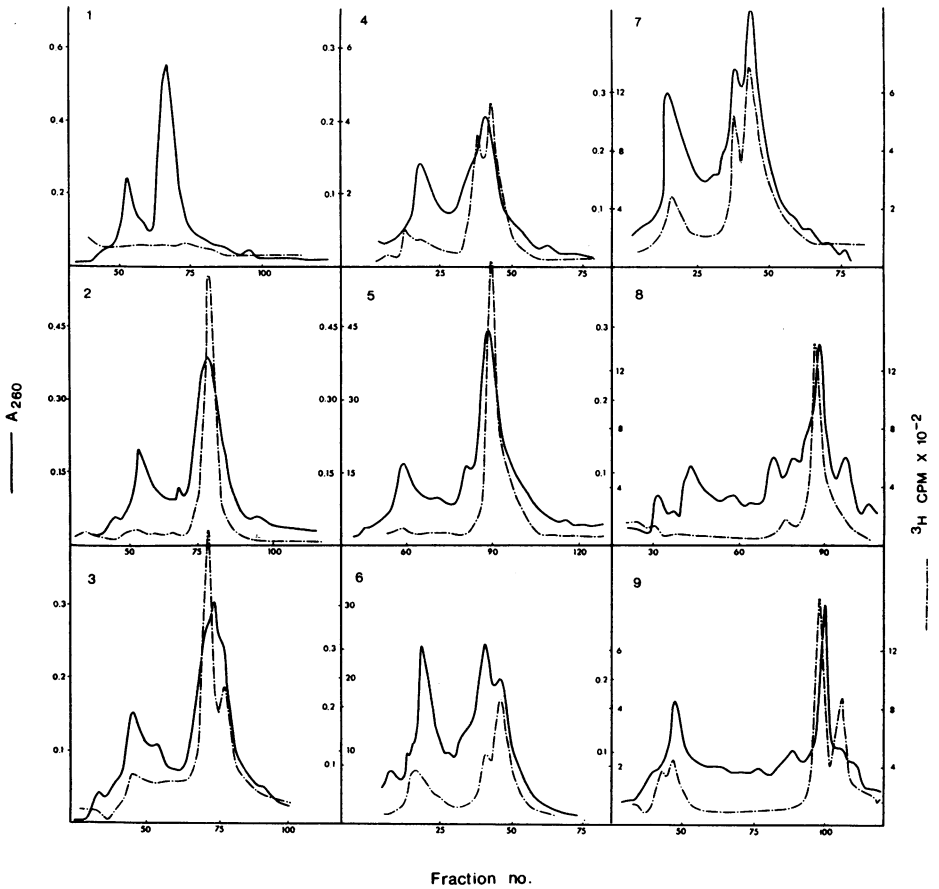

Figure 6. RPC-5 chromatography of guanylated fractions from Fig. 5 (1-9).

guanylating enzyme first described by Farkas and co-workers [9,10,16] suggested that it was involved in Q-biosynthesis. The finding that the γ forms of the Q family of tRNAs in Drosophila are substrates and the δ forms are not, is consistent with this suggestion. The presence of a guanylating activity in extracts of adult flies and its absence in larvae (unpublished results) is also consistent with this in light of the developmental changes of the Q family of tRNAs[2].

Dubrul and Farkas[9] first postulated that the guanylating enzyme might be involved in Q-biosynthesis because the major rabbit reticulocyte tRNA substrate, $tRNA_3^{His}$, was the Q-lacking form. However, because they could not chase out the [^{3}H] guanine by incubation with cold guanine they concluded

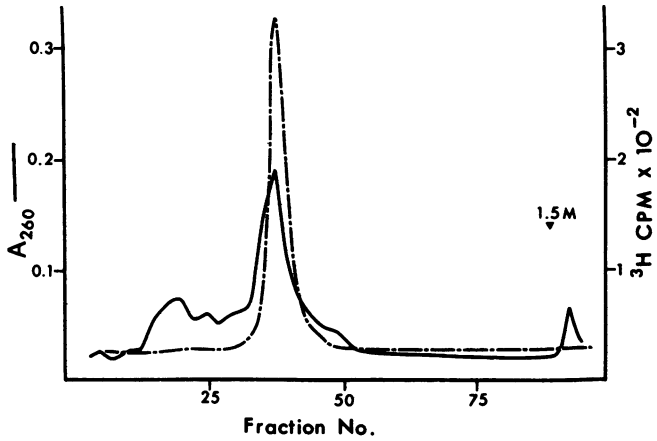

Figure 7. RPC-5 chromatography of guanylated purified $tRNA^{Asp}_{2\gamma}$. 3 A_{260} units of $tRNA^{Asp}_{2\gamma}$ were guanylated and chromatographed as in Fig. 3 followed by a 1.5 M NaCl wash.

that the reaction was irreversible and this led them to discount the possibility of the enzyme being involved in Q-biosynthesis. Since the extent of guanylation represents only 1-2% conversion of substrate, it follows that subsequent incubation of guanylated tRNA with unlabelled guanine would not chase out previously incorporated [^{3}H] guanine. The inability to chase out the label, therefore, should not be used in discussions on the role of this enzyme in Q-biosynthesis.

The Q-lacking form of these tRNAs is apparently the normal substrate for homologous guanylating enzymes in all systems so far examined. In rabbit reticulocytes the Q-lacking forms of $tRNA^{Asn}$ and $tRNA^{His}$ are substrates while rabbit liver tRNA which contains solely Q-containing forms is not a substrate[10]. Yeast tRNA contains no Q yet serves as a substrate for both the reticulocyte and *E. coli* enzymes[11]. The *E. coli* enzyme will not normally guanylate *E. coli* tRNA (which is entirely in the Q-containing form), but will guanylate Q-lacking tRNA obtained from methyl-deficient *E. coli*[12]. The only instance in which Q-containing tRNAs have been shown to be substrates for guanylation was with a heterologous enzyme source[11].

Using tRNA from methyl-deficient *E. coli*, Okada *et al.*[12] identified 3 derivatives of Q that may be possible precursors. All of these precursors were shown to have the 7-deazaguanosine structure with a side chain at C-7. Okada *et al.*[12] have suggested that a precursor (pre-Q) is synthesized from GTP free of the polynucleotide chain, by a mechanism similar to that for the

7-deazaadenosine antibiotic toyocamycin[20] and that the guanine insertion enzyme actually replaces a G in the anticodon with this pre-Q. The very slow rate of the guanylation reaction using either guanine or guanosine as a substrate also supports the idea that a pre-Q might be the normal substrate of the enzyme. The evidence presented here for Drosophila tRNA supports this model. The validity of this biosynthetic scheme can only be established by the use of radioactive Q or pre-Q as a substrate. If confirmed, we propose that the guanine insertion enzyme be more appropriately termed a pre-Q insertion, or guanine replacement enzyme.

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REFERENCES

1. Harada, F. and Nishimura, S. (1972) Biochemistry 11, 301-308.
2. White, B.N., Tener, G.M., Holden, J., and Suzuki, D.T. (1973) J. Mol. Biol. 74, 635-651.
3. White, B.N. (1974) Biochim. Biophys. Acta. 353, 283-291.
4. Kasai, H., Ohashi, Z., Harada, F., Nishimura, S., Oppenheimer, N.J., Crain, P.F., Liehr, J.G., von Minden, P.H., and McCloskey, J.A. (1975) Biochemistry 14, 4198-4208.
5. Kasai, H., Nakanishi, K., MacFarlane, R.D., Torgerson, D.F., Ohashi, A., McCloskey, J.S., Gross, H.J., and Nishimura, S. (1976) J. Am. Chem. Soc. 98, 5044-5046.
6. Kasai, H., Kuchino, Y., Nihei, K., and Nishimura, S. (1975) Nucl. Acid Res. 2, 1931-1939.
7. Wosnick, M.A., and White, B.N. (1978) Biochem. Biophys. Res. Commun. 81, 1131-1138.
8. Hankins, W.D., and Farkas, W.R. (1970) Biochim. Biophys. Acta. 213, 77-89.
9. Dubrul, E.F., and Farkas, W.R. (1976) Biochim. Biophys. Acta. 442, 379-390.
10. Farkas, W.R. and Chernoff, D. (1976) Nucl. Acid Res. 3, 2521-2529.
11. Okada, N., Harada, F., and Nishimura, S. (1976) Nucl. Acid Res. 3, 2593-2603.
12. Okada, N., Yasuda, T., and Nishimura, S. (1977) Nucl. Acid Res. 4, 4063-4075.

13. Kirby, K.S. (1956) Biochem. J. 64, 405-408.
14. Kelmers, A.K., Novelli, G.D., and Stulburg, M.P. (1965) J. Biol. Chem. 240, 3979-3983.
15. Twardzik, D.R., Grell, E.H., and Jacobson, K.B. (1971) J. Mol. Biol. 57, 231-245.
16. Farkas, W.R., Hankins, W.D., and Singh, R. (1973) Biochim. Biophys. Acta. 294, 94-105.
17. Pearson, R.L., Weiss, J.F., and Kelmers, A.D. (1971) Biochim. Biophys. Acta. 228, 770-774.
18. Wosnick, M.A., and White, B.N. (1978) Biochim. Biophys. Acta. (in press).
19. Nishimura, S. (1972) Progr. Nucl. Acid Res. Mol. Biol. 12, 49-85.
20. Suhadolnik, R.J., and Uematsu, T. (1970) J. Biol. Chem. 245, 4365-4371.