INACTIVATION OF TRANSFER RNA WITH FORMALDEHYDE-- A TEST OF THE TRIPLET PAIRING MODEL

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The identification of the biochemical role of transfer RNA has led to the proposal by Spencer, et. al. (1962) and McCully and Cantoni (1962) of a simple model for the structure of transfer RNA and for the mechanism by which the transfer RNA functions as an adaptor. In this model the transfer RNA chain is thought to be folded back on itself like a hairpin, the two halves forming a nearly perfect double helix with each other. Since a number of unpaired nucleotides are necessary at the fold, these are thought to be available for specific hydrogen bonding with the appropriate messenger RNA, thus reading the genetic code and positioning the attached amino acid for incorporation into protein. The specificity of the transfer RNA for acceptance of its particular amino acid is thought to lie in a separate portion of the molecule. This hypothesis of the separation of recognition sites is supported by the contrast between the species specificity of the amino acid accepting abilities of transfer RNA and the lack of such specificity in the amino acid transfer step, reported by Rendi and Ochoa (1962).

It is possible to test this model by use of formaldehyde as an agent to inactivate phenylalanyl and lysyl transfer RNAs. On the simple hypothesis of base pairing of a triplet on the transfer RNA to its complementary triplet on the messenger, we would expect phenylalanyl transfer RNA to have an exposed triplet of adenine nucleotides while lysyl transfer RNA would replace these with uracil nucleotides. Formaldehyde reacts readily with all of the nucle-

otide bases except uracil, and thus would distinguish readily between these anti-codon triplets.

Penniston and Doty (1963b) used formaldehyde to inactivate yeast transfer RNA and showed that inactivation of the amino acid acceptor ability was exponential when the secondary structure of the RNA was partly melted out. However, when the secondary structure was stabilized by low concentrations of magnesium ions, the transfer RNAs lost less than half of their amino acid accepting ability in the first ten hours of reaction, with no further inactivation taking place thereafter. Since the existence of secondary structure protected the amino acid accepting ability against inactivation, it should be possible to test for amino acid transferring ability under these same conditions, and thereby to demonstrate the presence or absence of exposed triplets in the transfer RNA. An exposed triplet of adenine nucleotides, which were capable of reading the polyuridylic acid messenger in the phenylalanine transfer step, would rapidly have this ability destroyed by reaction with formaldehyde. A triplet of uracil nucleotides would be immune to such inactivation.

EXPERIMENTAL METHODS

E. coli transfer RNA was prepared by a modification of the method of Zubay (1962). After the first ethanol precipitation of the RNA, the precipitate was thoroughly dispersed in 800 ml of cold 0.01M magnesium chloride plus 0.02M Tris buffer, pH 7.6. This was centrifuged for three hours at 100,000 x g in the Spinco Model L Ultracentrifuge at 5°C. The supernate was poured off and made 1M in sodium chloride by adding the solid salt. This was then cooled in an ice bath to near 0°C. If a precipitate formed, it was centrifuged 15 minutes at 1°C and 30,000 x g in a refrigerated Servall centrifuge. The transfer RNA was then precipitated by ethanol and the phenol extraction repeated twice more. The final RNA solution was dialysed against 1M sodium chloride and then against three changes of distilled water, lyophilized and stored dry.

Enzyme preparations S-30 and S-100 were prepared according to the method of Nirenberg and Matthaei (1961) from fresh E. coli grown by Grain Processing Corporation, Muscatine, Iowa.

Inactivation of transfer RNA at 25°C by 0.3% formaldehyde was performed as previously described by Penniston and Doty (1963a), except that a potassium acetate-acetic acid buffer solution (total concentration 0.005M) was used to maintain the low pH reaction mixtures at pH 4.6, and the magnesium chloride concentration in the neutral solutions was raised to 0.002M.

For each assay, 0.15 mg of RNA was reacted with formaldehyde in 0.15 ml of water. Best results were obtained when the inactivated RNA solutions were immediately frozen in liquid nitrogen, and stored there until assayed. Before assay, the RNA was thawed and precipitated; it was found that precipitation by making the solution 0.1M in both hydrochloric acid and potassium chloride gave more consistent results than the previously used alcohol precipitation. The RNA precipitate was centrifuged down and redissolved in a small amount of 0.1M acetate buffer, pH 5. The concentration of each portion of RNA was determined by optical density measurements on a portion, and 30-50 µgm of RNA were added to the assay mixture.

Assays of amino acid accepting ability were performed as described by Penniston and Doty (1963a), except that the crude yeast enzyme was replaced by the S-100 fraction from E. coli. The concentrations of reagents were the same, but the reaction volume was reduced to 50 microliters.

Assays of transfer activity were patterned after those of Nirenberg and Matthaei (1961). The reaction mixture contained 0.1M tris buffer, pH 7.6, 0.01M magnesium chloride, 0.05M potassium chloride, 0.002M mercaptoethanol, 2 µgm/ml creatine phosphokinase, 0.002M ATP, 0.013M creatine phosphate, 0.0002M GTP, 0.2 mg/ml polyuridylic acid, 0.0004M ^{14}C-phenylalanine, ca. 2 mg/ml S-30 enzyme and 0.5-1 mg/ml RNA. The reaction volume was 60 microliters. Standard incubation time was 45 minutes at 37°C, after which protein and RNA were precipitated with 10% trichloracetic acid, incubated 15 minutes

at 90°C, and then filtered and counted on millipore filters, using a gas flow counter. This assay system responded linearly to the added RNA, up to 1 mg/ml.

For assay of inactivation of transfer activity at low pH, an RNA loaded with ^{14}C-phenylalanine was prepared by incubation in a scaled-up acceptor assay system, and then isolated by phenol extraction, alcohol and acid precipitation. This was done as previously described for preparation of RNA (Penniston and Doty, 1963b) but enough 1M acetate buffer, pH 5, was added to bring the pH to between 5 and 6 in order to prevent hydrolysis of the amino acyl-RNA bond. The methoxyethanol treatment was omitted. About 200 µgm of this RNA were used in the transfer assay.

RESULTS

This communication reports only the results of inactivation of phenylalanyl acceptor and transfer ability. A fuller report is in preparation. The inactivation of phenylalanyl acceptor ability follows a pattern very similar to that already reported for other amino acid acceptors in the yeast system. At pH 4.6, where secondary structure is partly melted out, the loss of activity on reaction with formaldehyde is exponential, as shown in Figure 1, indicating that a single reaction at a sensitive site is sufficient to inactivate the transfer RNA. If we assume that E. coli transfer RNA reacts with formaldehyde at the same rate as does the yeast material, we may calculate the number of such sensitive sites in the manner previously described. The exponential inactivation constant γ given when the logarithmic data for reaction times less than 15 hours are fitted by a least squares straight line is $-0.086 \pm .024$ hr^{-1} (95% confidence limits). This rate of inactivation indicates that there are four to eight sensitive sites on the phenylalanyl acceptor RNA. As was true with the yeast system, reaction at the large majority of sites causes no loss of activity, but reaction at any one of the few sensitive sites is inactivating.

If the secondary structure of the transfer RNA is protected during reaction by use of triethanolamine buffer at pH 7.6 and by presence of 0.002M

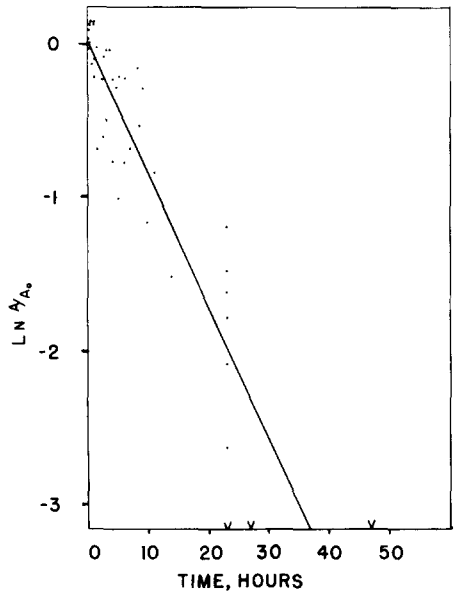

Figure 1. Inactivation by 0.3% Formaldehyde of Phenylalanyl Accepting Ability of E. coli Transfer RNA; in 0.005M Acetate Buffer, pH 4.6, at 25°C.

The solid line is a least squares fit to the points for less than 18 hours; subsequent points are close to background level and their probable error is too large to include them; the arrows represent points indistinguishable from background.

$MgCl_2$, then 51 ± 6 percent of the molecules are completely protected against inactivation. The phenylalanyl acceptor activity shows a gradual fall for the first ten hours and then levels off, showing no further loss of activity over a period of one hundred hours (Figure 2). This is once again similar to the case with the yeast system, and the same conclusions may be drawn. The presence of secondary structure protects the sensitive sites and therefore these sites must be among those deeply involved in secondary structure.

All of these comments simply repeat those which have been drawn with respect to the yeast system. However, the study of the inactivation of phenylalanyl transfer ability leads us to further conclusions. Since the inactivation at neutral pH was done on transfer RNA free of amino acids, the inactivation pattern for the transfer step would be expected either to follow the pattern found for the acceptor step (since the acceptor step must precede

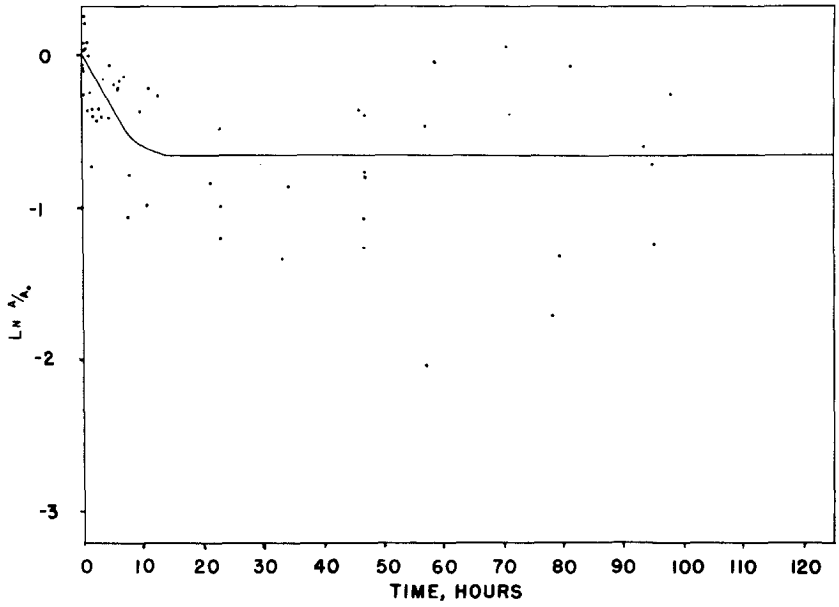

Figure 2. Inactivation by 0.3% Formaldehyde of Phenylalanyl Accepting Ability of E. coli Transfer RNA; in 0.01M Triethanolamine Buffer, 0.002M $MgCl_2$, pH 7.6, at 25°C.

The solid line represents the average of all points for more than 10 hours.

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the transfer step) or to be inactivated more completely or more rapidly. The former pattern is observed with phenylalanyl transfer activity; it falls slowly for ten hours and then levels off with 52 ± 11 percent of the original activity remaining (Figure 3). A similar result is observed in preliminary experiments with lysyl transfer RNA.

At low pH, hydrolysis of the amino-acyl RNA bond is very slow, and it is possible to inactivate the transfer RNA with radioactive amino acid attached and then to measure transfer of the amino acid to polypeptide. These results are not limited by the inactivation of the acceptor ability, but none the less the inactivation under these conditions is approximately exponential and closely follows that observed for the acceptor step (Figure 4). The least squares fit to our results shows a γ of $-0.10 \pm .05$ hr^{-1}. The fact that no free radioactive amino acids are added in these assays gives a very

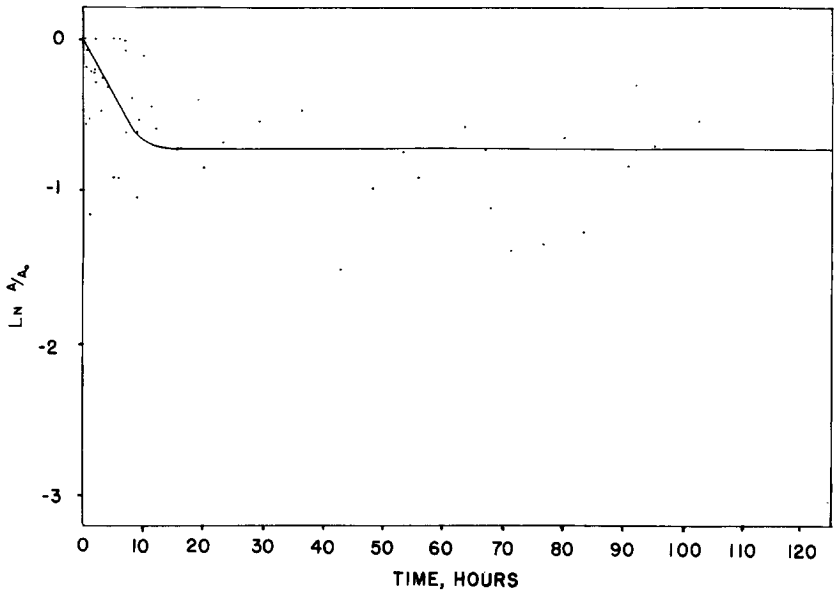

Figure 3. Inactivation by 0.3% Formaldehyde of Phenylalanyl Transferring Ability of E. coli Transfer RNA; in 0.01M Triethanolamine Buffer, 0.002M $MgCl_2$, pH 7.6, at 25°C.

The solid line represents the average of all points for more than 10 hours.

low background count; this enables the detection of deviations from true exponential behavior which could well be hidden in the acceptor assays. The sort of deviation found here is expected when the number of sites at which reaction can cause inactivation is less than eight. The reason for this is evident when one considers that a 0.3% formaldehyde solution will reach equilibrium at any given site with only about half of that site reacted; this case has been dealt with by Penniston and Doty (1963a).

CONCLUSIONS

These results make it evident that the critical active sites of the transfer RNA behave in a very similar manner for both the amino acid acceptor and transfer steps. From this similar inactivation behavior we may infer that there are essential recognition sites on the transfer RNA which are common to both steps and, furthermore, that there are no essential ex-

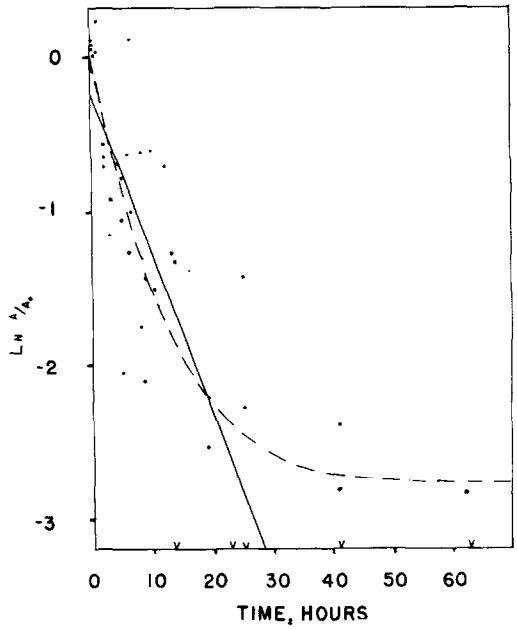

Figure 4. Inactivation by 0.3% Formaldehyde of Phenylalanyl Transferring Ability of E. coli Transfer RNA; in 0.005M Acetate Buffer, pH 4.6, at 25°C.

The solid line represents the least squares fit to the points for less than 15 hours. The dashed line represents the course of inactivation predicted for the case of five sensitive sites reacting with average rate and equilibrium constants. This line is calculated from equation 4 (Penniston and Doty, 1963a) and uses the same constants as were used in calculating the curves for Figure 5 in that paper. The arrows represent points indistinguishable from background.

posed recognition sites such as would be required on the hypothesis of an exposed coding triplet.

The apparent species specificity of the acceptor step would appear to rule out such common recognition sites. However, the recent work of Loftfield and Eigner (1963) has shown that in the cases of valyl and isoleucyl transfer RNAs, these specificities apply only to the rates of reaction and not to the Michaelis binding constants. The specific activating enzyme "recognizes" its appropriate RNA from any species with equal facility, but in some cases the subsequent reaction is slow. Thus, as they also conclude, there is no need to postulate separate recognition sites for acceptor and transfer abilities. The results presented here give positive evidence that

these sites are similar, and that an exposed triplet complementary to that of the messenger is not involved.

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