JOURNAL OF BACTERIOLOGY, Feb. 1973, p. 825-833

Vol. 113, No. 2
Printed in U.S.A.

Analysis of Isoaccepting Transfer Ribonucleic Acid Species of *Bacillus subtilis*: Chromatographic Differences Between Transfer Ribonucleic Acids from Spores and Cells in Exponential Growth

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Received for publication 9 November 1972

Differences between the transfer ribonucleic acid (tRNA) of spores and exponentially growing cells of *Bacillus subtilis* 168 were compared by co-chromatography on reversed-phase column RPC-5. This system gave excellent resolution of isoaccepting species in 1 to 2 hr using a 200-ml gradient. Two methods were used to extract spore tRNAs, a procedure using a Braun homogenizer and a pretreatment with dithiothreitol followed by lysis with lysozyme. Where changes were observed, column elution profiles of spore tRNAs were independent of the extraction method used. Three kinds of changes between the profiles of vegetative cell tRNA and spore tRNA were observed: (i) no change; phe-, val-, ala-, asp-, ileu-, pro-, met-, fmet-, and his-tRNAs, (ii) a change in the ratio of existing peaks; gly-, tyr-, leu-, ser-, thr-, aspn-, and arg-tRNAs, and (iii) the appearance or disappearance of unique peaks; lys-, glu-, and trp-tRNAs.

It has been postulated that alterations in transfer ribonucleic acid (tRNA) control certain aspects of cellular development, and the tRNAs of several differentiating systems have been examined in an effort to substantiate this hypothesis. The first step in such experiments is to establish what kinds of changes in tRNA species occur in differentiating cells. A useful technique is the comparison of the column elution profiles of isoaccepting tRNA species isolated from cells at two distinct stages of differentiation. The most commonly used systems for separating aminoacylated species have been methylated albumin-kieselguhr (18) reversed phase (24) and benzoylated diethylaminoethyl (β-DEAE)-cellulose (8) column chromatography. A new technique in reversed-phase chromatography utilizing Plaskon as a support matrix has been developed at the Oak Ridge National Laboratory which offers excellent resolution of aminoacylated tRNA isoaccepting species (13). The column analysis can be completed in about 90 min instead of the 3 days originally required with the 240- by 1-cm RPC-2 columns (21) and requires a gradient of 200 ml instead of 3 or 4 liters. The results are savings in time and the amount of tRNA required.

I have used the RPC-5 system to reevaluate the kinds of changes which occur in tRNAs of *Bacillus subtilis* during growth. Previously, the only column comparisons of isoaccepting species of *B. subtilis* tRNAs at different growth stages were done on methylated albumin-kieselguhr (MAK) columns. Such a study by Kaneko and Doi (11) showed multiple isoaccepting peaks for only 6 of the 15 aminoacyl-tRNAs tested. The authors themselves point out that the resolving power of MAK was very limited. However, studies by Kaneko and Doi (11), Lazzarini (15), and Arceneaux and Sueoka (1) have revealed differences in valyl-, lysyl-, and tyrosyl-tRNAs respectively, so that more extensive studies on tRNAs in *B. subtilis* seemed justified. Using RPC-5, I have been able to resolve multiple isoaccepting species of tRNAs for 16 of the 19 aminoacyl-tRNAs which were examined. In most cases, the tRNA species were well resolved. With this degree of resolution, several significant changes have

been detected between the tRNAs of spores and exponentially growing cells which have previously been overlooked.

MATERIALS AND METHODS

Column chromatography. Column chromatography was performed on a reversed-phase column, RPC-5, as described by Kelmers and Heatherly (13) using tRNAs which were aminoacylated in vitro with a radioactively labeled amino acid before chromatography. After aminoacylation, tRNAs were purified by stepwise elution from a 1-cm³ DEAE-cellulose column before application to the RPC-5 column. Columns of dimensions 0.6 by 16 cm were run at about 300 psi with a flow rate of 2 ml/min, and fractions were collected at 1-min intervals. Various concentrations of NaCl were used to generate linear NaCl gradients, 200-ml total volume, in an eluent buffer consisting of 10 mM $MgCl_2$, 10 mM sodium acetate, *p*H 4.5, 2 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 ml of isoamylacetate per liter. Initial and final NaCl concentrations are given in Table 1. All columns were run at 37 C except those for prolyl- and histidyl-tRNAs which were run at 16 C to prevent excessive hydrolysis. Recoveries for each kind of aminoacyl-tRNA are also given in Table 1. Recoveries were higher at 16 C than 37 C as evidenced by the following recovery figures for three aminoacyl-tRNAs run at 16 C or 37 C, respectively: methionyl-tRNA, 93% or 58%; histidyl-tRNA, 55% or 38%; and prolyl-tRNA, 53% or 18%. Eluate fractions from the RPC-5 column were precipitated with cold trichloroacetic acid after the addition of a deoxyribonucleic acid carrier. The precipitates were collected on glass fiber filters, washed with 5% trichloroacetic acid, washed with 95% ethanol, and dried. Filters were measured for radioactivity of ^3H and ^{14}C simultaneously by liquid scintillation spectroscopy in toluene scintillation fluid containing 4 g of Omnifluor (New England Nuclear Corp.) per liter of toluene. Background and 18% channel overlap were corrected by calculation.

Aminoacylation reactions. The various aminoacylation reactions used were as previously described (22) except that 1-ml reaction volumes were employed. The concentration of ^{14}C or ^3H amino acid was adjusted so that there was no difference in amino acid concentration between tRNA samples receiving a ^{14}C or ^3H label. Aminoacyl-tRNA synthetases were prepared from *B. subtilis* 168 *trpC2* grown to logarithmic phase on tryptone-yeast extract medium as previously described (22). Radioactive amino acids were tested for purity on thin-layer cellulose chromatograms developed with butanol, acetic acid, and water (60:15:25). All amino acids were greater than 90% pure except methionine and tryptophan which were greater than 80% pure.

Formylation reaction. ^{14}C-formylmethionyl-tRNA was prepared in a 1-ml reaction mixture containing 10 nmoles of tRNA, 1 mg of protein (from the aminoacyl-tRNA synthetase fraction), 100 mM *N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid (*p*H 7.5), 10 mM MgAc, 5 mM KCL, 2 mM adenosine 5'-triphosphate (ATP), 4 mM 2-mercaptoethanol, 1.4 nmoles of ^{14}C-$N^{5,10}$-methenyl-H_4-folate (MTHF) and 6 nmoles of L-[^{12}C]methionine. The reaction mixture also contained a mixture of 19 other L-amino acids at a concentration of 8 μM for each. ^{14}C-MTHF was kindly provided by G. D. Novelli.

TABLE 1. *Chromatographic conditions*

Figure no.	Isoaccepting group	% Recovery	Gradient (M NaCl)
1	fmet	76	0.4–1.0
	pro	53	0.3–1.2
2	ala	55	0.3–1.2
	asp	40	0.3–1.2
	ileu	89	0.4–0.9
	his	55	0.4–0.6
3	thr	73	0.3–1.0
	gly	35	0.4–0.8
4	tyr	62	0.7–1.0
	ser	84	0.4–1.2
5	arg	79	0.4–1.2
	aspn	50	0.3–1.2
6	trp	48	0.5–1.2
	glu	60	0.4–0.8
—[a]	met	58	0.4–0.7
	val	87	0.4–0.9
	phe	68	0.7–1.0
	leu	58	0.3–1.2
	lys	52	0.5–0.8

[a] These figures have been published elsewhere (22).

Preparation of tRNA. Cells were grown to exponential growth phase and extracted, and spores were prepared and purified as previously described (22, 23). Two methods were used to disrupt spores: dithiothreitol pretreatment followed by lysis with lysozyme and homogenizing with a Braun homogenizer. We have described these methods in detail elsewhere (22, 23).

After extraction, tRNAs were purified by phenol and $CHCl_3$: isoamyl alcohol extraction, treatment with deoxyribonuclease, and chromatography on Sephadex G-100, as previously described (22, 23). All tRNA preparations were deacylated of endogenous amino acids by incubation at 37 C in 1.8 M tris(hydroxymethyl)aminomethane (Tris), *p*H 8.0, at 37 C.

RESULTS AND DISCUSSION

Comparisons of 19 aminoacyl-tRNAs isolated from *B. subtilis* spores or cells in exponential growth are presented in Fig. 1–6. The aminoacyl-tRNA elution profiles are grouped according to the types of changes which were observed. Figures 1 and 2 are elution patterns for tRNAs where no changes in isoaccepting species were noted; Fig. 3, 4, and 5 present data

where changes in certain peak ratios were noted; and in Fig. 6 are presented elution profiles of tRNAs where unique species appear or disappear.

These changes and those which have been studied by others are discussed below. Increases and decreases described for isoaccepting species are relative since the exact quantity of each species has not been measured.

Formylmethionyl- and methionyl-tRNAs. Formylmethionyl-tRNA is a particularly interesting species because of its function as initiator of the reading of messenger RNA on 70S ribosomes (c.f. review by P. Lengyel, 17). In *Escherichia coli*, 64% of the total methionine acceptance of the tRNAs can also be formylated (19). Reports of the number of isoaccepting species of $tRNA^{Met}$ and $tRNA_f^{Met}$ in *E. coli* vary between 1 and 2. However, for *B. subtilis* tRNAs, only one isoaccepting species of $tRNA^{Met}$ and one of $tRNA_f^{Met}$ have been reported (10). The synthesis of *N*-formylmethionyl-tRNA in *B. subtilis* seems to be the same as that in *E. coli* (10), and the complete system for the synthesis of *N*-formylmethionyl-tRNA in extracts of *B. subtilis* spores has also been reported (5). In accordance with Bishop et al. (2), I found one peak of $tRNA^{Met}$ and one peak of $tRNA_f^{Met}$ in vegetative cells and spores (Fig. 1). Other figures illustrating the cochromatographic comparisons of methionyl-, phenylalanyl-, valyl-, lysyl-, and leucyl-tRNAs have been published elsewhere (22). Of the two peaks of methionyl-tRNA, the major peak was capable of being formylated whereas the minor peak was not. Because of the importance of $tRNA_f^{Met}$ in initiation, I also examined the chromatographic profiles of methionyl-tRNAs from two asporogenous mutants in the stationary phase of growth, *B. subtilis spoA12*, an early blocked mutant, and *B. subtilis* Rog22 (*spoC*), a mutant with a later block. I estimated the ratio of $tRNA_f^{Met}$ to $tRNA^{Met}$ by integrating the areas under peaks 1 and 2, and also determined the ratio of ^{14}C-methionyl-tRNA to ^{14}C-formylmethionyl-tRNA by assaying the tRNAs in vitro with either ^{14}C-methionine or nonradioactive methionine and ^{14}C-formyl donor. By either estimation, the ratio of $tRNA_f^{Met}$ to $tRNA^{Met}$ in logarithmically growing cells, the asporogenous mutants in stationary phase, or spores was 66% ± 5%. Therefore, I conclude that there is no difference in isoaccepting species of methionyl-tRNA and formylmethionyl-tRNA during sporulation in this system.

Valyl-tRNAs. A change in the ratios of two isoaccepting valyl-tRNAs during the early stages of sporulation was reported by Kaneko and Doi (11). However, the change in the ratios of valyl-tRNAs which they reported returned to that of logarithmically growing cells when cells late in sporulation were examined. Doi et al. (7) also reported similar but quantitatively smaller variations in the ratios of these two valyl-tRNA species during step-up and step-down shifts in culture media, although no differences in valyl-tRNA patterns were observed between tRNAs from cells grown on Penassay or SCM media (6).

DEAE-cellulose chromatography of T1 ribonuclease digests of ^{3}H-valyl-$tRNA_1^{Val}$, and ^{14}C-valyl-$tRNA_2^{Val}$ done by Doi et al. (7) suggested that those two species had different primary sequences. Heyman et al. (9) further observed that these two isoaccepting species had different kinetics of aminoacylation. At low concentrations of valine, this difference in the rate of aminoacylation caused an apparent difference in the ratio of these two species. However, even at high valine concentrations, Heyman et al. substantiated that a change in ratio of valyl-tRNA occurred during early stationary phase and returned to the profile characteristic of logarithmic cells during late stationary phase. Therefore, the findings (22) that there are two isoaccepting species of valyl-tRNA and that there is no difference in the ratio of these species between tRNAs of spores and cells in logarithmic phase are in agreement with those of Doi et al. (5) and Heyman et al. (9).

Prolyl-, phenylalanyl-, alanyl-, aspartyl-, isoleucyl-, and histidyl-tRNAs. Profiles for these aminoacyl-tRNAs are shown in Fig. 1, 2, and 3. I did not observe any changes in these aminoacyl-tRNAs, and no changes in chromatographic profiles of these species from sporulating *Bacillus subtilis* have been reported by others, although two isoaccepting species of histidyl-tRNA extracted from vegetative cells of *B. subtilis* were reported by Stulberg et al. (20). These authors reported the relatively high lability of histidyl-tRNAs to hydrolysis, as I have observed.

Leucyl-tRNA. This is a much studied species in *E. coli* tRNA because of the changes which occur in isoaccepting species of leucyl-tRNA during infection of *E. coli* by phage T_2 (12). Five isoaccepting leucine tRNA species have been observed in *E. coli* (14). No changes in leu-tRNA during sporulation in *Bacillus subtilis* have been reported. However, the only study which investigated this lack of changes employed MAK columns which could only resolve one peak of leucyl-tRNA (5). The chro-

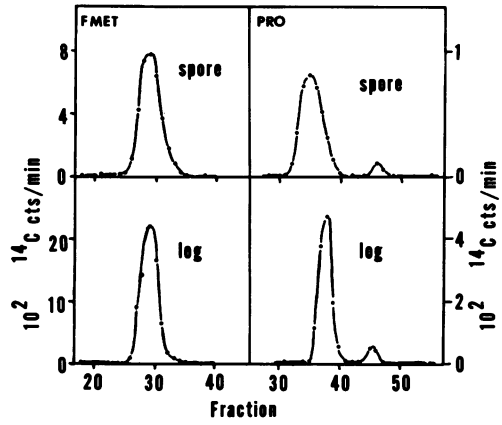

FIG. 1. *Single-label chromatographic elution profiles for formyl-, methionyl-, and prolyl-tRNAs. Refer to Table 1 for conditions. Unlike the double-label comparisons shown in all remaining figures, the tRNA samples from exponentially growing cells (bottom) or spores (top) were run sequentially.*

matography of leucyl-tRNAs on RPC-5 has been previously published (22). Six isoaccepting peaks of leucyl-tRNA are clearly resolved, and some columns indicate a split peak in the major peak. Leucyl-tRNAs prepared from spores contained more material in the fourth and sixth peaks relative to tRNAs prepared from exponentially growing cells (numbering peaks from left to right and not counting the shoulder on the first peak).

Glycyl-tRNA. Glycyl-tRNAs can be resolved into two major and one minor isoaccepting species. Glycyl-tRNAs prepared from spores contained more material in the first peak and lower amounts in the second and third peaks compared to tRNAs prepared from exponentially growing cells (see Fig. 3).

Tyrosyl-tRNA. Arcenaux and Sueoka (1) studied a change in the ratio of two species of tyrosyl-tRNA during sporulation in *B. subtilis* W168 grown on Penassay medium. The change in the two major species of tyrosyl-tRNAs shown in Fig. 4 is identical to the phenomenon they report, although I have detected some minor species and a shoulder on the second major peak. On MAK columns, the major tyrosyl-tRNA peaks were separated by about four tubes out of 120 collected. RPC-5 columns were able to completely separate the two major peaks by 45 tubes out of 100 collected.

Arceneaux and Sueoka (1) determined the ratio of $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ at 12 different time points during sporulation and found a constantly changing ratio between these two species, with $tRNA_1^{Tyr}$ the predominant species in exponentially growing cells and $tRNA_2^{Tyr}$ the predominant species in stationary-phase cells. They also examined several biochemical and biological properties of these two species and compared $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ for incorporation of ^{14}C-methyl-*S*-adenosylmethionine, the effect of mild oxidation with iodine, dialysis against water, heating in the presence of Mg^{2+} or EDTA, rates of acylation and deacylation, synthetase recognition, codon recognition, and frequency of usage by polyribosomes in vivo. No differences between the two species were found. Their results would imply that the major isoaccepting species of tyrosyl-tRNAs are very similar and perhaps only differ from each other in degree of modification. We have investigated the total amount of tyrosine acceptor activity in tRNAs prepared from logarithmically growing cells, stationary-phase cells, and spores and found no differences (22). Therefore, although the relative amount of $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ changed throughout the course of sporulation, the total amount of tyrosine tRNA did not.

Seryl-tRNAs. Seryl-tRNAs prepared from cells in exponential growth had relatively more material in isoaccepting peaks 3 and 4 and relatively less in peak 5 than the seryl-tRNAs prepared from spores (Fig. 4). Doi et al. (5) found no changes in seryl-tRNA between vegetative cells and sporulating cells although they did find a difference in the seryl-tRNAs between *B. subtilis* W23 cells grown on Penassay broth or SCM medium (6). They found three isoaccepting peaks of seryl-tRNAs from cells grown on Penassay media but found greatly reduced amounts of the third peak of seryl-tRNA prepared from cells grown on SCM medium. However, since Doi et al. (5) used MAK columns, it is not possible to compare their results directly with mine.

Arginyl- and asparaginyl-tRNAs. Profiles for these species are shown in Fig. 5. In all cases, one of the isoaccepting species differed in ratio relative to the other species between tRNA prepared from cells in exponential growth or spores. No changes in these species have been previously reported for *B. subtilis* tRNAs.

Lysyl-tRNAs. Lazzarini and co-workers (15) have observed a change in the relative amount of two isoaccepting tRNA species of lysyl-tRNAs between tRNAs prepared from spores or vegetative cells of *B. subtilis*. Lazzarini (15) reported that spore lysyl-tRNAs chromatographed on a MAK column into two major peaks, whereas vegetative cell lysyl-tRNAs showed one major and one minor peak. Laz-

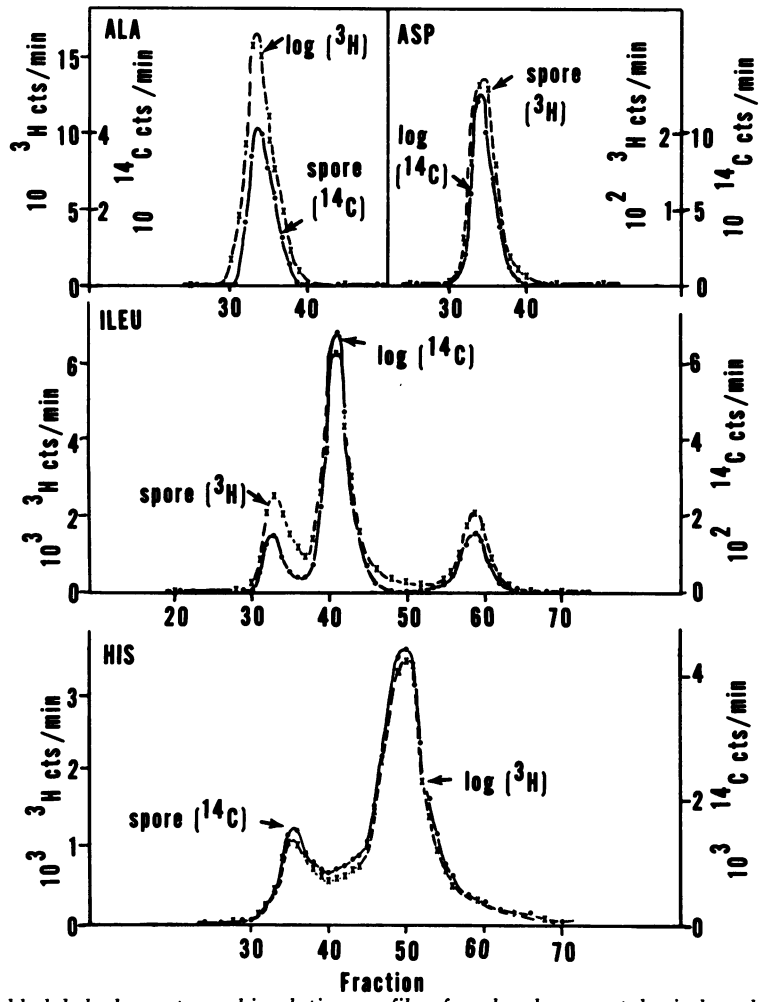

FIG. 2. *Double-label chromatographic elution profiles for alanyl-, aspartyl-, isoleucyl-, and histidyl-tRNAs. Refer to Table 1 for conditions.*

zarini and Santangelo (16) also reported that this change in lysyl-tRNAs was influenced by the medium in which the cells were grown. I have not investigated the effects of media, but, by using a complex media, the chromatography of lysyl-tRNAs from spores and exponentially growing cells were compared on MAK and RPC-2 (21). Again, it is difficult to compare my results with those of others, for, although I did observe a change of ratio between two species of lysyl-tRNAs on RPC-2, I could not resolve these species on MAK. It is probable, however, that this is the same phenomenon as that observed by Lazzarini. Chuang et al. (4) have also chromatographed vegetative lysyl-tRNAs on B-DEAE cellulose columns and reported the presence of two lysyl-tRNAs which they compared in hybridization experiments and concluded that they came from different cistrons. More recently, Chuang and Doi (3) have reported that spores of *B. subtilis* W23 contain only $tRNA_1^{Lys}$, although two isoaccepting species of $tRNA^{Lys}$ were present at a constant ratio during sporulation.

Using RPC-5, I was able to resolve four isoaccepting species of lysyl-tRNAs prepared from exponentially growing cells: two major and two minor peaks. The major species in lysyl-tRNAs from exponentially growing cells was $tRNA_3^{Lys}$, whereas the major species in the lysyl-tRNAs prepared from spores was $tRNA_1^{Lys}$. The presence of the minor species varied from one tRNA preparation to another. However, minor species were never found in lysyl-tRNAs prepared from spores. This may be due to variation with stage of growth. It is also possible that the minor peak is an artifact unique to a particular growth stage.

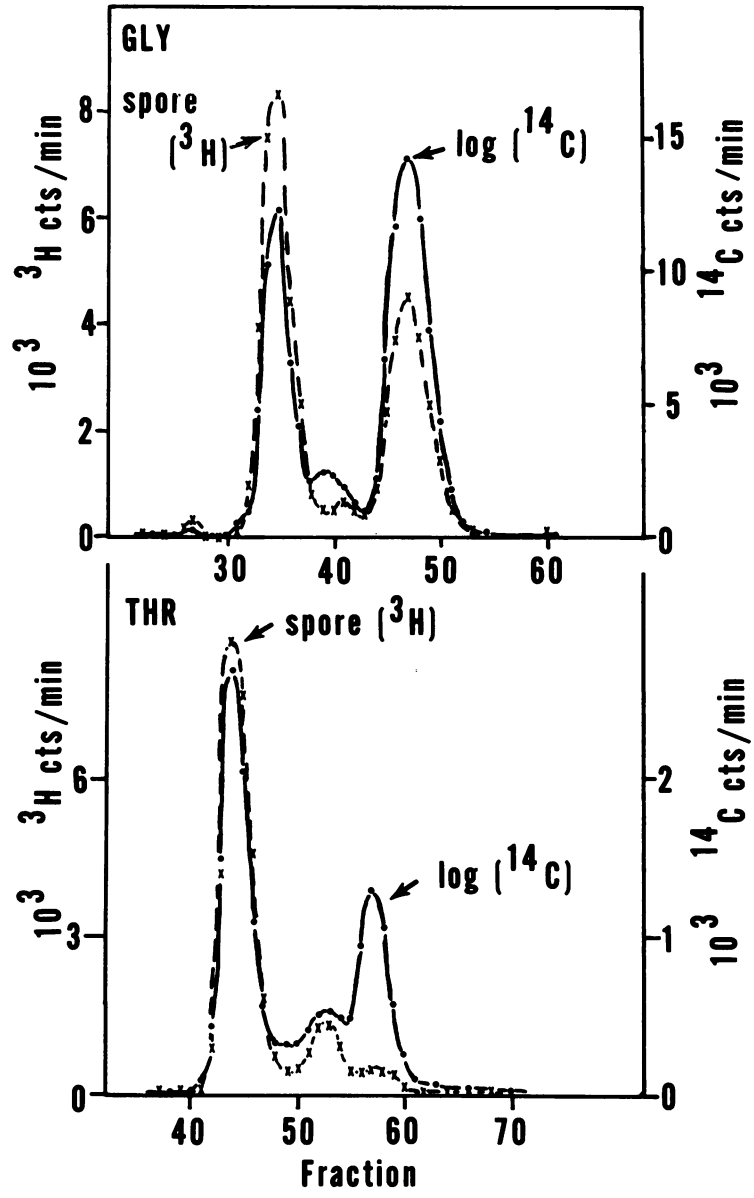

FIG. 3. *Double-label chromatographic elution profiles for glycyl- and threonyl-tRNAs. Refer to Table 1 for conditions.*

Tryptophanyl- and glutamyl-tRNAs. Tryptophanyl-tRNAs have a unique minor species in spore tRNA preparations (Fig. 6) and glutamyl-tRNAs have a unique minor species in tRNAs prepared from exponentially growing cells (Fig. 6). The minor peak of spore tryptophanyl-tRNAs seemed quite reproducible. However, the minor peak of glutamyl-tRNAs found in exponentially growing cells seemed somewhat variable, similar to the minor peaks of lysyl-tRNA from exponentially growing cells.

Analysis. In an analysis of column comparisons, the changes considered significant are somewhat arbitrary. For instance, occasionally I would observe small peaks containing less than 2% of the total radioactivity in the elution profile (i.e., in Fig. 6, there is a very small peak at about tube number 8). Although these kinds of variations may have biological significance, they are difficult to verify and I have chosen to ignore them. A change was considered significant if the difference in the integrated areas of a

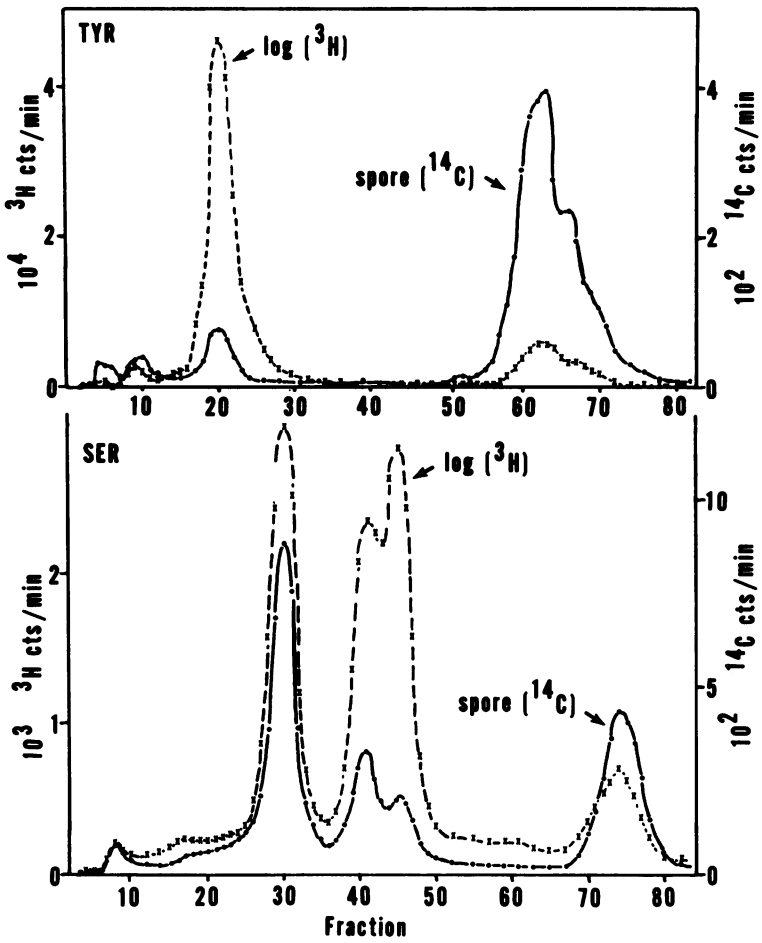

FIG. 4. *Double-label chromatographic elution profiles for tyrosyl- and seryl-tRNAs. Refer to Table 1 for conditions.*

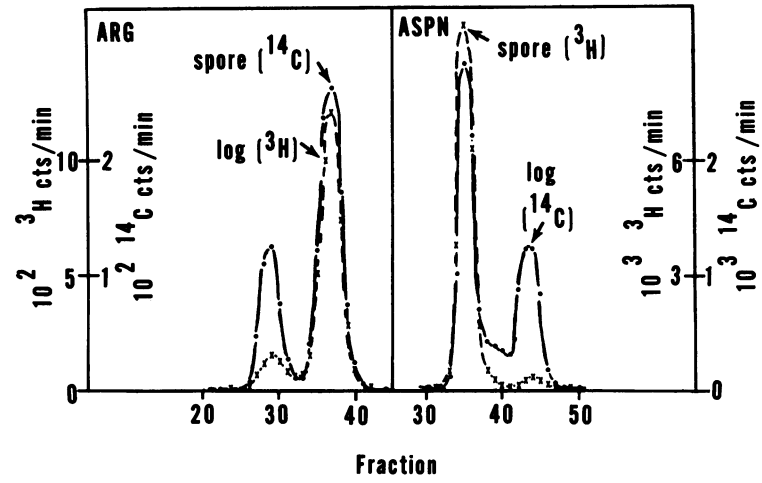

FIG. 5. *Double-label chromatographic elution profiles for arginyl- and asparaginyl-tRNAs. Refer to Table 1 for conditions.*

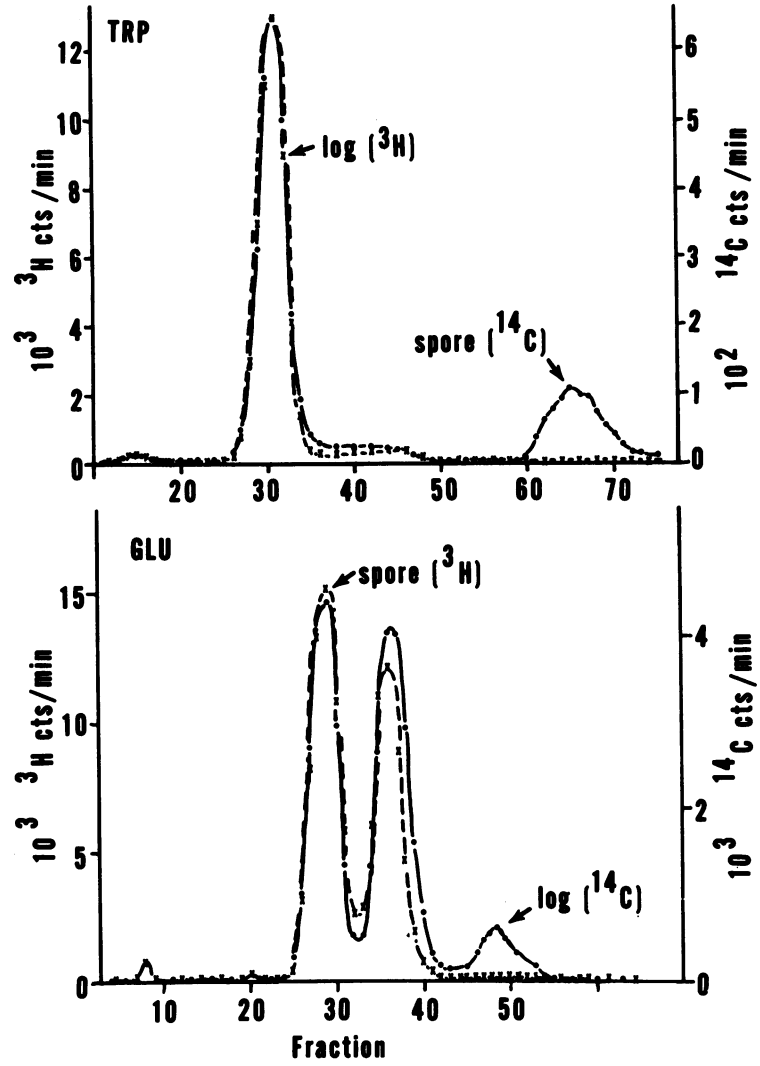

FIG. 6. *Double-label chromatographic elution profiles for tryptophanyl- and glutamyl-tRNAs. Refer to Table 1 for conditions.*

particular peak comparing the tRNAs prepared from exponentially growing cells (log tRNA) or spores (spore tRNA) was greater than the variability observed between sequential runs comparing "log" tRNAs to "log" tRNAs or spore tRNAs to spore tRNAs. Transfer RNAs extracted from spores by a Braun homogenizer or by pretreatment with dithiothreitol followed by breakage with lysozyme showed identical chromatographic profiles, indicating that the profiles of the spore tRNAs were not artifacts of a particular extraction procedure. The purity of all radioisotopes was checked on TLC chromatography before use, as mentioned in Materials and Methods, but, since methionine and tryptophan were of 80% purity, I repeated the profiles for methionyl- and tryptophanyl-tRNAs by using the labeled amino acids and tRNA preparations in reversed positions. The same profiles were obtained. The majority of the other aminoacyl-tRNAs were also repeated with reversed labels and with increased concentrations of aminoacyl-tRNA synthetase and amino acids.

Differences in the elution patterns of aminoacyl-tRNAs in column chromatograms such as those shown here could be caused by differences in the nucleotide sequence, differences in degree of modification of the nucleotide residues, or alterations in the secondary or tertiary structure. Although this present study cannot

differentiate between these alternatives, it does demonstrate several differences between the tRNAs of spores and exponentially growing cells which have not been described before and which merit further investigation.

ACKNOWLEDGMENTS

I thank Kristi Peterson for student technical assistance. This investigation was supported by Public Health Service research grant 5 RO1 GM-17421 and Public Health Service research career development award 1-K4-GM-23,736-01 from the National Institute of General Medical Sciences.

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