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Isolation and Characterization of Serine Transfer Ribonucleic Acids from Rat Liver*

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ABSTRACT: Partition chromatography yields two peaks of rat liver serine tRNA. One of them is 70–80% pure, as judged from its serine acceptor activity. However, coding experiments and sequence studies show that this material is still heterogeneous. By additional partition chromatography and reversed-phase chromatography, four chemically different serine tRNAs could be purified and characterized. In coding experiments species I responds to UCU, UCC, and UCA, species IIa to UCG, and species III to AGU and AGC. Species IIb apparently responds only to UCU and UCA.

Serine is one of the three amino acids coded for by six different triplets. Whereas the six codons of the other two amino acids, arginine and leucine, have at least one common base, in the case of serine no single base is common to all six codons. By carrying out ribosomal binding experiments with trinucleotides Caskey *et al.* (1968) have obtained evidence suggesting that there are three species of guinea pig liver serine tRNA. One species was bound to ribosomes in the presence of UCU, UCC, and UCA, the second in the presence of UCG, and the third in the presence of AGU and AGC. However, there is chemical and genetic evidence indicating the existence of a great many tRNAs with identical coding properties but different primary sequences (*e.g.*, Zachau *et al.*, 1966, and Goodman *et al.*, 1968). On the other hand, only one seryl-tRNA synthetase has been found in rat liver (Rouge, 1969). It therefore seemed of interest to isolate the various species of serine tRNA present in rat liver in order to study their structural differences. This paper describes the separation of several species of rat liver serine tRNA as well as their coding responses to different trinucleotides.

Methods and Materials

tRNA was prepared as described by Rogg *et al.* (1969) from the livers of Sprague–Dawley rats of both sexes. Partition chromatography was carried out according to Wehrli and Staehelin's modification (1971) of the method devised by Muench and Berg (1966). Several grams of rat liver tRNA were fractionated on columns (12 × 60 cm) at 20°. Fractions from these large columns were rechromatographed on columns measuring 2.5 × 90 cm; in this instance a temperature gradient was used. The tRNA was recovered from pooled fractions and the serine acceptor activity determined as described by Wehrli and Staehelin (1971).

Reversed-phase chromatography was carried out according to Weiss and Kelmers (1967) (system 2) at pH 4.5 in the absence of $MgCl_2$, and Weiss *et al.* (1968) (system 4). When tRNA was to be aminoacylated before rechromatography, the fractions from the partition chromatography were desalted on small (1 × 1 cm) DEAE-cellulose columns, which were washed with 0.1 M potassium acetate (pH 5) and eluted with 1 M potassium acetate. The tRNA was precipitated with 2 volumes of ethanol and aminoacylated as described by Wehrli and Staehelin (1971), 1.0 ml of the assay mixture being used for the aminoacylation of 100 A_{260} units of tRNA. The specific activity of labeled serine varied as indicated in the figures. The aminoacylation mixture was extracted with phenol, and the tRNA was precipitated with 2 volumes of ethanol and dissolved in 5–10 ml of the starting elution buffer. tRNA fractions that were not aminoacylated were dialyzed overnight at 4° against the starting elution buffer. For analytical purposes, aliquots of each fraction were precipitated with HCl, with 0.25 mg of carrier tRNA added to facilitate precipitation, and counted as described previously (Wehrli and Staehelin, 1971). Fractions to be recovered were diluted 10-fold with water and absorbed onto small (1 × 1 cm) DEAE-cellulose columns. These were washed with an equal volume of 0.2 M potassium acetate (pH 5.0) and eluted with 3 ml of 1 M potassium acetate (pH 5.0). tRNA was precipitated with 2 volumes of ethanol.

Methylated albumin kieselguhr columns (3.0 × 40 cm) were prepared in three layers as described by Mandell and

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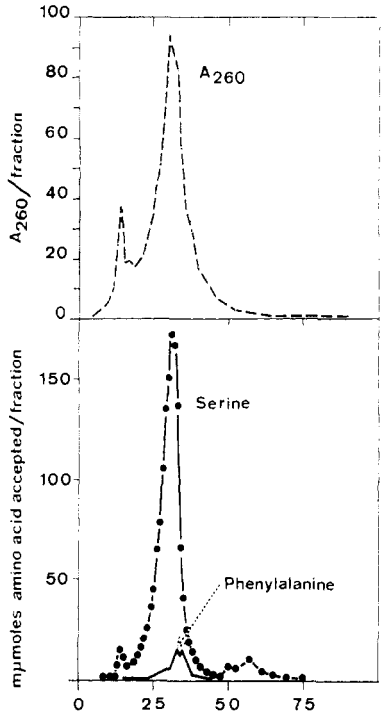

FIGURE 1: Fractionation of 1451 A_{260} units from fractions 88–177 (Figure 1 of Wehrli and Staehelin, 1971) on a partition column (2.5 × 90 cm) as described by Wehrli and Staehelin (1971). A linear temperature gradient was applied running from 30.0° at fraction 1 to 28.2° at fraction 77. Fraction size, 25 ml; 92% of the A_{260} units applied were reisolated and the recovery of serine-accepting activity was 81%.

Hershey (1960). Elution was performed at 0° (by means of a jacket with circulating ice water) in the presence of 0.05 M sodium phosphate (pH 5.8) with a linear gradient from 0.2 to 0.75 M NaCl.

Ribosomal binding was carried out as described by Leder (1968) with *E. coli* ribosomes. The trinucleotides AGU and AGC were prepared by chromatography on DEAE-cellulose of a pancreatic RNase digest of high molecular weight yeast RNA, as described by Staehelin (1963); UCG was prepared from high molecular weight yeast RNA after digestion with ribonuclease T_1 (Aoyagi and Inoue, 1968); UCU, UCC, and UCA were kindly donated by Dr. Marshall Nirenberg, National Institutes of Health, Bethesda, Md. Poly(U,C) (1:1) was purchased from Miles Laboratories, Elkhart, Indiana; [^{3}H]serine and [^{14}C]serine from the Radiochemical Centre, Amersham, England. Purified seryl-tRNA synthetase was prepared as described by Rouge (1969).

Results

The initial fractionation of tRNA was accomplished by partition chromatography. As shown in the preceding paper (Wehrli and Staehelin, 1971), large amounts of crude tRNA can be separated by this method. Since serine tRNA is one of the most lipophilic tRNAs and is therefore eluted very quickly, it can be purified to a very high degree.

The elution pattern of rat liver tRNA on a large partition column is shown in Figure 1 of the preceding paper (Wehrli and Staehelin, 1971). Serine acceptor activity appears mainly in two well-separated peaks. At the very beginning of the elution profile a large peak A (fractions 121–190) consisting of up to 80% serine tRNA is eluted. A serine tRNA shown by sequence analysis to contain less than 10% impurities was used to determine the extent of aminoacylation of a pure tRNA. Within the first 40% of tRNA eluted from the column, a second peak B (fractions 320–440) appears, which is about 10–30% pure in serine tRNA. Peak A contains about 65% of the total amount of serine tRNA, peak B 35%.

Peak A was further purified on a longer partition column eluted with a temperature gradient (Figure 1). A sharp serine acceptor peak appears that is almost homogeneous in terms of serine acceptor activity and corresponds well to the major A_{260} peak.

Although the amino acid acceptor activity indicated that this peak consisted of highly purified serine tRNA, partial digests by specific enzymes showed that it contained a mixture of at least two species (Rogg and Staehelin, 1971). An attempt at further separation was therefore made by reversed-phase chromatography (Weiss and Kelmers, 1967) (system 2).

First the behavior of the two serine peaks from partition chromatography on reversed-phase chromatography was investigated. Samples from peaks A and B were charged with [^{14}C]serine and mixed with total rat liver tRNA charged with [^{3}H]serine. As shown in Figure 2a,b, serine tRNA from peak A corresponds to the second peak on reversed-phase chromatography and that of peak B to the first. Peaks A and B were then examined separately. Figure 3a shows that most of the tRNA from peak A is eluted in a broad peak. The specific activity was tested in several fractions and found to be equally high throughout the peak, although the ultraviolet-absorption profile indicated that the peak was not symmetrical, suggesting considerable heterogeneity. This confirms the earlier notion that peak A consists of a mixture of various serine tRNAs, which is also borne out by coding experiments done with individual fractions. As can be seen from Figure 3a, the serine tRNA from the later fractions is coded for by UCU, UCC, and UCA, whereas that from the initial fractions is mainly coded for by UCG. These two fractions of serine tRNA will hereafter be designated as serine tRNA I and II, respectively.

Peak B was obtained in a less pure form from the partition columns and, judging from the specific activity, contained only 10–30% serine tRNA. Upon reversed-phase chromatography, however, it yielded a fairly symmetrical peak well separated from the other tRNAs (Figure 3b). It showed coding responses to AGU and AGC, but none to any of the UC codons. This fraction will be referred to as serine tRNA III.

To determine whether the various serine tRNA peaks isolated upon reversed-phase chromatography corresponded to defined entities, they were also investigated on a different chromatographic system, *i.e.*, columns of methylated albumin kieselguhr. Species I, II, and III were isolated as described above and aminoacylated with [^{14}C]serine. These were then cochromatographed with total liver tRNA aminoacylated with [^{3}H]serine. The results reported in Figure 4 show that the three species are eluted in different positions on methylated albumin kieselguhr columns and correspond more or less to the three major serine tRNA fractions of total tRNA. But whereas serine tRNAs I and III give rise to a major, fairly symmetrical peak, serine tRNA II still seems heterogeneous, since it is eluted in a very broad and asymmetrical peak.

A further attempt to separate the different species of serine tRNA present in fraction II was therefore made by means of an additional reversed-phase chromatography system (Weiss *et al.*, 1968) (system 4). When serine tRNA II was

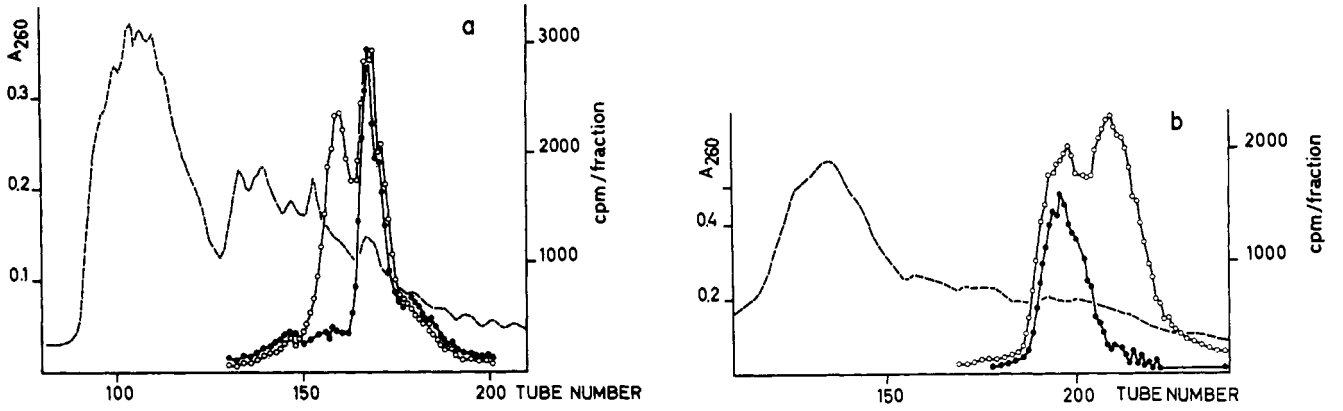

FIGURE 2: Reversed-phase chromatography of total tRNA with tRNA. (a) From peak A. Total rat liver tRNA (300 A_{260} units) was aminoacylated with [^{14}C]serine (5 μCi/μmole); 10 A_{260} units of serine tRNA corresponding to an aliquot of the 6 peak fractions in Figure 1 were aminoacylated with [^{3}H]serine (100 μCi/μmole) as described in Methods. The two samples were extracted with phenol, precipitated with 2 volumes of ethanol, and adsorbed onto a 1 × 250 cm reversed-phase chromatography column (system 2 of Weiss and Kelmers, 1967). Elution was carried out with a linear gradient from 0.2 to 1.0 M NaCl in 0.01 M sodium acetate, pH 4.5 (total volume 3 l.). Fractions of 10 ml were collected at a flow rate of 133 ml/hr at 25°. Aliquots of 3 ml were analyzed as described in Methods. (– – – – –) A_{260}, (O—O—O) counts per minute of ^{14}C, and (●–●–●) counts per minute of ^{3}H. (b) From peak B. Total rat liver tRNA (450 A_{260} units) was aminoacylated with [^{14}C]serine (5 μCi/μmole); 2.5 A_{260} units of serine-tRNA from peak B (fractions 350–420) in Figure 1 of Wehrli and Staehelin (1971) were aminoacylated with [^{3}H]serine (100 μCi/μmole). Chromatography and analysis as in part a. (– – – – –) A_{260}, (O–O–O) counts per minute of ^{14}C, and (●–●–●) counts per minute of ^{3}H.

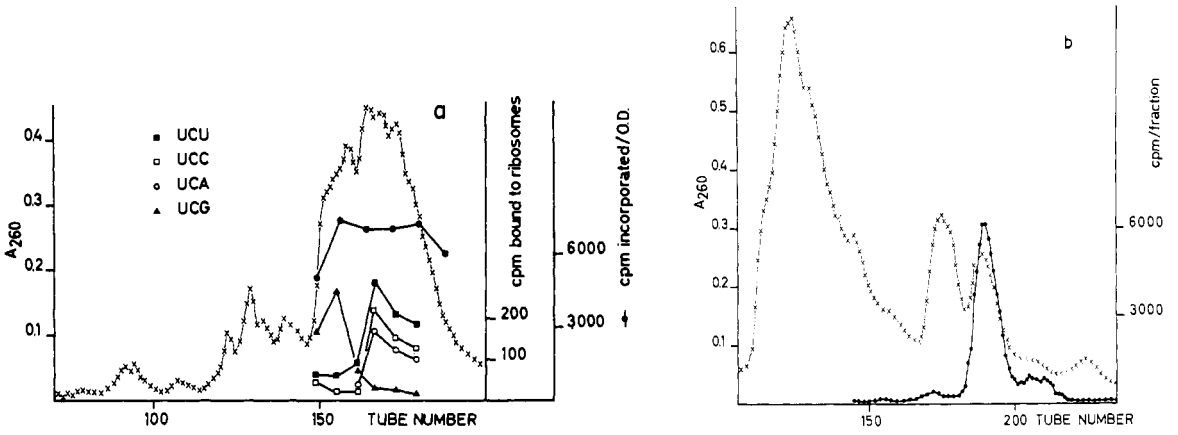

FIGURE 3: Reversed-phase chromatography. (a) Of peak A. 370 A_{260} units corresponding to the six peak fractions of Figure 1 were dissolved in 10 ml of water and dialyzed overnight at 4° against the starting solution. Chromatography was carried out as in Figure 2a. tRNA from individual fractions was isolated and assayed for acceptor activity (●–●–●, cpm/A_{260} unit) and the coding properties were determined as described in Methods. (b) Of peak B. 300 A_{260} units of tRNA from fractions 350-420 of peak B (Figure 1 of Wehrli and Staehelin, 1971) were aminoacylated with [^{14}C]serine (50 μCi/μmole) and chromatographed as described in Figure 2a. (×–×–×) A_{260} and (●–●–●) counts per minute of ^{14}C.

treated in this way, two well separated peaks, IIa and IIb, were observed (Figure 5). Both had very high serine acceptor activity, but they differed in their coding properties, fraction IIa coding for UCG and poly(U,C), and fraction IIb for poly(U,C), UCU, and UCA, but less well for UCG and not at all for UCC. To ascertain whether tRNA IIb was different from serine tRNA I, the two species were cochromatographed. An aliquot of a mixture of fraction IIa and IIb was aminoacylated with [^{3}H]serine, and a tRNA fraction from the initial part of peak A (fractions 121–140, Figure 1 of Wehrli and Staehelin, 1971), containing mainly species I and in lesser amounts IIa, was aminoacylated with [^{14}C]-serine. The elution profile of a reversed-phase chromatography column, in which these tRNAs were cochromatographed, is shown in Figure 6. It is apparent that both the ^{3}H- and ^{14}C-labeled serine tRNA IIa are eluted at the same place (fraction 118), but that species IIb (fraction 134) lags behind species I (fraction 133). The final proof that species I and IIb are chemically different is furnished by sequence studies (Staehelin, 1971). The additional serine tRNAs (fractions 140 and 154) are present in very small amounts in total rat liver tRNA but are markedly enriched in this tRNA fraction arising from the ascending part of peak A (fractions 121–140, Figure 1 of Wehrli and Staehelin, 1971). Their nature has not been examined.

The small peak eluted very early (fraction 98) represents an additional serine tRNA designated as species IV. It is found in variable amounts on long, thin partition columns. In the chromatogram shown in Figure 1 it is present only in very small amounts (fractions 50–60), but in many chromatograms it appeared in considerably larger amounts. In contrast to the main serine tRNA from peak A it is eluted later, *i.e.*, after phenylalanine tRNA. This serine tRNA IV was also examined on methylated albumin kieselguhr columns.

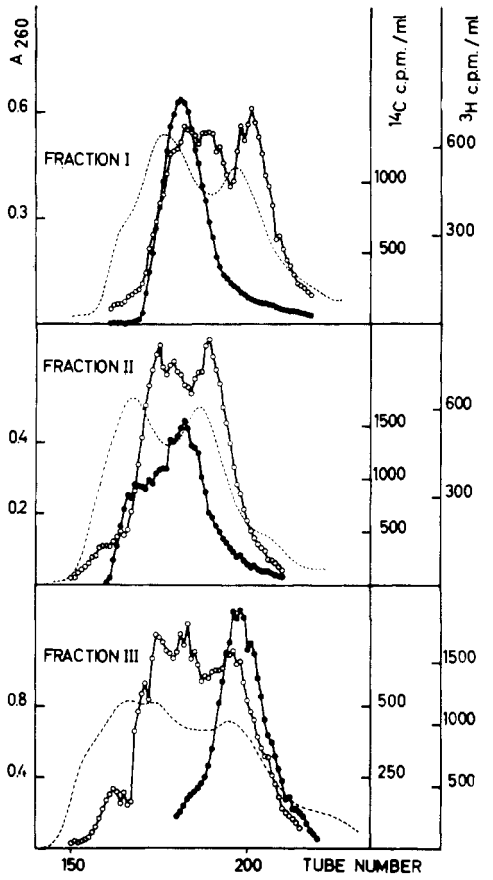

FIGURE 4: Methylated albumin kieselguhr column chromatography of serine tRNA fractions I, II, and III. Total rat liver tRNA was aminoacylated with [^{3}H]serine (100 μCi/μmole) and the isolated fractions I, II, or III with [^{14}C]serine (50 μCi/μmole). Chromatography was carried out as described in Methods. Fractions (5 ml) were collected at a flow rate of 45 mg/hr. Aliquots (3 ml) of each fraction were analyzed as in Figure 2a. (●-●-●) counts per minute of ^{14}C and (○-○-○) counts per minute of ^{3}H. Top: 1 A_{260} unit of serine tRNA (pooled fractions 162–175 from Figure 3a) aminoacylated with [^{14}C]serine and 120 A_{260} units of total tRNA aminoacylated with [^{3}H]serine were mixed with 120 A_{260} units of untreated total tRNA. Middle: 1 A_{260} unit of serine tRNA fraction II (pooled fractions 149–161 from Figure 3a) was aminoacylated with [^{14}C]-serine and combined with total tRNA as above. Bottom: 30 A_{260} units of serine tRNA III (fractions 350–420 of peak B from Figure 1 of Wehrli and Staehelin, 1971) were aminoacylated with [^{14}C]-serine and combined with 300 A_{260} units of total tRNA aminoacylated with [^{3}H]serine.

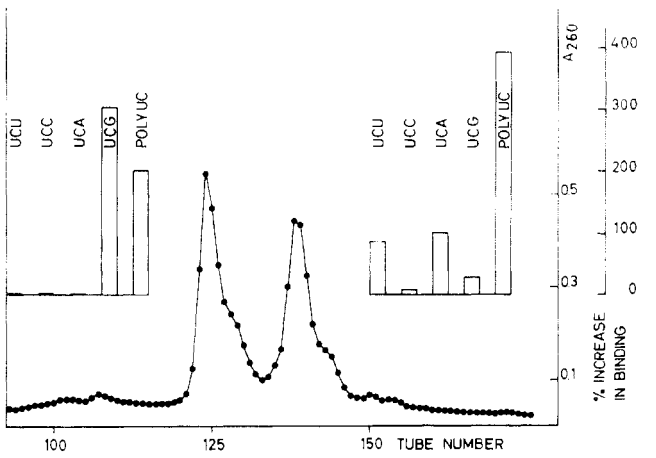

FIGURE 5: Reversed-phase chromatography of serine tRNA fraction II. tRNA corresponding to fractions 150–163 of Figure 3a was pooled from three column chromatograms. 88 A_{260} units were applied to a reversed-phase chromatography column (system 4 of Weiss *et al.*, 1968) and eluted with a linear gradient from 0.45 to 0.75 M NaCl in 0.01 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, and 0.001 M mercaptoethanol. Fractions of 10 ml were collected at a flow rate of 40 ml/hr at 37°. Fractions 122–128 (serine tRNA IIa) and 134–142 (serine tRNA IIb) were pooled, desalted on small DEAE-cellulose columns, and aminoacylated with [^{14}C]serine (50 μCi/μmole). Ribosomal binding was examined with 0.5 A_{260} unit of poly(U, C) and 0.05 A_{260} unit of trinucleotides in 0.02 M $MgCl_2$/total volume, as described by Leder (1968). The left insert represents the coding responses of serine tRNA IIa, the right insert the coding responses of serine tRNA IIb.

It is eluted very early and coincides with a variable shoulder also found in total tRNA (Figure 7). Analysis of the base composition of this tRNA shows that it does not contain N^6-(Δ^2-isopentenyl)adenosine (Rogg and Staehelin, 1971). Results from partial digests with specific enzymes indicate that it might still be heterogeneous.

Thus, a number of rat liver serine tRNAs can be fractionated. The flow sheet depicted in Figure 8 summarizes the fractionation procedures used.

Discussion

The existence of multiple species of isoaccepting tRNAs was recognized a number of years ago. Weisblum *et al.* (1962, 1965) were able to separate five different leucine tRNAs from *E. coli* by countercurrent distribution; some of them showed different but some identical coding properties. Recently, Roy and Söll (1970) have separated five different species of serine tRNA from *E. coli*. The different physicochemical properties of isoaccepting species reported in the literature may be partly due to modifications at the 3′ end. Lebowitz *et al.* (1966) have found that the serine tRNA lacking the terminal adenosine is separated by countercurrent distribution from that containing the complete C-C-A end. Furthermore, Maxwell *et al.* (1968) have observed that on benzoylated DEAE-cellulose aminoacylated tRNAs sometimes have different chromatographic properties from the unesterified tRNAs.

Therefore, in order to decide whether the multiple tRNA species correspond to components with different base sequences or represent artefacts due to aggregation, partial degradation, or aminoacylation, their nucleotide sequences have to be compared.

The main purpose of this work has been to separate the various rat liver serine tRNAs and purify them sufficiently for sequence studies in order to determine how many chemically distinct serine tRNA species exist. The results show that four different species, I, IIa, IIb, and III, could be highly purified and characterized. They differ in their coding properties, and three of them correspond to the species expected on the basis of the wobble hypothesis, *i.e.*, species I is coded for by UCU, UCC, and UCA, IIa by UCG, and III by AGU and AGC, in accordance with the findings of Caskey *et al.* (1968). In addition, species IIb was found to have different coding properties, *i.e.*, it corresponds only to UCU and UCA. If the low degree of response to UCC is not due to an inherent shortcoming of the system (Roy and Söll, 1970), this coding result would indicate a wobble mechanism additional to those known so far (Crick, 1966). Chemical studies show the presence of a modified base at the 5′ side of the anticodon (Staehelin, 1971), demonstrating

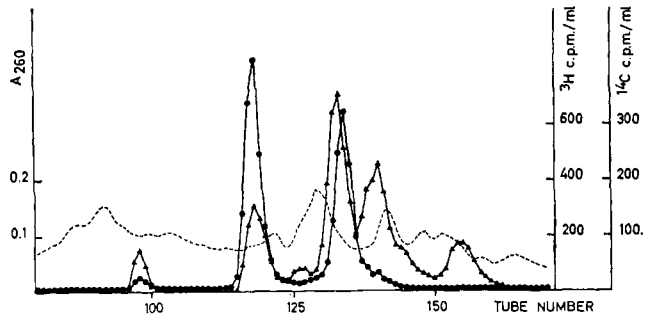

FIGURE 6: Reversed-phase chromatography of serine tRNAs IIa, IIb, and I. 1 A_{260} unit of tRNA from the same pool as in Figure 5 was aminoacylated with [^{3}H]serine (100 μCi/μmole) and cochromatographed with 139 A_{260} units of tRNA corresponding to the first 25% of serine tRNA in peak A of Figure 1 of Wehrli and Staehelin (1971) (fractions 121–140). 3 A_{260} units of this material were aminoacylated with [^{14}C]serine (50 μCi/μmole). Chromatography was carried out as described in Figure 5. The radioactivity peaks do not correspond to the A_{260} peaks because, in contrast to reversed-phase chromatography system 2, in reversed-phase chromatography system 4 aminoacylated tRNAs are eluted about 10 fractions earlier than uncharged tRNAs. (------) A_{260}, (●–●–●) counts per minute of ^{3}H, and (▲–▲–▲) counts per minute of ^{14}C.

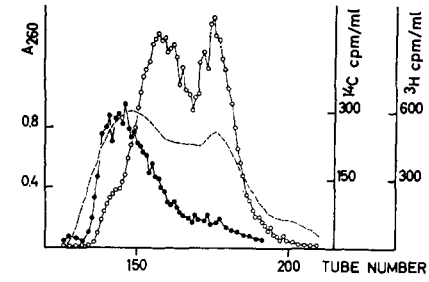

FIGURE 7: Methylated albumin kieselguhr column chromatography of serine tRNA IV. 8 A_{260} units of tRNA corresponding to fractions 50–60 in Figure 1 were aminoacylated with [^{14}C]serine (5 μCi/μmole), and 140 A_{260} units of total tRNA were aminoacylated with [^{3}H]serine (100 μCi/μmole). Column chromatography as in Figure 4. (●–●–●) counts per minute of ^{14}C, (○–○–○) counts per minute of ^{3}H, and (------) A_{260}.

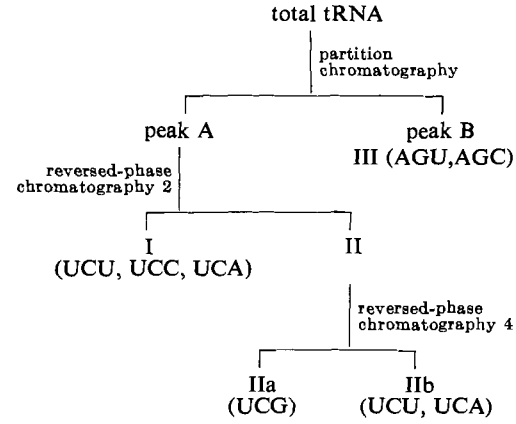

FIGURE 8: Scheme of fractionation of various serine tRNAs in different chromatographic systems. Coding properties of the serine tRNA fractions are indicated in parentheses.

that IIb is a distinct species chemically different from I. Another deviation from the wobble mechanism due to the modified base in the anticodon has recently been found by Yoshida *et al.* (1970) in yeast glutamine tRNA.

Preliminary data on sequence analysis (Staehelin, 1971) of the four species corroborate the fact that they are chemically distinct, since they all differ in the sequence in the anticodon as well as in other parts of the molecule.

One interesting result is presented by the coding responses to poly(U,C) which is expected to provide the codons UCU and UCC only. Caskey *et al.* (1968) have already observed that in their separation of guinea pig liver tRNA the species responding to UCG but not to UCU, UCC, or UCA also responded to poly(U,C). The same responses were found in the present investigation with species IIa.

A fifth serine-acceptor tRNA (species IV) has been found in peak A that is eluted upon repartitioning after the phenylalanine tRNA (Figure 1). In its base composition it very closely resembles species I, but it contains no N^6-(Δ^2-isopentenyl)adenosine. Since it occurs in variable amounts from preparation to preparation, it seems conceivable that it arises from species I by mild oxidation, due to a possible peroxide content of the partition chromatography system (Robins *et al.*, 1967). On the other hand, it could be that the adenosine adjacent to the anticodon has not been completely modified to N^6-(Δ^2-isopentenyl)adenosine, by analogy with the findings of Gefter and Russell (1969). Recently Sharma and Borek (1970) also found a marked increase in a similar mammalian serine tRNA species under conditions of undermethylation. This species was characterized by its very early elution from methylated albumin kieselguhr columns, in which respect it closely resembles species IV.

Some additional minor serine-accepting tRNA species have been identified (Figure 6). Their properties, however, could not be studied, since they account for only a few per cent of total rat liver serine tRNA.

One of the most significant findings appears to be the fact that the various serine tRNAs coding for UCX triplets all have very similar physicochemical properties and can therefore only be separated by a combination of the various chromatographic methods. If this should be true of all tRNAs, it might have certain consequences with regard to the identification of the primary sequence of a specific tRNA. Frequently, tRNA fractions from different preparative processes have to be used to carry out all necessary digests. According to the fractions chosen one or the other species may be enriched in different preparations. The fragments obtained might then not always represent the same tRNA species. The finding that three different serine tRNAs are present in a highly purified serine tRNA obtained by two subsequent chromatographic separations clearly indicates the possible pitfalls in establishing primary sequences.

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Separation of *Escherichia coli* Ribosomal Ribonucleic Acids by Reversed-Phase Chromatography*

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ABSTRACT: Reversed-phase chromatography has been applied to the separation and purification of 16S and 23S rRNAs from *Escherichia coli* on a 50-mg scale. The column consisted of methyltrialkyl(C_8–C_{10})ammonium chloride in 1,1,1,3-tetrachlorotetrafluoropropane or other similar solvents, supported on dimethyldichlorosilane-treated diatomaceous earth, and was developed by sodium chloride gradient elution. The products were characterized and the purity verified by ultracentrifugation, polyacrylamide gradient gel electrophoresis, base composition, and end-group analysis. The 16S RNA had only adenosine as the 3′-terminal nucleoside, and had a molecular weight of about 530,000. RNA preparations from rat spleen and Novikoff hepatoma cells were resolved into several component peaks in similar chromatographic runs. Smaller RNAs elute before rRNAs and can also be resolved.

Three techniques commonly used for separating rRNAs are ultracentrifugation (McConkey, 1967), gel electrophoresis (Dingman and Peacock, 1968; Loening, 1967; Eliceiri and Green, 1970), and chromatography on methylated albumin kieselguhr columns (Murakami, 1967; Osawa and Sibatani, 1967; Mandell and Hershey, 1960). Fractionation of rRNAs as the cetyltrimethylammonium salts has has also been reported (Young, 1968).

We have applied reversed-phase chromatographic systems, which have proven useful for tRNA separations (Kelmers *et al.*, 1965, 1970; Weiss and Kelmers, 1967; Weiss *et al.*, 1968), to the separation of 16S and 23S rRNAs from *Escherichia coli*. The system employs a column material consisting of methyltrialkylammonium chloride extractant dissolved in an organic solvent and supported on an inert support. The RNAs are eluted from the column with a buffered NaCl gradient. Reversed-phase chromatography gives as good or better resolution of a wide range of RNAs as the other techniques, and its scale-up capability has already been demonstrated for the separation of tRNAs (Weeren *et al.*, 1970). This technique provides a simple, inexpensive method for separating a variety of RNAs on both an analytical and preparative scale.

Materials and Methods

Materials. Adogen 464, methyltrialkyl(C_8–C_{10})ammonium chloride, was obtained from Ashland Chemical Co., Columbus, Ohio. The equivalent weight of the material used was 487, determined by titration with $HClO_4$ in the presence of mercuric acetate (Fritz, 1952; Pifer and Wollish, 1952). Trioctylpropylammonium bromide was obtained from Eastman Organic Chemicals. Dimethyldilaurylammonium chloride (Aliquat 204) was obtained from General Mills. 1,1,1,3-Tetrachlorotetrafluoropropane was obtained from DuPont as Freon 214, and also from Peninsular Chemresearch, Gainesville, Fla. Urea (Ultra Pure) was obtained from Mann Research Laboratories. Acid-washed, dimethyldichlorosilane-treated Chromosorb W (100–120 mesh) was obtained from Johns-Manville Products Co. Another support material, a fluorohalocarbon resin, Plaskon CTFE 2300 powder, was obtained from Allied Chemical Co. The powder was sieved through stainless steel screens to obtain an 80–120 mesh material which gave better flow properties.

Polynucleotides and calf thymus DNA were obtained from General Biochemicals. Novikoff hepatoma 4–8S nuclear RNA was kindly provided by Dr. Harris Busch

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