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Specificity of Serine Transfer Ribonucleic Acids in the Synthesis of Hemoglobin*

Fabio Gonano†

ABSTRACT: Partition chromatography on Sephadex G-25 has been used to separate *Escherichia coli* transfer ribonucleic acids (tRNAs). Three fractions of serine tRNA have been obtained. A rabbit reticulocyte cell-free system has been used to test whether differences in the specificity of these serine tRNAs could be observed in the synthesis of a natural protein. Incorporation studies using [^{14}C]serine attached to tRNA$^{Ser\ I}$ and tRNA$^{Ser\ II}$ as a source of this amino acid indicated that serine is selectively incorporated into some of the tryptic peptides of hemoglobin. As has been shown in the case of leucine tRNAs, the specificity of transfer of serine into various positions of hemoglobin depends on a particular tRNA. This indicates that in a natural messenger there are different code words for the same amino acid. At least three distinct serine tRNAs are present in *E. coli* and are able to recognize different degenerate code words.

It has been clearly established from the work of Nirenberg (Nirenberg *et al.*, 1965) and Khorana (Söll *et al.*, 1965) that the genetic code is highly degenerate. For the majority of the amino acids there are at least two coding triplets that differ in the third base (or in some cases the first).

Evidence has been presented by Bernfield and Nirenberg (1965) that a single species of Phe-tRNA recognizes both UpUpU and UpUpC. The mechanism by which a single tRNA molecule is able to recognize more than one triplet has not been experimentally determined. The "wobble hypothesis" of Crick[1] suggests that certain anticodons can match more than one synonym codon because of a "certain amount of wobble in the base pairing of the third letter."

* From the International Laboratory of Genetics and Biophysics, Naples, Italy. *Received November 17, 1966.* Work was carried out under the Association Euratom-CNR-CNEN Contract No. 012-61-12 BIAI.

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[1] F. H. C. Crick (1965), personal communication to Informational Exchange Group No. 7.

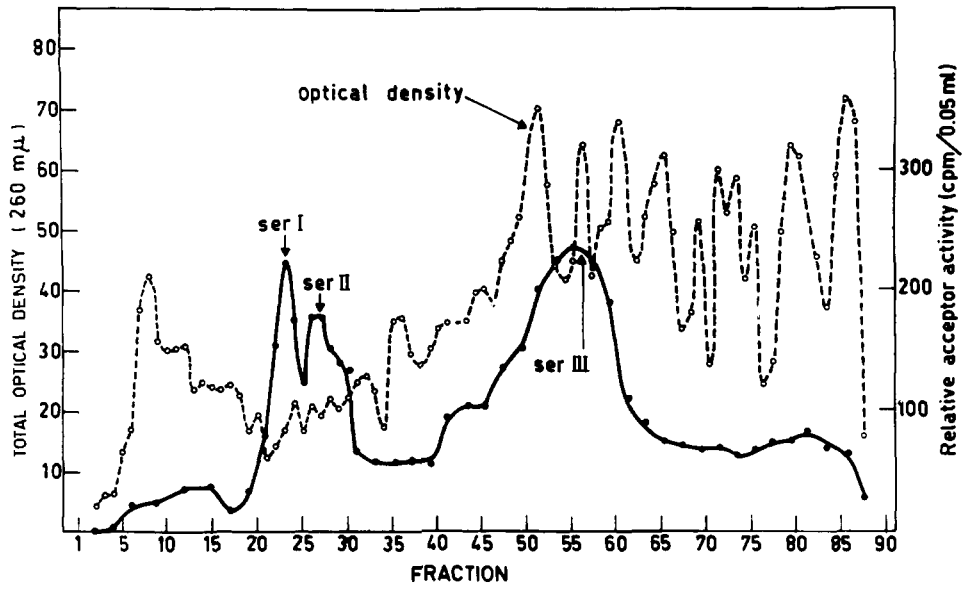

FIGURE 1: Partition chromatography of *E. coli* sRNA on Sephadex G-25 (conditions in Materials and Methods). Each fraction was dissolved in 4 ml and the serine acceptor activity was tested on 0.05-ml aliquots.

The basis for another mechanism by which synonym codons can be recognized, namely heterogeneity within a set of tRNA molecules with the same acceptor activity, was suggested by the work of Weisblum *et al.* (1962), Von Ehrenstein and Dais (1963), and Bennet *et al.* (1965). These authors have shown that multiple peaks of Leu-tRNA isolated by countercurrent distribution possess different coding properties when tested with synthetic polynucleotides. The experiments of Weisblum *et al.* (1965) have shown that two Leu-tRNAs are able to transfer leucine into different positions of the hemoglobin chain synthesized in a cell-free system. In this paper evidence is presented that some Ser-tRNAs are destined to transfer serine into specific positions of a natural polypeptide chain.

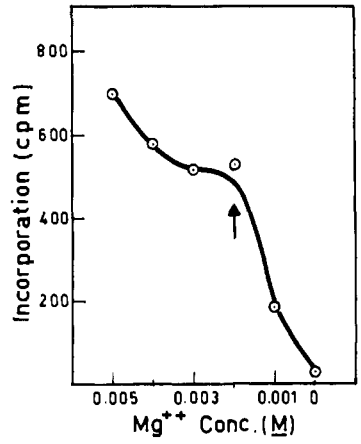

FIGURE 2: Effect of Mg^{2+} concentration on the synthesis of hemoglobin in a cell-free system. [^{14}C]Serine attached to unfractionated sRNA was used (2000 cpm input for each fraction). The incubation mixture (0.5 ml) contained the same proportion of components as described in Materials and Methods except that the Mg^{2+} concentration (molar) was varied as indicated. After 15 min the mixture was precipitated with 10% TCA heated at 90° for 5 min and passed through a Millipore filter, which was glued on a planchet and counted in a gas-flow counter.

Materials and Methods

Partition Chromatography of tRNA. Escherichia coli strain B sRNA was purchased from General Biochemicals (Chagrin Falls, Ohio). Gradient partition chromatography according to Muench and Berg (1966) was used to separate the tRNA species. Sephadex G-25, fine, in bead form (Pharmacia, Uppsala, Sweden), was suspended in the lower phase obtained after equilibration at 23° of the following solvents: six volumes of 1.25 M potassium phosphate buffer (pH 6.8), two volumes of ethoxyethanol, one volume of butoxyethanol, and 0.01 volume of triethylamine. A jacketed column (50 cm^2 × 200 cm) kept at 23° was packed with Sephadex, equilibrated with the lower phase, and washed with the upper phase until no trace of the lower phase remained in the effluent. *E. coli* sRNA (1 g), dissolved in the upper phase, was added to the column and eluted with a linear gradient made with 30 l. of upper phase containing 0.01 volume of triethylamine as a starting buffer and with 30 l. of upper phase containing 0.05 volume of triethylamine as a limiting buffer. Fractions of 450 ml were collected every 30 min. The tRNA was extracted by adding one-fourth volume of butanol; the lower phase was dialyzed three times against a solution of 0.01 M neutral

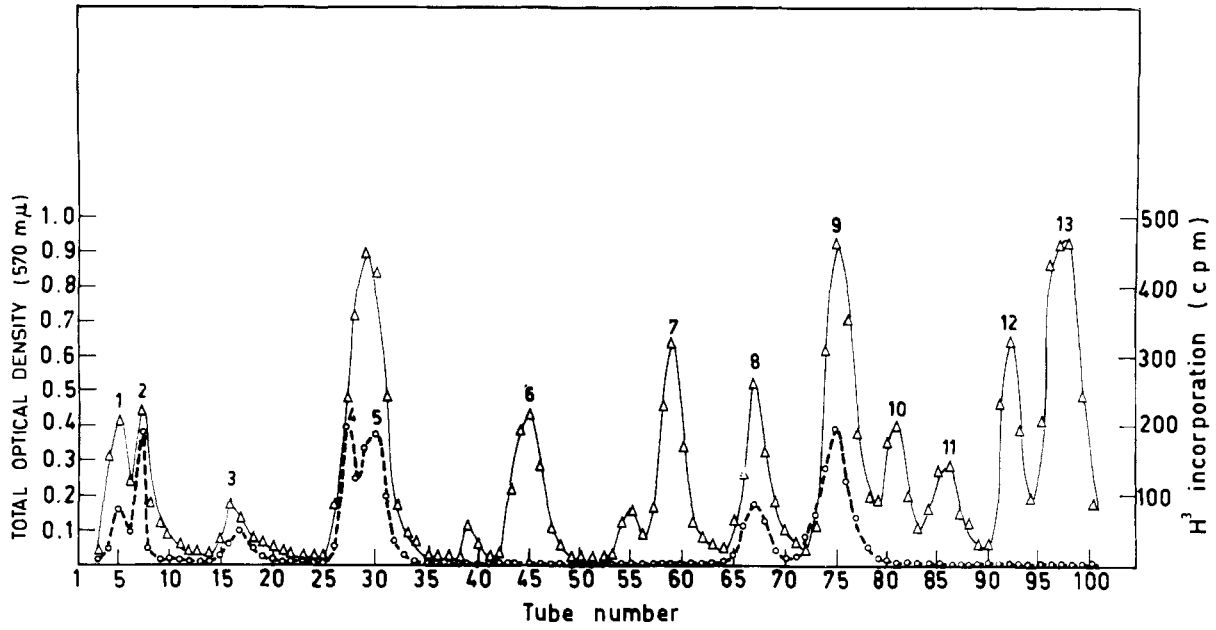

FIGURE 3: Elution profile of peptides obtained from a tryptic digest of an α chain labeled *in vivo* with [^{3}H]serine. Chromatography was carried out on Dowex 50 H^+ columns and 5-ml fractions were collected. Aliquots (0.5 ml) were assayed for ninhidrin. The radioactivity was determined in a Tri-Carb scintillation counter. Fractions (3 ml) were dried in counting vials and redissolved with 0.2 ml of Hyamine, and 15 ml of a standard toluene scintillation mixture was added. Between 70 and 80% of the radioactivity was recovered from the column. The interesting tubes containing the label were pooled (for identification each peak is numbered from 1 to 13) and further fractionated by means of paper electrophoresis. An aliquot of the purified peptides (localized on paper by staining a strip guide with ninhydrin) was counted. The peptides containing the label were hydrolyzed and analyzed for their amino acid composition. The chemical identification of the peptides containing serine is reported in Table I. (Δ—Δ), ninhydrin. (O- -O), [^{3}H]serine.

EDTA, 10% ethanol, and 10% polyethylene glycol 6000 (Carbowax), made 2% with respect to potassium acetate and then precipitated with two volumes of ethanol. Each fraction was finally dissolved in 4 ml of deionized water.

Acceptor activity was determined on 0.05-ml aliquots according to von Ehrenstein and Dais (1963). Uniformly labeled [^{14}C]serine (125 mc/mmole) and DL-[^{3}H]-serine (27 mc/mmole) were obtained from New England Nuclear Corp. The isotopic purity had been checked by autoradiographic paper chromatography which indicated that less than 1% of radioactive impurities were present.

Seryl-tRNA Preparation. The tubes with the maximum acceptor activity were charged with labeled serine using as a source of activating enzymes fraction S-100 prepared free of tRNA (von Ehrenstein and Dais, 1963). A mixture of 19 cold amino acids (minus serine) was added to dilute out trace contaminations of other radioactive amino acids that might have been present in the commercial preparation of labeled serine.

Preparation of Cell-Free System from Rabbit Reticulocytes. Reticulocytes from phenylhydrazine-treated rabbits were lysed as described by Weisblum *et al.* (1965). The stroma-free supernatant fluid was acidified to pH 5 by adding 0.2 M acetic acid as described by Arnstein *et al.* (1965). The precipitate was removed quickly by centrifugation at 5000 rpm for 5 min. It was then resuspended in a standard Tris-HCl buffer (buffer C of Weisblum *et al.*, 1965). A few additional drops of 1.0 M Tris buffer (pH 7.5) were necessary to dissolve the pellet completely. Since the "pH 5" pellet obtained by this method lost activity upon repeated freezing and thawing, it was always used immediately after preparation.

Transfer of Labeled Aminoacyl-tRNA into Hemoglobin. The reaction mixture described by Weisblum *et al.* (1965) was used except that the final volume was 3 ml and contained 6 μmoles of $MgCl_2$, 6 μmoles of [^{12}C]serine, 1 μmole of each of the 19 amino acids (minus serine), "pH 5" pellet 200–300 A_{260} units, and radioactive seryl-tRNAs: [^{14}C]Ser-tRNA$^{Ser\ I}$ containing a total of 34,400 cpm/21 A_{260} units; [^{14}C]Ser-tRNA$^{Ser\ II}$ containing a total of 45,000 cpm/25 A_{260} units; and [^{3}H]Ser-tRNA$^{Ser\ III}$ containing a total of 150,000 cpm/135 A_{260} units. After 15 min at 37° the mixture was acidified to pH 5 and the precipitate, which contained the ribosomes, was removed by centrifugation.

Analysis of the Synthesized Hemoglobin. The supernatant fluid was dialyzed against a solution which was 0.2 M with respect to formic acid and 0.02 M with respect to pyridine (Dintzis, 1961). After 5 hr, the

solution was diluted with 100 ml of the same buffer and applied to a 2 × 30 cm column of carboxymethylcellulose (0.78 mequiv/g, Serva Entwikelungslabor, Heidelberg, Germany). The globin was eluted with a linear gradient according to Dintzis (1961). It has been found unnecessary to split the heme from the hemoglobin to separate the α and β chains under these conditions, because the heme remains tightly bound to the absorbent (L. Felicetti, personal communication). The α and β chains were precipitated with 5% TCA,[2] washed with ethanol–ether, and redissolved in a small volume of water. The α chain was digested with trypsin according to the method described by Baglioni *et al.* (1965). After precipitation of the trypsin-resistant core, the digest was made 1% with respect to formic acid and the mixture was applied to a Dowex 50 H^+ column (Jones, 1964) and eluted by a concave gradient produced with the aid of a nine-chambers Technicon apparatus. Chambers 1–5 each contained 150 ml of pH 3.1 buffer (Jones, 1964), while chambers 6–9 contained 150 ml of pH 5.0 buffer (Jones, 1964). Fractions of 5 ml were collected and aliquots of 3 ml were counted in a Packard Tri-Carb Model 4000 liquid scintillation counter with an efficiency of 30% for ^3H and 55% for ^{14}C with less than 1% of ^3H contamination in the ^{14}C channel. Aliquots of 0.2 ml were assayed with ninhydrin according to Moore and Stein (1964). The fractions corresponding to each serine-containing peak were pooled, taken to dryness, and examined by means of electrophoresis on paper (Weisblum *et al.*, 1965). The purified peptides were analyzed after acid hydrolysis for their amino acid composition and identified with the peptides reported by Diamond and Braunitzer (1962).

Results

Separation of Serine sRNAs. Figure 1 shows the pattern of distribution of serine acceptor activity obtained by partition chromatography (see Materials and Methods). Three peaks of acceptor activity are resolved, two closely associated peaks showing maximum acceptor activity in tubes 23 and 27 and a broad peak with the maximum in tube 55. The last peak also possesses a shoulder at tube 43, indicating a possible fourth peak not well separated. This latter peak will not be considered in this work because of its poor resolution from peak III. Samples taken from the tubes with maximum acceptor activity will be referred to as $tRNA^{Ser\ I}$, $tRNA^{Ser\ II}$, and $tRNA^{Ser\ III}$.

Effect of Mg^{2+} Concentration on Incorporation of Serine. Because of the ambiguities in the translation process encountered with synthetic polynucleotides at high Mg^{2+} concentrations (Szer and Ochoa, 1964; So and Davie, 1965), attempts were made to reduce the Mg^{2+} concentration in these experiments to the lowest level compatible with good incorporation. A concentration of 0.002 M was chosen, since under these conditions the incorporation is still 75% of the value obtained at the standard concentration of 0.005 M, whereas at lower concentrations the incorporation activity drops precipitously (Figure 2).

Incorporation of Serine from Three tRNA Fractions into Specific Peptides of Hemoglobin. In a preliminary experiment, hemoglobin was labeled uniformly with [^3H]serine in intact cells (Dintzis, 1961) and subjected to the analytical procedures described under Materials and Methods. As a reference a typical chromatogram of an α-chain digest is shown in Figure 3. Seven of the thirteen peaks separated by the Dowex 50 column contain serine and have been identified by amino acid analysis (Table I).

TABLE I: Chemical Identification of the Serine-Containing Peptides.

Peak No.[a]	Tryptic Peptide No.[b]
1	Unfractionated material
2	αT 1
3	αT 9
4	αT 4
5	αT 10
8	αT 6
9	αT 8 + 9, αT 6 (ambiguous)[c]

[a] Peak number is referred to the numbers of Figures 3 and 4a,b indicating the peaks containing radioactive serine, eluted from the Dowex 50 column and further analyzed for amino acid composition. [b] According to Diamond and Braunitzer (1962). [c] The amino acid composition of this peptide corresponds to that of the peptide indicated as "^{14}C peptide" by Weisblum *et al.* (1965).

In order to test the specificity of the three serine-tRNA fractions, incorporation experiments were carried out and the serine peptides were analyzed for radioactivity. To ensure identical conditions for comparison, [^3H]Ser-$tRNA^{Ser\ III}$ was used as an internal standard by mixing it with (a) [^{14}C]Ser-$tRNA^{Ser\ I}$ and (b) with [^{14}C]Ser-$tRNA^{Ser\ II}$. Each mixture was incubated and analyzed separately. The results in Figure 4 show that all seven serine-containing peaks are labeled with ^3H derived from Ser-$tRNA^{Ser\ III}$. In contrast only one peak is labeled, when either Ser-$tRNA^{Ser\ I}$ or Ser-$tRNA^{Ser\ II}$ are used as ^{14}C donors: peak 2 in the case of Ser-$tRNA^{Ser\ I}$ and peak 9 in the case of Ser-$tRNA^{Ser\ II}$; in both cases a small amount of label is also associated with peak I which contains mostly undigested material. The small amount of [^{14}C]serine in peak 2 (Figure 4b) can be accounted for by contamination of Ser-$tRNA^{Ser\ I}$ with the Ser-

[2] Abbreviation used: TCA, trichloroacetic acid.

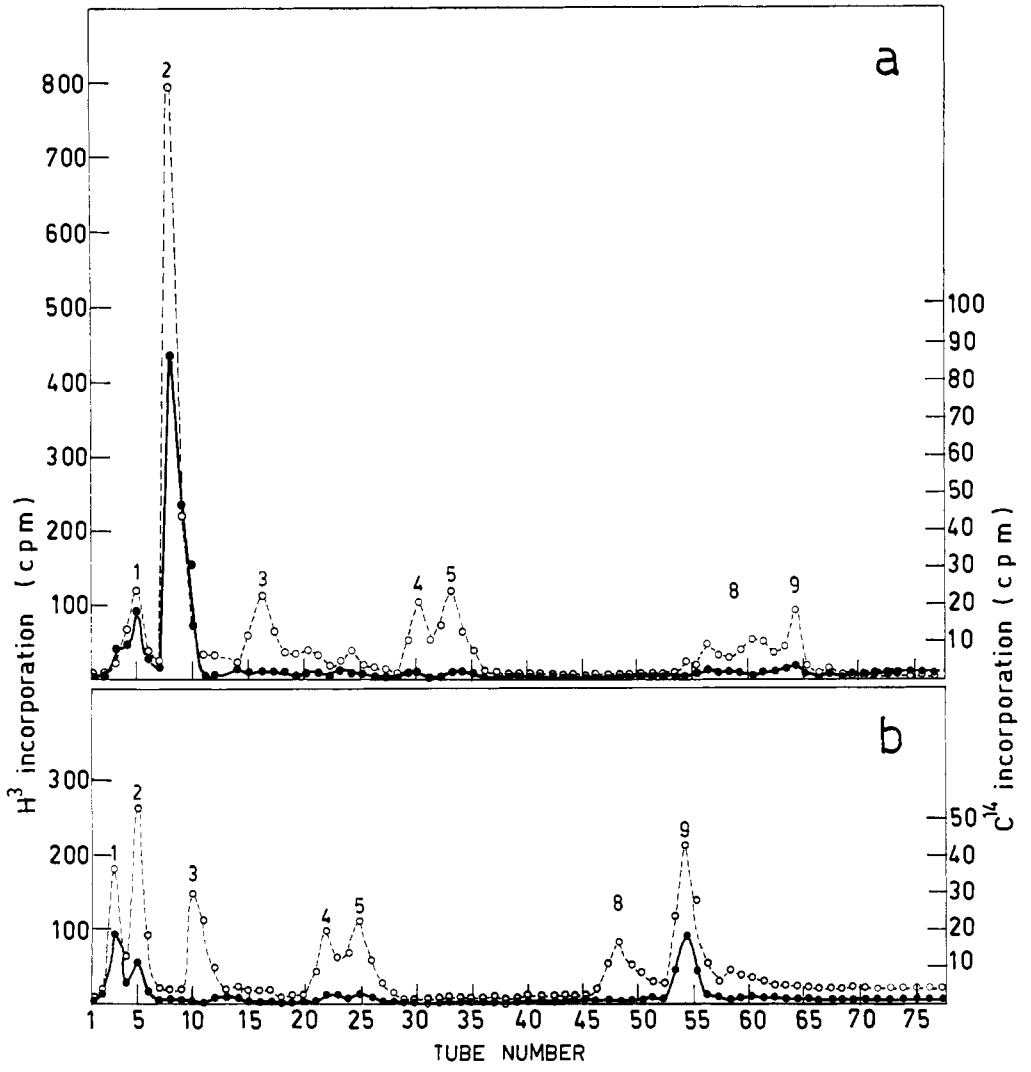

FIGURE 4: Radioactive profile of tryptic digest of hemoglobin α chain on a Dowex 50 column (experimental details given in Materials and Methods). The labeled hemoglobin was obtained incubating [^{14}C]serine attached to tRNA$^{Ser\ I}$ and [^{3}H]serine attached to tRNA$^{Ser\ III}$ in (a) and [^{14}C]serine attached to tRNA$^{Ser\ II}$ and [^{3}H]serine attached to tRNA$^{Ser\ III}$ in (b). Ninhydrin was omitted for clarity and it is similar to that of Figure 3. Fractions of 5 ml were collected and aliquots of 3 ml counted in a scintillation counter. ^{3}H and ^{14}C incorporations in counts per minute are plotted in the figure. Only the serine-containing peaks are numbered according to Figure 3. The chemical identification of the serine-containing peptides is reported in Table I. (O- -O), [^{3}H]serine from peak III, and (●—●), [^{14}C]serine from peak I (a). (O- -O), [^{3}H]serine from peak III; (●—●), [^{14}C]serine from peak II (b).

tRNA$^{Ser\ II}$ since the two tRNAs are not completely separated by the procedure used.

The chemical identification of the peptides containing serine (Table I) has been done in a separate run, since the low amount of radioactivity incorporated in the transfer experiments did not permit further analysis of the radioactive peaks. The peaks of the chromatograms shown in Figure 4a,b have been identified also with the ninhydrin reaction on the same carrier α chain used in another run for the chemical identification of the peptides (Figure 3).

Because of the high reproducibility of the Dowex 50 H^+ column we can therefore compare the peaks obtained in the transfer experiments with those used to identify the peptides (Table I). With this assumption we can deduce which are the serine positions on the α chain served by the two *E. coli* serine tRNAs.

Under peak 2 (Figure 4a), labeled when Ser-tRNA$^{Ser\ I}$ is used as a donor of [^{14}C]serine, there is one peptide only and this is obviously the peptide labeled. This peak when analyzed by high-voltage ionophoresis showed the presence of one peptide only containing serine; the amino acid composition of this peptide was identical with that of αT 1.

There are two peptides under peak 9, labeled when Ser-tRNA$^{Ser\ II}$ is used as a donor of [^{14}C]serine:

αT 8 + 9 and αT 6 "ambiguous" (Table I). αT 8 + 9 is a dipeptide that has the same sequence of peptide αT 9 with an additional lysine in His NH_2 terminal. The complete sequence of this particular region of the rabbit hemoglobin α chain has been elucidated by von Ehrenstein (1966).

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-Gly-Lys-Lys-Val-Ser-Glu-Ala-Leu-Thr-Lys-
 59  60/  61  /62 63  64  65  66  67  68
 αT7      αT8          αT9
```

This sequence can be splitted by trypsin: (a) between residues 60 and 61 and residues 61 and 62. This gives peptide αT 7, peptide αT 8 (free lysine), and peptide αT 9. (b) A split between residues 60 and 61 only. This gives peptide αT 7 and peptide αT 8 + 9 (αT 9 with an additional lysine in the NH_2 terminal). Under peak 9 (Figure 4a,b) has been found peptide αT 8 + 9, resulting from the action of trypsin as described in b. The two peptides resulting from the splitting of trypsin described in a are observed separated in the chromatogram. Peptide αT 8 is free lysine and peptide αT 9 runs under peak 3. It is clear that in the chromatograms shown in Figure 4a,b that peptide αT 9 (peak 3) is not labeled with [^{14}C]serine. Hence, peptide αT 8 + 9 that chromatographs under peak 9 in Figure 4b is not labeled and, therefore, we can conclude that under this peak the only peptide labeled is αT 6 "ambiguous" when Ser-tRNA$^{Ser\ II}$ is used as a donor of [^{14}C]serine.

Discussion

Weisblum *et al.* (1965) have shown that there are two leucine tRNAs in *E. coli*, one (Leu-tRNA$^{Leu\ I}$) that recognizes all the leucine triplets in the messenger for the rabbit hemoglobin α chain; the other (Leu-tRNA$^{Leu\ IIb}$) recognizes only one triplet for leucine in position 48. In the case of the transfer of serine from *E. coli* tRNA, a similar although different situation is observed. Of two serine tRNAs (Ser-tRNA$^{Ser\ I}$ and Ser-tRNA$^{Ser\ II}$) each recognizes a different triplet and a third serine tRNA (Ser-tRNA$^{Ser\ III}$) recognizes all possible triplets for serine of reticulocyte mRNA. Since the complete amino acid sequence of the rabbit hemoglobin α chain has recently been established (von Ehrenstein, 1966), it is possible to pinpoint the positions which are served by the two specific serine-tRNAs: Ser-tRNA$^{Ser\ I}$ transfers serine into position 3 (peptide αT 1) and Ser-tRNA$^{Ser\ II}$ into either position 49 or 52 (peptide αT 6 "ambiguous"). The present data do not permit a decision between positions 49 and 52, since peptide αT 6 "ambiguous" served by Ser-tRNA$^{Ser\ II}$ contains two serine residues (49 and 52). It is interesting to note, however, that position 49 is characterized by an as yet obscure ambiguity, for it has been found that two different amino acids, threonine and serine, are encountered with equal frequency in this position (von Ehrenstein, 1966). Moreover, in the adjacent position 48, a similar ambiguity with respect to phenylalanine and leucine is encountered. The fact that the leucine in this position is inserted by only one particular tRNA might suggest an analogy to the present observation.

Any assignment of the significance of the present findings has to take into account the fact that the tRNA used here is derived from *E. coli* and thus heterologous to the hemoglobin-synthesizing system. Nevertheless, within the framework of present concepts, it must be concluded from these findings that the messenger coding for hemoglobin chain uses different synonym triplets for serine and leucine and, perhaps, for other amino acids as well.

It would be of great interest to identify the triplet(s) recognized by each serine tRNA species. Bennet *et al.* (1965) have shown that of the two serine tRNA fractions which they isolated by countercurrent distribution, one was more responsive to C-rich UC polymers, and the other to U-rich UC polynucleotides. Unfortunately, the serine tRNA species described here cannot be related to the fractions of Bennet *et al.* (1965) since different fractionation methods were employed. Identification of the triplets recognized by serine tRNA fractions reported here must, therefore, await experiments with polynucleotides of the defined sequence.

Acknowledgments

The author wishes to thank Dr. C. Baglioni for his helpful discussion throughout the study and Dr. H. Noll for help in the preparation of the manuscript.

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A New Method for Preparing Ribosomal Ribonucleic Acid*

D. L. Weller and R. S. Morgan

ABSTRACT: The reaction of a soluble sulfonated polystyrene (molecular weight approximately 100,000) with *Escherichia coli* ribosomal subunits has been studied. Concentrations of the reagent in the range 0.1–0.8% lead to essentially complete dissociation of protein from nucleic acid. By varying the concentration of alcohol the ribonucleic acid (RNA) can be almost quantitatively separated from the protein. The nucleic acid isolated by this method is contaminated with 1–5% protein. Yields of the nucleic acid are usually about 90%. Characterization of the nucleic acid prepared from unfractionated subunits by this new method showed 16S (18.4S) and 23S (24.6S) RNA components in the usual mass ratio (*i.e.*, 1:2, slow:fast). From purified 50S subunits a main 23S component was obtained. The approximate molecular weights of these components are estimated to be 0.65 (16 S) and 1.1 $\times$ 10^6 (23 S).

Most methods used today for preparation of ribosomal RNA make use of phenol or a combination of sodium lauryl sulfate and phenol (Petermann, 1964). In this paper we report a new and simple method for the isolation of high molecular weight ribosomal RNA that employs a soluble sulfonated polystyrene (molecular weight approximately 100,000). Earlier studies had shown this polymer was a good RNase inhibitor (Danner, 1964), and that it had the ability to dissociate some of the protein from ribosomes (Morgan, 1966).

Our experiments show that concentrations of the polyanion in the range 0.1–0.8% lead to essentially complete dissociation of protein from *Escherichia coli* ribosomal subunits. The nucleic acid could be separated from the ribosomal protein by a combination of differential centrifugation and sucrose density gradient centrifugation. Yields by this method, however, were very low. The new procedure employs differential precipitation of RNA and of protein. The nucleic acid is quantitatively precipitated from solution by the addition of NaCl (0.01 M) and alcohol (52%). Under these conditions essentially all the protein remains soluble. RNA isolated by this method has chemical and physical properties similar to phenol-prepared nucleic acid.

Methods and Materials

The ribosomes used in these experiments were prepared from early log phase *E. coli B* (Grain Processing, Muscatine, Iowa) either by a method employing bentonite (D. L. Weller, manuscript in preparation) or by the $(NH_4)_2SO_4$ method (Elson, 1958; Kurland, 1966). Purified 50S ribosomes were isolated by differential centrifugation of mixtures of ribosomal subunits. The sulfonated polystyrene used was obtained as a free sample from Hagen Chemicals, Pittsburgh, Pa. This was the same batch employed in the previous study (Morgan, 1966). A sulfonated polystyrene (ET-409) of higher molecular weight can now be obtained from Dow Chemical, Midland, Mich. We have not yet investigated the properties of this compound.

Sedimentation analyses were done with a Spinco Model E ultracentrifuge at room temperature. The sedimentation coefficients were corrected to the viscosity and density of water at 20°.

The viscosity of solutions was measured with a constant-volume Ubbelohde viscometer (Cannon Instruments, State College, Pa.) with a solvent flow time of 305 sec at 20°. Flow-time measurements were made at 20°. Dust and large aggregates were removed from solutions, before the measurement of flow time, either by filtering through an HA Millipore filter (Millipore Filter Corp., Bedford, Mass.) or by centrifugation.

Absorption measurements were made either with a Beckman Model DB or Cary 15 recording spectrophotometer. Light path for all measurements was 1 cm.

Concentrations of ribosomes and RNA were estimated from A_{260} using an $E_{1\text{ cm}}^{0.1\%}$ of 16 and 23, respectively. Alternatively RNA concentrations were estimated by the orcinol method (Mejbaum, 1939) with yeast RNA (Schwartz BioResearch, Inc., Orangeburg, N. Y.) as a standard.

The protein content of the RNA was determined by direct analysis of the nucleic acid samples (Rama-

* From the Children's Cancer Research Foundation, Boston, Massachusetts. *Received October 26, 1966.* This work was supported by Grant GB-4238 from the National Science Foundation.