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Presence and Function of Sulfur-containing Transfer Ribonucleic Acid of *Bacillus subtilis*

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Bacillus subtilis transfer ribonucleic acid (tRNA) was analyzed for the occurrence of thionucleotides by in vivo labeling with ^{35}S and fractionation by methylated albumin kieselguhr column chromatography. Alkaline hydrolysates of tRNA were also examined by column chromatography and paper electrophoresis, and the amino acid-accepting ability of thionucleotide-containing tRNA was tested after iodine oxidation. The results showed that *B. subtilis* tRNA contains 4-thiouridylate, a second nucleotide with properties similar to 2-thiopyrimidine, and a third unidentified thionucleotide. The amino acid-accepting ability for serine, tyrosine, lysine, and glutamic acid was markedly inhibited after oxidation of the tRNA with iodine, suggesting the presence of thionucleotides in these tRNA species. This inhibition could be reversed by thiosulfate reduction. The iodine treatment totally inactivated all lysine tRNA species, partially inactivated the serine tRNA species, and did not affect the accepting ability for valine. A comparison of tRNA from cells in the log and stationary phases and from spores revealed similar iodine inactivation patterns in all cases. The thionucleotide content in *B. subtilis* tRNA differed from that in *Escherichia coli*, both in extent and in distribution. A possible function of the thionucleotides in tRNA is discussed.

Transfer ribonucleic acid (tRNA) contains several minor nucleotides in addition to the usual four major nucleotides found in RNA. These unusual nucleotides include pseudouridylate, 5,6-dihydrouridylate, and various substituted nucleotides including thionucleotides (12). Thionucleotides are not present in tRNA universally. Carbon et al. (1) demonstrated the presence of 2-thiopyrimidine in *Escherichia coli* tRNA and in rabbit liver tRNA. Lipsett (8) observed that *E. coli* and *Salmonella typhimurium* tRNA contained 4-thiouridylic acid; this nucleotide was apprently absent from yeast and rat liver tRNA. In this paper we submit detailed data for the presence of sulfur-containing nucleotides in the tRNA from *Bacillus subtilis*. A comparison of the amino acid-accepting ability of several tRNA species after iodine oxidation indicated that the thionucleotide pattern of tRNA did not vary significantly as a function of the growth phase of *B. subtilis*.

MATERIALS AND METHODS

Organisms and media. *B. subtilis* W23 was employed in this investigation as the source of tRNA and of aminoacyl-tRNA synthetase; *E. coli* Q13 was used for the preparation of heterologous aminoacyl-tRNA synthetase. Both organisms were grown at 37 C in Penassay medium (Difco). For the preparation of ^{35}S-labeled tRNA, cells were grown for 2 to 3 generations in a synthetic medium of the following composition: NH_4Cl, 2 g; NaCl, 5 g; KCl, 370 mg; $MnCl_2$, 20 mg; $MgSO_4$, 12 mg; Na_2HPO_4, 155 mg; NaH_2PO_4, 195 mg; tris(hydroxymethyl)aminomethane (Tris), 6 g; $FeCl_3$, 1 mg; glucose, 3 g; sodium citrate, 1 g; sodium glutamate, 1 g; $(NH_4)_2{}^{35}SO_4$, 7 to 19 mc; distilled water, 1,000 ml; *p*H 7.2.

Preparation of tRNA. The cells were grown to the desired growth phase, harvested by centrifugation, and washed once with 0.44 M sucrose-5 mM Mg acetate-1 mM Tris buffer, *p*H 7.7. Stationary-phase cells were harvested 2 hr after the end of the log phase of growth. Spores were prepared and cleaned as described previously (2). Extraction of the tRNA from vegetative cells was performed according to the phenol method of von Ehrenstein and Lipmann (3). Protein was removed from the extract by three additional phenol extractions. The tRNA was obtained from spores by freezing a pellet of water-washed spores in liquid nitrogen and then grinding the frozen spores with glass beads in a prechilled mortar (−20 C) for 10 min. A solution containing 1 mM Tris-chloride, 10 mM Mg acetate, and 1% sodium dodecylsulfate (*p*H 7.2) and an equal volume of phenol was added to the mortar, and the grinding was continued for an additional 3 min. The frozen mixture was allowed to

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thaw in the cold room and then the crude mixture was extracted by the phenol method (4). The isolated tRNA was treated with 0.5 M Tris buffer, *p*H 8.8, for 60 min at 35 C to remove bound amino acids. For ^{35}S-labeled tRNA this treatment was extended to 3 hr. The final precipitate was dialyzed against 10 mM Tris buffer, *p*H 8.0, and stored in the frozen state. The alkaline *p*H was used to stabilize the 4-thiouridylate. In one experiment a tRNA preparation was stored for 2 months at *p*H 5 and was found to have lost its sensitivity to iodine treatment which inhibits the amino acid accepting-ability of many of the tRNA species by oxidation of 4-thiouridylate.

The iodine oxidation and thiosulfate reactivation of tRNA were performed according to the method of Carbon et al. (1). Periodate oxidation of tRNA for the removal of thio groups from 4-thiouridylate was described by Lipsett and Peterkofsky (10) and modified by incubating the reaction for 5 hr.

Preparation of aminoacyl-tRNA and aminoacyl-tRNA synthetases. The enzyme was prepared from mid-log phase cells, by the procedure outlined by Zubay (14). For the preparation of aminoacyl-tRNA, 0.1 to 0.15 mg of enzyme protein was employed in a reaction mixture which contained (μmoles per 0.5 ml): Tris-chloride buffer, *p*H 7.3, 40; adenosine triphosphate (ATP), 1; KCl, 5; $MgCl_2$, 5; phosphoenolpyruvate, 5; 1 μmole each of 19 unlabeled amino acids; pyruvate kinase, 10 μg; ^{14}C- or ^{3}H-labeled amino acids (1 μc), 5 to 10 mμmoles; and tRNA, 0.5 to 1.0 mg. After incubation of the mixture at 37 C for 10 min, the reaction was stopped by adding an equal volume of water-saturated phenol and by rapid cooling in an ice bath. Aminoacyl-tRNA was removed by the phenol method (4) and stored at −20 C.

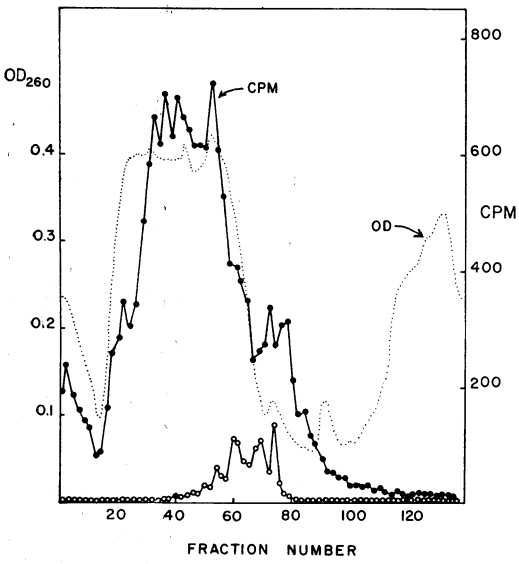

FIG. 1. *Elution profile of ^{35}S-labeled RNA from a methylated albumin column. The ^{35}S-labeled RNA was prepared from cells grown in a synthetic medium containing $^{35}SO_4^=$. All uneven-numbered fractions (●) were precipitated with cold trichloroacetic acid and the radioactivity was determined by scintillation counting. The even-numbered fractions (○) were treated with pancreatic ribonuclease (10 μg/ml) at 37 C for 30 min before acid precipitation and counting. See Materials and Methods for details concerning the column and assay procedures.*

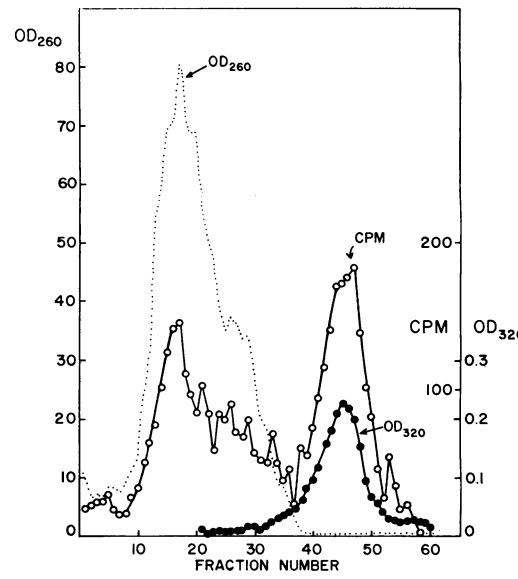

FIG. 2. *Fractionation of an alkaline hydrolysate of ^{35}S-labeled and carrier tRNA by a DEAE cellulose column. Carrier tRNA (500 mg) was mixed with ^{35}S-labeled tRNA (0.05 mg; 75,000 counts/min) and hydrolyzed in 0.35 N KOH for 18 hr at 30 C. Elution with NH_4HCO_3, pH 8.6, in 7 M urea was carried out according to the method employed by Lipsett (10). Fractions (10 ml) were collected and 1-ml portions were used to determine the radioactivity. Symbols: ○, counts per minute; ●, optical density at 320 mμ.*

Fractionation of aminoacyl-tRNA by methylated albumin kieselguhr (MAK) columns. The method of Sueoka and Yamane (13) was used with the modifications previously described (7). The aminoacyl-tRNA was eluted from the column with 0.3 to 1.1 M NaCl linear gradient in 0.05 M phosphate buffer, *p*H 6.8. Then 2 ml fractions were collected and analyzed for optical density (at 260 mμ) and radioactivity as described previously (2).

Column chromatography of alkaline hydrolysates of tRNA. Mixtures of unlabeled carrier tRNA and ^{35}S-labeled tRNA were hydrolyzed in 0.35 M KOH at 37 C for 18 hr. Further treatment depended upon the type of column used. For separation on Dowex-1-formate (system 1), the hydrolysate was diluted with distilled water before application to the column. Here, elution was accomplished by a stepwise increase in the concentrations of formic acid and ammonium formate (6). In system 2 the hydrolysate was brought to *p*H 8 with Dowex-50 (H^+ form) and eluted from a Dowex-1-formate column by two exponential gradi-

ents of formic acid (1). For separation on diethylaminoethyl (DEAE) cellulose columns, the procedure employed by Lipsett (8) was followed except that a smaller scale was used.

Ultraviolet spectra were obtained with a Cary recording spectrophotometer (model 14). Paper electrophoresis was carried out with a Gilson High Voltage Electrophoretor (model D) employing 0.05 M borate buffer, *p*H 9.55, with a potential of 4 kv for 30 min, and Whatman no. 1 paper (7.5 × 61 cm).

Materials. ATP, phosphoenolpyruvate, and pyruvate kinase were obtained from Calbiochem, Los Angeles, Calif.; $H_2{}^{35}SO_4$ was from New England Nuclear Corp., Boston, Mass.; ^{14}C-L-arginine (172 mc/mmole), ^{14}C-L-glycine (116 mc/mmole), ^{14}C-L-threonine (167 mc/mmole), ^{14}C-L-proline (146 mc/mmole), ^{14}C-L-tryptophan (16 mc/mmole), ^{14}C-L-valine (160 mc/mmole), ^{3}H-L-valine (500 mc/mmole), ^{14}C-L-glutamine (16 mc/mmole), ^{14}C-L-histidine (201 mc/mmole), ^{14}C-L-phenylalanine (302 mc/mmole), ^{14}C-L-leucine (170 mc/mmole), ^{14}C-L-aspartate (160 mc/mmole), ^{14}C-L-isoleucine (201 mc/mmole), ^{14}C-L-methionine (187 mc/mmole), ^{14}C-L-alanine (117 mc/mmole), ^{3}H-L-alanine (4.8 c/mmole), ^{14}C-L-serine (112 mc/mmole), ^{3}H-DL-serine (82.3 mc/mmole), ^{14}C-L-tyrosine (360 mc/mmole), ^{14}C-L-lysine (240 mc/mmole), ^{3}H-L-lysine (800 mc/mmole), and ^{14}C-L-glutamate (200 mc/mmole) were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y.

RESULTS

Data for the presence of thionucleotides in tRNA of *B. subtilis* were obtained by MAK column chromatography of RNA extracted from cells grown for several generations in the presence of $^{35}SO_4^{=}$. Bound amino acids were removed prior to chromatography by incubation of the bulk RNA at 35 C in 0.5 M Tris buffer, *p*H 8.8, for 3 hr. When the typical elution profile of bulk RNA was effected (Fig. 1), we found radioactivity to be associated only with the tRNA peak (fractions 15 to 85), whereas the ribosomal RNA (fractions 100 to 140) contained negligible counts.

To demonstrate that the radioactive sulfur was a component of the tRNA and was not contained in contaminating proteins, every other fraction collected was treated with pancreatic ribonuclease (10 μg/ml) for 30 min at 37 C before it was acid-precipitated and counted. Then the remaining fractions were precipitated and counted as eluted from the column. The acid-precipitable counts were reduced in most fractions by the ribonuclease treatment to about 5% of the untreated control (Fig. 1). In some fractions the reduction of counts was about 50%. This was probably caused by incomplete digestion of resistant cores by the ribonuclease or possibly protein contamination. In another MAK column experiment, similar results were obtained after hydrolysis of the fractions in 0.35 M KOH for 18 hr at 30 C. Only 5% or less of the ^{35}S counts remained acid-precipitable after the alkali treatment which hydrolyzes RNA.

Lipsett (8) observed that *E. coli* tRNA had a

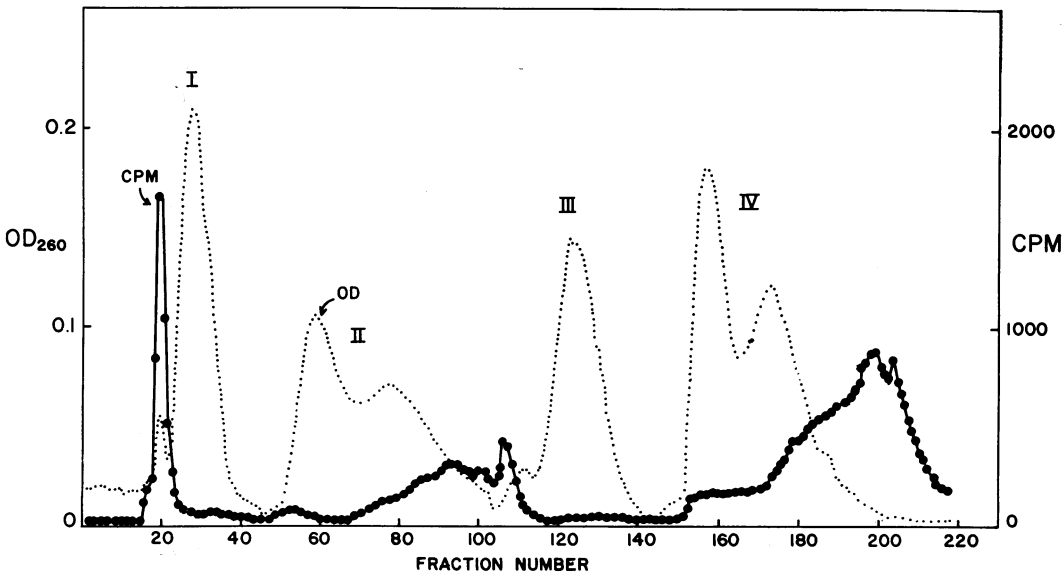

FIG. 3. *Dowex-1-formate chromatography of an alkaline digest of ^{35}S-labeled tRNA (0.25 mg; 287,000 counts per min) and carrier tRNA (1.86 mg). Hydrolysis was carried out in 0.35 M NaOH at 37 C for 18 hr. For elution, system 1 (see Materials and Methods) was employed. The nucleotides eluted as follows in this system: (I) cytidine monophosphate, (II) 2'- and 3'-adenosine monophosphate, (III) uridine monophosphate, (IV) 2 - and 3'-guanosine monophosphate. The closed circles represent radioactivity.*

minor absorption peak at 330 mμ caused by 4-thiouridylate in addition to the absorption maximum at 260 mμ. The ultraviolet spectrum of *B. subtilis* tRNA in 10 mM phosphate buffer, *p*H 6.8, revealed a shoulder at 335 mμ, amounting to approximately 0.3 to 0.4% of the absorption at 260 mμ. This suggested that 4-thiouridylate was present in tRNA of *B. subtilis*, although in a smaller amount than in *E. coli* tRNA.

To confirm the presence of 4-thiouridylate, an alkaline hydrolysate of ^{35}S-labeled tRNA was prepared and fractionated by passage through a DEAE cellulose column (Fig. 2). Most of the mononucleotides were eluted in this system in one large peak (fractions 8 to 40), absorbing strongly at 260 mμ but not at 320 mμ. Only the fractions after this peak (fractions 41 to 60) displayed absorption at 320 mμ. When the absorption spectra were obtained, these fractions were associated with a distinct peak of radioactive sulfur and showed maximal absorption at 333 mμ in 0.1 M HCl and at 315 mμ in 0.1 M NaOH. These values were almost identical to those found for 4-thiouridine under the same conditions. Comparison of this material with uridine monophosphate (UMP) by paper electrophoresis also exhibited its identity with 4-thiouridine monophosphate. In 0.05 M borate buffer, *p*H 9.55, this material has a mobility 1.17 times greater than UMP, which is the value observed for 4-thiouridylate (8).

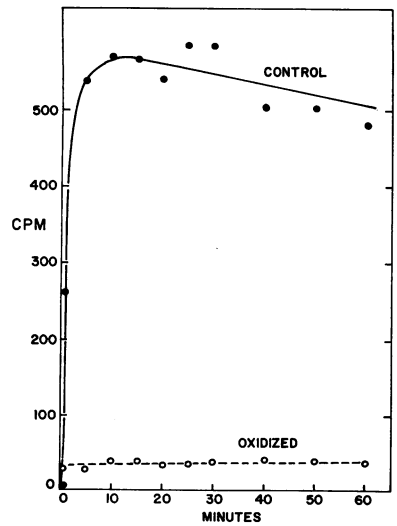

FIG. 4. *Rate and extent of lysine charging to oxidized and untreated tRNA. A 0.1-ml portion was taken from each reaction mixture containing equal amounts of either iodine-oxidized or untreated tRNA and assayed for acid-precipitable counts at the indicated times (see Materials and Methods for details). The symbols represent the counts incorporated for control (●) and iodine-treated (○) tRNA.*

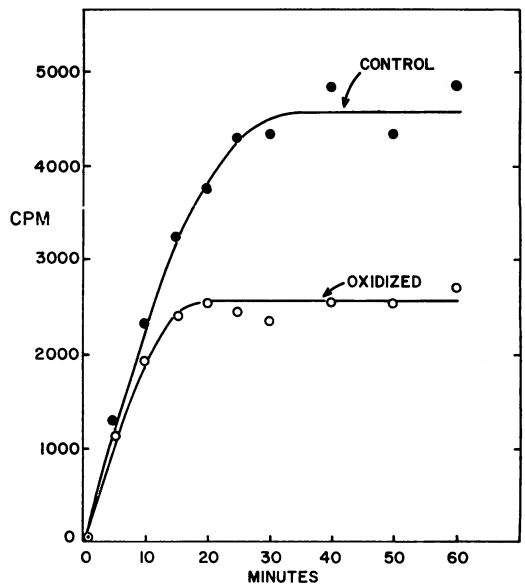

FIG. 5. *Rate and extent of serine charging to iodine-oxidized and untreated tRNA. See Fig. 4 and Materials and Methods for details. The symbols represent the serine counts incorporated for control (●) and oxidized (○) tRNA.*

It is evident from Fig. 2 that the nucleotides eluting in the main absorption peak (fractions 8 to 40) contained considerable radioactivity. The possible presence of other sulfur-containing nucleotides was investigated by fractionating alkaline hydrolysates of ^{35}S-labeled tRNA on Dowex-1-formate columns. Two different elution systems were employed with essentially the same results. In both instances a very distinct peak of radioactivity was eluted just before the cytidylate peak (Fig. 3). This elution position agreed with that obtained by Carbon et al. (1) for a 2-thiopyrimidine derivative from *E. coli* tRNA. The spectra of this material had maximal absorptions at 267 mμ in 0.1 N HCl and at 265 mμ in 0.1 N NaOH. In both columns at least one more small radioactive peak was present, between adenylate and guanylate with system 2 (*see* Materials and Methods) and between adenylate and uridylate in Fig. 3. The radioactivity observed in the last fractions (170 to 200) in Fig. 3 coincides with the elution of free $^{35}SO_4^{=}$.

To understand the relationship between the radioactive nucleotides eluted from the DEAE-cellulose and Dowex-1-formate columns, the nucleotides from fractions 10 to 38 in Fig. 2 were rechromatographed on Dowex-1-formate columns. These radioactive nucleotides eluted from the Dowex-1-formate column in a major peak just

before the cytidylate peak as shown in Fig. 3. Later two minor peaks eluted in the regions between adenosine monophosphate and guanosine monophosphate. This behavior suggested that most of the ^{35}S-labeled nucleotides in the major peak from the DEAE cellulose column were similar or identical to the 2-thiopyrimidine derivative. The identity of the two other peaks is unknown.

For *E. coli* tRNA, a relationship between the susceptibility of tRNA to oxidation by iodine and an inhibition of its amino acid-accepting ability was demonstrated by Carbon et al. (1). Their data suggested that an easily oxidized 2-thiopyrimidine nucleotide was the basis for this inactivation. After treating *B. subtilis* tRNA according to their technique, we determined the difference in amino acid-accepting ability between control and oxidized samples. Three types of results were observed upon iodine oxidation. The amino acid-accepting ability was either completely lost as with lysine (Fig. 4), partially lost as with serine (Fig. 5), or unaffected as with valine (Fig. 6).

The effects of iodine oxidation on the aminoacylation process were also studied by MAK column chromatography. Little or no attachment of lysine to the oxidized tRNA was noted. With serine tRNA it was apparent that a selective in-

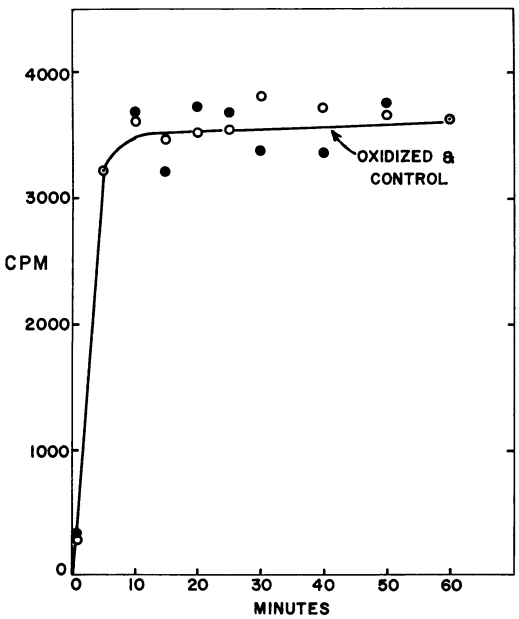

FIG. 6. *Rate and extent of valine charging to iodine-treated and untreated tRNA. See Fig. 4 and Materials and Methods for details. The symbols represent control* (●) *and oxidized* (○) *tRNA.*

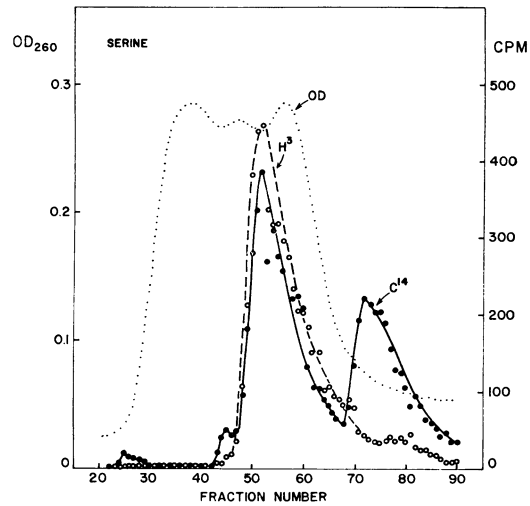

FIG. 7. *Elution profile of oxidized and control seryl-tRNA from a MAK column. Oxidized tRNA was charged with ^{3}H-serine* (○) *and control tRNA was charged with ^{14}C-serine* (●). *Only one peak of seryl-tRNA is shown in the oxidized preparation.*

activation of a particular tRNA species occurred during oxidation. While control tRNA showed at least two species of tRNA charged with serine, only one of these still accepted the amino acid after oxidation (Fig. 7). When the iodine treatment was followed by thiosulfate reduction prior to aminoacylation, both seryl-tRNA peaks reappeared (Fig. 8). As with serine specific tRNA, complete reactivation of lysine tRNA was observed. The relative amounts of the two valyl-tRNA peaks eluted from a MAK column and the elution patterns were unchanged after iodine oxidation (Fig. 9). These results confirmed the data obtained from kinetic studies (Fig. 4 to 6) and clarified the reasons for the partial inactivation observed with serine specific tRNA.

A summary of the results with other amino acids and tRNA from various phases of growth is presented in Table 1. Marked reductions of amino acid-accepting ability after oxidation were observed for serine, tyrosine, lysine, and glutamic acid. In contrast, oxidation did not affect the ability of the tRNA to accept arginine, glycine, valine, proline, threonine, and tryptophan. For glutamine, histidine, phenylalanine, leucine, aspartic acid, isoleucine, methionine, and alanine, the results were not consistent, and the degree of inhibition depended in part on the tRNA preparation. However, partial inactivation was noted in several tests for these amino acids. When tRNA from log- and stationary-phase cells and from spores were compared, no significant dif-

ference in inactivation pattern by iodine treatment was observed among the three tRNA preparations.

The structural change caused by iodine oxidation is recognized by the *E. coli* aminoacyl-tRNA synthetases. The enzymes from both *B. subtilis* and *E. coli* showed a similar, but not identical, charging pattern with oxidized and control *B. subtilis* tRNA. The per cent inhibition of charging oxidized *B. subtilis* tRNA with *E. coli* enzyme was 28%, 43%, and 86% for serine, glutamate, and lysine, respectively (see Table 1 for *B. subtilis* data). The extent of charging of the treated *B. subtilis* tRNA with the *E. coli* enzyme was similar except for glutamate in which the inhibition was reduced.

Furthermore, the removal of thio groups from 4-thiouridylate by periodate treatment (10) may render tRNA insensitive to iodine oxidation. This possibility was investigated by treating aminoacylated tRNA first with periodate to remove the thio groups and then oxidizing the deacylated tRNA with iodine. Lysine specific tRNA, which was unable to accept lysine after only iodine treatment, received lysine to the same extent as the control tRNA (i.e., 100%) when the iodine treatment had been preceded by periodate oxidation. Also, the serine-accepting ability

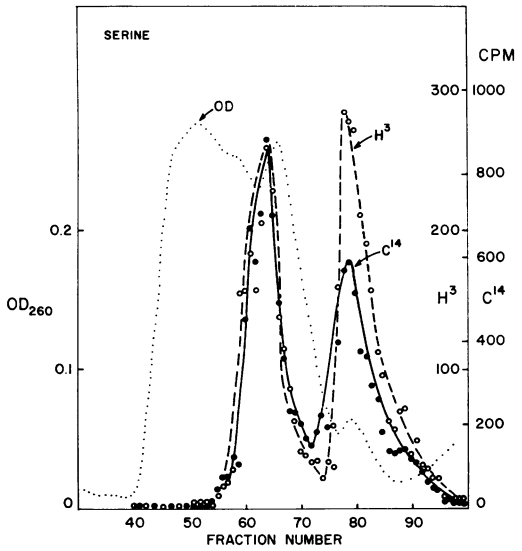

FIG. 8. *MAK column profile of reactivated and control tRNA charged with serine. Oxidized tRNA was treated with thiosulfate (reactivated) before charging with ^{14}C-serine (●). The control tRNA was charged with ^{3}H-serine (○). Two peaks were observed again after reactivation with thiosulfate (compare with Fig. 7).*

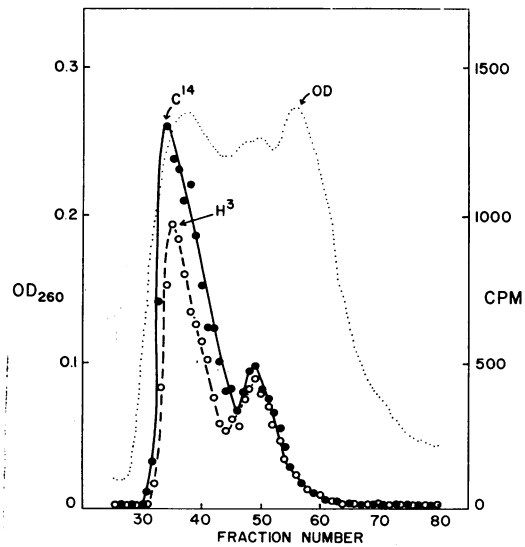

FIG. 9. *MAK column profile of oxidized and untreated tRNA charged with valine. Oxidized and untreated tRNA were charged with ^{3}H-valine (○) and ^{14}C-valine (●), respectively. The elution profile was unaltered by iodine treatment.*

TABLE 1. *Inhibition by iodine oxidation of the amino acid-accepting ability of tRNA preparations from various growth phases of Bacillus subtilis*[a]

Amino acids	Inhibition (%)		
	Log-phase cells	Stationary-phase cells	Spores[b]
Arginine	0	0–5	—
Glycine	0	—	—
Threonine	0	—	—
Proline	0	—	—
Tryptophan	0	—	—
Valine	0	—	20
Glutamine	0–3	—	—
Histidine	0–8	—	—
Phenylalanine	0–15	0–14	11
Leucine	0–16	—	—
Aspartic acid	0–23	—	—
Isoleucine	0–25	0–3	—
Methionine	0–26	0–13	—
Alanine	0–45	0–21	—
Serine	30–50	30–50	26
Tyrosine	60–70	80–90	—
Lysine	80–90	90–95	85
Glutamic acid	90–95	70–90	—

[a] An equal quantity of tRNA was tested for amino acid-accepting capacity before and after iodine oxidation (*see* Materials and Methods). Per cent inhibition = $100 - \frac{\text{accepting capacity after oxidation}}{\text{accepting capacity before oxidation}} \times 100$.

[b] Average of two experiments.

of the periodate-treated tRNA was not partially inhibited by iodine oxidation and was charged to 100% even after iodine treatment. These results suggest that the removal of thio groups presumably from 4-thiouridylate by periodate treatment precluded oxidation by iodine and therefore allowed proper recognition between the tRNA and the aminoacyl synthetase.

DISCUSSION

These results illustrate that *B. subtilis* tRNA species contain 2 and possibly 3 thionucleotides. The presence of thionucleotide was determined previously for tRNA preparations from *E. coli* (1, 8, 11), rabbit liver (1), and *S. typhimurium* (8). The relative amount of 4-thiouridylate was similar in *B. subtilis* and in *E. coli* tRNA preparations. Lipsett (8) observed that the absorption at 335 mμ amounted to about 1.5 to 2.0% of that at 260 mμ. In *B. subtilis* tRNA the absorption at 335 mμ was 0.3 to 0.4% of the absorption at 260 mμ. The distribution of the bases in *B. subtilis* tRNA species is different from that found in *E. coli* when analyzed by iodine oxidation. In *E. coli* it was found that iodine oxidation inhibited the amino acid-accepting ability of only one of the two lysine specific tRNA species (1), whereas in *B. subtilis* preparations all lysine specific tRNA were inhibited. These data suggest that all *B. subtilis* lysine tRNA species contain thionucleotides, but only one of the two species contained thionucleotides in *E. coli*. However, according to the data presented in Lipsett (9), a tRNA may contain thionucleotides and not be inactivated by iodine. Therefore, caution must be taken in interpreting these results.

From the results given from iodine oxidation studies (Table 1) and those shown in Fig. 1, it seems that thionucleotides are not distributed randomly among all tRNA species. All the lysine tRNA but none of the valine tRNA species appear to contain thionucleotides. In addition, the pattern of thionucleotide distribution among tRNA species did not appear to be a function of the growth phase because the iodine oxidation studies gave similar results with tRNA from cells in the log and stationary phases and from dormant spores.

The function of the thio bases is still unknown. However, the ability of the tRNA to be charged with lysine after removal of thio groups by periodate treatment suggests that the thio groups themselves do not act in the recognition process between the tRNA and aminoacyl-tRNA synthetase. Nevertheless, the fact that some tRNA species are unable to accept amino acids after iodine oxidation implies that the thio groups are essential for the proper conformation of the tRNA; the oxidation of the thio groups may alter the conformation of the tRNA so that the aminoacyl synthetase is unable to recognize it. Also, Goehler and Doi (5) illustrated that the oxidation of an amino-acylated tRNA affected its binding properties to a ribosome-messenger RNA complex. They found that the binding efficiency of oxidized lysyl-tRNA to a polyadenylate-ribosome complex was reduced 65 to 75% after iodine treatment. These results suggested that a change in conformation of the lysyl-tRNA prevented its binding to the messenger RNA-ribosome complex. This effect was reversible because thiosulfate reduction restored the binding efficiency to 100%. These results illustrate that both the aminoacylation of tRNA and the binding of an aminoacyl-tRNA to ribosomes can be inhibited by thionucleotide oxidation and suggest two possible sites for the regulation of tRNA activity during protein synthesis.

ACKNOWLEDGMENTS

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