INACTIVATION OF ALANINE ACCEPTANCE ACTIVITY OF YEAST TRANSFER RNA BY PHOTOCHEMICAL REDUCTION OF URACIL

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Received May 8, 1967

Despite very considerable work relating modification of bases in transfer RNA (tRNA) with inactivation of its functions, no clear picture of the sites of these functions has emerged except that the integrity of the anti-codon is essential for mRNA induced amino acid incorporation [1]. This report records the first part of a new effort in which modifications of single bases are carried out in a progressive manner so that eventually the bases crucial to activity can be identified. In the work reported here inactivation of alanine acceptance by unfractionated yeast tRNA was examined to determine the sensitivity of this activity towards the photochemical reduction of uracil. It is found that modification of about 8 uracil bases per tRNA molecule, that is about half of those present, are required for inactivation.

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1) For reviews, see Brown, 1963; Brown and Lee, 1965; Miura, 1967; Engelhardt and Kisselev, 1966.

RESULTS

Assay conditions were selected so that the incorporation of C^{14}-alanine into tRNA was a nearly linear function of tRNA concentration (Figure 1), and the incubation time with yeast extract gave maximum incorporation (Figure 2). Prior to incubation with yeast extract, tRNA was allowed to react with $NaBH_4$ in the pres-

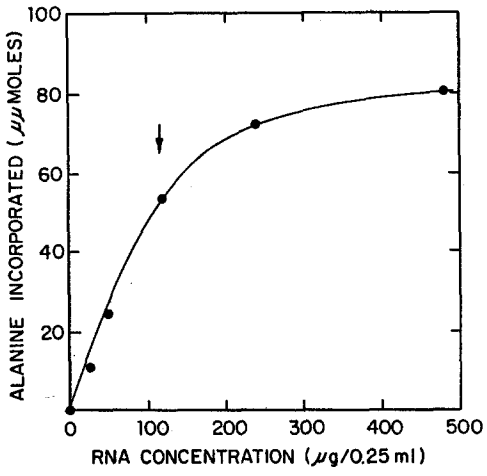

Fig. 1

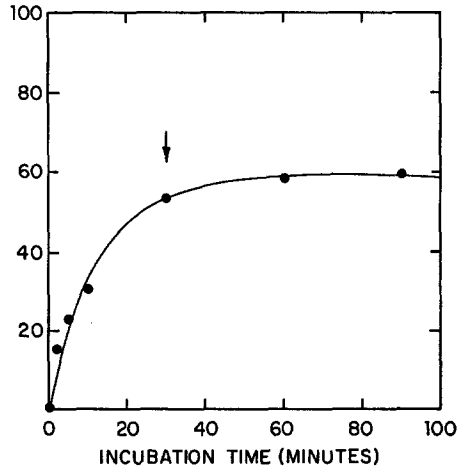

Fig. 2

Figure 1. Incorporation of alanine as a function of tRNA concentration. Mixtures contained 0.025M Tris-chloride, pH 7.5, 0.005M $MgCl_2$, 0.0025M ATP, 0.0005M EDTA, 0.020mM C^{14}-alanine (specific activity, 7400 cpm/mμmole), 1.0 mg/ml of yeast extract (prepared by the method of Stephenson and Zameenik, 1961, as modified by Hoskinson and Khorana, 1965), the indicated amount of yeast tRNA (prepared from Fleischman's yeast by the method of Monier, Stephenson, and Zameenik, 1960, as modified by Holley, 1963), in a volume of 0.25 ml, and were incubated at 37° for 30 minutes. Reactions were terminated by placing the tubes in ice and immediately adding 4 ml of ice-cold 5% trichloroacetic acid (TCA). The tubes were decanted onto glass fiber filters (Whatman GF/B, 2.4 cm diameter) in a stainless steel holder under suction. The tubes were rinsed 3 times with 4 ml portions of cold 5% TCA, and the filters rinsed 3 times with 4 ml portions of cold 5% TCA, followed by 1 ml of acetone to speed drying of the filters. The filters were placed in glass vials containing 10 ml of scintillation fluid (toluene containing 0.5% PPO and 0.02% POPOP), and the C^{14} incorporated into the tRNA determined using a Packard scintillation counter. Results in this figure are the mean of duplicates. The arrow indicates the usual tRNA level.

Figure 2. Incorporation of alanine as a function of incubation time. All mixtures contained 120 μg of tRNA, and reactions were terminated at the indicated times. Other details of the assay procedure are given for figure 1. The arrow indicates the usual incubation time for the later experiments.

ence of ultraviolet light, conditions which should result in the specific conversion of uracil to 5, 6-dihydrouracil (Cerutti, Ikeda, and Witkop, 1965). At intervals, aliquots were removed from the reaction mixture for determination of alanine acceptance (Figure 3). Zero order inactivation was observed.

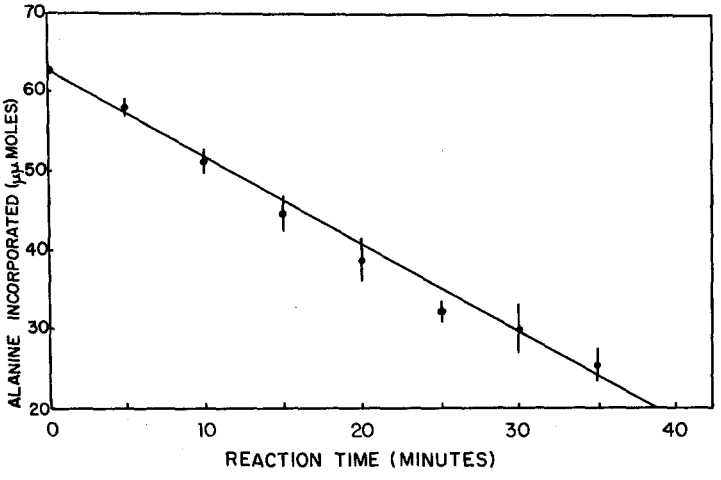

Figure 3. Inactivation of alanyl aminoacylation activity. Reaction conditions based on method of Cerutti et al. (1965). A solution containing 6.0 mg/ml of yeast tRNA, 0.017M $MgCl_2$, 0.10M $NaBH_4$, in 0.60 ml total volume was irradiated in a stoppered 3 ml quartz cuvette with a Vycor filter at 1 cm from a 15W General Electric germicidal lamp. At 2.5 minute intervals, the mixture was shaken, and at 5.0 minute intervals, a 20 μl aliquot was taken for the alanine acceptance assay. The excess $NaBH_4$ was neutralized with 2 μl of 0.8M HCl, and activity then determined directly, as described for figure 1. Data represents 3 separate experiments, and the dispersion of the data is given as the standard error of the mean (68% confidence limits).

To check the specificity of the reaction under these conditions, aliquots were removed, subjected to alkaline hydrolysis (0.3 M KOH, 18 hr, 37°), and the four major nucleotides separated using paper electrophoresis at pH 3.5 (in 0.05M $HCOONH_4$ at 25 V/cm for 8 hours; Davidson and Smellie, 1952), at which pH ψMP is not resolved from GMP. The reaction specificity may be inferred from the apparent base composition of tRNA following reduction

(Table I), as only the UMP is observed to decrease. Minor nucleotides are not detectable by this method.

Table I. Composition of tRNA (%).

Reaction time	0 min	40 min	% at 40 min / % at 0 min
UMP	22.0±0.8	15.7±0.4	0.72±0.04
GMP + ψMP	32.0±0.7	35.7±0.3	1.11±0.03
AMP	20.2±0.2	21.7±0.2	1.07±0.02
CMP	25.8±0.2	27.0±0.3	1.05±0.02

Reduction was carried out using $NaBH_4^3$ to determine the number of hits per tRNA molecule (Figure 4). Following the initial

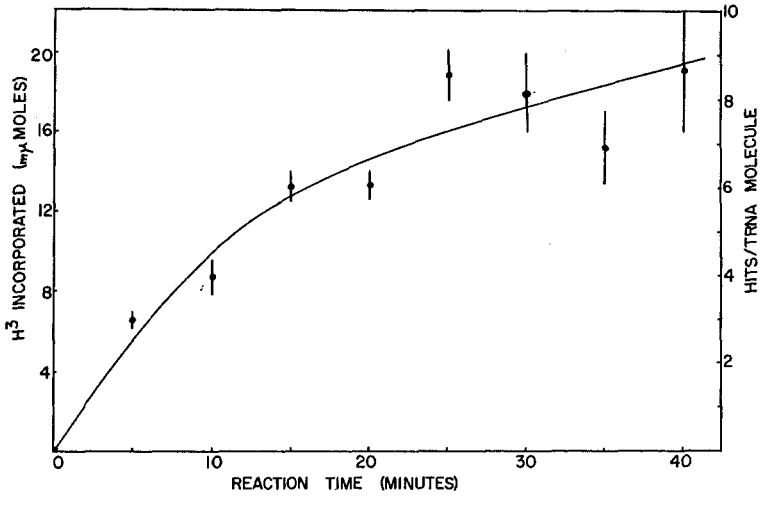

Figure 4. Uptake of H^3 during reduction of tRNA using $NaBH_4^3$. Reaction conditions are given for figure 3. At the indicated times, duplicate 10 μl aliquots were removed, spotted on pre-moistened glass fiber filters, and rinsed with ten 4 ml portions of cold 5% TCA, and 1 ml of acetone. Data represents the mean of 4 experiments, and the dispersion of the data is given as the standard error of the mean.

rapid uptake of H^3, corresponding to 3 to 4 hits per tRNA molecule, the rate decreased six-fold. At the maximum extent of reaction, about 8 hits per tRNA molecule had been introduced. Assuming a

chain length of 80 for the average tRNA molecule, there are about 18 UMP residues available for reaction.

To determine the extent of disruption of secondary structure of the tRNA optical rotary dispersion spectra were obtained. At 0, 20, and 40 minutes, the reaction was stopped by the addition of 54µl of 1M HCl, then diluted into 26.8 ml of a buffer containing 0.01 M $MgCl_2$ and 0.005 M Tris-chloride, pH 7.5, to give an A260 of 1.5. Spectra were obtained with a Cary 60 spectropolarimeter. The decrease in magnitude of the rotation difference (Table II) is so marked as to indicate that the secondary structure is being altered throughout the region where inactivation is observed.

TABLE II. Change of optical rotation: $[\alpha]_{278m\mu} - [\alpha]_{252m\mu}$

Reaction time	$[\alpha]$
0 min	3920 deg
20 min	3660 deg
40 min	3200 deg
0 min (hydrolysate)	1070 deg

That the magnitude of this rotational difference may be taken as an index of secondary structure is suggested by the observation that this difference decreases with increasing temperature of tRNA solutions (Sarin, Zamecnik, Bergquist, and Scott, 1966).

DISCUSSION

The number of hits per tRNA molecule required to inactivate alanine acceptance can be estimated by assuming that the hits are introduced at random, and that their distribution within the population of tRNA molecules is described by the Poisson function.

On this basis the fraction of molecules, N/N_o, which can still accept alanine when an average of λ hits per molecule have been introduced is taken as the fraction containing fewer than λ hits, and may be calculated using the expression,

$$N/N_o = \sum_{x=0}^{\lambda-1} \frac{\lambda^x}{e^{\lambda}\, x!}$$

for x hits, which varies from zero to $(\lambda-1)$ for the surviving molecules. The time required to introduce λ hits, the number postulated to be necessary for inactivation, is obtained from the H^3 uptake curve (Figure 4): with this, the theoretical zero

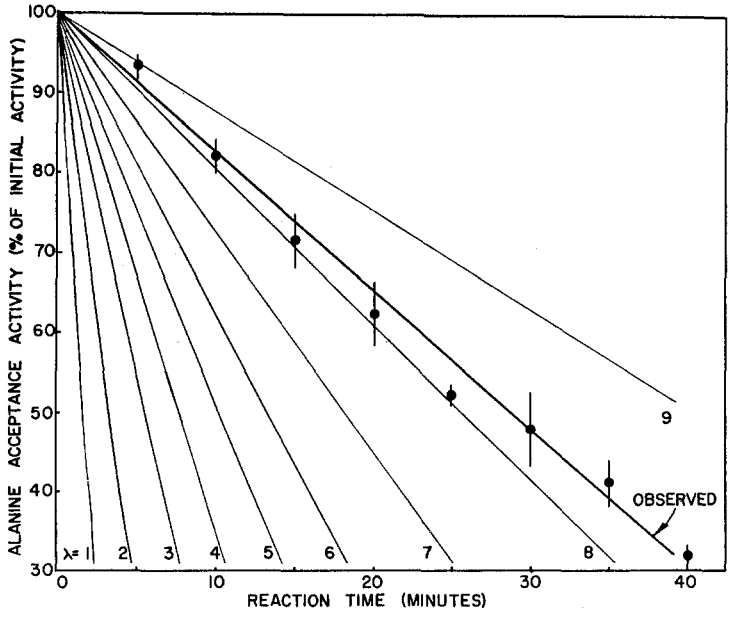

Figure 5. Observed and predicted rates of inactivation of alanine acceptance. Curve designated observed is the actual decrease of acceptance expressed as a percentage of initial activity. Curves designated by λ are the calculated inactivation curves assuming that λ hits per molecule are required for inactivation. The time needed to introduce λ hits per molecule was obtained from the H^3 uptake experiment shown in figure 4.

order inactivation curves can be calculated for comparison with the observed rate of inactivation, as shown by Figure 5. This analysis rests on the fundamental assumption that the species of tRNA which accept alanine are affected in the same way as the population of tRNA molecules as a whole. On this basis, about 8 uracil residues must be reduced before alanine acceptance is inactivated, but by then, disruption of tRNA secondary structure has progressed rather far. Further work with purified tRNA will be required to improve the correlation of the number of hits with inactivation of acceptance activity, and to locate the site of the hits.

A number of other workers have determined the effects of specific chemical modifications of bulk yeast tRNA on alanine acceptance (Cerna, Rychlik, and Sorm, 1964 and 1966; Rake and Tener, 1966; Yoshida, Duval, and Ebel, 1966; Muto, Miura, Hayatsu, and Ukita, 1965; Kikugawa, Muto, Hayatsu, Miura, and Ukita, 1967; Kisselev, Zaitseva, and Frolova, 1965). Their results are not strictly comparable since differences exist for sources of tRNA and yeast extract, as well as assay conditions. Three of the reports, however, mention modification of UMP. Cerna et al. (1964) found that treatment of yeast tRNA with hydroxylamine at pH 9, a reaction fairly specific for uracil which results in ring opening (Schuster, 1961), gave only about 5% inactivation of alanine acceptance although an average of 6 hits per molecule were introduced, and the other acceptance activities assayed were decreased by 20 to 60%. Kisselev et al. (1965) also found that alanine ac-

ceptance was relatively resistant towards this modification. And from the data of Yoshida et al. (1966), using ICl to iodinate uracil, it can be calculated that 8 to 9 hits per tRNA molecule were required to inactivate alanine acceptance.

Thus alanine acceptance activity is rather insensitive towards modification of uracil, and it is likely that the loss of this activity following photochemical reduction is due to general disruption of the ordered tRNA structure.

Acknowledgement. This work was completed during the tenure of a postdoctoral fellowship to R. A. from the National Institute of General Medical Sciences, U. S. Public Health Service and was supported by National Institutes of Health Grant HD 01229

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