

ON THE HETEROGENITY OF INDIVIDUAL tRNA^{Val}₁ THAT ARISES IN THE COURSE OF MODIFICATION WITH CME-CARBODIIMIDE*

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1. Introduction

It was demonstrated recently in this laboratory that valine tRNA affords on modification with CME-carbodiimide a heterogeneous population of molecules that differ considerably in the extent of modification [1]. The fractionation method applied [1], however, could not provide good yields of fractions homogenous with respect to the extent of modification.

It appeared that fractionation of modified tRNA^{Val}₁ by chromatography on BDEAE-cellulose provides much better resolution.

2. Materials and methods

Valine tRNA₁ from baker's yeast was isolated by a combination of three chromatographic procedures: (a) chromatography on DEAE-Sephadex A-25 at pH 6.5 [2]; (b) chromatography in the RPC-2 system of Kelmers and Weiss [3]; (c) chromatography on BDEAE-cellulose at neutral pH in the presence of Mg²⁺ ions [4]. The valine tRNA₁ obtained incorporated 1800–1900 pmoles of valine per 1 A₂₆₀ unit (optical density at 260 nm A₂₆₀ was measured in 0.1 M Tris-HCl, pH 7.5, – 0.02 M MgSO₄) with rat liver valine: tRNA ligase. The terminal guanylyl-ribonuclease test [5] showed a relative content of tRNA^{Val}₁ of about 85%.

* CME-carbodiimide: *p*-toluenesulfonate of *N*-cyclohexyl, *N'*-β-(4-methylmorpholinium)ethylcarbodiimide.

[³H]CME-carbodiimide, labelled at the methyl group (about 20 Ci/mole) was obtained from [³H]methyl *p*-toluenesulphonate as described earlier [6].

BDEAE-cellulose was obtained by the method of Gillam et al. [4]. Radioactivity was counted using a Mark I "Nuclear Chicago" scintillation counter with Bray's dioxan scintillator. The extent of tRNA^{Val}₁ modification and the acceptor activity

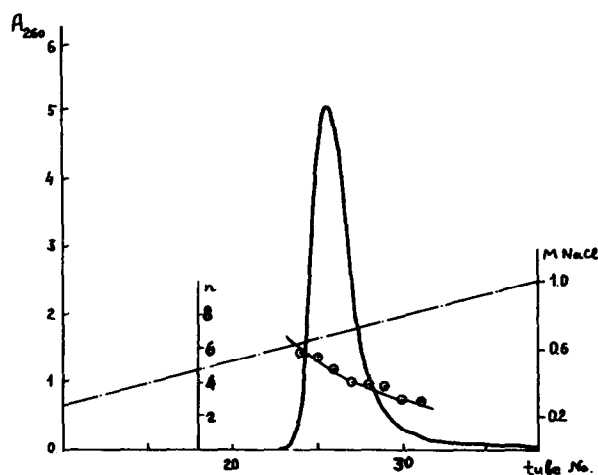


Fig. 1. Chromatography of modified tRNA^{Val}₁ on BDEAE-cellulose. DEAE-cellulose (TLC, "Serva"). Column 1.2 × 15 cm; linear gradient, 150 ml of each 0.02 M sodium acetate buffer, pH 5, and the same buffer with 1 M NaCl; flow 15.6 ml per hr; fraction volume 7.8 ml. Application was 150 A₂₆₀ units of tRNA^{Val}₁ modified with CME-carbodiimide to *n* = 6 (1.1 × 10⁷ cpm). — A₂₆₀; —○— extent of modification, *n*, moles of CME-nucleoside residues per mole of tRNA; - - - concentration of NaCl, M.

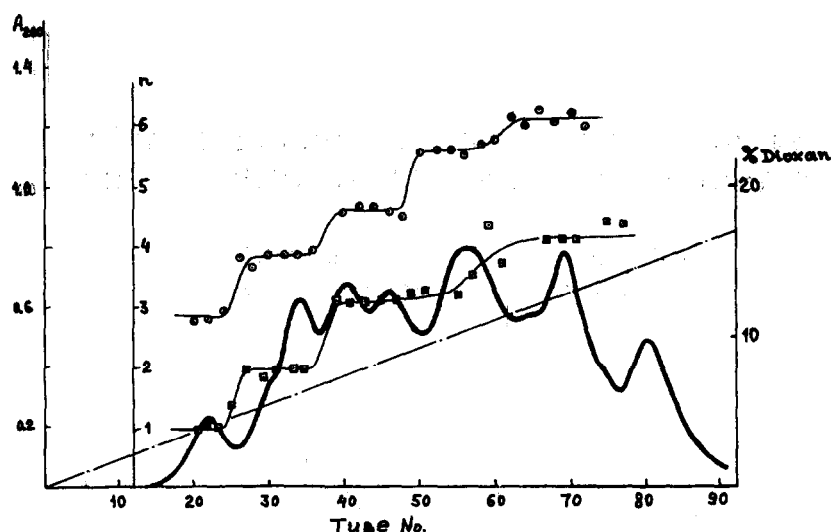


Fig. 2. Chromatography of modified $\text{tRNA}_1^{\text{Val}}$ on BDEAE-cellulose. Rechromatography of fractions 25–29 (fig. 1). Column 1×20 cm; linear gradient, 180 ml of each 1 M NaCl and 1 M NaCl in 30% dioxan (peroxide free); fraction volume 128 drops; flow about 10 ml per hr. Application was 106 A_{260} units of modified $\text{tRNA}_1^{\text{Val}}$ average $n = 4.9$. — A_{260} ; —○— extent of modification n , moles per mole of $\text{tRNA}_1^{\text{Val}}$; —□— content of CME-nucleosides stable to mild alkaline hydrolysis (see text); - - - concentration of dioxan, percent.

were determined as described earlier [1]; the reaction conditions are given in the text and in figure legends. The isolation of modified $\text{tRNA}_1^{\text{Val}}$ from reaction mixtures was by gel-filtration on Sephadex G-50 in 0.1 N NaCl, unless otherwise stated. To estimate the content of CME-nucleosides stable to mild alkaline hydrolysis (presumably, CME-pseudouridine residues [7]) 1/10 volume 1 M carbonate-bicarbonate buffer pH 10 was added, the solution incubated for 15 hr at room temperature and subjected to gel-filtration on Sephadex G-50 in 0.1 N NaCl. The known extent of modification of a given $\text{tRNA}_1^{\text{Val}}$ fraction was multiplied by the ratio of polymer to monomer radioactivities.

3. Results and discussion

The fractionation discussed below was done with modified $\text{tRNA}_1^{\text{Val}}$ obtained in a preparative experiment and intended initially for structural studies. Valine tRNA_1 was modified by incubation for 30 hr in 0.15 M CME-carbodiimide at 25° ,

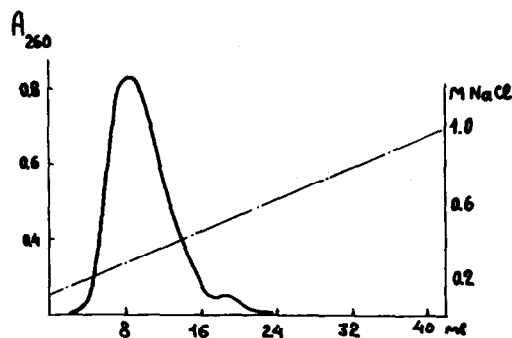


Fig. 3. Chromatography of native $\text{tRNA}_1^{\text{Val}}$ on BDEAE-cellulose. Column 0.4×18 cm; linear gradient, 20 ml each of 0.02 M MgSO_4 , 0.1 M NaCl and 0.02 M MgSO_4 , 1 M NaCl, flow about 1 ml/hr. Application was 8 A_{260} units of native $\text{tRNA}_1^{\text{Val}}$. — A_{260} ; - - - concentration of NaCl, M.

pH 8.0, in 0.1 N *N*-methylmorpholinium chloride–0.02 M MgSO_4 . In these conditions, 9 moles of CME-carbodiimide added per mole of tRNA , as revealed by gel-filtration of a portion of the reaction mixture. Isolation of the major part of the modified

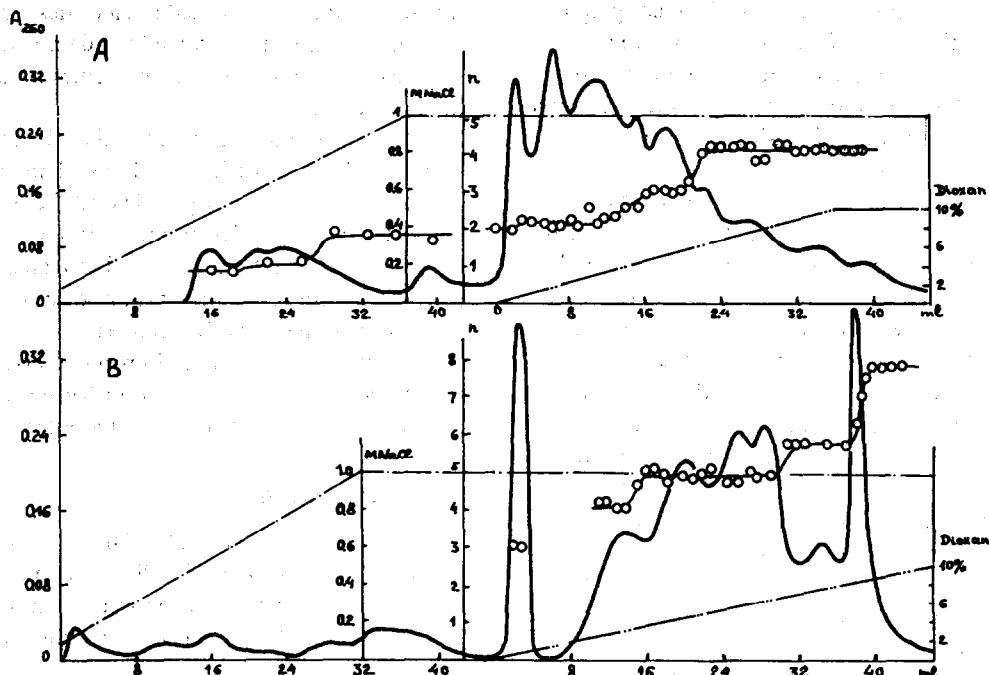


Fig. 4. Chromatography of modified tRNA^{Val} on BDEAE-cellulose. Column 0.4×18 cm; two linear gradients, first, 20 ml each of 0.02 M MgSO_4 , 0.1 M NaCl and 0.02 M MgSO_4 , 1 M NaCl; second, 20 ml each of 0.02 M MgSO_4 , 1 M NaCl and 0.02 M MgSO_4 , 1 M NaCl in 30% dioxan, flow about 1 ml/hr. A: Application was 6 A_{260} units modified $\text{tRNA}_1^{\text{Val}}$, $n = 2.6$, see text. B: Application was 6 A_{260} units modified $\text{tRNA}_1^{\text{Val}}$, $n = 5.2$, see text. — A_{260} ; —○— extent of modification n , moles per mole $\text{tRNA}_1^{\text{Val}}$; - - - Concentration of NaCl, M; - . - - Concentration of dioxan, percent.

$\text{tRNA}_1^{\text{Val}}$ from the reaction mixture was effected by ethanol precipitation. A 30% loss of nucleotide material occurred* and the average extent of modification dropped to 6 moles of CME-nucleoside residues per mole. The modified tRNA obtained was subjected to chromatography on DEAE-cellulose as described earlier [1] (see fig. 1). The use of CME-carbodiimide of higher specific radioactivity provided greater accuracy. It appears that the specific activity of modified tRNA in the right part of the peak continuously decreases and does not remain constant as claimed in our previous paper [1].

The central part (from $n = 5.6$ to $n = 3.8$) of the peak was rechromatographed on BDEAE-cellulose (see fig. 2). Unlike native $\text{tRNA}_1^{\text{Val}}$, modified $\text{tRNA}_1^{\text{Val}}$ is not eluted with NaCl up to 1 M in the absence of dioxan. Elution with a dioxan gradient from 0 to 30% on a 1 M NaCl background afforded 7 peaks of opti-

cal density, as seen in fig. 2. The extents of modification increase stepwise along this profile from $n = 3$ in the first peak to $n = 6$ in the last one. The relative content of CME-nucleosides stable to mild alkaline hydrolysis* also changes stepwise from $n = 1$ to $n = 4$. It is noteworthy that two fractions, the third and the fourth peaks, are modified to the same extent.

Acceptor activity of modified $\text{tRNA}_1^{\text{Val}}$ measured after chromatography on DEAE-cellulose was about 1500 pmoles of valine per A_{260} unit. Studies of acceptor activity along the profile obtained on BDEAE-cellulose did not reveal inactive fractions. All of them exhibited acceptor activity higher than 1400 pmoles of valine per A_{260} unit.

* CME-pseudouridine is stable to mild alkaline hydrolysis [7]; the stability of CME-dihydrouridine [8] is unknown; all the other CME-nucleosides will be completely converted to starting nucleosides under the conditions applied [9, 10] (see "Materials and methods").

* Probably, because of insufficient cooling.

The good resolution obtained in chromatography of modified tRNA^{Val} on BDEAE-cellulose appeared not to be an artefact since rechromatography of one of the peaks (the fourth one) on an analytical column afforded a single major peak with maximum at the same concentration of dioxan.

Having obtained these somewhat unexpected results we subjected to chromatographic analysis, with the new method, preparations of modified tRNA^{Val} isolated at the beginning and the end of the reaction. Figs. 3 and 4 show elution patterns on an analytical column of BDEAE-cellulose. It is seen that native tRNA^{Val} is eluted as a single peak in the NaCl gradient with maximum about 0.28 M NaCl (fig. 3). Figs. 4A and 4B show the chromatographic patterns of modified tRNA^{Val} preparations isolated by gel-filtration from the reaction mixture (0.055 M CME-carbodiimide; 0.1 M *N*-methylmorpholinium chloride; 0.02 M MgSO₄; 25°; pH 8.0) at 23 and 80 hr after the beginning of reaction. It is seen that even at a small extent of modification ($n = 2.6$; fig. 4A) there is a shift of nucleotide material to fractions eluted by higher concentrations of NaCl and by the dioxan gradient. Quite clearly seen is the heterogeneity of fractions modified to the same extent ($n = 1$, two peaks; $n = 2$, three peaks). At the higher extent of modification ($n = 5.2$), corresponding to the kinetic "plateau" (see [11]), modified tRNA^{Val} does not become less heterogenous, although fractions of small extents of modification ($n = 1-2$) practically disappear (fig. 4B). Again clearly seen is the heterogeneity of fractions of the same extent of modification ($n = 5$, three peaks).

We have already pointed out [1, 11] that the extensive heterogeneity of individual RNA's which arises in the course of modification with CME-carbodiimide and which has been followed by several methods [1, 11-14], finds a ready explanation in a model [11] that assumes changes in the conformation of RNA in the course of modification due to electrostatic interaction of positively charged CME-nucleoside residues with the negatively charged polyribose phosphate backbone. According to this model, modification begins with random attack of a few "exposed" nucleosides that are different in different tRNA molecules. Due to above mentioned interactions these different molecules will appear in different conformations that have greater or

smaller numbers of exposed nucleosides. The above data support the model since they demonstrate extensive heterogeneity even of molecules modified to the same extent.

In conclusion it will be noted that the affinity of modified tRNA to BDEAE-cellulose depends mainly on the presence of hydrophobic cyclohexyl moieties of CME-nucleosides on the surface of the macromolecule since fractions modified to a greater extent are eluted at higher concentrations of dioxan in spite of their lower net negative charges.

The fractionation method proposed affords a wide variety of active modified tRNA molecules for comparative structural and functional studies.

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