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Biochemistry

Selenium-containing tRNAs from *Clostridium sticklandii*: Cochromatography of one species with L-prolyl-tRNA

(seleno-tRNAs/^{75}Se labeling *in vivo*/L-proline acceptor activity)

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ABSTRACT **^{75}Se-Labeled tRNAs were synthesized by *Clostridium sticklandii* cultures supplemented with 1 μM sodium [^{75}Se]selenite or [^{75}Se]selenocysteine. This process is highly specific for selenium; it occurred in the presence of 1.2 mM sodium sulfide and was not decreased by the further addition of a 500-fold molar excess of cysteine. The ^{75}Se in these tRNAs was located in the polynucleotide portion of the molecules and not in esterified (alkali-labile) selenocysteine. Inhibition of cell multiplication by antibiotics that block either protein synthesis or DNA-dependent RNA synthesis did not prevent this ^{75}Se incorporation. Three [^{75}Se]tRNAs were separated from *C. sticklandii* cells labeled in the presence of chloramphenicol and were partially purified by chromatography on benzoylated DEAE-cellulose and DEAE-Sephadex A-50 columns. These were designated seleno-tRNAs I, II, and III according to their elution sequence from benzoylated DEAE-cellulose. Cochromatography of purified seleno-tRNA II on DEAE-Sephadex A-50 with an L-proline-accepting species suggests that it is a selenium-containing L-prolyl-tRNA.**

Selenium has been shown to be an essential component of a member of enzymes, and in addition several selenoproteins of unknown function have been discovered (1, 2). In three of the selenoenzymes—glycine reductase (3), glutathione peroxidase (4), and formate dehydrogenase (5)—selenium occurs in the form of a specific selenocysteine residue in the polypeptide chain. The selenium analogs of methionine, *S*-adenosylmethionine, oxytocin, several sulfur-containing coenzymes, and various thiopurines and thiopyrimidines have been prepared in the laboratory or isolated from biological systems after incubation with radioactive selenium. However, the occurrence of these derivatives in the form of *essential* biological constituents produced in the cell by reactions highly specific for selenium is not established. In *Escherichia coli* it is known that selenium can be incorporated into tRNA (6, 7), and a ^{75}Se-labeled nucleoside that cochromatographed with 4-selenouridine was isolated from enzymic digests of ^{75}Se-labeled tRNA. In this case it was concluded that selenium incorporation had occurred by the known pathway of sulfur transfer to a specific uracil residue of *E. coli* tRNA (8), with the result that some labeled 4-selenouracil was formed instead of the normal 4-thiouracil. However, no dilution effects of varying ratios of sulfur to selenium in the medium were reported. The sulfur transferase that catalyzes this reaction utilizes either cysteine or selenocysteine as donor with similar efficiency (M. Lipsett, personal communication), and thus if the observed ^{75}Se labeling had occurred by this process it should have been markedly decreased by the addition of high levels of sulfur.

In the present study it was observed that selenium is incorporated into certain of the tRNAs of *Clostridium sticklandii* and a few other anaerobic bacteria by a process highly specific for selenium. This communication describes the partial purification of the three [^{75}Se]tRNAs produced by *C. sticklandii* and the tentative identification of one of these as L-prolyl-tRNA.

MATERIALS AND METHODS

Materials. The following were purchased from commercial sources: Benzoylated DEAE-cellulose and ATP (Boehringer Mannheim); DEAE-cellulose, (Whatman); DEAE-Sephadex A-50 (Pharmacia); L-[*U*-^{14}C]proline (ICN); L-[2,3,4,5-^{3}H]-proline, amino acid mixture TRK.440 containing 15 ^{3}H-labeled amino acids, amino acid mixture TRK.550 containing 5 ^{3}H-labeled amino acids and L-[^{75}Se]selenocystine (Amersham); and H_2 $^{75}SeO_3$ (New England Nuclear). An unfractionated aminoacyl-tRNA synthetase preparation from *E. coli* was a generous gift from D. Hatfield.

Methods. Cultures of *C. sticklandii* that had been allowed to attain maximum turbidity in a selenium-deficient complex medium (9, 10) were diluted into equal volumes of fresh media supplemented with $Na_2{}^{75}SeO_3$ or L-[^{75}Se]selenocystine (0.5–1 μM final concentrations). All cultures contained 1.2 mM sodium sulfide as reducing agent and in some instances cysteine (0.1–1 mM) also was added. Chloramphenicol (100 mg/liter final concentration) was added where indicated to prevent cell multiplication. Cultures were incubated anaerobically at 30°C or 35°C for the times specified in the various experiments. Cells were collected by centrifugation and sonic extracts were prepared as described (9, 10). Acidic proteins and tRNAs were separated from crude extracts by adsorption to DEAE-cellulose and elution with phosphate buffers containing 1 M NaCl. After concentration with ammonium sulfate, the ^{75}Se-labeled fraction was subjected to molecular sieve chromatography on Bio-Gel P-30 polyacrylamide columns (Bio-Rad) (9).

For isolation purposes the ^{75}Se-labeled tRNAs were extracted from cells or acidic protein fractions with phenol and purified by the procedure of Holley (11) modified to include chromatography on benzoylated DEAE-cellulose (12) and DEAE-Sephadex A-50 (13).

Amino acid acceptor activity of ^{75}Se-labeled tRNA fractions was assayed by a filter paper disc method (14) after incubation with ^{3}H- or ^{14}C-labeled amino acids and a preparation of unfractionated *E. coli* aminoacyl-tRNA synthetases as described in the legend of Table 2.

The biological activity of selenoprotein A was determined in the glycine reductase assay (10). To avoid interference from ^{75}Se, unlabeled glycine was used as substrate and acetate formation was estimated by subsequent reaction with acetate kinase. Selenoprotein A activity is expressed as arbitrary units based on a colorimetric assay of the amount of acethydroxamate formed (15).

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RESULTS

Observations of an unknown selenium-containing macromolecule

Selenoprotein A, one of the components of *C. sticklandii* glycine reductase, is a 12,000 dalton acidic protein that contains a single selenocysteine residue (3, 16). During a series of investigations on the biosynthesis of this protein it was noticed that conspicuous amounts of a second acidic, selenium-containing macromolecule of about twice the size accumulated under certain conditions. A representative profile that shows the elution pattern of this substance and selenoprotein A from a polyacrylamide P-30 column is shown in Fig. 1. In this instance the selenium-labeled macromolecules were produced by selenium-deficient cells that had been incubated in fresh culture medium containing [^{75}Se]selenocysteine and sufficient bacitracin to partially arrest cell multiplication. The same amounts of these labeled products were isolated from an identical culture incubated with [^{75}Se]selenite instead of [^{75}Se]selenocysteine (see legend of Fig. 1). The prominent radioactive peak that was eluted ahead of selenoprotein A was inactive as a component of the glycine reductase system (10). In parallel cultures incubated with [^{75}Se]selenite in the absence of bacitracin approximately equal amounts of the two labeled materials were formed during a short incubation period (Fig. 2, left), whereas by the time the cells had doubled in number there was a relatively greater increase in the amount of [^{75}Se]selenoprotein A present

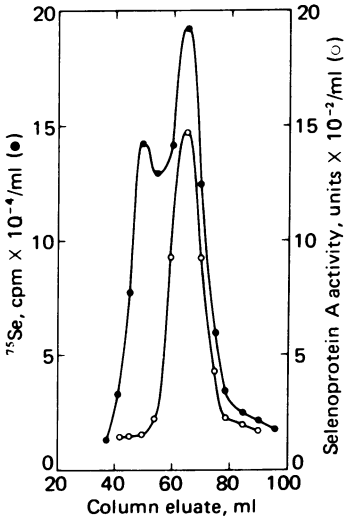

FIG. 1. Polyacrylamide P-30 chromatography of an ^{75}Se-labeled acidic protein fraction from *C. sticklandii*. A 1-liter selenium-deficient culture of *C. sticklandii* was diluted with 1 liter of fresh medium containing [^{75}Se]selenocysteine (2 μmol, 37 × 10^6 cpm/μmol) and bacitracin (28 mg). During incubation for 5 hr at 30°C the cell density increased from 83 (determined turbidimetrically in a colorimeter with a 660-nm filter immediately after dilution) to 137 at the time of harvest. A crude [^{75}Se]selenoprotein A fraction was prepared from the extract of these cells (9) and chromatographed on a polyacrylamide P-30 column (2.7 × 31 cm bed) equilibrated with 100 mM potassium phosphate, pH 7.2, containing 1 mM dithiothreitol. The breakthrough volume of this column was 35 ml, the brown ferredoxin band eluted in the fractions between 80 and 95 ml, and salt emerged after 130 ml. The peak of ^{75}Se-labeled material in fractions 40–54 ml contained 1.47 × 10^6 cpm (39.8 nmol of Se) and the selenoprotein A peak contained 2.49 × 10^6 cpm (67.5 nmol of Se). An identical culture containing bacitracin that had been incubated with [^{75}Se]selenite (1.5 μmol, 107 × 10^6 cpm/μmol) instead of selenocysteine was analyzed in the same manner. The 40–54 ml peak fractions contained 4.16 × 10^6 cpm (38.9 nmol of Se) and the selenoprotein A peak contained 7.86 × 10^6 cpm (73.5 nmol of Se). Selenoprotein A activity was estimated as described in ref 10.

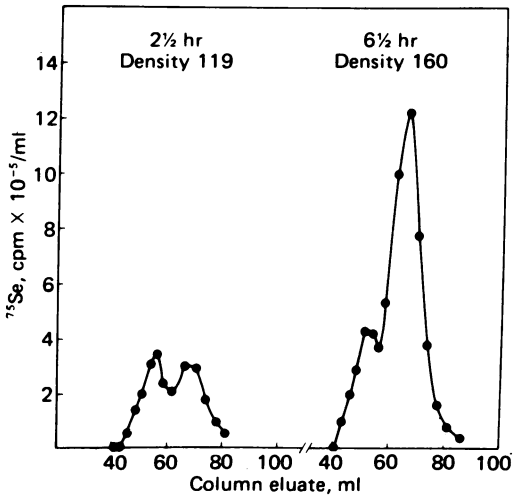

FIG. 2. Polyacrylamide P-30 profiles of ^{75}Se-labeled acidic macromolecules from *C. sticklandii*. One-liter samples of the same selenium-deficient culture used for the experiments of Fig. 1 were diluted with equal volumes of fresh media containing [^{75}Se]selenite (1.5 μmol, 107 × 10^6 cpm/μmol) and incubated in the absence of bacitracin for the times indicated on the figure. Indicated cell densities were measured in a Klett colorimeter with a 540-nm filter. Labeled extracts from these cells were fractionated as described in the legend of Fig. 1, using the same polyacrylamide P-30 gel column. The radioactivity in the leading ^{75}Se peaks of the profiles was equivalent to 40.3 nmol and 46 nmol of Se for the 2½- and 6½-hr cultures, respectively, as compared to 46 nmol and 154.5 nmol of Se, respectively, in the selenoprotein A peaks (60–80 ml eluate fractions).

(Fig. 2, right). When cell multiplication was completely inhibited by inclusion of rifampicin or chloramphenicol in the radioactive dilution media, only the ^{75}Se-labeled substance that was eluted in the peak near the front of the P-30 column profiles was formed, and [^{75}Se]selenoprotein A synthesis was completely blocked (data not shown). Addition of 50- to 500-fold molar excess L-cysteine to cultures incubated with L-[^{75}Se]selenocysteine in the presence of chloramphenicol did not decrease the amount of ^{75}Se incorporation into this material (Table 1). This lack of a dilution effect of the sulfur amino acid analog indicated the highly specific nature of the observed ^{75}Se incorporation.

Rechromatography of the radioactive material from several pooled polyacrylamide P-30 column fractions, after concen-

Table 1. Incorporation of ^{75}Se from L-[^{75}Se]selenocysteine in the presence of excess L-cysteine

Addition to culture	Incubation time, min	Cell density of culture at harvest, Klett units*	Amount of ^{75}Se in P-30 peak fraction, cpm
None	150	160	926,000
Cysteine, 0.1 mM	150	170	886,000
Cysteine, 1 mM	210	160	1,138,000
None†	240	157	1,131,000

One-liter portions of a 22-hr selenium-deficient culture (cell density 270 Klett units) were diluted with equal volumes of fresh media, and chloramphenicol (200 mg), L-[^{75}Se]selenocysteine (2 μM), and the indicated amounts of L-cysteine were added. All cultures also contained 1.2 mM sodium sulfide. At the indicated times the cells were harvested and extracts were fractionated as described in the legend of Fig. 1. In all cases a single radioactive peak that emerged from the polyacrylamide P-30 column in fractions between 40 and 54 ml was present; no selenoprotein A was formed.

* Measured with a 540-nm filter.

† One liter of a 22-hr selenium-deficient culture grown in the presence of 10 μM K_2TeO_3 was used; cell density of the inoculum prior to dilution = 260 Klett units.

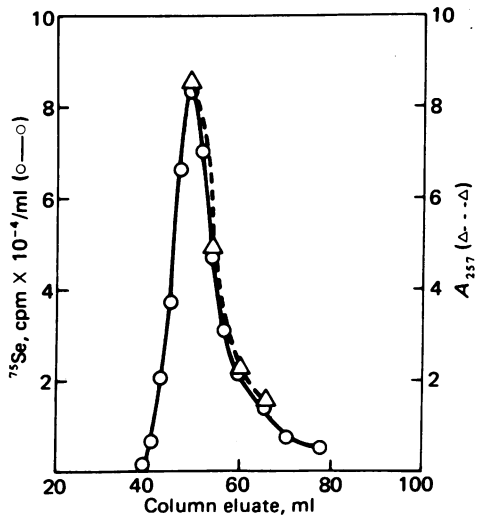

FIG. 3. Cochromatography of ^{75}Se and 257-nm-absorbing material. The ^{75}Se-labeled material in pooled fractions from several polyacrylamide P-30 columns was adsorbed to a DEAE-cellulose pad and, after washing with 50 mM potassium phosphate, pH 7.2, was eluted with a small volume of buffer containing 1 M NaCl. The NaCl eluate was rechromatographed on the polyacrylamide P-30 gel column described in the legend of Fig. 1. The ^{75}Se content of 100-μl aliquots of each fraction was measured and UV spectra of appropriate dilutions of the fractions were recorded between 240 and 350 nm. The fractions eluted between 50 and 60 ml exhibited a major absorbance peak at 257 nm and a low broad peak at 333 nm.

tration by adsorption to DEAE-cellulose, achieved further resolution from contaminating unlabeled protein that emerged at the front of the elution profile. The ^{75}Se-containing fractions from this column (Fig. 3) exhibited high absorbance in the ultraviolet region with a maximum at 257 nm, and there was a constant ratio of ^{75}Se to A_{257} units in the fractions from the back of the peak. The electronic absorption spectra of these samples exhibited a second low maximum at 333 nm with a ratio of absorbance at 257 nm to 333 nm of about 50. A similar electronic absorption spectrum is exhibited by unfractionated tRNA preparations from *E. coli*, and in this case the 335-nm absorbance reflects the presence of a 4-thiouracil residue in the individual tRNA species.

During further purification of the ^{75}Se-labeled material from *C. sticklandii* by repeated DEAE-cellulose and molecular sieve chromatography, a constant ratio of 257-nm absorbance units to ^{75}Se was maintained. Incubation with pancreatic ribonuclease caused extensive degradation to low molecular weight fragments and 80–90% of the ^{75}Se and 90–100% of the A_{257} units were recovered in the salt fractions after gel filtration of treated samples. Both the 257-nm-absorbing material and the ^{75}Se in these digests adsorbed readily to charcoal and were eluted with equal efficiency with 50% ammoniacal ethanol. Thin-layer chromatographic analyses of the smaller molecular weight fractions from the ribonuclease digests revealed the presence of several ultraviolet-absorbing components that cochromatographed with ^{75}Se in both acidic and basic solvent systems. When the intact purified material was extracted with phenol (11), both the 257-nm-absorbing material and the ^{75}Se remained in the aqueous phase and were quantitatively precipitated by the addition of 2 vol of ethanol. These and several other chemical properties suggested that the approximately 25,000-dalton selenium-containing macromolecular material might be tRNA. The ^{75}Se clearly was not bound in the form of an esterified ^{75}Se-containing amino acid (e.g., [^{75}Se]selenocysteine) because incubation at pH 8–9 did not dissociate it from the intact labeled material. Although ^{75}Se-labeled low molecular weight fragments were produced by treatment with 0.1 M KOH for several hours, extensive cleavage of the 257-nm-absorbing material also had occurred and, in fact, the conditions used were sufficient to degrade tRNA to oligonucleotides.

Purification and characterization of [^{75}Se]tRNA

For further characterization of the ^{75}Se-labeled material described above, fractions from a number of polyacrylamide P-30

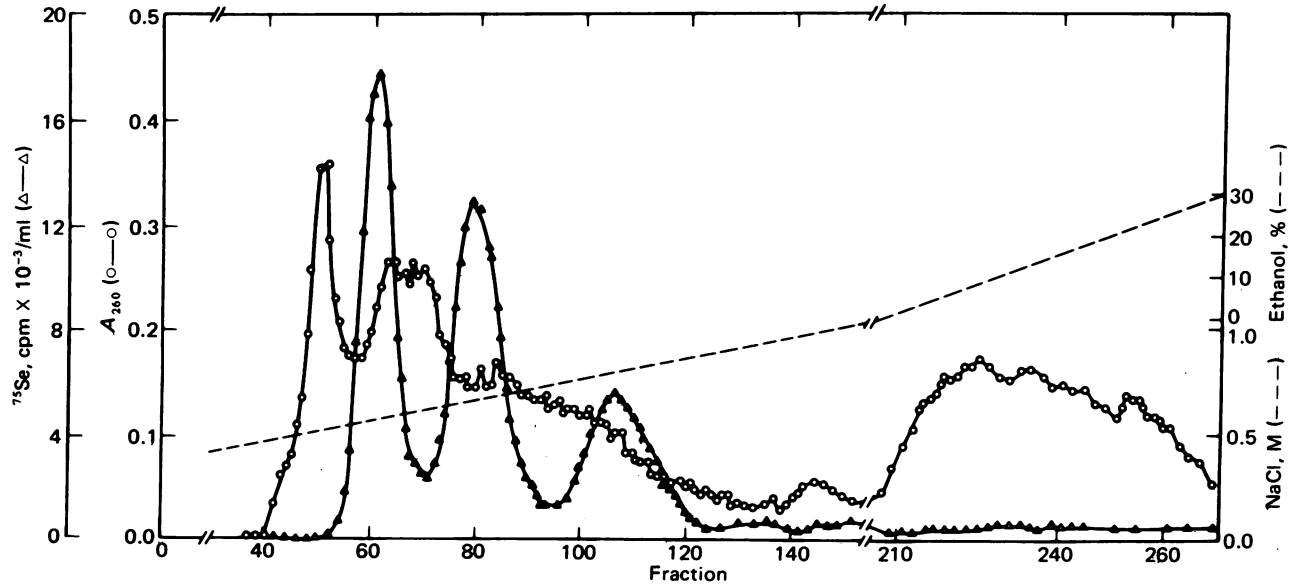

FIG. 4. Chromatography of a bulk [^{75}Se]tRNA mixture on benzoylated DEAE-cellulose. A [^{75}Se]tRNA fraction (5.14 X 10^6 cpm, 235 A_{260} units) that had been eluted from a DEAE-cellulose column with 1 M NaCl in 100 mM Tris·HCl at pH 7.5 and 1 mM dithiotreitol was diluted with 50 mM sodium acetate at pH 5 containing 10 mM $MgCl_2$ and 1 mM dithiotreitol (buffer A) and applied to a benzoylated DEAE-cellulose column (1.6 X 90 cm) equilibrated with 0.35 M NaCl in buffer A. The column was eluted at 5°C with a linear gradient (2 liters total volume) of 0.35–1.5 M NaCl in buffer A followed by a linear gradient (1140 ml total) of 0–30% (vol/vol) ethanol in buffer A plus 1.5 M NaCl. Fractions (10 ml) were collected at a flow rate of 1 ml/min. The absorbance of even-numbered fractions was measured spectrophotometrically at 260 nm, and 50-μl aliquots were assayed for ^{75}Se by scintillation spectrometry in 10 ml of Aquasol. The first radioactive peak that emerged, in fractions 55–68 (seleno-tRNA I), contained 1.35 X 10^6 cpm and 34 A_{260} units; the second peak (seleno-tRNA II), in fractions 72–90, contained 1.47 X 10^6 cpm and 31 A_{260} units; and the third (seleno-tRNA III), fractions 98–118, contained 0.81 X 10^6 cpm and 30 A_{260} units.

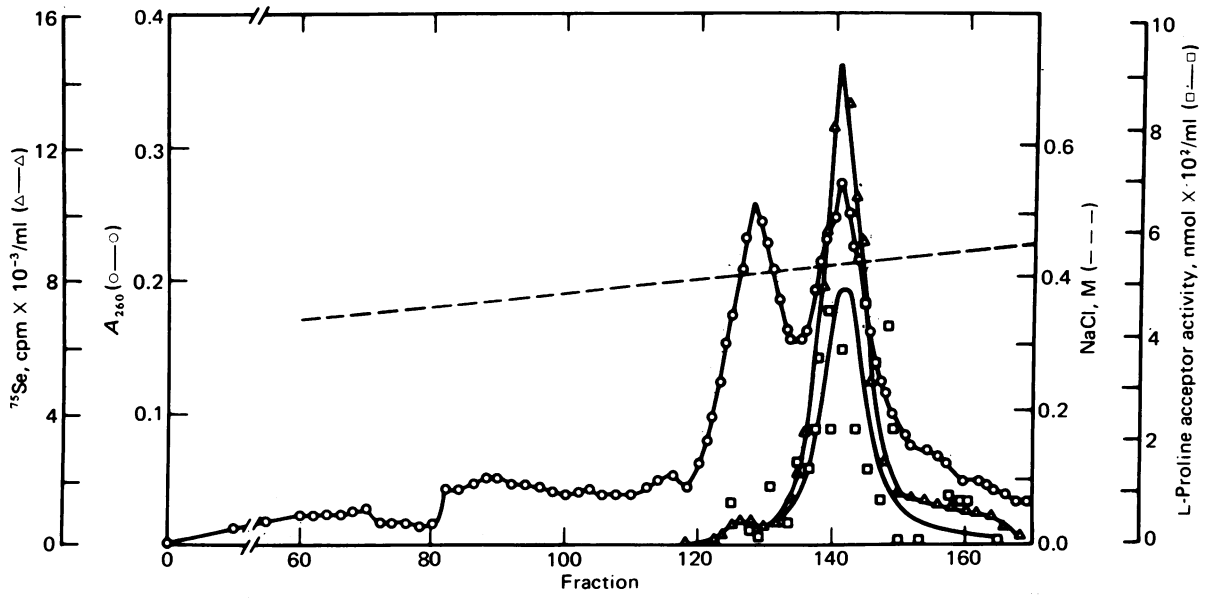

FIG. 5. Chromatography of [^{75}Se]tRNA II on a DEAE-Sephadex A-50 column. Pooled fractions of the seleno-tRNA II peak from a benzoylated DEAE-cellulose column were dialyzed against 20 mM Tris·HCl at pH 7.5 containing 8 mM $MgCl_2$, 1 mM dithiothreitol, and 0.3 M NaCl (buffer B). The dialyzed sample (757 × 10^3 cpm, 31 A_{260} units) was applied to a DEAE-Sephadex A-50 column (1.6 × 90 cm) that had been equilibrated with buffer B. The column was eluted at 5°C by using a linear gradient of 510 ml of buffer B and 510 ml of 20 mM Tris·HCl at pH 7.5 containing 16 mM $MgCl_2$, 1 mM dithiotreitol, and 0.525 M NaCl (buffer C) in the reservoir. Fractions (5 ml) were collected at a flow rate of 0.25 ml/min. Absorbance at 260 nm and ^{75}Se were measured as described in the legend of Fig. 4. The tRNA recovered in the radioactive fractions 135–150 contained 533 × 10^3 cpm and 16.8 A_{260} units. The L-[U-^{14}C]proline acceptor activity of fractions of the labeled peak was assayed as described in the legend of Table 2.

column profiles were pooled and extracted with phenol. After ethanol precipitation and an additional fractionation by adsorption to DEAE-cellulose and elution with 1 M NaCl in 100 mM Tris·HCl, pH 7.5, containing 1 mM dithiothreitol, the resulting ^{75}Se-labeled mixture was chromatographed on a benzoylated DEAE-cellulose column (12). Alternatively, fresh *C. sticklandii* cells that had been incubated with [^{75}Se]selenite in the presence of chloramphenicol were extracted directly with phenol and the ^{75}Se-labeled bulk tRNA preparation was purified by the same procedure. A typical elution profile from a benzoylated DEAE-cellulose column is shown in Fig. 4. The three radioactive peaks of this profile were eluted in the gradient between 0.6 and 0.9 M NaCl and are designated seleno-tRNA I, seleno-tRNA II, and seleno-tRNA III according to the order of their elution sequence from the column.

The fractions corresponding to each of the three radioactive peaks were pooled and further purified by chromatography on DEAE-Sephadex A-50. The seleno-tRNA II profile showed only one major ^{75}Se peak, with two small shoulders at positions corresponding to seleno-tRNA I and III (Fig. 5). Similarly, the profiles of seleno-tRNA I and seleno-tRNA III indicated they contained traces of seleno-tRNA II as the only radioactive contaminant (data not shown). The limited distribution of ^{75}Se among the 260-nm-absorbing species separated by benzoylated DEAE-cellulose chromatographed (Fig. 4) is indicative of the relative specificity of the observed selenium incorporation.

Table 2. L-Proline acceptor activity of seleno-tRNA II

Omission	L-[^{14}C]Proline acceptor activity, pmol/A_{260} unit ± SD
None	97.4 ± 2.6
Enzyme	11.3
Seleno-tRNA II	0
ATP	7.8 ± 3.2
None; + L-proline, 2 μmol	11.3 ± 1.4

The complete reaction mixture (0.1 ml) contained 50 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$ 10 mM KCl, 2 mM ATP, 1 mM dithiothreitol, 3.54 nmol of L-[U^{14}C]proline (282 nCi/nmol), 0.0115 A_{260} unit of seleno-tRNA II, and 27 μg of *E. coli* aminoacyl-tRNA synthetase. After 10 min at 37°C, 70-μl aliquots were assayed for 5% trichloroacetic acid-insoluble ^{14}C (10).

To determine the amino acid acceptor activity of the seleno-tRNA II fraction, samples from the DEAE-Sephadex A-50 column (Fig. 5) were incubated with an *E. coli* aminoacyl-tRNA synthetase preparation and appropriate mixtures of ^{3}H-labeled amino acids plus the corresponding nonradioactive amino acids added individually. L-Proline proved to be the only amino acid that was esterified to a significant extent, and the data of Table 2 show that omission of aminoacyl-tRNA synthetase, seleno-tRNA II, or ATP from the reaction mixture almost abolished L-proline acceptor activity. There was a linear relationship between L-proline acceptor activity and amount of purified seleno-tRNA II, in the range of 0.1–2 μg. Furthermore, the addition of a 565-fold molar excess of unlabeled L-proline markedly decreased the amount of L-[^{14}C]proline bound. The low L-proline acceptor activity (97 pmol/A_{260} unit) of the preparation might be attributed to a number of factors, including use of a heterologous aminoacyl-tRNA synthetase preparation in addition to the impurity of the tRNA sample. The profiles of L-proline acceptor activity and of ^{75}Se content of the seleno-tRNA peak in Fig. 5 were similar, and after further purification by rechromatography on a second DEAE-Sephadex A-50 column the two profiles were coincident (Fig. 6*B*). The A_{260} profile of the concentrated samples (Fig. 6*B*), which is undoubtedly more accurate than that measured in the individual dilute samples (Fig. 6*A*), indicates the presence of other unlabeled tRNA species in the fractions at the front of the peak.

DISCUSSION

More than 50 modified nucleosides have been identified in tRNAs, and most or perhaps all of these are formed by specific enzymes, each of which recognizes and reacts with a particular base in the parent polynucleotide (17). Certain of the modified

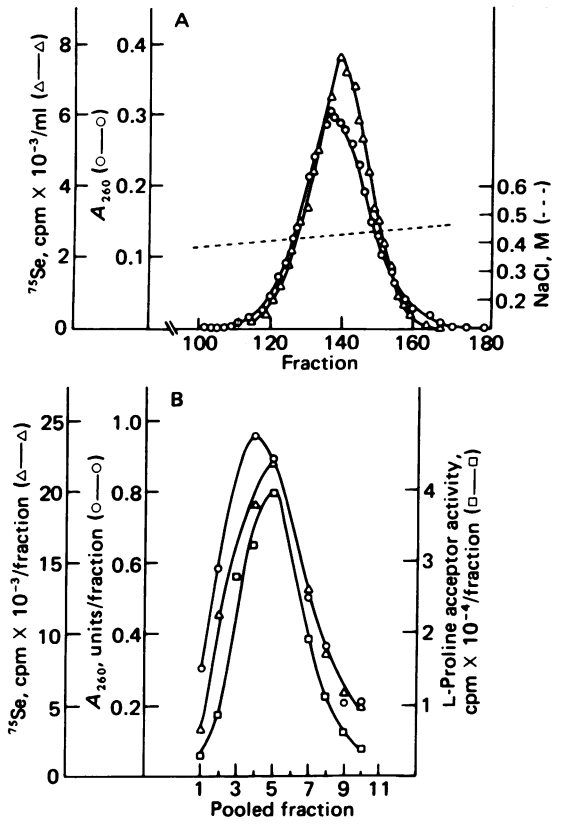

FIG. 6. Rechromatography of seleno-tRNA II on a DEAE-Sephadex A-50 column. A portion of the pooled seleno-tRNA II fractions from the column of Fig. 5 was dialyzed against buffer B and reapplied to a 1.6 × 23 cm DEAE-Sephadex A-50 column, which was developed with a gradient consisting of 120 ml each of buffer B and buffer C as described for Fig. 5. Absorbance at 260 nm and ^{75}Se, determined in aliquots of the 1-ml fractions as described for Fig. 4, are shown in *A*. Of the 117 × 10^3 cpm of ^{75}Se applied to the column, 96 × 10^3 cpm was recovered in fractions 120–160. For determination of proline acceptor activity, every five successive fractions starting with fraction 116 were pooled and concentrated to about 1 ml in dialysis sacs immersed in dry Sephadex G-200. (Fraction 1 = pooled fractions 116–120 from profile *A* and so on.) Aliquots of the resulting concentrated fractions were assayed for L-proline acceptor activity, absorbance at 260 nm, and ^{75}Se content, and the results are shown in *B*. The L-proline acceptor activity is expressed as cpm of [^{14}C]proline fixed per fraction; 1 nmol of proline is 314 × 10^3 cpm.

nucleosides are common to most of the tRNAs of a given organism, for example 4-thiouridine in *E. coli* tRNA (18), whereas others may occur in only one isoaccepting species of a given amino acid tRNA. In a few instances the biological significance of the particular modification is known (17), but for the most part there are only general indications that some type of regulatory role is probably involved.

In view of the fact that several sulfur-containing nucleosides have been identified in tRNAs of bacteria and mammals (8, 17, 19), it is not surprising that selenium-containing tRNAs should also occur. However, in the case of *C. sticklandii* and a few other anaerobic bacteria that we have examined, selenium is incorporated into tRNA highly specifically and is not merely replacing sulfur as a normal component. Although the site of selenium attachment has not been determined, preliminary stability and compositional studies indicate that it is likely to be present as a substituent on the ring of a purine or pyrimidine base. The effect of the introduction of a selenium atom, even more so than a sulfur, should be to increase the hydrogen bonding potential of the particular base in question. In view of the fact (unpublished data) that selenium-containing tRNAs are not restricted to amino acid fermenting anaerobic bacteria but also occur in *Methanococcus vannielii*, a methane-producing organism that grows in an inorganic salts medium containing formate as the sole organic compound, it appears that some general type of regulatory phenomenon may be involved. The relative importance of these seleno-tRNAs to survival of the various anaerobic bacteria is unknown because at present we have no practical way to completely prevent seleno-tRNA formation during their growth.

The amount of ^{75}Se incorporated into the bulk tRNA from several different batches of selenium-deficient cells of *C. sticklandii* varied from 0.05 to 0.1 equivalent per mole of the average tRNA population. Subsequent chromatographic analysis showed this to be distributed among three readily separable tRNA species. Assuming these represent 3 of the 20 different amino acid tRNAs, it is likely that minor isoaccepting species account for the total selenium that is present. One of the three labeled fractions, designated seleno-tRNA II, cochromatographed with prolyl-tRNA during subsequent purification steps, and at the state of purity shown in Fig. 5 the peak fractions contained 0.17 nmol of selenium per nmol of tRNA. For this calculation one A_{260} unit was assumed to be equivalent to 1.66 nmol of tRNA (20) and the specific radioactivity of the ^{75}Se administered to the culture was used. This should be a minimal value in view of the fact that the contribution of unlabeled selenium in the initial selenium-deficient culture was ignored in the calculation. If *C. sticklandii*, like *E. coli*, contains multiple isoaccepting prolyl-tRNA species, the occurrence of selenium in at least 17% of the tRNA molecules of the impure L-proline-accepting fractions could be equivalent to one seleno-base per mole of one isoaccepting species. Further purification of the *C. sticklandii* seleno-tRNAs by reversed-phase chromatography (21) should provide answers to these and related questions.

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