Moore, P. B. (1966), *J. Mol. Biol. 22*, 145.
Nirenberg, M., and Leder, P. (1964), *Science 145*, 1399.
Salas, M., Smith, M. A., Stanley, W. M., Jr., Wahba, A. J., and Ochoa, S. (1965), *J. Biol. Chem. 240*, 3988.
Saunders, G. F., and Campbell, L. L. (1966), *J. Bacteriol. 91*, 332.
Schell, P. L. (1966), *Z. Naturforschung 21*, 1245.
Sheard, B., Miall, S. H., Peacocke, A. R., Walker, I. O., and Richards, R. E. (1967), *J. Mol. Biol. 28*, 389.
Stanley, W. M., and Wahba, A. J. (1967), *Methods Enzymol. 12*, 524.
Tamaoki, T., and Miyazawa, F. (1966), *J. Mol. Biol. 17*, 537.
Tamaoki, T., and Miyazawa, F. (1967), *J. Mol. Biol. 23*, 35.
Tissières, A., Watson, J. D., Schlessinger, D., and Hollingworth, B. R. (1959), *J. Mol. Biol. 1*, 221.
Traub, P., and Nomura, M. (1968), *J. Mol. Biol. 34*, 575.
Traut, R. R., and Haenni, A. L. (1967), *European J. Biochem. 2*, 64.
Wang, J. H., and Matheson, A. T. (1966), *Biochem. Biophys. Res. Commun. 23*, 740.

Hydrogen-Exchange Measurements on *Escherichia coli* Transfer Ribonucleic Acid before, after, and during Its Aminoacylation*

R. R. Gantt,† S. W. Englander,‡ and Melvin V. Simpson§

ASBTRACT: The conformation of transfer ribonucleic acid has been compared with aminoacyl transfer ribonucleic acid by using the Sephadex hydrogen–tritium-exchange technique. The exchange-out kinetics of the acylated and unacylated transfer ribonucleic acid are almost identical, suggesting that secondary structures are closely similar. However, a small but consistent difference was observed. The aminoacyl transfer ribonucleic acid seems to possess three or four more stabilized hydrogens than the unacylated transfer ribonucleic acid, representing approximately 4% the measurable structure. Thus, the aminoacyl transfer ribonucleic acid has at least as much structure involving hydrogen bonds as has unacylated transfer ribonucleic acid and perhaps slightly more.

In addition, a new application of hydrogen exchange is described. It was possible to carry out measurements that should detect changes in transfer ribonucleic acid structure that might occur during the transient interval in which it binds to the amino acid–adenosine monophosphate–synthetase complex, undergoes acylation, and then dissociates.

In the last few years evidence has accumulated suggesting that tRNA may have a regulatory function in addition to its adaptor function. For example, the appearance of a new tRNA upon phage or virus infection (Kano-Sueoka and Sueoka, 1966; Hay *et al.*, 1967), the appearance in bacterial spores of a new tRNA component not found in vegetative cells (Doi and Kaneko, 1966), the formation of aminoacyl-tRNA as a required step for the possible control of RNA synthesis (Böck *et al.*, 1966), and the repression of enzymes involved in amino acid biosynthetic pathways, *e.g.*, those involved in the histidine, valine, and isoleucine pathways (Schlesinger and Magasanik, 1964; Eidlic and Neidhardt, 1965; Freundlich, 1967), all implicate tRNA in a role other than that of simply an adaptor molecule.

The latter type of control, that is, by a tRNA–aminoacyl-tRNA interconversion, could be effected by induction of a change in the three-dimensional structure of the tRNA upon aminoacylation or deacylation. Such a change in conformation could also explain the preferential binding to ribosomes of the aminoacylated form of tRNA (Seeds *et al.*, 1967). That tRNA can assume one or more conformational states (which affect the acceptor activity of the molecule) has been shown by Gartland and Sueoka (1966) and Lindahl *et al.* (1966). Here, we ask the question: "Does the conformation of tRNA differ from the conformation of aminoacyl-tRNA?" The answer to this question is sought using the method of hydrogen exchange (Hvidt and Nielsen, 1966; Englander, 1968) to compare the structure of tRNA before and after the attachment of the amino acid.

Hydrogen atoms covalently bound to nitrogen or oxygen exchange rapidly with those of water. However, when these hydrogens are hydrogen bonded, the

* From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire. *Received September 23, 1968.* Supported by grants from the National Science Foundation (GB352) and Public Health Service (AM07853).

† Postdoctoral fellow of the American Cancer Society. Present address: National Cancer Institute, Laboratory of Biology, Bethesda, Md. 20014.

‡ Present address: Department of Biochemistry, University of Pennsylvania School of Medicine, Philadelphia, Pa.

§ Present address: Biochemistry Section, Department of Biological Sciences, State University of New York at Stony Brook, Stony Brook, N. Y. 11790.

rate of exchange is much reduced. The rate of exchange of a particular hydrogen-bonded hydrogen depends, among other things, upon the stability of the local three-dimensional structure (Printz and von Hippel, 1965; Englander and Englander, 1965). Hence, if the exchangeable hydrogen sites of tRNA are labeled with ^3H, the ^3H exchange-out kinetics yields information on the total number of hydrogen bonds and on the distribution of structural stabilities. A change in the three-dimensional structure of tRNA would be expected to result in an alteration of either or both of these parameters so that a difference in conformation between tRNA and aminoacyl-tRNA should manifest itself by altered hydrogen-exchange kinetics.

In addition to the static situation in which the structure of tRNA is compared before and after aminoacylation, an experiment is described in which the conformation of tRNA *during* the process of aminoacylation is probed. This experiment serves to focus attention on an aspect of the hydrogen-exchange technique in which it may be feasible to detect transient changes in the conformation of a specific macromolecule *while* it is functioning, even in a complex biological system containing numerous macromolcular components.

Materials and Methods

Materials. Escherichia coli B whole cells, harvested at mid-log phase, were purchased from General Biochemicals, Inc. tRNA was either prepared from these whole cells or was purchased from General Biochemicals. DL-[1-^{14}C]Leucine (17–25 mCi/mmole), uniformly labeled [^{14}C]algal protein hydrolysate (1.39 mCi/mg), and tritiated water (1 Ci/ml) were obtained from the New England Nuclear Corp. Analytical grade Tris (Trizma Base) was purchased from Sigma Chemical Co. DEAE-cellulose was obtained from Bio-Rad.

Purification of tRNA. Most of the work reported here was done with tRNA prepared in our laboratory from commercially obtained *E. coli* cells stored for several months at −70 or −130°. The frozen cells were extracted with phenol solution and were precipitated with ethanol according to the procedure of Holley *et al.* (1961). The material was centrifuged in 250-ml glass bottles at 2000 rpm for 15 min in the International PR-2 centrifuge. The combined pellets were extracted with 1 M NaCl and the extract was chromatographed on DEAE-cellulose (Holley *et al.*, 1961). Fractions containing the tRNA peak (260 mμ) were pooled and precipitated by the addition of 2.5 volumes of ice-cold 95% ethanol. After centrifugation as before, the combined material was recentrifuged in a 150-ml bottle at 5000*g* for 30 min in the GSA rotor of the Sorvall centrifuge. The hard pellet was dissolved in a volume of 1 M NaCl to give a final concentration of 25–30 mg of RNA/ml and 10 ml of this solution was applied to a 2.5-cm diameter column of G-100 Sephadex (Schleich and Goldstein, 1964) or 40 ml to one of 5.0-cm diameter. The column was run at room temperature but the eluent fractions were stored in the cold. This procedure completely separates rRNA, small oligonucleotides, and free nucleotides from tRNA and largely resolves 5S RNA, aggregated tRNA, and other possible low molecular weight RNA fragments (Figure 1). The fractions in the tRNA peak were combined except that those fractions overlapping the adjacent faster moving peak were not included (see Figure 1). The combined fractions were again precipitated with 2.5 volumes of ice-cold ethanol and permitted to stand in the cold for several hours. The precipitate was collected as after the previous DEAE-cellulose step and was stored at −20°. In routine preparations, 500 g of frozen cells yields 250–300 mg of purified tRNA. Such preparations contain essentially 100% terminal adenosine at the free 3′-OH end.

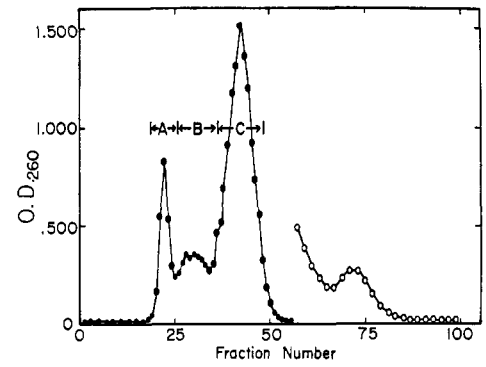

FIGURE 1: Chromatograph of tRNA on Sephadex G-100. A total of 250 mg of tRNA was chromatographed in 1 M NaCl at room temperature. Each fraction contains 10 ml. Peak C contains more than 85% of the amino acid acceptor activity. Peak A is devoid of activity, while peak B has about 25% of the specific acceptor activity of C when measured as counts per minute of [^{14}C]algal protein hydrolysate incorporated per milligram of RNA. (●) Samples diluted 30-fold; (○) undiluted samples.

Some experiments were done with selected lots of commercially obtained tRNA prepared by the procedure of Holley *et al.* (1961). These preparations, after chromatography on G-100 Sephadex as described above, were found to be indistinguishable in amino acid acceptor activity, terminal adenosine content, and spectrophotometric constants from those completely prepared in our laboratory. However, the quality of other commercial preparations varied widely from lot to lot after Sephadex G-100 purification. Only those lots which gave high amino acid acceptor activity were selected for further use. Lots which showed poor amino acid loading were always less than 55% aminoacylated while those which loaded well were greater than 63%. The poor preparations were invariably characterized by an unusual Sephadex G-100 profile in which peak B (Figure 1) almost diappears, and the material that remains partly merges with the tRNA peak. Some of these poor preparations had less than 100% terminal adenosine content and neither this parameter nor their ability to accept amino acids was enhanced by carrying out the aminoacylation in the presence of ATP and CTP.

Aminoacylation of tRNA. The aminoacylation of tRNA was carried out in maleate buffer as suggested by Cherayil and Bock (1965). The reaction mixture contained 100 mg of tRNA, 1 mmole of ATP, 0.1 mmole of each of 20 L-amino acids, 2 mmoles of $MgCl_2$,

4 mmoles of KCl, 0.05 mmole of EDTA, 0.1 mmole of dithiothreitol, 5 mmoles of potassium maleate buffer (pH 6.9). and 3–5 ml of enzyme solution containing 8–10 mg of protein/ml in a total volume of 100 ml. All reagents were neutralized with KOH before addition. The reaction mixture was incubated at 37° for 20 min. When labeled aminoacyl-tRNA was desired, the amino acid mixture was replaced by 0.07 mg of a [^{14}C]-algal protein hydrolysate or the L-leucine was replaced by 0.025 mg of 1-DL-[^{14}C]leucine. After incubation, the solution was chilled in ice water and 10 ml of 2 M potassium acetate (pH 5.2–5.3) added. After the addition of 110 ml of redistilled phenol–water (90%, w/w) the mixture was shaken in the cold for 10–15 min. The aqueous upper phase was removed after low-speed centrifugation and the lower phenol phase shaken with 90 ml of water. Both aqueous phases were combined and 2.5 volumes of cold ethanol was added and the solution was stored overnight at −20°. The fluocculant precipitate was removed by low-speed centrifugation, dissolved in 10–20 ml of 0.02 M triethylammonium formate buffer (pH 4.6), and dialyzed at 0° against 2 l. of the same buffer for 20 hr with four changes of buffer. Before use, the dialysis bags (Visking Corp.) were boiled 15–20 min in sodium carbonate–EDTA, exhaustively rinsed in distilled water, and finally subjected to several rinses in the triethylammonium formate buffer. The aminoacyl-tRNA was stored at −20° in triethylammonium formate solution and if needed in more concentrated form, it was reprecipitated with ethanol, centrifuged at high speed (10,000*g*, 30 min) to remove the maximum amount of ethanol, and dissolved in a smaller volume of buffer.

Deacylation of Aminoacyl-tRNA. The amino acids were hydrolyzed from the tRNA by either of two methods. The first involved dialysis of the sample for 30 min against a solution containing 0.06 M KCl and 0.01 M magnesium acteate, adjusted with NH_4OH to pH 10.5 at room temperature, followed by immediate neutralization of the tRNA solution. When tRNA was charged with a ^{14}C-labeled algal protein hydrolysate to test this method, it was found that this procedure resulted in the total removal of the amino acids but a loss of 20% of the acceptor activity. In the second method (Sarin and Zamecnik, 1964), the aminoacyl-tRNA was incubated in 2 M Tris-HCl (pH 8.0, measured at room temperature) at 37° for 15 and 45 min. After 15 min, more than 87% of the radioactivity from the [^{14}C]algal hydrolysate-charged tRNA was lost and more than 96% after 45 min. There was little or no loss of acceptor activity.

Preparation of Activation Enzymes. All operations were carried out at 0–5°. Frozen *E. coli* cells weighing 7 g were ground in a chilled mortar and pestle with 14 g of washed alumina. The paste was extracted with 21 ml of buffer containing 0.01 M Tris-HCl (pH 7.8), 0.01 M magnesium acetate, 0.06 M KCl, and 0.006 M β-mercaptoethanol. The alumina and cell debris were removed by low-speed centrifugation. The supernatant solution was decanted and further centrifuged for 2 hr at 150,000*g*. The supernatant fluid, including the turbid material in the lower one-third of the tube, was decanted and dialyzed against 2 l. of the buffer for 16 hr. The activating enzymes were prepared fresh for each acylation experiment.

Amino Acid Acceptor Assay. The routine assays for terminal adenosine content or total aminoacylation were performed on a pancreatic RNase digest of the tRNA using the DEAE-cellulose column chromatography procedure of Wolfenden *et al.* (1964). This method resolves aminoacyl adenosine and adenosine from the remainder of the digest (which does not emerge) and from each other. The optical density (260 mμ) of the combined fractions from each peak was measured and used to calculate the adenosine and aminoacyladenosine content of the peaks. An E_M at 260 mμ of 15.0×10^3 was used for adenosine at pH 4.6, and 6×10^5 for tRNA at pH 7.0.

Hydrogen–Tritium Exchange. A. EQUILIBRATION. Exchangeable hydrogen sites of unacylated, acylated, and deacylated tRNA were labeled with tritium by equilibration in tritiated water using the procedure of Englander and Englander (1965). A tracer amount of tritiated water (about 10 mCi/ml of tRNA solution) was introduced into a solution of tRNA (0.4–15 mg/ml) containing 0.02 M triethylammonium formate (pH 4.6). To ensure equilibration, the solution was warmed to 35–40° for 15 min, after which the pH was adjusted to 6.5 by the addition of $^1/_{100}$ volume of 1 M cacodylate buffer containing 1 M magnesium acetate. The solution was then chilled to 0°. This procedure results in no detectable deacylation as measured by loss of radioactivity from preparations acylated with amino acids from an algal [^{14}C]protein hydrolysate.

B. TWO-COLUMN RUNS. With the exception of early time points (7 min or less), all runs were of the two-column type (Englander, 1963). Here, the tritiated tRNA was rapidly separated from the unbound tritiated water on a previously calibrated Sephadex G-25 column and eluent tRNA was collected in a single tube. The [^{3}H]tRNA was then incubated under appropriate conditions. At various times, samples were removed and each was subjected to a second Sephadex run, a separate column being used for each time point. The second column run separates the [^{3}H]tRNA from the tritiated water formed by exchange during the incubation period. Fractions were taken through the RNA peak and their optical density and tritium content were measured, and the amount of ^{3}H still bound to tRNA was computed. A series of such data points in time define the exchange-out kinetics of the tRNA.

The first column was 3 cm in diameter by 7 cm high and the second was 3 × 4.5 cm. All columns were jacketed with polyethylene cups containing crushed ice, and the experiments were carried out in a cold box at 0°. Sephadex G-25 (fine beads) was sieved (dry) using No. 325 U. S. standard sieve to remove the fine particles. It was then swelled in water, stirred, permitted to settle for about 10 min, and then decanted, to remove remaining fine particles before pouring the columns. This procedure was repeated about ten times.

C. SINGLE-COLUMN RUNS. When early time points were required, single-column runs were used. Here, the large excess of tritiated water is separated from the [^{3}H]tRNA in the upper portion of the column; the flow is then

stopped for the required incubation period and then resumed, thereby separating the tritium that exchanged-out while the flow was interrupted. The procedure used was a scaled-down modification of that described previously (Englander, 1963) requiring five to ten times less material. Columns were 1 cm in diameter, and 0.3-ml samples were collected and analyzed.

D. TREATMENT OF DATA. Hydrogen-exchange data are plotted as number of exchangeable hydrogens remaining per molecule against time of exchange. The parameter hydrogen/molecule is calculated from the ratio, R, of tritium activity to optical density in samples of Sephadex column effluent through the tRNA peak region. Results are computed as follows (Englander, 1963): H/molecule $= 111E_M(R/C_0) = 6.66 \times 10^7\ R/C_0$, where R is defined above, 111 is the gram-atom concentration of hydrogen in water, E_M is the molar extinction coefficient of tRNA at 260 mμ, taken here as 6×10^5/cm, and C_0 is the tritium count rate per milliliter of initial equilibration mixture. Zero time was taken as the moment of complete entry of the equilibrated sample into the first column. True zero time, *i.e.*, the time at which sufficient reduction of solvent tritium occurs to render back-exchange of tritium insignificant, differs slightly from the above nominal zero time (Englander and Englander, 1965). In certain experiments, rates of hydrogen exchange are compared as a function of some independent variable. In making such comparisons, one would like to compare what are presumably the same hydrogens. Hence, these rates are obtained by noting the absolute times at which a given number of hydrogens still remain unexchanged. The ratio of these time values is considered equivalent to the ratio of the rates. Thus, in Figure 5, the time taken to reach a value of 15.5 remaining H's at 2.5° is 58 min, while at 16°, it takes 17 min to reach this same level. The difference between the rates is then 3.3-fold.

E. HYDROGEN-EXCHANGE MEASUREMENTS DURING CHARGING OF tRNA. In this experiment, samples of the aminoacylation reaction mixture were removed periodically and the pretritiated tRNA was assayed for unexchanged ^{3}H. In general, measurements were carried out in the same manner as in the static experiments, but two differences should be noted. First, the exchange-out and acylation reactions were carried out in potassium maleate buffer at pH 6.9, but the "second columns" were equilibrated with potassium cacodylate as usual in order to remove the maleate, the very high ultraviolet absorbance of which interferes with the optical density measurements. Second, a correction factor for the 260-mμ absorbance contributions of the heated and unheated enzyme extracts to the tRNA peak from the Sephadex G-25 was determined. This was done after the experiment by establishing that all the absorbance in the cell extracts appeared in the macromolecular peak and then calculating the relative absorbance contributions of the tRNA and the cell extract. The tRNA usually contributed about two-thirds of the total absorbance. The extract loses about 10% of its absorbance after heat treatment and centrifugation.

At the low temperature (0°) at which the aminoacylation was carried out in this experiment, the extent of charging obtained is about 33%, and the reaction reaches a plateau in 6 min.

Assay of Radioactivity. Tritium samples were counted in Bray's (1960) solution in a Packard Tri-Carb liquid scintillation counter at a counting efficiency of about 8% and a background of about 15 cpm; ^{14}C-labeled aminoacyl-tRNA was washed and counted by the slightly modified (Dietz *et al.*, 1965) paper disc method of Mans and Novelli (1961) with an efficiency of 50% and background of 12 cpm.

Results

Purity of tRNA and Aminoacyl-tRNA. Studies on tRNA which deal with its function, *e.g.*, its amino acid acceptor or donor activity, do not usually require highly purified tRNA. In such studies, the presence of contaminating protein, other RNA species such as rRNA, or functionally inactive tRNA may usually be tolerated, inasmuch as these contaminants often are inert in the reaction being studied. This is not true for hydrogen-exchange measurements, however, since such contaminants may incorporate tritium as well as or better than the substance actually being studied. Since the object of these experiments was to compare the hydrogen-exchange behavior of tRNA and aminoacyl-tRNA, it was necessary to obtain tRNA preparations with demonstrably high terminal adenosine content and high ability to accept amino acids.

The preparations used in these studies contained 100 ± 3% terminal adenosine based on the measurement of total moles of terminal adenosine and total moles of tRNA used. The extent of charging with a complete amino acid mixture varied from 63 to 67%. A DEAE-cellulose column chromatography assay of a typical charged tRNA preparation after RNase hydrolysis is shown in Figure 2. The faster moving peak represents

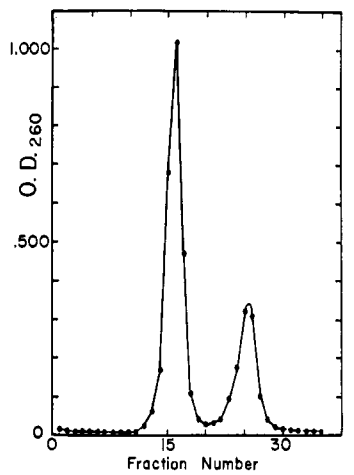

FIGURE 2: Typical assay for acceptor activity and terminal adenosine content of tRNA. After digestion of 25 mg of aminoacyl-tRNA with 0.5 mg of pancreatic ribonuclease for 15 min in 2 ml of 0.02 M triethylammonium formate (pH 4.6) at room temperature, the digest was chromatographed on a 100-ml bed volume of DEAE-cellulose with the same buffer. The faster moving peak contains 0.67 μmole of aminoacyladenosine and the trailing peak contains 0.31 μmole of adenosine.

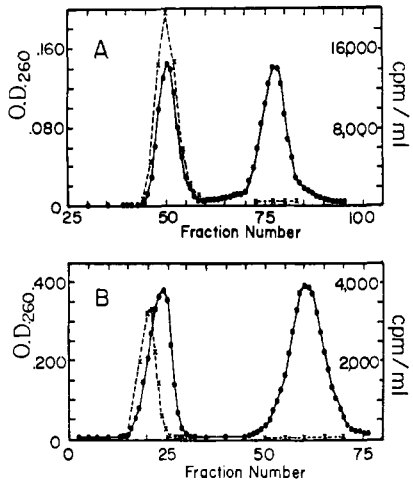

FIGURE 3: Separation of adenosine from aminoacyladenosine on DEAE-cellulose after ribonuclease digestion (see Figure 2). (●——●) Optical density 260 mμ; (×- - -×) radioactivity. (A) 4.0 mg of aminoacyl-tRNA labeled with [^{14}C]algal protein hydrolysate. (B) 10.0 mg of aminoacyl-tRNA labeled with [^{14}C]leucine in the presence of the remaining unlabeled amino acids.

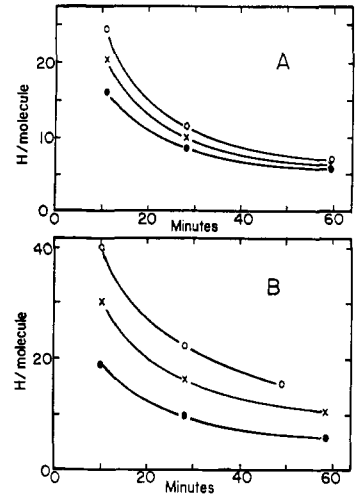

FIGURE 4: Effect of some solvent conditions on hydrogen exchange. (A) Effect of pH. Solvent conditions were 0.01 M potassium cacodylate–0.01 M magnesium acetate at 11°; (O——O) pH 6.5, (×——×), pH 6.0, (●——●) pH 7.49. (B) Effect of buffer salts. Solvent conditions were pH 7.0, 2.5°, and 0.01 M magnesium acetate; (O——O), 0.01 M potassium cacodylate, (×——×) 0.01 M Tris-Cl, (●——●) 0.1 M Tris-Cl.

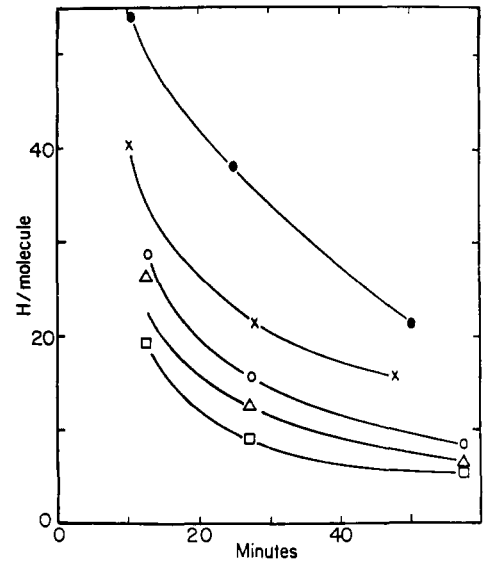

FIGURE 5: Effect of temperature on hydrogen exchange. Solvent conditions were 0.01 M potassium cacodylate buffer (pH 7.0), containing 0.01 M magnesium acetate. (●) 0°, (×) 2.5°, (O) 6.2°, (△) 11°, and (□) 16°.

mixed aminoacyladenosines while the trailing peak is free adenosine itself (Wolfenden *et al.*, 1964). To further substantiate the identity of these peaks, tRNA preparations charged with a labeled amino acid mixture and with labeled leucine were also assayed by the DEAE technique. It can be seen (Figure 3A) that the faster peak, shown by Wolfenden *et al.* (1964) to be aminoacyladenosine, is labeled while the slower peak, shown (Wolfenden *et al.*, 1964) to be adenosine, contains no labeled amino acids. Further, the content of the adenosine peak was analyzed spectrophotometrically in acid and alkali and by paper chromatography and found to be homogeneous. In the case of leucyl-tRNA (Figure 3B), similar results were obtained except that leucyladenosine chromatographs a bit faster than the average of the aminoacyladenosines (*cf.* Wolfenden *et al.*, 1964).

Effect of Solvent Conditions on Hydrogen Exchange. Hydrogen-exchange rates are known to be sensitive to solvent conditions, and it was thought desirable to study the effect of these parameters before choosing conditions for the tRNA–aminoacyl-tRNA comparison experiment. Some exploratory data are given concerning the effect of pH (Figure 4A), buffer salts (Figure 4B), and temperature (Figure 5).

The effect of Mg^{2+} and Na^{+} salts has already been studied (Englander and Englander, 1965) and it is clear, in accord with the results obtained in many laboratories using a variety of methods, that cations, and in particular divalent cations, stabilize the tRNA molecule; removal of salts results in hydrogen exchange at a rate too fast to measure.

The exchange-out rate as a function of pH values near the physiological region (Figure 4A) goes through a minimum somewhere near pH 6.5 and then increases rapidly when the pH is either raised or lowered. This situation is qualitatively similar in ribosomes (Page *et al.*, 1967) and proteins (Hvidt and Nielsen, 1966) in which the exchange is both acid and base catalyzed.

The results in Figure 4B show that at constant pH, the nature of the buffer can also result in appreciable effects on the rate of hydrogen exchange. The exchange rate in 0.01 M Tris is 1.5 times the rate with an equivalent concentration of cacodylate; in 0.1 M Tris, the rate rises to 3.6 times that in cacodylate. This catalytic effect of Tris on hydrogen exchange was also seen in the case of ribosomes (Page *et al.*, 1967) and the peptide model, *N*-methylacetamide (Klotz and Frank, 1965). To avoid this effect, tRNA–aminoacyl-tRNA comparison experiments were carried out in cacodylate buffer.

The temperature dependence of the tRNA exchange-

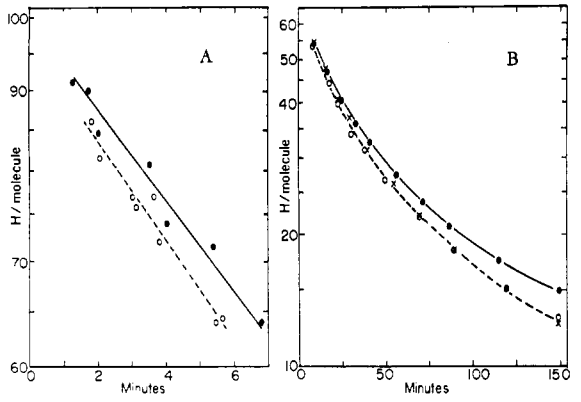

FIGURE 6: Comparison of hydrogen exchange-out of aminoacyl-tRNA with that of unacylated tRNA. Solvent conditions were 0.01 M potassium cacodylate buffer (pH 6.5), 0.06 M KCl, and 0.01 M magnesium acetate at 0°. (A) Exchange-out at early times. The points on these curves are the result of grouping separate experiments consisting of two to four determinations over several days. (●) Aminoacyl-tRNA; (○) unacylated tRNA. (B) Exchange-out for longer times. These experiments were done simultaneously taking samples alternately from the incubation solutions for determination; (●) aminoacyl-tRNA; (○) unacylated tRNA. The unacylated tRNA experiment (×) was repeated the following day to test reproducibility.

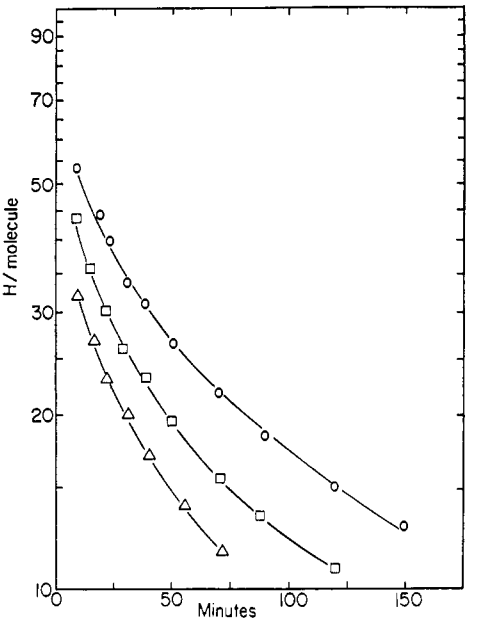

FIGURE 7: Hydrogen-exchange kinetics of tRNA incubated at pH 10.5. tRNA was incubated at 23° in 0.06 M KCl and 0.01 M magnesium acetate adjusted to pH 10.5 with NH_4OH, neutralized, and reisolated by ethanol precipitation. Hydrogen-exchange solvent conditions were the same as given in Figure 6. (○) Untreated tRNA; (□) incubation time, 30 min; (△) incubation time, 1 hr.

out rates is shown in Figure 5. The rapidity with which the exchange occurs at the higher temperatures makes it difficult to follow the exchange of the faster hydrogens. Hence, most of the studies reported here were done at low temperatures.

Comparison of tRNA with Aminoacyl-tRNA. Figure 6 shows a comparison between the hydrogen-exchange properties of tRNA and aminoacyl-tRNA. The experiments in A and B were done with two different tRNA preparations, each at a different portion of the hydrogen-exchange curve. Figure 6A focuses on the early portion of the curve. These "faster" hydrogens presumably originate from the less stable parts of the molecule where conformational change might be induced more easily. The curves in Figure 6B reflect the exchange-out character of the presumably more stable parts of the molecule.

At neither of the time periods studied are large differences evident. However, the data do suggest a small difference, no more than a few hydrogens per molecule between the two forms of tRNA. Other results on independent preparations of tRNA, at time intervals overlapping those presented here, tend to confirm this small difference.

Originally, it was intended to do hydrogen-exchange studies on aminoacyl-tRNA after deacylation to serve as an additional control in the above experiments. Two commonly used methods were employed for the deacylation: hydrolysis at pH 10.5 at 23° and Tris-catalyzed hydrolysis at pH 8.0 (adjusted to pH 8.0 at room temperature) at 37°. However, when uncharged tRNA was subjected to the conditions of the former procedure, a drastic change occurred in its hydrogen-exchange behavior (Figure 7), indicating the occurrence of some gross structural change in the tRNA. Substitution of the Tris hydrolysis procedure also produced changes in the hydrogen-exchange pattern, albeit of considerably smaller magnitude, with an accompanying slight decrease in amino acid acceptor activity. Because of these effects, neither procedure was considered acceptable for the purposes of these experiments, and these controls were abandoned.

Hydrogen-Exchange Measurements during Charging of tRNA. In order to test the possibility that a transient conformational change occurs in tRNA in the course of binding to the activating enzyme, perhaps as part of the mechanism of recognition between tRNA and the aminoacyl-AMP–synthetase complex, hydrogen-exchange measurements were carried out during the process of enzymatic aminoacylation of tRNA. The results (Figure 8) show that no change in the hydrogen-exchange pattern was observed. That is, no appreciable amount of extra hydrogens have been "spilled out" during the aminoacylation. The small differences found previously (Figure 6) when comparing tRNA with aminoacyl-tRNA are not apparent here, probably because the amount of tRNA aminoacylated under these conditions is only half (33%) that in the experiments described in Figure 6.

Discussion

The results of the experiments in which the hydrogen-exchange pattern of tRNA and aminoacyl-tRNA are compared make it improbable that there are gross differences between the three-dimensional structures of these two molecules. However, the results are consistent with the possibility that a small region of the tRNA has been stabilized upon aminoacylation. Another possibil-

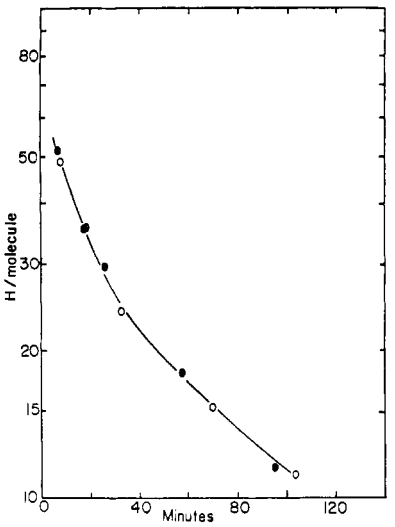

FIGURE 8: Hydrogen-exchange-out kinetics during aminoacylation. The composition of the aminoacylation medium was as described in the text except that 67.2 optical density units (260 mμ) of [^{3}H]tRNA and 33.9 units (260 mμ) of enzyme extract were used in a final volume of 7.0 ml. In the control sample the equivalent amount of enzyme was first heat treated at 100° for 2 min, centrifuged, and the optical density was determined before addition to the aminoacylation medium. The hydrogen-exchange experiments were run simultaneously, the control and experimental flasks being sampled alternately. (●) Aminoacylating sample; (○) heat-inactivated control.

ity is that a major conformational change occurs in only a few specialized species or subspecies of the tRNA, perhaps those which may be involved in repression, and that the magnitude of this change is masked by the presence of large amounts of unaltered molecules.

The change observed involves no more than two to three measurable hydrogens per molecule. Actually, this number should be increased by about half, to three or four hydrogens since the tRNA is only about two-thirds aminoacylated. This represents less than 5% of the measurable character of the molecule and, though the observed difference is formally outside of experimental error, its small magnitude invites the exercise of caution as to its meaningfulness.

Two reports have presented evidence for a change in conformation of tRNA after aminoacylation. Sarin and Zamecnik (1965), using optical rotatory dispersion, found both large changes in amplitude and a shift in the peak of the Cotton effect when comparing tRNA and aminoacyl-tRNA at pH values decreasing from 7 to 3. They concluded that aminoacyl-tRNA has less helical content than tRNA. In contrast, our results would indicate that if there is any change at all, it is very small and in the direction of increased hydrogen bonding. Some differences in experimental conditions may play a role here. The hydrogen-exchange curves were obtained at 0°; the optical rotatory dispersion experiments were done at 21°. Also, the experiments of Sarin and Zamecnik (1965) were performed in citrate buffer in the absence of Mg^{2+}. It is plausible that, in the absence of Mg^{2+}, an interaction between the positively charged amino acid ester and negative phosphates effects a structural change that would not occur in the presence of phosphate-bound Mg^{2+}. In any case, the absence of Mg^{2+} does have other effects on the destabilization of the tRNA structure, as evidence by a large change in the position and shape of the tRNA melting curve (Monier and Grunberg-Manago, 1962). In other optical rotatory dispersion experiments (Lamborg *et al.*, 1965) carried out at the same laboratory, but here *with* Mg^{2+}, aminoacylated valyl-tRNA was indistinguishable from unacylated tRNA (mixed).

Kaji and Tanaka (1967), using preparative sucrose density gradients, have reported slight changes in sedimentation behavior of certain species of tRNA after the attachment of labeled amino acids. They observed a decrease in the sedimentation velocity of the lysyl-, seryl-, phenylalanyl-, tyrosyl-, and aspartyl-tRNA peaks as compared with the uncharged tRNA peaks, but saw no change for valyl-, methionyl-, and isoleucyl-tRNA. These results were interpreted to be consistent with the observations of Sarin and Zamecnik (1965) indicating a less helical molecular structure for the aminoacylated tRNA. Further work is necessary to resolve these differences.

In order to best compare the structures of tRNA and aminoacyl-tRNA by the hydrogen-exchange technique, it is desirable to assay as large a proportion of the molecule as possible and this requires the measurement of as many stabilized hydrogens as is technically feasible. For this reason, the lowest practical temperature, 0°, was chosen for our experiments to take advantage of the large temperature effect on the hydrogen-exchange rate and, in addition, to minimize the hydrolysis of the labile aminoacyl esters. However, this introduces the problem of tRNA aggregation and the consequent possibility of an experimental artifact in the exchange-out kinetics.

Judging from the work of Millar and Steiner (1966) and Henley *et al.* (1966), the tRNA, under the conditions of many of our experiments, is probably in the dimer state. A number of hydrogen-exchange experiments were carried out with rather dilute tRNA preparations to establish whether conditions which alter the dimer–monomer equilibrium would also alter the exchange-out kinetics. In these studies concentrations were as low as 0.05 mg/ml during exchange-out on the columns. Further, the magnesium concentration was decreased from 10^{-2} to 10^{-3} M while the KCl was increased to 0.2 M. These conditions, which reduce aggregation, resulted in no change of the exchange-out kinetics. This suggests that occurrence of aggregation does not effect the data.

The hydrogen-exchange experiment carried out during aminoacylation failed to detect a transitory opening of hydrogen bonds during the aminoacylation reaction. It is conceivable, of course, that hydrogen bonds were in fact broken, but that the life of the transient open structure was too short to permit the detection of a change in exchange rate. Further work is required to adequately evaluate this approach to the study of transient conformational changes in complex functioning biological systems.

References

Adams, A., Lindahl, T., and Fresco, J. R. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1684.

Böck, A., Faiman, L. E., and Neidhardt, F. C. (1966), *J. Bacteriol. 92*, 1076.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Brown, G. L. (1963), *Progr. Nucleic Acid Res. 2*, 259.

Cherayil, J. D., and Bock, R. (1965), *Biochemistry 4*, 1174.

Coles, N., Bukenberger, M. W., and Meister, A. (1962), *Biochemistry 1*, 317.

Dietz, G. W., Jr., Reid, B. R., and Simpson, M. V. (1965), *Biochemistry 4*, 2340.

Doi, R. H., and Kaneko, I. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 581.

Eidlic, L., and Neidhardt, F. C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 539.

Englander, S. W. (1963), *Biochemistry 2*, 798.

Englander, S. W. (1968), *Methods Enzymol. 12*, 379.

Englander, S. W., and Englander, J. J. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 370.

Freundlich, M. (1967), *Science 157*, 823.

Gartland, W. J., and Sueoka, N. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 948.

Hay, J., Subak-Sharpe, H., and Shepherd, W. M. (1967), *Biochem. J. 103*, 69P.

Henley, D. D., Lindahl, T., and Fresco, J. R. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 191.

Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., and Merrill, S. H. (1961), *J. Biol. Chem. 236*, 200.

Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 288.

Kaji, H., and Tanaka, Y. (1967), *Biochim. Biophys. Acta 138*, 645.

Kano-Sueoka, T., and Sueoka, N. (1966), *J. Mol. Biol. 20*, 183.

Klotz, I. M., and Frank, B. H. (1965), *J. Am. Chem. Soc. 87*, 2721.

Lamborg, M. R., Zamecnik, P. C., Li, T.-K., Kagi, J., and Vallee, B. L. (1965), *Biochemistry 4*, 63.

Lindahl, T., Adams, A., and Fresco, J. R. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 941.

Mans, R. J., and Novelli, G. D. (1961), *Arch. Biochem. Biophys. 94*, 48.

Millar, D. B., and Steiner, R. F. (1966), *Biochemistry 5*, 2289.

Monier, R., and Grunberg-Manago, M. (1962), *Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 106*, 163.

Page, L. A., Englander, S. W., and Simpson, M. V. (1967), *Biochemistry 6*, 968.

Printz, M. P., and von Hippel, P. H. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 363.

Sarin, P. S., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91*, 653.

Sarin, P. S., and Zamecnik, P. C. (1965), *Biochem. Biophys. Res. Commun. 20*, 400.

Schleich, T., and Goldstein, J. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 744.

Schlessinger, S., and Magasanik, B. (1964), *J. Mol. Biol. 9*, 670.

Seeds, N. W., Retsema, J. A., and Conway, T. W. (1967), *J. Mol. Biol. 27*, 421.

Sueoka, N., Kano-Sueoka, T., and Gartland, W. J. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 571.

Wolfenden, R., Rammler, D. H., and Lipmann, F. (1964), *Biochemistry 3*, 329.