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Selenium-containing tRNA^{Glu} from *Clostridium sticklandii*: Correlation of aminoacylation with selenium content

(seleno-nucleotide/clostridial tRNA)

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ABSTRACT Selenium-containing amino acid tRNAs are normal components of several bacterial tRNA populations. In *Clostridium sticklandii* seleno-nucleotides occur in at least four different tRNA species which account for 5–8% of the total tRNA population. One of these has been isolated in a highly purified form and shown to be an isoaccepting tRNA^{Glu}. Experimental evidence indicates that the presence of the seleno-nucleotide in this tRNA^{Glu} is essential for its acylation with glutamate.

Selenium, an essential micronutrient for mammals, birds, and several bacteria (1), is now known to be a normal component of at least six different enzymes (2). When selenium was detected earlier in the tRNAs of *Escherichia coli* (3, 4) in the form of selenium-modified bases, it was thought to have resulted from the nonspecific substitution of the element for sulfur, but recent studies show that selenium in these tRNAs is a normal component (5) and is incorporated by means of a highly specific process (6). The tRNAs of two strictly anaerobic bacteria, *Clostridium sticklandii* and *Methanococcus vannielii*, have a higher content of selenium and this is distributed among four to six seleno-tRNA species (7, 8). That this is a normal level is indicated by the fact that incorporation of selenium into the tRNAs of these organisms is not decreased even when the molar ratio of sulfur to selenium in the culture medium is increased by 2 to 3 orders of magnitude.

Among the 50 or more different modified bases that have been detected in tRNAs (9), some are believed to play an important role in codon recognition and a few have been shown to exert other types of regulatory functions. It seems likely that the biological consequences of selenium modification of certain tRNAs will prove to be similar in nature.

The present communication describes the isolation of one of the selenium-containing tRNAs present in *C. sticklandii* and its identification as an isoaccepting tRNA^{Glu}. The direct correspondence between selenium content and glutamate-accepting activity suggests that selenium occurs at a site on this tRNA that is important for interaction with its cognate synthetase.

MATERIALS AND METHODS

Materials. The following were purchased from commercial sources: bulk tRNA from *E. coli* MRE 600 (Boehringer Mannheim), ATP (Sigma), DEAE-cellulose (Whatman), organomercurial agarose Affi-Gel 501 (Bio-Rad), radioactive amino acids (Amersham), $H_2{}^{75}SeO_3$ (New England Nuclear), and disc membrane filters, type HA (Millipore). Plaskon CTFE 2300 powder and Adogen 464 (methyltrialkylammonium chloride) for RPC-5 column packing were gifts from D. Novelli.

Bacterial ^{75}Se-Labeled tRNAs. Labeling of *C. sticklandii* cells with 0.5 μM $Na_2{}^{75}SeO_3$ (1 m Ci/μmol; 1 Ci = 3.7×10^{10} becquerels) in the presence of 1.25 mM Na_2S was essentially as described (7). Bulk tRNAs were extracted from cells by the procedure of Holley (10).

Aminoacyl-tRNA Synthetase Preparation. Unfractionated aminoacyl-tRNA synthetases from *C. sticklandii* were prepared by a slight modification of the method of Hatfield and Portugal (11). Frozen cells thawed in 2 vol of buffer A [50 mM Tris·HCl, pH 8/10 mM $MgCl_2$/0.2 M NaCl/1 mM dithiothreitol/50 μM phenylmethylsulfonyl fluoride and 10% (vol/vol) glycerol] were broken in a French pressure cell at 10,000 psi (69 MPs). After centrifugation at 200,000 × g for 2 hr, the cell extract was diluted with an equal volume of buffer B [150 mM Tris·HCl, pH 7.1/10 mM $MgCl_2$/1 mM dithiothreitol/50 μM phenylmethylsulfonyl fluoride/10% (vol/vol) glycerol]. The extract was freed of tRNA by treatment with an equal volume of DEAE-cellulose previously equilibrated with buffer C [100 mM Tris·HCl, pH 7.4/0.1 M NaCl/10 mM $MgCl_2$/1 mM dithiothreitol/50 μM phenylmethylsulfonyl fluoride/10% (vol/vol) glycerol]. After stirring for 15 min, the DEAE-cellulose was removed and the extract was brought to 75% saturation with $(NH_4)_2SO_4$. The precipitated protein was collected, dissolved in an equal volume of buffer C, and dialyzed overnight against buffer C. Glycerol was added to the dialyzed sample to a final concentration of 50%. When stored in liquid nitrogen, the synthetase activities of the preparation were stable for at least 9 months.

Purification of tRNAs. Sulfur- or selenium-containing tRNAs in the bulk tRNA were selectively enriched by affinity chromatography on an organomercurial agarose gel (Affi-Gel 501). The gel was equilibrated with 0.1 M sodium acetate buffer at pH 5.0. About 56–78% of the tRNAs in the applied sample, depending on flow rate, could be washed off by the equilibrating buffer. Bound molecules, consisting of thiolated tRNA in addition to seleno-tRNA, were readily eluted by 0.1 M sodium acetate, pH 5.0/1 mM dithiothreitol. The presence of selenium in this tRNA population was determined by atomic absorption using a Perkin–Elmer atomic absorption spectrophotometer (model 603), and tRNAs containing 4-thiouridine were detected by absorbancy measurements at 335 nm.

Rapid reversed-phase chromatography of tRNA was carried out according to the method of Kelmers and Heatherly (12). All isolation procedures were carried out in dim light.

Difference spectra were recorded on a Hewlett–Packard spectrophotometer (model 4850A) which had a built-in microprocessor.

RESULTS AND DISCUSSION

The selenium content of tRNAs isolated from *C. sticklandii* is sufficient to modify 5–8% of the total tRNA population (assum-

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Abbreviations: Se^{4}Ura, 4-selenouracil; S^{4}Ura, 4-thiouracil; Se^{2}Ura, 2-selenouracil; Se^{2}Thy, 2-selenothymine.

ing one selenium-modified nucleotide per tRNA molecule). These seleno-nucleotides occur in at least four different tRNA species. A typical reversed-phase chromatographic profile of seleno-tRNAs from *C. sticklandii* cells labeled with ^{75}Se is shown in Fig. 1. Initally, the two major seleno-tRNAs of this profile were isolated in a highly purified form as nonacylated species by a combination of chromatographic techniques—e.g., reversed salt gradient on Sepharose 4B, ion exchange on DEAE-Sephadex A-50, and reversed phase on RPC-5. These isolated tRNAs, however, failed to be acylated by an aminoacyl-tRNA synthetase preparation from *E. coli* and thus their identification by this method was unsuccessful. Also, acylation of these purified seleno-tRNAs with a homologous aminoacyl-tRNA synthetase preparation was barely detectable, although several nonselenium tRNAs from *C. sticklandii* were charged by both the homologous and heterologous synthetase preparations.

We investigated the possibility that the seleno-tRNAs were preferentially inactivated during purification by assaying the ability of the *C. sticklandii* aminoacyl-tRNA synthetases to esterify each of their 20 individual cognate tRNAs in bulk tRNA. For comparative purposes, an identical set of assays using bulk *E. coli* tRNA and the *C. sticklandii* synthetase preparation also was performed. The results of these experiments are summarized in Table 1. In general the amino acid-accepting activities of individual tRNAs in the *E. coli* population were greater than the corresponding ones in the *C. sticklandii* bulk tRNA under the assay conditions used. The one striking exception was glutamate. The capacity of the *C. sticklandii* tRNA preparation to be acylated by glutamate was almost 10 times greater than that of *E. coli* bulk tRNA. In other experiments (data not shown) in which assays were performed with *E. coli* synthetases, the observed glutamate-accepting activity of *E. coli* bulk tRNA was more than 6-fold greater than that measured with *C. sticklandii* synthetases. In contrast, the values for other amino acids—e.g.,

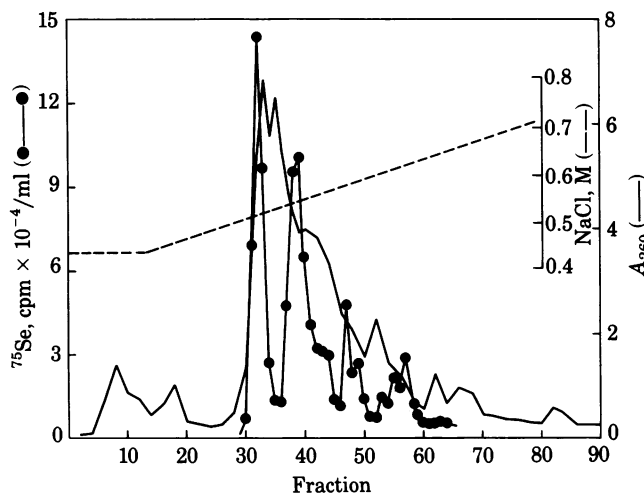

FIG. 1. Reversed-phase chromatography of seleno-tRNAs from *C. sticklandii*. Bulk tRNA (3.6 × 10^6 cpm; 288 A_{260} units) prepared from ^{75}Se-labeled *C. sticklandii* cells was dissolved in 6 ml of buffer D (10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/0.45 M NaCl) and applied to a RPC-5 column (0.9 × 41 cm bed). After a washing step with 10 ml of buffer D, the adsorbed tRNA was eluted with a linear gradient (total volume, 200 ml) of buffer D and buffer E (same as buffer D but containing 0.8 M NaCl). Fractions (2 ml) were collected at a flow rate of 1.6 ml/min. The column pressure was 500 psi at the beginning and was gradually increased to 550 psi as the salt concentration increased. Aliquots (20 μl) of each fraction were diluted with 0.5 ml of H_2O and assayed for ^{75}Se by scintillation spectrometry in 10 ml of Aquasol.

Table 1. Extent of aminoacylation of *E. coli* tRNA and *C. sticklandii* tRNA by a *C. sticklandii* tRNA synthetase preparation

Amino acid	Conc. μM	Amino acid incorporated,* pmol/A_{260} *E. coli*	*C. sticklandii*	Relative activity†
Ala	98	12.3 ± 0.2	10.1 ± 1.9	1.20
Arg	69	36.6 ± 1.0	13.6 ± 0.5	2.68
Asn	123	27.1 ± 0.2	0.70 ± 0.26	38.5
Asp	84	27.7 ± 1.8	1.62 ± 0.53	17.0
Cys	205	19.4 ± 1.2	12.2 ± 1.2	1.60
Glu	75	2.61 ± 0.52	20.2 ± 3.1	0.13
Gln	163	57.0 ± 0.8	9.2 ± 1.9	6.20
Gly	128	37.4 ± 0.1	15.8 ± 0.3	2.37
His	69	16.8 ± 1.1	3.07 ± 0.12	5.48
Ile	69	85.9 ± 0.5	32.1 ± 1.2	2.67
Leu	70	123 ± 1.1	47.1 ± 0.9	2.60
Lys	70	48.5 ± 1.8	6.19 ± 2.0	7.83
Met	75	59.1 ± 1.7	44.5 ± 1.9	1.33
Phe	59	30.8 ± 0.9	36.3 ± 3.3	0.85
Pro	75	31.2 ± 4.7	6.61 ± 0.26	4.73
Ser	97	41.0 ± 5.5	32.4 ± 2.5	1.27
Thr	84	32.7 ± 0.9	8.18 ± 0.59	4.00
Trp	209	10.4 ± 1.5	12.7 ± 1.9	0.81
Tyr	60	32.7 ± 0.5	26.2 ± 0.7	1.25
Val	74	93.0 ± 0.7	34.3 ± 3.3	2.71

The complete reaction mixture (51 μl) contained 50 mM Tris·HCl (pH 7.5) 10 mM $MgCl_2$, 10 mM KCl, 2 mM ATP, 1 mM dithiothreitol, 0.82 A_{260} unit of tRNA, 26 μg of the *C. sticklandii* synthetase preparation, the indicated amount of ^{14}C-labeled amino acid, and the 19 other unlabeled amino acids (40 μM each). After 10-min incubation at 37°C, reactions were terminated by the addition of 0.8 ml of ice-cold water followed by 6 ml of 5% (wt/vol) trichloroacetic acid. After mixing, the samples were held in ice for 20 min, filtered on membrane filter discs, washed three times with 5-ml portions of 5% trichloroacetic acid, and dried under a heating lamp. The radioactivity bound to the dried discs was measured in 10 ml of Econofluor (New England Nuclear).
* Shown as mean ± SD.
† Ratio of *E. coli* value to *C. sticklandii* value.

aspartate, lysine, threonine, and valine—were similar with both synthetase preparations. This suggested that the *C. sticklandii* glutamyl-tRNA synthetase might be relatively specific for its own $tRNA^{Glu}$ and this could be a reflection of the presence of a greater amount of a particular glutamate isoaccepting species.

A $tRNA^{Glu}$ species containing a relatively labile seleno-nucleotide would be a reasonable candidate in view of earlier indications that one of the seleno-tRNAs appeared to purify with a glutamate-accepting species during the initial steps of the isolation procedure. To test this hypothesis, an alternative procedure was developed to facilitate isolation of seleno-tRNA and $tRNA^{Glu}$ species. Thiolated tRNAs and selenium-containing tRNAs were first separated from the bulk tRNA by adsorption to an organomercurial gel. After elution and concentration, these tRNAs were incubated in a mixture containing the *C. sticklandii* synthetase preparation and [^{14}C]glutamate to acylate any $tRNA^{Glu}$ that might be present. The tRNAs recovered from the incubation mixture then were chromatographed on a RPC-5 column (Fig. 2). Three or four [^{14}C]glutamate-associated tRNA species were eluted from this column in the salt gradient between 0.5 and 0.57 M NaCl. The major peak of ^{14}C that emerged in fractions 70 to 74 coincided with an A_{260} peak and also a selenium-containing peak. Results of the analyses performed on each of the fractions of this peak after recovery of the tRNA by ethanol precipitation are shown in Table 2. The high degree of correlation among the amounts of glutamate and selenium and absorbancy at 260 nm in these fractions indicates

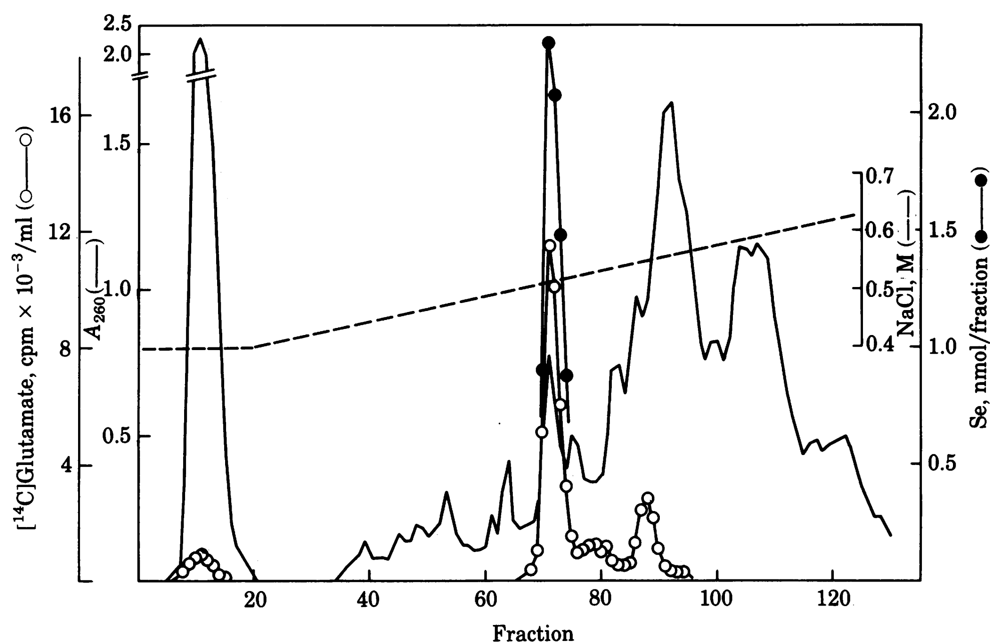

FIG. 2. Reversed-phase chromatography of glutamyl-tRNA^{Glu}. Bulk tRNA (500 A_{260} units from nonradioactive *C. sticklandii* cells) was applied to an Affi-Gel 501 column. The sulfur and selenium containing tRNAs which were selectively absorbed were eluted with sodium acetate buffer containing 1 mM dithiothreitol. These tRNAs were incubated with 2 mM [^{14}C]glutamate (specific activity, 6.13 Ci/mol) and an unfractionated aminoacyl-tRNA synthetase preparation from *C. sticklandii*. The reaction conditions were as described in the legend of Table 1 except that 40 mM NaCl was included in the total mixture of 8 ml, the 19 other amino acids were omitted, and the incubation time was 15 min. The tRNAs (155 A_{260} units) recovered from the incubation mixture as described by Chiu *et al.* (13) were dissolved in buffer F (10 mM sodium acetate, pH 4.5/10 mM magnesium acetate/1 mM EDTA, 1 mM dithiothreitol/0.4 M NaCl) and chromatographed on a RPC-5 column (0.9 × 49 cm bed). The sample was eluted at about 400 psi with a linear gradient (total volume, 240 ml) of buffer F and buffer G (same as buffer F but containing 0.65 M NaCl). Fractions (2 ml) were collected at a flow rate of 1.0 ml/min. Aliquots (50 μl) of each fraction were assayed for ^{14}C in 10 ml of Aquasol by liquid scintillation spectrometry. The amount of selenium in the major ^{14}C peak (fractions 70–74) was determined by atomic absorption. The ratio of glutamate to selenium and the amount of selenium per A_{260} unit in the fractions of this peak were determined after ethanol precipitation of the tRNA and are listed in Table 2.

that this major isoaccepting tRNA^{Glu} species is a seleno-tRNA.

To characterize this material further, fractions 71 and 72 were combined, deacylated, and then tested for the ability to be reaminoacylated with [^{3}H]glutamate. After this treatment the glutamate-accepting activity of the tRNA sample was only about half of that expected from its A_{260} value (assuming 1.66 nmol of tRNA per A_{260} unit). Moreover, analysis of the amount of selenium in the sample showed that half had been lost during the process of deacylation. Before treatment the average amount of selenium per A_{260} unit in fractions 71 and 72 was 1.58 nmol but after deacylation it was only 0.8 nmol (Table 3). This fortuitous loss of half of the selenium during the deacylation process and the concomitant 50% decrease in glutamate-accepting activity strongly suggest that selenium is essential for the acylation. The ratio of esterified glutamate to selenium was approximately 0.8 for the isolated charged tRNA^{Glu}; after the deacylation treatment, the ratio was 1.14. These data indicate a stoichiometry of one selenium atom per active tRNA^{Glu} molecule. The lower initial ratio could have been due to loss of glutamate from the charged tRNA^{Glu} during the overall isolation procedure.

From these results it seems likely that a selenium-modified nucleotide in this tRNA^{Glu} species is essential for recognition by its cognate glutamyl-tRNA synthetase. There is suggestive evidence that the second major seleno-tRNA peak in the profile of nonacylated tRNAs (Fig. 1) contains this seleno-tRNA^{Glu}. Whether this was inactivated during recovery in the earlier experiments or was present along with other seleno-tRNAs and

Table 2. Amounts of glutamate and selenium in fractions from [^{14}C]glutamate-tRNA^{Glu} peak of Fig. 2

Fraction	[^{14}C]Glu, nmol	Se, nmol	[^{14}C]Glu/Se	Se, nmol/A_{260}
70	0.50	0.56	0.89	0.89
71	1.29	1.63	0.79	1.53
72	1.00	1.29	0.78	1.62
73	0.62	0.93	0.67	1.58
74	0.32	0.53	0.59	1.13

Table 3. Correlation of glutamate-accepting activity and selenium content of tRNA^{Glu}

tRNA^{Glu} preparation	Se, nmol/ A_{260}	Glu, nmol/ A_{260}	Glu/Se
Isolated as charged species	1.58	1.24	0.79
After deacylation and reaminoacylation	0.8	0.91	1.14

The peak glutamate-accepting fractions (nos. 71 and 72) from the RPC-5 column profile shown in Fig. 2 were combined and the tRNA was deacylated by incubation in 1 M Tris·HCl (pH 8.5) at 37°C for 90 min. This treatment released 96% of the ^{14}C which was found in the supernatant solution after ethanol precipitation of the tRNA. The selenium content of the deacylated tRNA preparation, determined by atomic absorption, was 12 pmol per 0.015 A_{260} unit. Aliquots containing 4.5 to 29 × 10^{-3} A_{260} unit of the tRNA were reaminoacylated under conditions described in the legend of Table 1, except that 2 mM [^{3}H]glutamate (specific activity, 97.6 Ci/mol) and 40 mM NaCl were included. The amount of glutamate incorporated was a linear function of the amount of selenium in each aliquot over this entire range.

therefore was not detected in the aminoacylation assays is not known at present. The lability of selenium in seleno-tRNAs, particularly at alkaline pH values, has been observed in this laboratory and by others (4). In the modified isolation procedure that finally was used in this study, either the lower pH or acylation of the $tRNA^{Glu}$ may have been responsible for the increased stability of the seleno-base in the molecule.

The modified base 4-thiouracil (S^4Ura), which occurs at the eighth position from the 5′ end of a large number of *E. coli* tRNA species, can be detected because of its characteristic electronic absorption maximum at about 335 nm (14). Its selenium analog, Se^4Ura, exhibits an absorption maximum at about 366 nm (15). Similar bathochromic shifts in the spectra of other modified nucleotide bases characteristically result from replacement of sulfur by selenium (16). To determine if the seleno-$tRNA^{Glu}$ exhibited any unusual absorbancy in the ultraviolet range that might furnish a clue to the nature of its selenium-modified base, difference spectra of the purified sample were recorded; as the reference sample, we used a population of sulfur- and selenium-deficient tRNAs from *C. sticklandii* that were not retained on the organomercurial agarose gel. The difference spectrum of the glutamyl-$tRNA^{Glu}$ sample from fractions 71 and 72 of Fig. 2 is shown in Fig. 3. The two positive absorbance maxima at 293 nm and 327 nm are equivalent to about 3% of the absolute absorbance of the sample at λ_{max} 258 nm. Assuming a molar extinction coefficient of the unknown seleno-base is similar to those reported (16) for Se^2Ura, Se^2Thy and 6-selenopurine (12,000–16,200 M^{-1} cm^{-1}) and 1.66 nmol of tRNA per A_{260} unit, the absorbance contributed by one seleno-base per $tRNA^{Glu}$ molecule would be about 2% to 3% of the total absorbancy at 260 nm. This is in good agreement with the value calculated from the experimental data presented above.

Whether the two absorbance peaks observed in the difference spectrum represent the contribution of one seleno-base and one thiobase present in the same tRNA molecule or the contribution of an additional substituent on the selenium-modified base itself or result from the interactions of the seleno-base with other bases is unknown. At present we are unaware of any published spectral data for known thiolated bases or selenium containing bases that would explain these observations. Although selenium is known to be able to replace sulfur in the enzymic reactions leading to the synthesis of S^4Ura in tRNA (1, 17) and the occurrence of Se^4Ura in *E. coli* has been reported (3, 4) based on the argument of cochromatography with authentic Se^4Ura, no rigorous identification of a naturally occurring seleno-nucleotide from tRNA has been made. Digestion of ^{75}Se-labeled bulk tRNA preparations from either *C. sticklandii* or *Methanococcus vannielii* with nucleases liberates a radioactive nucleotide that has been partially purified by a series of chromatographic procedures (8). In DEAE-cellulose chromatographic profiles this ^{75}Se-labeled nucleotide is eluted in LiCl or ammonium acetate gradients after a guanine nucleotide and slightly ahead of 4-thiouridylic acid. The latter is an abundant nucleotide in the tRNAs of both microorganisms. This type of elution pattern, which has been observed repeatedly, serves to distinguish the selenium-containing nucleotide from 4-selenouridylic acid. The latter, which is more acidic than its sulfur analog, should follow rather than precede 4-thiouridylic acid in elution profiles from DEAE-cellulose. Attempts to purify the selenonucleotide further by electrophoretic and thin-layer chromatographic procedures have been hampered by problems of instability, suggesting that its final identification may depend on the development of alternative isolation procedures.

The *C. sticklandii* $tRNA^{Glu}$ described in this report was initially detected in bulk tRNA by its greater glutamate-accepting activity compared to *E. coli* $tRNA^{Glu}$ (Table 1). In contrast, in this study, the aspartate- and asparagine-accepting activities of the *C. sticklandii* bulk tRNA were much lower than their *E. coli* counterparts. Although unexplained at present, these observations suggest the presence of unusual aspartate- and asparagine-accepting tRNA species in *C. sticklandii*.

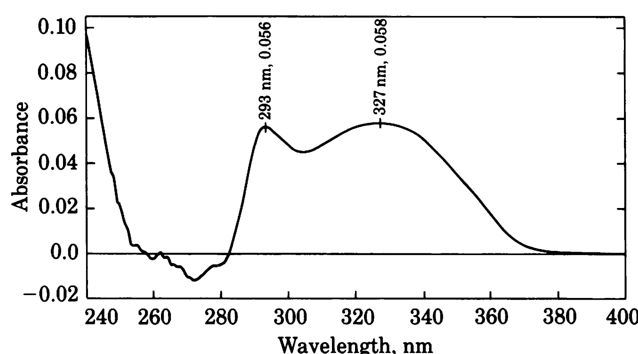

FIG. 3. Difference spectrum of seleno-$tRNA^{Glu}$ against tRNAs deficient in sulfur and selenium. Seleno-$tRNA^{Glu}$ (A_{258} = 1.908 units) from fractions 71 and 72 (Fig. 2) was dissolved in 50 mM sodium acetate buffer, pH 4.9/100 mM NaCl/10 mM $MgCl_2$. The reference sample (A_{258}= 1.901 units) in the same buffer was those tRNAs that had no affinity for Affi-Gel 501. The small difference in absorbance at 258 nm (λ_{max}) was corrected for. The differences in absorbance at 293 and 327 nm were 0.056 and 0.058 respectively, which was about 3% of the absorbance at λ_{max}.

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