ULTRA-RAPID QUANTITATIVE ISOLATION OF SPECIFIC TRANSFER RIBONUCLEIC ACIDS A SOLID-PHASE METHOD

D.J. Goss[1] and L.J. Parkhurst
Department of Chemistry
University of Nebraska
Lincoln, Nebraska 68508

Received April 24,1974

Summary. The following procedure is used in a rapid isolation of a specific (isoacceptor) tRNA from a mixture of deacylated tRNAs: (1) Acylation with the amino acid corresponding to the desired specific tRNA (2) Reaction of the specifically acylated tRNA with a new bi-functional reagent (PAMBSYL) (3) Binding of the pambsylated tRNA to a sulfhydryl-containing resin (4) Release of the specific tRNA from the resin after removal of the non-reacted tRNA. Steps 3 and 4 can be carried out in 20 min. One ml of the resin can bind approximately 200 mg of reacted tRNA.

In the course of preparing transfer RNA for rapid-reaction kinetic studies in our laboratory, it occurred to us that it should be possible to prepare specific tRNAs using solid-phase synthetic methods. The basic idea was to subject the crude mixture of tRNAs to aminoacylation using a single amino acid to select the corresponding specific tRNA, couple that specific aminoacyl-tRNA to a solid-phase substrate with a bi-functional reagent (thus separating it from the nonaminoacylated tRNAs) and then, after separation, simply raise the pH so that deacylation would result in the release of the desired specific tRNA from the substrate.

A search of the literature revealed that reactions at the amino acid site in aminoacyl-tRNAs had been used in spin-label studies (1) and in separation methods (2-4). Zamecnik, et al. (2) used periodate oxidation of the nonaminoacylated tRNA and reaction with a hydrazine derivative to remove it from the aminoacylated tRNA. Brown et al. (3) exploited the reactivity of the aminoacyl group of tRNA to give an insoluble derivative. Gillam et al. (4) have used substitution at the aminoacyl group to alter the pattern of

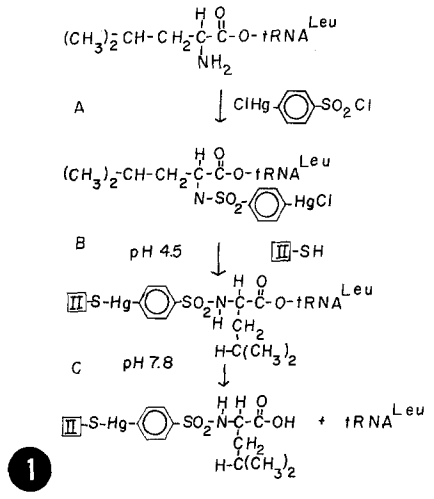
[1]Taken in part from a thesis to be presented to the University of Nebraska in partial fulfillment of the requirements for the Ph.D. degree.

elution from BD-cellulose. Other commonly used separation techniques include: counter-current distribution (5-6), chromatography on DEAE cellulose (7) and benzoylated DEAE (BD) cellulose (8), reverse phase chromatography (9), and chromatography on methylated serum albumin on kieselguhr (MAK) columns (10). The scheme used in this study is depicted in figure 1.

Materials. tRNA from E. coli, strain B, was prepared as described by Goldstein (11). Amino acids were of the highest purity commercially available (leucine and tyrosine were products of Mann Research Laboratories, arginine was a product of General Biochemicals). Aminoacyl-tRNA synthetases were prepared as described by Muench (12). [^{14}C] Leucine was from International Chemical Nuclear. [^{14}C] Arginine and tyrosine were from New England Nuclear. Sepharose 4B was purchased from Pharmacia. N-acetyl-homocysteinethiolactone and p-chloro-mercuribenzene sulfonic acid (PMBS) were purchased from Sigma. All other chemicals were reagent grade.

Preparation of Bi-Functional Reagent, p-chloro-mercuribenzenesulfonyl chloride (PAMBSYL). PCl_5 (0.6 gm) and 0.2 gm of PMBS were added to 4 ml of $POCl_3$. The mixture was refluxed at 110°-120° for 2 hours. The product was poured over ice and the resulting white precipitate was collected by filtration, dried and stored in a dessicator, (yield 0.103 gm, 52%). The product was insoluble in water and gave the expected colorimetric reaction with beta-mercaptoethanol (13). Mass spectroscopic analysis resulted in a pattern from m/e 408 to m/e 416 to be expected for $ClHgC_6H_4SO_2Cl^+$. Fragments containing Hg were readily identified as $C_6H_4HgSO_2Cl^+$, $C_6H_4HgCl^+$, $HgCl_2^+$, $HgCl^+$, and Hg^+. In addition, the most abundant fragments were $C_6H_4SO_2^+$, and $C_6H_4^+$.

Preparation of Solid-Phase Resin. Sepharose 4B was reacted sequentially with ethylene diamine, succinic anhydride and ethylene diamine to form an amine resin (I) and then converted to a sulfhydryl resin (II) by reaction with N-acetyl-homocysteinethiolactone, all according to Cuatrecasas (14) (see figure 2). The resulting resin had a capacity of 10 microequivalents of SH per ml of settled gel.



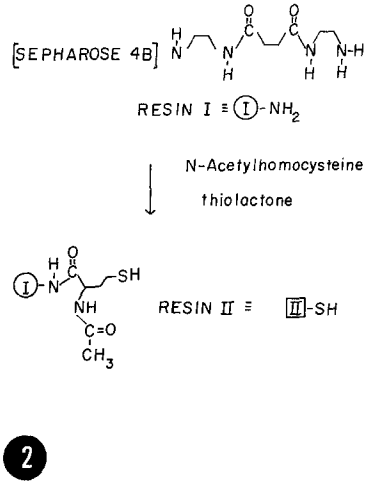


Figure 1. The steps in the isolation of a specific aminoacylated tRNA. (A) Reaction of aminoacylated tRNA (e.g., leu-$tRNA^{Leu}$) with PAMBSYL; (B) Reaction of the aminoacyl-tRNA-PAMBSYL with the sulfhydryl resin (II) at pH 4.5 and elution from the column of nonaminoacylated tRNA; (C) Deacylation of the resin-bound amino acid which liberates the desired specific tRNA, pH 7.8.

Figure 2. Preparation of sulfhydryl-resin (II) from amino-resin (I).

Aminoacylation of tRNA. Aminoacylation of tRNA was carried out in 0.5 ml of a reaction mixture 100 mM in tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM ATP, and containing 100 A_{260} units of unfractionated tRNA, 5 mg of leucine (or the desired amino acid) and 0.1 ml of aminoacyl-synthetase solution (12 mg of protein /ml). The reaction was carried out at 37° for 10 min and was terminated by addition of 0.2 M acetic acid-acetate buffer, pH 4.5, 1 M in NaCl. The tRNA was recovered by precipitation with ethanol after 4 hrs., -20° (final solution, 70% in ethanol). This procedure followed Muench (12).

Reaction of Aminoacyl-tRNA with Bi-Functional Reagent. The ethanol precipitate from the previous step was dissolved in 1 ml of 0.1 M triethanolamine hydrochloride, pH 4.3, and cooled to 0° C. To this solution was added dropwise 0.5 mg of PAMBSYL (with stirring at 0° within 10 seconds) dissolved in 0.2 ml of tetrahydrofuran. The pH of the solution was rapidly adjusted to 8.0 with 2 N NaOH and stirred for 2.5 min. The pH was then adjusted to

4.5 with acetic acid to quench the reaction and inhibit deacylation. Insoluble precipitate was removed by centrifugation. The tRNA was recovered by precipitation from ethanol as before.

Solid-Phase Separation. Into a 5 mm I.D. column (Pasteur disposable pipet) was poured 0.4 ml of the resin (II) above a small glass wool plug. The column was connected to an ISCO UV analyzer (254 nm, model UA-2, ISCO, Lincoln, Nebraska) through 1 meter of 0.066" I.D. polyethylene tubing. A strip-chart recorder (Hewlett-Packard 680) was connected to the analyzer. The column was washed with 0.2 M, pH 4.5 acetate buffer, 1 M in NaCl, until equilibrated. The ethanol precipitate from the previous step containing reacted aminacyl-tRNA was dissolved in 0.2-0.5 ml of the same buffer, pH 4.5, and was applied to the column at room temperature (20^{o}). In 6 min the first large peak was eluted. This contained about 88% of the initial material absorbing at 260 nm and about 5% of the leucine acceptor activity. When the column was washed for a further 10-15 min with acetate buffer, (20-40 ml) no further peaks were eluted. The elution buffer was then changed to 0.1 M tris-HCl, pH 7.85, 1 M in NaCl. In 4 minutes a second broader peak appeared and was completely eluted after a further 9 min. This contained 12% of the initial A_{260} units and about 95% of the initial leucine acceptor activity. When commercial sRNA (General Biochemicals) was used the absorbance of the $tRNA^{Leu}$ fraction was 9.6% of the initial A_{260}. The assay for amino acid acceptor activity was carried out as described by Nishimura et al. (15).

Results and Discussion. The results of a typical separation for $tRNA^{Leu}$ are shown in figure 3. Similar results have been obtained for arginine and tyrosine. The 5% $tRNA^{Leu}$ found in band I undoubtedly derives from that $tRNA^{Leu}$ which was not aminoacylated. As a measure of resolution, band II was analyzed for both alanine and leucine acceptor activity. Less than 0.5% of the acceptor activity of band II could be attributed to $tRNA^{Ala}$. The leucine acceptor activity of band II was 1.98 ± 0.08 nmoles/A_{260}. The purity of the separated specific tRNAs should be limited only by the purity of the

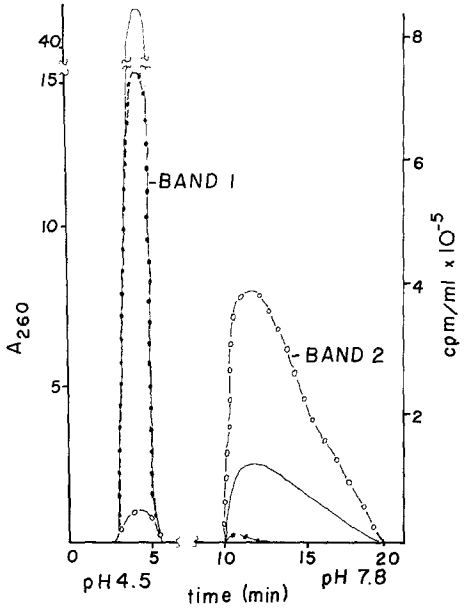


Figure 3. Elution pattern from resin (II). Solid lines (———) depict absorbance at 260 nm, calculated from measurements made on a Beckman DU Spectrophotometer (left vertical axis). The right vertical axis refers to acceptor activity for (^{14}C) labelled alanine (.-.-.-.) and (^{14}C) labelled leucine (o-o-o-o-) for a separation in which the crude tRNA preparation was reacted with leucine according to the scheme in figure 1.

synthetases and the amino acids. In our work, unfractionated synthetases were used which may well have contained small amounts of amino acids other than that added for the specific tRNA desired. The alanine acceptor activity in band II may well have arisen from this source. When the procedure was carried out without synthetase, no second peak was eluted from the solid-phase resin. We cannot exclude the possibility that a small fraction of the tRNA might react with PAMBSYL at sites other than at the $-NH_2$ of the amino acid, but such material should then remain bound to the column. Preliminary experiments have shown that the two precipitations are probably not necessary, since in the leucine system, the elution pattern from the column is unchanged if the second precipitation to remove excess reagent is eliminated, and the first precipitation may be eliminated if one is willing to add sufficient PAMBSYL to remove the synthetases by binding them to the resin. In this

manner, steps (1) and (2) in the summary can be carried out in less than 25 min, and the complete separation, steps (1) through (4), in less than 45 min. This method should be generally applicable to the fractionation of any specific tRNA. In the case of $tRNA^{Cys}$, blocking of the -SH group of cysteine with p-hydroxymercuribenzoate (PMB) should be carried out immediately prior to reaction with PAMBSYL. Some slight modifications in pH may be necessary for those instances where the synthetases are not very active, or the tRNA is easily deacylated. As has been pointed out elsewhere (4) if further purification to separate the different tRNAs specific for the same amino acid is required, such separations are usually easily effected on BD-cellulose. In case pure $tRNA^{fMet}$ is required, it could probably be separated from $tRNA^{Met}$ after formylation, reaction with the reagent PAMBSYL, and elution. The $tRNA^{fMet}$ should then elute in the first peak.

If one were interested in purifying all 20 tRNAs, a simple branching pattern for the separation could be established such that for 10 of the tRNAs, 4 cycles of elution and reaction with PAMBSYL would be required, and for the remaining 10, 5 cylces would be required. In this manner all of the initial crude tRNA would be processed. It is of some interest to consider the cost of such an operation. If crude tRNA is isolated from cells, 4 gms of tRNA cost about $400. All 20 tRNAs could be isolated for an additional $200. The cost of the resin material, starting from Sepharose 4B, is $0.10 for 1 ml, sufficient to isolate 200 mg of specific tRNA. The cost of the bi-functional reagent PAMBSYL sufficient for 200 mg of specific tRNA is about $9.

Acknowledgements. This work was supported by grants from Research Council of the University of Nebraska, a Nebraska Heart Association Grant-in-Aid, and NIH Grant No. HL 15284-02. We wish to thank Prof. Fred Wagner for providing samples of resin (I) and Profs. R. Johnston and J. van Etten for samples of (^{14}C) amino acids for the assays of acceptor activity.

REFERENCES

1. Hoffman, B.M., Schofield, P. and Rich, A. (1969), Proc. Nat. Acad. Sci. 62, 1195.

2. Zamecnik. P.C., Stephenson, M.L., and Scott, J.F. (1960), Proc. Nat. Acad. Sci. U.S. 46, 811.

3. Brown, G.L., Brown, A.V.W., and Gordon, J. (1959), Brookhaven Symp. Biol. 12, 47.

4. Gillam, J., Blew, D., Warrington, R.C., von Tigerstrom, M., and Tener, G.M. (1968), Biochem. 7, 3459.

5. Apgar, J., Holley, R.W. and Merrill, S.H. (1962), J. Biol. Chem. 237, 796.

6. Goldstein, J., Bennett, T.P. and Craig, L.C. (1964), Proc. Nat. Acad. Sci. U.S.A. 51, 119.

7. Cherayil, J.D. and Brock, R.M. (1965), Biochem. 4, 1175.

8. Gillam, J., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G.M. (1967), Biochem. 6, 3043.

9. Kelmers, A.D., Novelli, G.D., and Stulberg, M.P. (1965), J. Biol. Chem. 240, 3979.

10 Mandell, J.D., and Hershey, A.D., (1960), Analyt. Biochem. 1, 66.

11 Goldstein, J., in "Methods in Molecular Biology" 1, Last, J.A., and Laskin, A.I., ed., Marcell Dekker, Inc. N.Y. (1971), pp. 235-265.

12 Muench, K.H. in "Methods in Molecular Biology" 1, Last, J.A. and Laskin, A.I., ed., Marcell Dekker, Inc. N.Y. (1971), pp. 213-233.

13 Boyer, P. (1954), J. Amer. Chem. Soc. 76, 4331.

14 Cuatrecasas, P. (1970), J. Biol. Chem. 245, 3059.

15 Nishimura, S., Harada, F., Narushima, U., and Seno, T. (1967), Biochem. Biophys. Acta. 142, 133.