# *THE POLYAMINE CONTENT OF THE tRNA OF E. COLI**

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*Abstract.*—A sensitive method of polyamine estimation has been adapted to the study of the organic cations of small amounts of nucleic acid. A procedure utilizing phenol extraction, alcohol precipitation, and separation on Sephadex G100 has been devised for the isolation of tRNA at low ionic strength. The procedure is applicable to the isolation of tRNA from liter batches of bacterial culture. With these methods we have examined the polyamines of tRNA isolated from polyauxotrophic strains of *E. coli* incubated under various physiological conditions and have found the following: (1) The tRNA from relaxed bacteria (TAU rel) harvested during exponential growth is heterogeneous with respect to polyamine content. Some portions of the population contain about one mole of spermidine per mole of tRNA. Some putrescine and an unknown amine are also present in low concentration. (2) After exponential TAU rel is incubated with thymine and uracil in the absence of arginine, the tRNA population is far more homogeneous with respect to polyamine content. The various fractions contain two moles of spermidine per mole of tRNA and a small amount of putrescine. (3) After exponential TAU rel is incubated in the absence of both arginine and uracil, the polyamine pattern of the tRNA resembles that isolated from exponential cells. (4) The tRNA from stringent bacterias harvested during exponential growth is heterogeneous with respect to polyamine distribution and some fractions contain relatively high concentrations of the unknown amine.

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We have been studying the relationship of synthesis of RNA and of the polyamines in polyauxotrophic strains of *E. coli*.[1, 2] In addition to known effects of spermidine on RNA synthesis *in vitro*,[3] our observations have led us to conclude that it is a controlling element in RNA synthesis *in vivo*. Our data have shown (1) the concomitant synthesis in bacteria of RNA and of this organic cation in numerous physiological conditions,[2] (2) the relaxation of synthesis of ribosomal RNA in stringent bacteria by exogenous addition of spermidine,[1] and (3) the relatively high rate of synthesis of spermidine in a relaxed mutant as compared to the stringent parent, a difference expressed even in the absence of RNA accumulation.[4] It may be asked whether these effects of spermidine arise mainly from the activation of transcription or by the reaction of this polyamine with the main RNA products, rRNA and tRNA.

That spermidine can serve to organize helical structure in both RNA[5] and DNA[6] is known. Indeed, it has been shown that polyamines can help to effect a conversion *in vitro* of the inactive configuration of tRNA to the active form.[7–9] Spermidine appears to be the most active organic cation in this conversion.[10] Furthermore, spermidine can replace $Mg^{++}$ in the methylation of tRNA by liver tRNA methylase[11] and in the transfer of an amino acid from a complex of enzyme-amino acid-adenylate to tRNA.[12] In seeking to define a physiological

role at the molecular level for this organic cation, which appears so ubiquitously in RNA-containing cellular structures, we undertook to determine the spermidine content of the tRNA isolated from a stringent and relaxed mutant pair of *E. coli* strain 15 TAU. As described earlier, the synthesis of both polyamine and RNA in these strains can be controlled by simple nutritional conditions.[4]

In order to minimize the exchange of cations from the native nucleic acids during extraction, we have used a relatively simple method of isolation of tRNA at low ionic strength. Extracts of the nucleic acid fractions were dansylated and the fluorescent derivatives were separated by thin-layer chromatography. The polyamines were estimated by scanning the fluorescent spots. With this sensitive assay we can determine $10^{-11}$ moles of polyamines, an amount which may be contained in the equivalent of 0.01 optical density unit of purified tRNA. It was found by these methods that the tRNA of cells incubated under various physiological conditions may contain significant amounts of spermidine. A particularly striking result was that the tRNA isolated from the relaxed strain of *E. coli* TAU incubated in a medium containing thymine and uracil but lacking arginine was found to contain about 2 moles of spermidine per mole of tRNA.

*Methods.—Bacterial strains:* The work reported in this paper was done with *E. coli* strain 15 TAU, requiring thymine, arginine, and uracil for exponential growth. The mutant pair, $RC^{st}$ (TAU st) and $RC^{rel}$ (TAU rel), were used and grown or incubated as described previously.[4]

*Isolation of tRNA:* The initial step was that described by Gutcho.[13] Pellets of bacteria, grown in 1 or 2 liter batches, were harvested by centrifugation, drained, and frozen. The extraction and precipitation of tRNA, scaled down proportionately to the weight of the pellets, were carried out as described through "Step 1." [13] Two minor changes were introduced. Precipitation of RNA with ethanol was effected in a solution containing 0.025 M potassium acetate. The RNA pellets were washed successively with 80%, 95%, and absolute ethanol and with ether. Yields of these crude tRNA fractions were about 20 to 25 mg/gm of frozen bacteria. In water, these fractions contained about 4 OD units at 260 mμ/mg and contained significant amounts of spermidine.

The crude fractions derived from exponentially grown TAU rel contained about twice the spermidine per unit OD (optical density) as did fractions derived from TAU st. At levels of 100 μg (about 1 mμmole of tRNA) in 0.3 ml of reaction mixture, the tRNA accepted 5–50 μμmoles of each of 5 different amino acids.[14, 15]

In the next step, the crude tRNA (40–80 OD) was dissolved in 1 ml of 0.01 M potassium acetate buffer at pH 7.0 and fractionated at 4° on a Sephadex G100 column (28 × 2 cm) equilibrated with this buffer at 4°. The separation of the main fractions into 3 peaks of absorbancy is presented in Figure 1. The initial fractions immediately following the void volume contain a small but significant amount of RNA, presumed to be rRNA, in addition to some cell wall material. The amount of RNA in this fraction was 14–27% of that in the two major peaks.

The initial shoulder to the main peak appears to contain material approaching 5S RNA. The main peak (the central three quarters) consists largely of tRNA by the following criteria: (a) enzymatic acceptance of phenylalanine is approximately proportional to optical density across the approximately symmetrical portion of the peak.[15] No amino acid acceptance is obtained with other fractions, (b) acceptance of phenylalanine per optical density of isolated tRNA is similar quantitatively to that of *E. coli* tRNA which had been purified on a methylated albumin Kieselguhr column,[16] (c) fractions in this peak accept methyl from S-adenosylmethionine in the presence of a liver tRNA methylase.[15] These fractions were more active in this test than was purified methyl-deficient *E. coli* tRNA, (d) fractions in this peak accept serine from the complex of seryl-adenylate-serine-activating enzyme.[15]

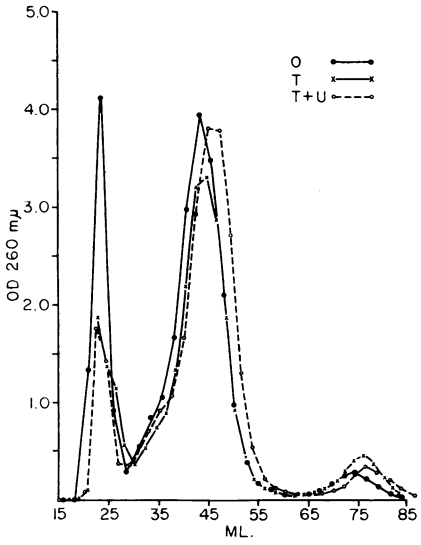


FIG. 1.—The purification of tRNA on Sephadex G100 in 0.01 M potassium acetate at pH 7.0. Three different samples of crude tRNA (step 1, Gutcho)[13] from TAU rel were fractionated on the same column at 4° on successive days. The O sample was derived from organisms harvested during exponential growth. Aliquots of this same culture were washed and incubated further for 1 hr in the absence of arginine in the mineral medium plus glucose supplemented with thymine (T) or with thymine plus uracil (T + U).

Fractions of tRNA derived from the various preparations were heated from 25 to 90° in the 0.01 M potassium acetate buffer in cuvettes in the Gilford spectrophotometer. The increase in ultraviolet absorption was followed at 260 mμ. The $T_m$ values were in the narrow range of 56 to 58°C, and were essentially independent of the spermidine contents of the isolated samples. The melting curves were smooth and reannealing was 70 to 78% complete in each instance. The observed hyperchromic effects at this wave length were 25.2 to 27.7%.

*Polyamine Analyses:* These were done by a modification of the method of Seiler and Wiechmann[17] as modified by Dion, Spaulding, and Herbst (unpublished). Perchloric acid is added to fractions containing about 0.5 OD units to a concentration of 3%. The final volume is 0.2 ml in a 12-ml glass-stoppered centrifuge tube. To this is added with mixing 0.4 ml of an acetone solution of 1-dimethylamino-naphthalene-5-sulfonyl chloride (30 mg dansyl chloride per ml) and about 50 mg of ground $Na_2CO_3 \cdot 10H_2O$. It is convenient to incubate the mixtures overnight at room temperature in the dark. In the morning 0.1 ml of an aqueous solution of proline (100 mg/ml) is added and the mixture is incubated for 30 min in the dark. One-half ml of benzene (Spectrograde) is added, mixed thoroughly for 30 sec and the emulsion is then centrifuged at 2000 rpm for 10 min at 10°C.

A thin-layer plate (Silica Gel G, 250 microns-Analtech, Inc.) is activated by heating at 110°C for 1 hr just before application of 10 μl of the benzene extract. For samples devoid of spermine, the chromatogram may be developed in ethyl acetate-cyclohexane (1/2) (v/v); the solvent mixture is prepared fresh daily. Development is carried out almost to the top of the plate. After removal the chromatogram is sprayed immediately with 10 ml triethanolamine-isopropanol (1:4) and is then placed in the dark in a vacuum (25–30 mm Hg) at room temperature overnight. The next morning the plate is equilibrated in air at atmospheric pressure in the dark for one hour.

The spots characteristics of putrescine, spermidine, and components of intermediate $R_f$ are scanned in the Turner fluorimeter model 111 and the areas under the curves of the recorded scan tracing are determined. On the same chromatogram these areas are directly proportional to concentration of putrescine and spermidine in the range of 0.03 to 0.2 mμmole. Under the conditions described above, identical aliquots of both polyamines may yield slightly different areas on different plates. Also the different polyamines show slightly different responses on different plates; thus the ratios of the fluorescence of

dansyl putrescine to that of dansyl spermidine may vary somewhat from plate to plate. It is therefore necessary to run standards of both polyamines on the same chromatogram as that used for the unknowns. The yield of fluorescence per mole of putrescine in this system tends to be slightly greater than that of spermidine.

Polyamine analyses may be run in this way directly on 0.2 ml of acidified aliquots of complete bacterial cultures at concentrations of $10^8$ organisms/ml, or on the media after filtration or centrifugation, or on extracts of separated cells.

*Chemicals:* Mallinckrodt's liquefied phenol (88%) was distilled in a nitrogen atmosphere over Zn immediately before use. Samples of putrescine dihydrochloride were obtained from Calbiochem. Occasional samples of spermidine were found which contained quite large amounts of impurities, dansyl derivatives of which migrated in the position of dansyl spermine. The samples used as standards had less than 5% of a detectable impurity which reacted with dansyl chloride. Dansyl chloride was obtained from Calbiochem and was dissolved in acetone immediately before use. Proline was obtained from Sigma Chemical Company and solutions were stored at 4°C and used for several weeks.

*Results.*—Samples of tRNA were isolated and fractionated from *E. coli* TAU st and TAU rel which had been incubated under various conditions. As presented in the Legend to Figure 1, organisms were collected in the exponential phase of growth. After such growth, some batches were further incubated for an hour in the absence of arginine, which would markedly inhibit synthesis of RNA in TAU st but permit synthesis of rRNA and tRNA for an hour in TAU rel.[4] It had been shown that in the latter system, i.e., TAU rel + T + U, the accumulation of cellular RNA is paralleled by an accumulation of cellular spermidine but not of putrescine.

The recovery of spermidine in the two major peaks presented in Figure 1 was greater than 80 per cent of that applied. The spermidine content of the combined fractions in the leading shoulder of the tRNA peak was about 10 per cent of that recovered in the two main peaks. About 1 per cent of the total polyamine was found in the last small peak of optical density.

The ratio of spermidine content to ultraviolet light absorption of the fraction containing tRNA was always severalfold higher than that of the tRNA fraction.

*RNA of TAU rel (exponential):* The pattern of fractionation on Sephadex G100 of crude tRNA derived from TAU rel in the exponential phase of growth is given in Figure 1 as TAU rel (O). Tracings of the dansylated polyamine patterns for the initial RNA and the tRNA fractions are shown in Figure 2. In the pattern for the fraction containing rRNA, the large peak is that of spermidine (spd). The small peak is that of putrescine (pu). The identity of the minor intermediate peak in this tracing is unknown.

Figure 2 also shows the tracings of dansyl polyamines found in the fractions [tubes 21 and 23 (see Table 1)] on either side of the tRNA peak. The front of the tRNA peak (tube 21) appears to be significantly lower in spermidine content than the later fraction (tube 23). Small amounts of putrescine and an unknown dansylated derivative (designated as X) are also seen in both fractions. The actual contents of spermidine per mole of tRNA of these fractions are summarized in Table 1.

*RNA of TAU rel incubated in thymine plus uracil:* In Figure 3 are presented the tracings of dansyl derivatives of the polyamines found in rRNA and tRNA fractions. The cells had been harvested in exponential growth and incubated

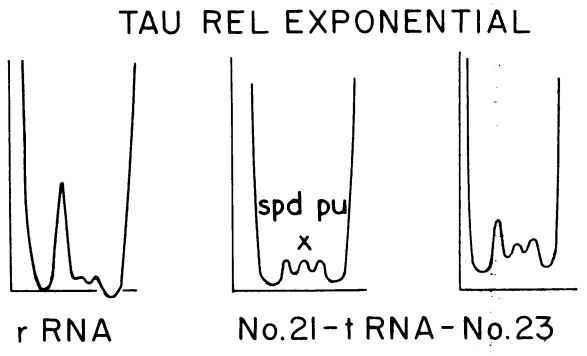


FIG. 2.—Tracings of dansylated polyamines of fractions containing rRNA and tRNA derived from TAU rel harvested during exponential growth (TAU O rel in Fig. 1). Spd = spermidine, pu = putrescine, × = unknown amine. Tube numbers and spermidine contents of the fractions are given in Table 1. No. 21 and 23 refer to tube numbers presented in Table 1.

for an hour in a medium containing thymine and uracil but lacking arginine. By comparing the tracings of this Figure with those of Figure 2, it can be seen that these fractions are relatively high in spermidine. Furthermore, spermidine is present in considerably larger amount than is putrescine, and the unknown dansylable constituent has disappeared from the tRNA fractions. The spermidine content of the various fractions in the peak approximate two moles per mole of tRNA, as presented in Table 1.

*RNA of TAU rel incubated in thymine alone:* When TAU rel is incubated in the absence of uracil, RNA does not accumulate. Nevertheless, the cells make the same quantity of spermidine, which, however, is excreted into the medium as acetylspermidine.[4] Thus, tRNA present in such a cell may be exposed to a continual supply of spermidine which conceivably might build to a very high concentration of this cation per mole of tRNA. Nevertheless the tracings shown in Figure 4 show that the polyamine content of rRNA and tRNA fractions resemble those derived from cells harvested in exponential phase (Fig. 2), being lower in spermidine and containing the material yielding the unknown dansyl derivative. Calculations of the spermidine content per tRNA are given in Table 1.

*RNA of TAU st (exponential):* The pattern of separation of tRNA fractions from the stringent strain is essentially superimposable on the patterns presented in Figure 1. Figure 5 presents tracings of dansyl derivatives of polyamines derived from RNA fractions of TAU st harvested during exponential growth. Fractions of both tRNA and rRNA contain very considerable amounts of the unknown constituent, designated as X in the Figure. Although the spermidine content of the "rRNA" fraction is fairly high, that of the tRNA fractions appear to be of the order of half or less that of the tRNA derived from relaxed organisms. The putrescine contents of the tRNA fractions are of the same low order as in tRNA fractions from the relaxed organisms.

*Discussion.*—The new method for measurement of polyamines exploited in this work clearly opens up the exploration of polyamine content and metabolism of relatively small amounts of biological material. Samples in short supply, that are nevertheless amenable to study by ultraviolet absorbance, can be examined for their content of these organic cations. We have used this procedure for the analysis of polyamine synthesis in small volumes of bacterial culture and have

TABLE 1. *Spermidine content of RNA fractions of TAU rel.*

| Incubation conditions | Sample | Tube No. | $OD_{260}$ | Moles spermidine per mole tRNA* |
|---|---|---|---|---|
| TAU rel O | rRNA | 11 | 3.68 | 3.25† |
| Exponential growth | tRNA | 21 | 2.31 | 0.72 |
| | | 22 | 3.16 | 1.52 |
| | | 23 | 3.77 | 1.08 |
| | | 24 | 3.75 | 1.14 |
| | | 25 | 3.11 | >0.97 <1.45 |
| | | 26 | 2.08 | <0.97 |
| TAU rel T + U | rRNA | 10 | 2.47 | 9.27 |
| Incubation in thymine + uracil | tRNA | 21 | 3.72 | 1.94 |
| | | 22 | 4.57 | 1.94 |
| | | 23 | 5.38 | 1.82 |
| | | 24 | 5.27 | 1.88 |
| | | 25 | 4.00 | 2.29 |
| | | 26 | 2.40 | 2.88 |
| TAU rel T | rRNA | 11 | 2.49 | 3.39 |
| Incubation in thymine | rRNA | 21 | 1.78 | 1.02 |
| | | 22 | 2.49 | 1.15 |
| | | 23 | 3.71 | 0.89 |
| | | 24 | 4.03 | 0.93 |
| | | 25 | 3.44 | 0.88 |
| | | 26 | 2.17 | <0.7 |

* The calculation assumes an approximate molecular weight equivalence of unfractionated tRNA of *E. coli* and of yeast,[10] i.e., approximately 77 nucleotides, and an equivalence of extinction coefficients for both structures, i.e. $\epsilon_p$ = 7.4 ml/μmole/cm[18] leading to a molar extinction coefficient of 570 ml/μmole/cm.

† The spermidine content of the fractions that contain rRNA is calculated using the same molar extinction coefficient used for tRNA. It is obviously inexact but permits comparison with tRNA samples.

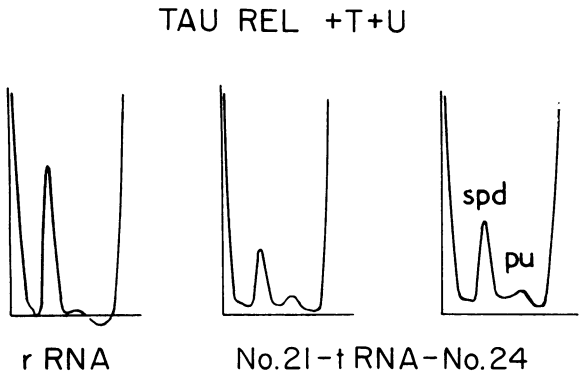


FIG. 3.—Tracings of dansylated polyamines of RNA fractions from TAU rel incubated in thymine and uracil in the absence of arginine. Spd = spermidine, pu = putrescine.

been able to avoid artifacts introduced in manipulating large volumes, e.g., centrifugation for significant periods at temperatures permitting metabolic alteration of the polyamines.

The sensitivity of the assay method has permitted us to work with relatively small amounts of RNA. It has therefore been feasible to bypass steps of concentration and purification on methylated albumin Kiesleguhr or ion exchange columns and to go directly from extracted tRNA to columns of Sephadex G100, equilibrated at low ionic strength (0.01 M K acetate). Not only has purification

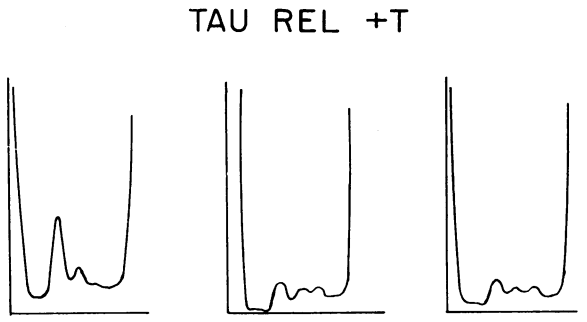


FIG. 4.—Tracings of dansylated polyamines of RNA fractions from TAU rel incubated in thymine.

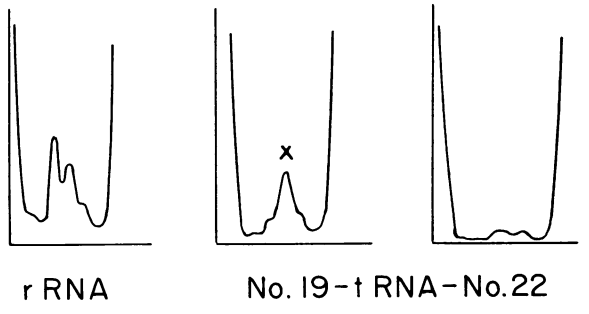


FIG. 5.—Tracings of dansylated polyamines of RNA fractions from TAU st harvested during exponential growth.

on Sephadex columns proven to be rapid and efficacious but it has provided demonstration of the relative nondissociability of the spermidine from tRNA under the conditions selected. It is quite possible that polyamines including some additional spermidine molecules may have been lost from tRNA as a result of the isolation procedure. Nevertheless, some spermidine is firmly bound (one to two molecules) and this poses the questions of the conditions under which these spermidine molecules can be removed, as well as the nature of the apparently specific sites to which they are bound.

The hypothesis of Liquori *et al.*,[6] in which spermidine may bind to three phosphates on the two polydeoxynucleotide chains lining the narrow groove in helical regions of DNA, appears possibly applicable to our observations. It can be imagined that spermidine spans a groove in helical double-stranded regions of tRNA comprised of perhaps four to six complementary base pairs. Models of tRNA developed from studies of nucleotide sequence and reactivity, as well as the physicochemical behavior of this nucleic acid, strongly suggest the existence of such helical regions in tRNA. Furthermore, it can be imagined that the presence of spermidine in such regions may serve to stabilize such conformations or even to facilitate their formation.

The presence of two spermidine molecules per tRNA throughout the population of molecules isolable from a cell making RNA and spermidine in the absence of protein synthesis suggests the upper limit of binding sites of tRNA which hold

polyamines tightly enough to survive the isolation procedure we have been using. It is possible that the saturation of these sites by spermidine occurs only at the time of synthesis of tRNA, since synthesis of spermidine in the absence of RNA synthesis does not lead to a significant change in the polyamine content of the isolable RNA.

The finding of less than one molecule of spermidine per mole of tRNA in a population of tRNA molecules isolable from stringent or relaxed cells harvested during exponential growth suggests a significant heterogeneity in these populations. It is possible that many molecules isolated under such conditions are inactive and in the presence of being degraded. Such populations also differ significantly between themselves in the type of polyamines found, e.g., spermidine in relaxed tRNA or X in stringent tRNA. The nature of the unknown dansylatable cation (X) present in stringent tRNA and apparently in various cells in specific physiological states has not yet been clarified. X is not cadaverine, 1,3 diaminopropane, agmatine, or S-adenosylmethionine.

Despite the marked differences in total spermidine content or in the types of polyamines found, no striking functional differences, i.e., amino acid acceptance, methyl acceptance, have yet been found among our tRNA samples. Such a result may merely reflect the quantitative inadequacies of the tests currently available in screening such tRNA populations. It is possible also that the necessity of estimating some types of functionality in the presence of an excess of essential cation, i.e., $Mg^{++}$ or spermidine, masks functional differences possibly detectable by more subtle tests.

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