REDUCED LEVELS OF 5,6-DIHYDROURIDINE IN FLUOROURACIL-CONTAINING TRANSFER RNAs FROM *SACCHAROMYCES CEREVISIAE*

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1. Introduction

When 5-fluorouracil is added to growing cultures of either bacteria or yeast, extensive replacement of uracil occurs in the RNA of these organisms (see review by Mandel [1]). In tRNA from *Escherichia coli*, the minor pyrimidine nucleosides of pseudouridine [2–4], ribothymidine [3, 4], 4-thiouridine [5] and 5,6-dihydrouridine [6] are also replaced to at least the same extent as uracil. In yeast, pseudouridine and ribothymidine are also replaced by FU [7], but the amount of 5,6-dihydrouridine was reported to be unaffected by the incorporation of FU [7]. This inconsistency, with respect to dihydrouridine, led to an examination of the dihydrouridine content of FU-tRNA from *Saccharomyces cerevisiae*. Results by two different methods indicate that the decrease in dihydrouridine content of FU-tRNA essentially parallels the decrease in uridine.

2. Methods

These studies were carried out with *Saccharomyces cerevisiae* (wild type), kindly provided by Dr. Martha Christensen. Cells were grown in a New Brunswick 14 l Microferm under forced aeration at 30° in media G of Galzy and Slonimski [8] with the modifications of Giege and Ebel [9]. Growth was followed by measuring turbidity at 420 nm. In the early exponential phase of growth a portion of the culture was harvested and used for the preparation of N-tRNA. FU was then added to a final concentration of 25 μg/ml and incubation continued for 6 (batch no. 1) or 10 (batch no. 2) hr. The cells were harvested, washed, and stored as previously described [10].

The tRNA was extracted from whole-cells, essentially as described for *E. coli* [10]. It was further purified by chromatography on long columns (0.9 × 230 cm) of Sephadex G-75.

DEAE-cellulose chromatography, base hydrolysis, and paper chromatography of the nucleotides from the tRNAs were carried out as previously reported [10]. The uridylic and 5-fluorouridylic acid spots were located under ultraviolet light, cut out, eluted, and their relative amounts calculated from their ultraviolet absorbance [10].

One assay for dihydrouridine was carried out as described by Molinaro et al. [11], using a Beckman DU monochromater thermostated at 25° and a Gilford model 222 photometer. The other assay was based on the colorimetric method of Hunninghake and Grisolia [12] as modified by Jacobson and Hedgcoth [13]. The latter procedure was performed exactly as described, except the assay was scaled up 3-fold so that the final volume was 1.08 instead of 0.36 ml.

The standard buffer was composed of 0.01 M potassium cacodylate (pH 7), 0.15 M KCl, 0.005 M $MgCl_2$ and 0.0005 M EDTA.

Abbreviations:

Hrd	: 5,6-dihydrouridine
FU	: 5-fluorouracil
FU-tRNA	: tRNA containing FU
N-tRNA	: tRNA from normal cells
A_{260}-unit	: a unit of material which in a volume of 1 ml will have an absorbance of one at 260 nm when measured in a cell of 1-cm path length.

3. Results and discussion

Transfer RNA isolated from FU-treated cells had 26–35% of the uracil replaced by FU (table 1). The extent of replacement in the sample could be increased by separating the N-tRNA from the FU-containing material as previously described for FU-tRNA from *E. coli* [10]. Fig. 1 shows the results of passing a sample of FU-tRNA, having 26% of the uracil replaced by FU, over DEAE-cellulose and eluting with a concave-upward NaCl gradient. The material eluting in fraction A had only 13% of the uracil replaced by FU, whereas fraction C had 52%.

Molinaro et al. [11] described the time-dependent loss of absorbance at 235 nm when tRNA was placed in 0.1 M KOH. This decrease in absorbance offered a semi-quantitative method for the estimation of the dihydrouridine content of RNA. Data from three different samples of tRNA, containing 0, 26, and 52% FU replacement of uracil are shown in fig. 2. Although this method is based on looking at a small change in a large number, which is inherently inaccurate, it seems

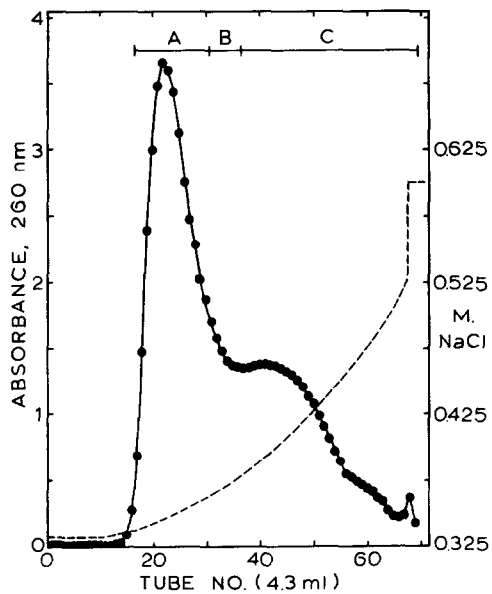

Fig. 1. DEAE-cellulose column chromatography of FU-tRNA. FU-tRNA from yeast (318 A_{260}-units) with 26% of the uracil residues replaced by FU was chromatographed as previously described for FU-tRNA from *E. coli* [10]. Recovery of A_{260}-absorbing material was 98%. Absorbance, •—•; NaCl gradient, - - - - -.

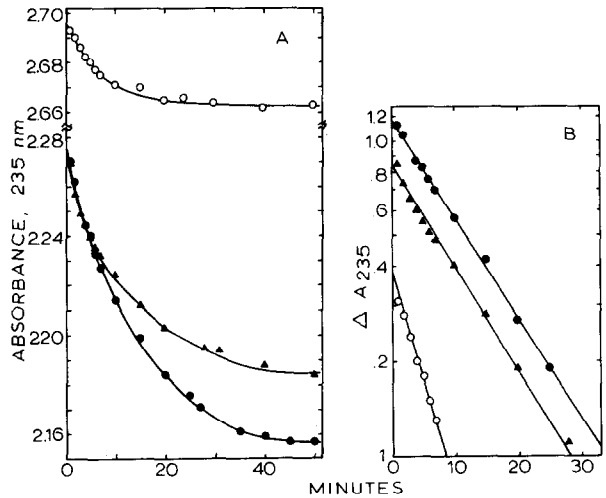

Fig. 2. (A) Plot of absorbance at 235 nm as a function of time for N-tRNA and FU-tRNAs in 0.1 M KOH. N-tRNA, •—•: FU-tRNA with 26% (▲—▲) and 52% (○—○) of its uracil replaced by FU. (B) Plot of A_{235} *minus* A_{235} at 50 min (final A_{235}) on a logarithmic scale. Data from (A). The ordinate intercept is Δ A_{235}. See paper by Molinaro et al. [11] for details. These samples of N-tRNA, FU-tRNA (26%), and FU-tRNA (52%) and A_{260}-values of 2.98, 3.12, and 3.81 respectively, in standard buffer.

clear that the dihydrouridine content decreases with increasing FU replacement of uracil. The mole percentage of dihydrouridine, as determined by this procedure, in several samples of tRNA is summarized in table 1 under assay 1.

The second method used for the determination of dihydrouridine in RNA was based on a colorimetric procedure specific for ureido groups [6, 7]. In this procedure, an RNA solution must first be treated with alkali to convert dihydrouridine to an open-ring structure (*N*-ribosyl-3-ureidopropionic acid). Since 5-fluoro-5,6-dihydrouracil is also very labile to alkali [14], any reduced FU should also be detected in this assay. The content of dihydropyrimidines found in several samples of tRNA containing varying amounts of FU shown in table 1 under assay 2.

The mole percentage values obtained by the two assay methods are somewhat different (table 1). This may be due, at least in part, to imprecision in the extinction coefficient for dihydrouridine, as pointed out by Molinaro et al. [11]. In these experiments however, the Hrd content of the samples may be ex-

Table 1
Dihydrouridine content in N- and FU-tRNAs from *Saccharomyces cerevisiae*.

tRNA Preparation[a]	Dihydrouridine[b]				
	Assay 1		Assay 2		
	Mole %[c,d]	$\frac{\text{Hrd} \times 100}{\text{Hrd N-tRNA}}$	Mole %[c,e]	$\frac{\text{Hrd} \times 100}{\text{Hrd N-tRNA}}$	$\frac{\text{U}}{\text{U + FU}} \times 100$[f]
Normal (commercial)	3.73	–	4.29 (±0.35)	–	–
Normal from normal cells	3.47	100	4.05 (±0.34)	100	100
Fraction A, fig. 1	3.33	96	3.92 (±0.37)	97	87 (±1.0)
FU-tRNA, batch no. 1	2.48	71	2.72 (±0.10)	67	74 (±0.6)
FU-tRNA, batch no. 2	1.98	57	2.90 (±0.24)	72	65 (±1.7)
Fraction B, fig. 1	2.14	62	2.45 (±0.20)	60	74 (±1.5)
Fraction C, fig. 1	0.89	26	1.60 (±0.08)	40	47 (±0.6)

[a] Normal (commercial) tRNA was purchased from Schwarz/Mann and purified on Sephadex G-75. Fractions A, B, and C correspond to those shown in fig. 1 after chromatography of FU-tRNA, batch no. 1. FU-tRNA, batch no. 1 and no. 2, represents material before DEAE-cellulose chromatography.

[b] Assay 1 was based on the time dependent loss of absorbance at 235 nm [11]. Assay 2 was the colorimetric method of Hunninghake and Grisolia [12] with the modifications of Jacobson and Hedgcoth [13].

[c] Mole % was calculated by assuming a tRNA solution of 1 mg/ml would give an A_{260} of 24 for a 1-cm light path at room temperature in standard buffer. The average weight used for a micromole of tRNA nucleotide was 340 μg [15].

[d] A value of 9.2×10^3 was used for the molar extinction coefficient of dihydrouridine at 235 nm, pH 13 [11].

[e] Standard deviations are shown in parentheses. 4 to 8 determinations were run on each preparation.

[f] Standard deviations are shown in parentheses. 3 to 5 determinations were run on each preparation.

pressed as a percentage of the control. When expressed in this manner there is generally good agreement in the percentage of dihydrouridine present in the different samples (table 1).

The amount of dihydrouridine found in FU-tRNA from *S. cerevisiae* essentially parallels the amount of remaining uridine. Similar results have been found with FU-containing tRNA from *E. coli* [6]. The remaining dihydrouridine presumably arises from unreplaced uridine and does not represent 5-fluoro-5,6-dihydrouridine, which should have been detectable by the assay methods used in these experiments. These findings suggest that fluorouridine cannot serve as a substrate leading to dihydropyrimidine formation in either yeast or bacteria. Additional support for this idea is provided by the more recent results of Giegé et al. [16] and also by those of Johnson and Horowitz [17], who have found a lower content of Hrd in FU-containing tRNAs from yeast.

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