TRANSFER RNA CODED BY THE T4 BACTERIOPHAGE GENOME

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Modifications in transfer RNA (tRNA) chromatographic patterns after virus infection have been described by several laboratories.[1-5] In a recent communication, we described distinct alterations in the chromatographic behavior of pulse-labeled S^{35}-tRNA after infection of *Escherichia coli* B with T-even bacteriophage.[6] The experimental data suggested that the observed S^{35} transition from the "normal" to the "infected" tRNA profile was virus-induced and that these changes were not the result of host tRNA modifications. The "infected" and "uninfected" sulfur-labeled profiles of tRNA as observed by chromatography on methylated albumin kieselguhr (MAK) are shown in Figure 1 where we have arbitrarily designated the radioactive regions as *peaks 1*, *2*, and *3*. In this communication we present evidence that "peak 3"-labeled tRNA from T4-infected cells is related to T4 DNA by sequence homology and that a portion of the amino acid acceptor activity in this tRNA region is T4-specific.

Experimental.—Preparation of T4 S^{35}-tRNA: *E. coli* B cells were grown in MCN medium which contained the following constituents per liter: 2 gm of NH_4Cl, 0.5 gm of NaCl, 8 gm of KCl, 1×10^{-6} gm of $FeCl_3 \cdot 6H_2O$, 2 gm of dextrose, 10^{-4} *M* Na_2SO_4, 10^{-4} *M* $CaCl_2$, 5×10^{-4} *M* $MgCl_2$, 2×10^{-2} *M* Na_2HPO_4 (pH 7.0) and 10^{-2} *M* tris-(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5). Into 400 ml of fresh MCN medium (minus sulfate) 50 ml of an overnight culture was transferred, and growth continued to 3×10^8 per ml (2-hr generation time). A mixture of 18 amino acids (methionine and cysteine were omitted) was added to give 0.1 μM/ml for each amino acid, and aeration continued until 4–5 $\times 10^8$ per ml. After another 30 min, tryptophan was added to 100 μg/ml, and cells were infected with T4 phage at a multiplicity of 5. These cells produce virus at about half the normal plaque-forming unit production in nonlimiting sulfate medium. At 4.5 min after infection, 17 mc of carrier-free S^{35}-H_2SO_4 (New England Nuclear Corporation) was added, and infection was stopped 5 or 15 minutes later, as previously described.[6] The cells were collected by centrifugation, and RNA was extracted as reported previously.[6] The RNA was subjected to chromatography on MAK, and the peak 3 fractions (Fig. 1) were combined and concentrated to 3 ml after overnight dialysis against deionized water.

Preparation of T4 P^{32}-RNA: Ribosomes were prepared from T4-infected *E. coli* B which had been pulsed with $^{32}P_i$ (1 mc/8 ml of cell suspension containing 2.5×10^{-4} *M* phosphate) between 4.5 and 9.5 min after infection. The ribosomes were washed once and RNA was extracted by the phenol procedure.[7] The P^{32}-RNA isolated was subjected to Sephadex G-100 chromatography as described below.

Hybridization: Procedure (A): Hybridization of DNA with RNA was essentially carried out as described by Gillespie and Spiegelman.[9] Denatured DNA fixed onto nitrocellulose filters was incubated in a 0.90-ml system (final volume) containing 0.30 ml of 6 × SSC (pH 6.7) and 0.60 ml of labeled tRNA at 67° for 15 hr. The filters were removed, washed with 2 × SSC, treated with RNase (20 μg of pancreatic and 1 μg of T1/ml) for 45 min at 37°, washed again, and then counted in a scintillation system. *Procedure (B):* DNA-filters were annealed in a 0.60-ml system containing 0.10 ml of 12 × SSC (pH 4.3), 0.20 ml of (H^3) aminoacyl-tRNA, and 0.30 ml of formamide. Annealing was at 33° for 3 hr. The filters were treated as described in (*A*), except that 2 × SSC

(pH 6) was used for washing and the RNase treatment consisted of T1 RNase only (2.5 μg/ml) in 2 × SSC (pH 6) for 30 min at room temperature. *Procedure (C)*: Annealing was carried out in the absence of filters. T4 DNA was denatured at 80° in 50% formamide. The annealing system (1.0 ml) contained 190 μg of denatured T4 DNA, 100–150 μg of (H^3) aminoacyl-tRNA, 0.17 ml of 12 × SSC (pH 4.3), and 0.50 ml of formamide. Annealing was at 33° for 3 hr, after which the mixture was chromatographed on Sephadex G-100 directly.

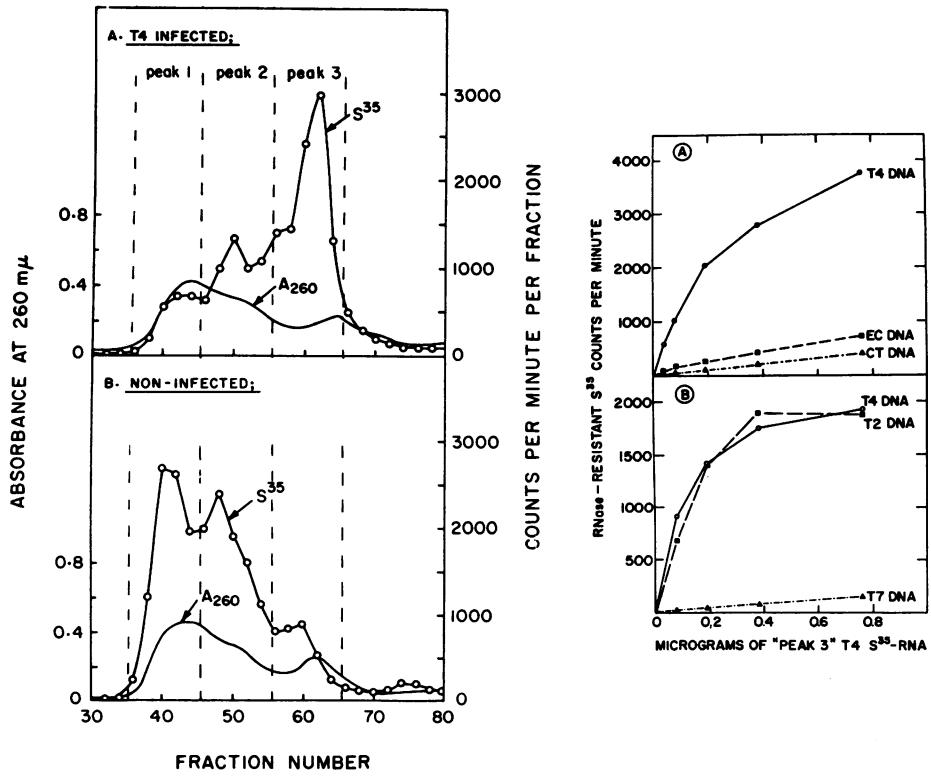
Chromatography: MAK chromatography was as previously described.[6] Sephadex G-100 chromatography was conducted on columns equilibrated with 0.05 *M* ammonium acetate, pH 5.4, the same solvent being used for elution. Chromatography of S^{35}- and P^{32}-RNA was at room temperature; chromatography of (H^3) aminoacyl-tRNA was at 4°.

Aminoacyl-tRNA: *E. coli* B extracts served as the source for amino acid synthetase and was prepared as described by Kelmers *et al.*[10] The enzyme was stored in 50% glycerol at −20° and was stable for several months. The reaction mixture for tRNA charging was as described by this same group. (H^3)L-leucine was purchased from Schwarz Bio-Research.

Results.—Although our earlier results[6] indicated that the S^{35} incorporated into peak 3 T4 tRNA (Fig. 1) represented *de novo* sulfur, it was not possible to determine whether *de novo* tRNA synthesis had occurred and, if so, whether it was host- or viral-directed. If indeed the S^{35} chromatographic transition were due to virus-directed tRNA synthesis, the sulfur-labeled tRNA from T4-infected cells should show some sequence homology to viral DNA, in which case both of the above queries would be immediately answered.

When highly labeled S^{35}-tRNA from T4-infected cells was employed in various annealing reactions, RNase resistance was imparted to the labeled RNA very effectively with T4 DNA but only slightly with *E. coli* DNA (Fig. 2*A*). Other annealing experiments not shown here indicate that approximately 30 per cent of the peak 3 T4 S^{35}-tRNA preparation is hybridizable to T4 DNA. Figure 2*B* shows that T4 S^{35}-tRNA hybridizes to T2 DNA equally as well as to T4 DNA; yet no significant hybridization occurs with T7 DNA. These results are not unexpected since the T7 phage does not show the enhanced peak 3 S^{35}-tRNA transition on MAK chromatography,[6] and it is also known that the T-even phages are genetically related to each other, but not to the T-odd viruses.[11] Since T4 tRNA preparations can be shown to contain partially degraded T4 mRNA (messenger RNA), the hybridization data could be accounted for if T4 mRNA contained organic sulfur, or if degraded T4 mRNA was subsequently sulfated *in situ*. To test these possibilities, it was necessary to devise a procedure which would clearly separate T4 mRNA from T4 tRNA; this is shown below.

When P^{32}-RNA from infected ribosomes was chromatographed on Sephadex G-100, almost all the label appeared with ribosomal RNA in the void volume; 5*S* and 4*S* RNA appear later and separate clearly from each other (Fig. 3*A*). Base analysis of the P^{32}-RNA in the ribosomal fractions indicates a nucleotide composition similar to T4 DNA (Fig. 3*A*, table insert). Over 70 per cent of this labeled RNA is hybridizable to T4 DNA and, when subjected to sucrose gradient centrifugation, most of the radioactivity sediments at a rate slower than 16*S* and is not coincident with the two major molecular species of ribosomal RNA (Fig. 3*B*). These results suggest therefore that the $^{32}P_i$ pulse-labeled RNA obtained from T4-infected cells, which is excluded with ribosomal RNA from Sephadex G-100, has chemical and physical properties similar to T4 mRNA.



(*Left*) FIG. 1.—MAK S^{35}-tRNA profiles from T4-infected and uninfected pulse-labeled cells. The chromatographic profiles in (*A*) and (*B*) are the same as in Fig. 3 of our earlier report.[6] The S^{35}-tRNA profiles have been arbitrarily divided into three regions (*dashed vertical lines*) designated as *peaks 1, 2,* and *3,* and referred to in the text.

(*Right*) FIG. 2.—Hybridization of T4 "peak 3" S^{35}-tRNA with T4 DNA. The conditions for hybridization were as described under *Methods* for *Procedure* (*A*). The nitrocellulose filters contained approximately 60 μg and 52 μg of denatured DNA in (*A*) and (*B*), respectively, for the various DNA's employed; T4, *E. coli* (*EC*), and calf thymus (*CT*). The S^{35}-content of the "peak 3" tRNA was 65,000 cpm per μg in (*A*) and 44,000 cpm per μg in (*B*).

When T4-infected cells are pulsed with S^{35}-sulfate and the total cellular RNA extract is chromatographed on Sephadex G-100, no S^{35} label is seen in the ribosomal RNA-mRNA fractions but all of the S^{35}-RNA appears in a position where 4*S* RNA is located (Fig. 4). Two radioactive peaks are observed in the 4*S* position, the first peak being equivalent to the viral peak 3 material seen on MAK chromatography and the second peak representing primarily host S^{35}-tRNA. Furthermore, Figure 5 shows that while T4 tRNA effectively competes with T4 S^{35}-tRNA for hybridization with T4 DNA, T4 mRNA and *E. coli* tRNA are not competitors. These experiments indicate that T4 mRNA does not contain sulfur and that there is no sequence homology between T4 mRNA and T4 S^{35}-tRNA; hence, it is unlikely that one RNA species is derived from the other.

An attempt was made to determine if any specific amino acid-acceptor activity is associated with T4 tRNA preparations. A comparison of normal and infected

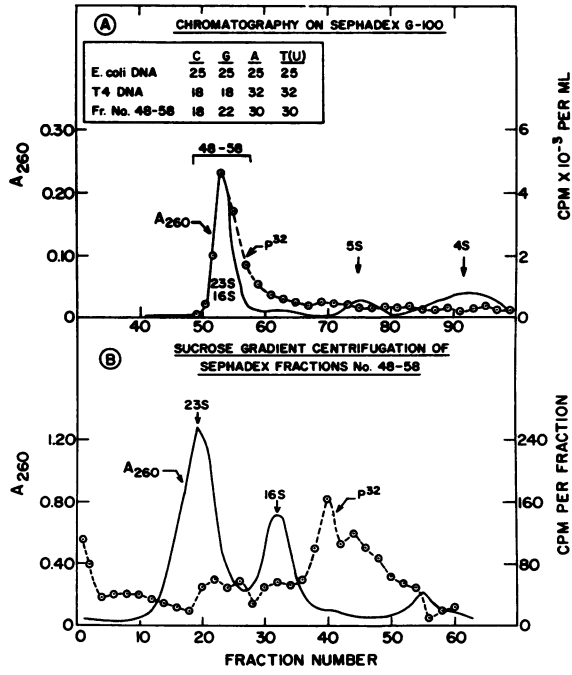


FIG. 3.—Identification of T4 mRNA by chromatography on Sephadex G-100.

Labeled RNA was isolated from the ribosomes of T4-infected cells as described under *Methods*. (*A*) Chromatographic distribution of the P^{32}-RNA extract on Sephadex G-100. The table insert gives the base composition analysis (moles %) of the combined fractions 48–58 as determined by examining the P^{32}-nucleotide content after NaOH hydrolysis and nucleotide separation by paper electrophoresis.[8] (*B*) Sucrose gradient (5–20%) centrifugation pattern of Sephadex fractions 48–58. Centrifugation was at 25,000 rpm, for 19 hr at 4°.

(H^3)leucyl-tRNA on MAK showed that "uninfected" leucyl-tRNA gave two radioactive peaks, the first of which is larger than the second; T4 leucyl-tRNA showed three distinct peaks, of which peaks 1 and 2 were now approximately equal in size while peak 3 occurred in a position similar to that in which infected peak 3 S^{35}-tRNA is usually found (Fig. 6). To test the possibility that the peak 3 leucyl-tRNA might be T4-specific, hybridization experiments with (H^3)leucyl-tRNA were carried out. When T4 (H^3)Leu_3-tRNA (peak *3*, Fig. 6*B*) was annealed with nitrocellulose-bound T4 DNA, a significant amount of T1 RNase-resistant counts was fixed to the filters, whereas annealing with calf thymus DNA gave only low levels of isotope binding (Fig. 7*A*). Over 90 per cent of the hybridized counts was released from the filters by treatment with 1 *M* Tris (pH 9) for 45 minutes at 33°. Under these conditions hybrids of S^{35}-tRNA and DNA are not dissociated. Similar annealings with T4 (H^3)Leu_1-tRNA (peak *1*, Fig. 6*B*)

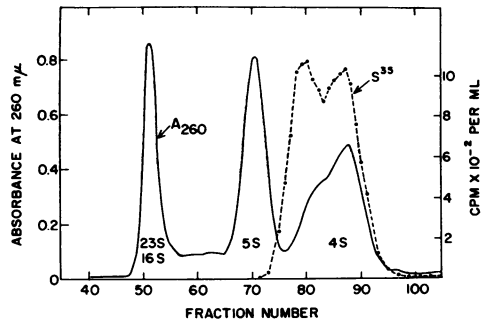


FIG. 4.—Chromatography of T4 S^{35}-tRNA on Sephadex G-100. T4-infected *E. coli* were pulsed with S^{35}-H_2SO_4 for 5 or 15 min as described under *Methods*. Samples of the total RNA extract isolated from these pulse-labeled cells were chromatographed on Sephadex G-100 (3 × 90-cm column) with carrier ribosomal RNA (23*S*, 16*S*, and 5*S*) and 4*S* RNA added; 3 ml fractions were collected. The optical density at 260 mμ and S^{35}-RNA profiles are shown above. The same chromatographic profiles were observed for the short and longer pulse-times.

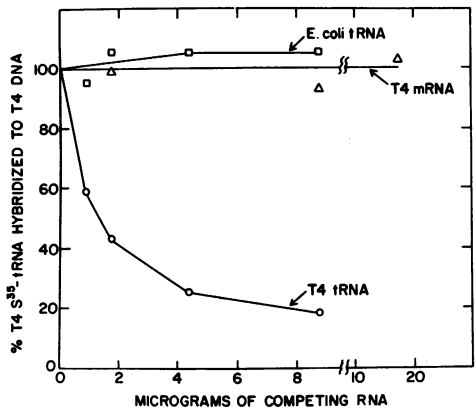


FIG. 5.—Competition of various RNA's with T4 "peak 3" S^{35}-tRNA for hybridization with T4 DNA.

The hybridization conditions were as described for *Procedure* (*A*) under *Methods*. Various RNA's (shown above) were added to annealing mixtures containing 0.19 μg of T4 "peak 3" S^{35}-tRNA (41,000 cpm per μg) and 54 μg of denatured T4 DNA immobilized on filters. The T4 mRNA used was prepared from T4-infected ribosomes and separated from low-molecular-weight RNA by Sephadex G-100 chromatography as in Fig. 3. This preparation competed effectively with T4 P^{32}-mRNA for hybridization with T4 DNA. The S^{35}-RNA bound to the filters after hybridization in the absence of any competing RNA (100%) was 1200 cpm.

showed essentially little or no hybridization with T4 DNA. The above hybridization appears to be T4-specific, because only T4 tRNA (crude or MAK peak 3 fraction) competes with T4 (H^3)Leu_3-tRNA for hybridization sites on T4 DNA; *E. coli* tRNA is not a competitor (Fig. 7*B*). As expected, the peak 3 T4 tRNA is three to four times more effective as a competitor than the crude T4 tRNA itself. The formation of a hybrid of aminoacyl-tRNA and DNA may be shown in another way without the use of filters.

When denatured T4 DNA (in solution) is annealed with T4 (H^3)Leu_1-tRNA in 50 per cent formamide-2 × SSC and then chromatographed on Sephadex G-100, there is no radioactivity associated with the excluded DNA in the void volume which precedes leucyl-tRNA, uncharged tRNA, and free leucine (Fig. 8*A*). On

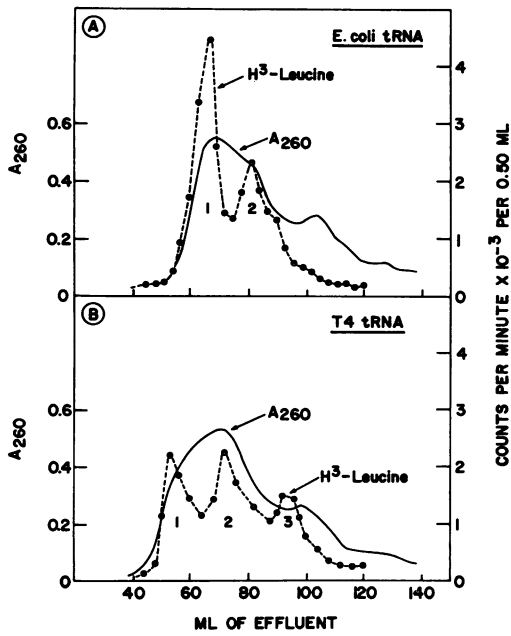


FIG. 6.—MAK chromatography of *E. coli* and T4 tRNA charged with (H^3)leucine.

E. coli and T4 tRNA (500 μg each) were charged with (H^3)leucine (2 c/mmole) as described under *Methods*. The reaction mixture was phenol treated three times, the RNA precipitated with ethanol, and then dialyzed exhaustively against distilled water. The labeled RNA was applied to 30-ml MAK columns (with 1.5 mg of *E. coli* tRNA added as carrier) and chromatographed with a linear salt gradient as previously described.[6] The chromatographic profiles of 260 $m\mu$ absorbing material and radioactivity are shown above.

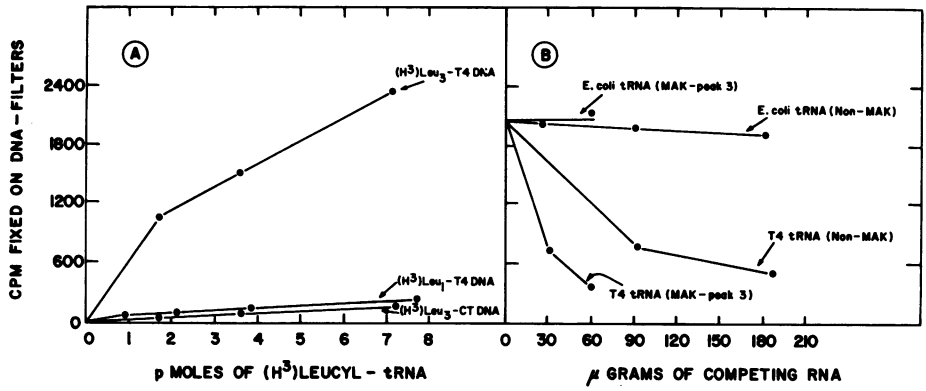


FIG. 7.—Hybridization and competition experiments with T4 (H^3)leucyl-tRNA.

Uncharged T4 tRNA was chromatographed on MAK and the fractions corresponding to the Leu_1 and Leu_3 tRNA regions (Fig. 6*B*) were combined separately, concentrated, dialyzed, and then charged with (H^3)leucine (22×10^6 cpm per mμmole). The isolated (H^3)leucyl-tRNA was used in the hybridization mixture described below; hybridization *Procedure* (*B*) (with formamide) was followed as described under *Methods*. For (*A*), the annealing mixture contained 64 or 70 μg of denatured T4 and calf thymus (*CT*) DNA, respectively, and varying amounts of T4 (H^3)Leu_1-tRNA or T4 (H^3)Leu_3-tRNA, as shown above. The amounts of (H^3)Leu-tRNA plotted were calculated from the radioactivity which remained acid-precipitable at the end of the annealing reaction. For (*B*), the annealing mixture contained 64 μg of denatured T4 DNA, 8 pmoles of T4 (H^3)Leu_3-tRNA (176,000 cpm), and varying quantities of competing RNA's, as shown above. The tRNA's designated as *non-MAK* represent the crude RNA extract which was soluble in 1 *M* NaCl in the cold. The tRNA's designated as *MAK-peak 3* correspond to MAK region 3 shown in Fig. 1.

the other hand, the chromatography of annealing reactions containing T4 (H^3)-Leu_3-tRNA and T4 DNA show a coincidence of labeled leucine with the excluded DNA from Sephadex G-100 (Fig. 8*B*). Over 90 per cent of the radioactivity applied to the column was recovered in an acid-precipitable form.

Discussion.—The hybridization experiments described in this report indicate that part of the S^{35}-tRNA formed after T4 infection is homologous to T4 DNA and must have been transcribed therefrom. Other experiments suggest that T4 S^{35}-tRNA is different from T4 mRNA since S^{35} pulse-labeling of infected cells shows no radioactivity in fractions containing T4 mRNA, and because T4 mRNA does not compete with T4 S^{35}-tRNA for hybridization with T4 DNA. Shubak-Sharpe and collaborators[4, 5] have suggested that the herpes virus genome specifies information for certain tRNA's; our hybridization data with S^{35}-tRNA suggested a similar possibility for T4 phage.

The hybridization test can be applied to tRNA's charged with radioactive amino acids if hybridization temperatures are lowered. This can be accomplished with solvents such as formamide[12] but requires a pH adjustment to below 7 in the annealing mixture to avoid excessive hydrolysis of aminoacyl-tRNA. Approximately 90 per cent of the aminoacyl-tRNA remains intact after three hours of incubation at 33° in 50 per cent formamide containing $2 \times$ SSC, at pH 6.3. Since the time of annealing is kept short to minimize aminoacyl-tRNA degradation, the hybridization reaction is less than 50 per cent of saturation as compared with S^{35}-tRNA annealing to T4 DNA. Using these conditions, we have observed

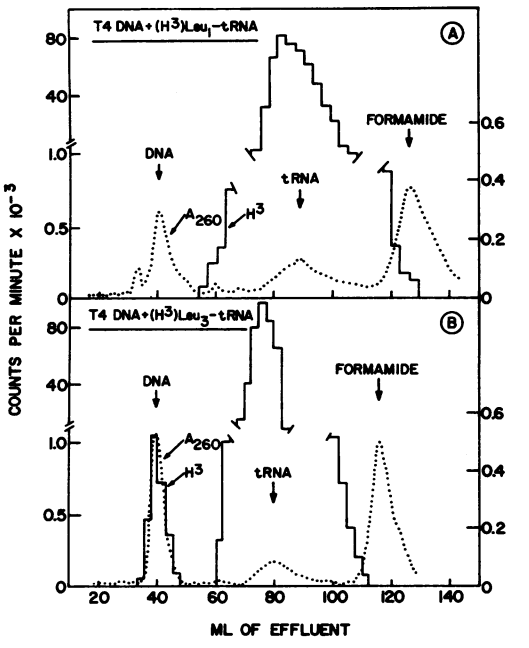


FIG. 8.—Sephadex G-100 chromatography of (H^3)leucyl-tRNA annealing mixtures.

The conditions for hybridization were as described for *Procedure* (*C*) under *Methods*. The reaction mixtures contained 190 μg of denatured T4 DNA, 480,000 counts of T4 (H^3)Leu_1-tRNA in (*A*), and 450,000 counts of T4 (H^3)Leu_3-tRNA in (*B*). The (H^3)leucine had an activity of 22×10^6 cpm/mμmole. After annealing at 33° for 3 hr, the mixture was added directly to a 1.5×70-cm column of Sephadex G-100 equilibrated at 4° with 0.05 *M* ammonium acetate, pH 5.4. Elution was carried out with the same solvent, and the fractions collected (0.8–1.0 ml) were examined for 260 mμ absorbing material and acid-precipitable radioactivity. The small 260-mμ peak just preceding the main DNA material in (*A*) is an anomaly of sample application to the column.

that T4 (H^3)Leu_3-tRNA hybridizes to T4 DNA whereas T4 (H^3)Leu_1-tRNA does not. The radioactivity fixed to the T4 DNA-filters with (H^3)Leu_3-tRNA appears to be specific in that T4 tRNA, and not *E. coli* tRNA, is a competitor for these hybridizations. Chromatography on Sephadex G-100 of similar annealing reactions, but without nitrocellulose filters, also provides evidence for hybrid formation between T4 (H^3)Leu_3-tRNA and T4 DNA. These experiments suggest that the T4 genome codes for at least one specific tRNA, leucine; hybridization of (H^3)prolyl-tRNA to T4 DNA has also been observed. Although our sulfur-labeling studies have directed us to search for T4-specific tRNA's, we cannot be absolutely sure that the viral leucyl-tRNA or prolyl-tRNA actually contain sulfur. Furthermore, our results do not exclude the possibility that viral infection also induces "modification" of host tRNA's.[1–3]

The (H^3)aminoacyl-tRNA hybridization and competition techniques offer a new tool for determining tRNA homology in developing systems even though small alterations in tRNA molecules go undetected. The use of aminoacyl-labeling, especially with unpurified tRNA preparations, provides specificity for the tRNA-DNA hybrid which might not obtain if the label were in the nucleic acid itself. Other problems should now be open to resolution by this technique, e.g., the homology and origin of mitochondrial tRNA's.

Summary.—A T4-specific S^{35}-tRNA unrelated to viral mRNA has been identified in T4-infected cells. Hybridization analysis with tRNA's charged with (H^3)-amino acids indicates the existence of T4 leucyl-and prolyl-tRNA's. Methods for annealing (H^3)aminoacyl-tRNA's are described.

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