INCORPORATION OF 4-THIOURIDINE INTO RNA IN GERMINATING RADISH SEEDS

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1. Introduction

Animal cells metabolize sulfur-containing analogues of nucleic acid precursors into RNA. Rat liver slices [1] and mouse myeloma cells [2] incorporate 2-thiouridine into RNA. BHK-21/C cells (baby hamster kidney cell line) [3] can incorporate 6-thioguanosine and 4-thiouridine into RNA. The relative incorporation of these sulfur-containing nucleosides into specific RNA species such as mRNA and rRNA appeared constant [2,3]. RNA containing sulfur-nucleosides can be separated from non-sulfur-containing RNA by affinity chromatography with mercurated-supports [2,3].

4-Thiouridine occurs naturally as a minor constituent in bacterial tRNA [4–6], but not in plant tRNA [7]. The 4-thiouridine absorbed during the germination of seeds has an inhibitory effect on chloroplast development [8–10]. However, the metabolism of 4-thiouridine in germinating seeds remains obscure. We now report the incorporation of 4-thiouridine into the RNA of germinating radish seeds and the separation of the 4-thiouridine-containing RNA by affinity chromatography on mercurated cellulose.

2. Materials and methods

4-Thiouridine was synthesized and purified as in [8]. The concentration of 4-thiouridine was determined using an absorbance coefficient of 21.5×10^3 $M^{-1} \cdot cm^{-1}$ at 331 nm [11]. [^{14}C]Methylamine–HCl (50.1 mCi/mmol) was obtained from New England Nuclear. Oligo(dT)-cellulose was a product of Boehringer. Mercurated cellulose (Hg-cellulose) was prepared by the method in [12].

2.1. *Germination with 4-thiouridine*

Radish seeds (*Raphanus sativus*) were germinated and grown with or without 0.5 mM 4-thiouridine in the dark for 4 days at 22–25°C, as detailed in [9].

2.2. *RNA preparation*

Excised radish cotyledons were homogenized in an ice-cold mortar with 0.05 M Tris–HCl (pH 9.0) containing 5 mM $MgCl_2$, 1% (w/v) sodium dodecyl sulfate, 0.2% (v/v) Triton X-100 and 5 μg/ml of polyvinyl sulfate (potassium salt). The homogenate was stirred vigorously for 30 min with an equal volume of phenol–chloroform [phenol (adjusted to pH 9.0 with Tris): chloroform = 1:1]. The aqueous phase was re-extracted with phenol–chloroform. RNA was precipitated from the aqueous phase by addition of 2 vol. ethanol and stored at −20°C. The precipitate was collected, washed with 80% ethanol, then dissolved in and dialyzed against water. The concentration of RNA was estimated assuming that 1 mg RNA/ml has an $A^{1\,cm}_{260\,nm}$ of 20 [13]. The fractionation of RNA into poly(A)-RNA, high M_r RNA and low M_r RNA was performed essentially by the methods in [3]. The RNA preparation dissolved in 10 mM Tris–HCl (pH 7.4) containing 0.5 M NaCl was applied to a column of oligo(dT)-cellulose equilibrated with 0.5 M NaCl in 10 mM Tris–HCl (pH 7.4). After washing the column with the same buffer to elute poly(A)-free RNA, the poly(A)-containing RNA was eluted with 10 mM Tris–HCl (pH 7.4). Poly(A)-free RNA was precipitated with 2 vol. ethanol and after which it was dissolved in 3 ml 10 mM Tris–HCl (pH 7.4) containing 10 mM NaCl and 10 mM EDTA. LiCl (2 ml, 5 M) was added and the mixture was stored overnight at 4°C before centrifugation at 10 000 × *g* for 10 min. The pelleted, high M_r RNA was dissolved in and dialyzed against water. The supernatant containing low M_r RNA was dialyzed against water.

2.3. *Derivertization of RNA*

4-Thiouridine-containing RNA (1.4 mg) was incubated with 7.8 mM [^{14}C]methylamine (1 μCi, pH 10.4) and 7.8 mM $NaIO_4$, which was added last to initiate the reaction [11], in a 1 ml total vol. at 40°C for 90 min. At specified intervals, samples (100 μl) were withdrawn and poured into 1.5 ml ice-cold 10% (w/v) trichloroacetic acid. The precipitate was collected on a Millipore paper and washed with 25 ml 10% trichloroacetic acid. Radioactivity incorporated into the RNA was measured in an aqueous scintillator system using an Aloka LSC-602 liquid scintillation counter.

2.4. *Mercurated cellulose chromatography*

RNA in 4 mM Tris–HCl (pH 7.4) containing 0.5 M KCl and 4 mM EDTA was applied to an Hg-cellulose column (0.9 × 5 cm) which had been equilibrated with the application buffer, monitoring the unbound RNA by A_{260}. The bound RNA was eluted with 50 mM 2-mercaptoethanol in 4 mM Tris–HCl (pH 7.4), monitoring the A_{330}. Under these conditions, at least, 100 μmol 4-thiouridine bound to 1 g Hg-cellulose (0.9 × 5 cm) and was eluted with 2-mercaptoethanol; a recovery of 80–90%.

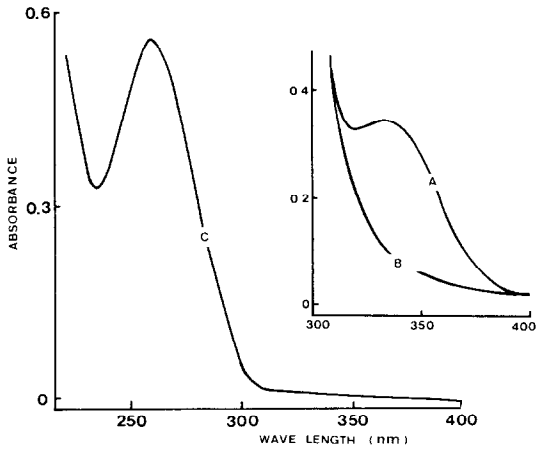

Fig.1. Absorption spectra of the RNA from radish cotyledons. Radish seeds were germinated and grown with 1 mM potassium phosphate (pH 7.0) with or without 0.5 mM 4-thiouridine, in the dark at 22–25°C for 4 days. The RNA isolated from 2 g excised cotyledons was dissolved in 5 ml water. (A) RNA from 0.5 mM 4-thiouridine-cultured radish cotyledons. (B) RNA from control cotyledons germinated and grown without 4-thiouridine. (C) The two RNA solutions diluted 100-fold with water.

3. Results

3.1. *Incorporation of 4-thiouridine into radish RNA during germination*

Radish seeds were germinated with 0.5 mM 4-thiouridine containing 1 mM potassium phosphate (pH 7.0) and were grown for 4 days in the dark. The concentrated RNA prepared from the 4-thiouridine-cultured radish cotyledons showed a distinct peak at 331 nm that was 0.6–0.7% of the peak at 260 nm (fig.1A). The RNA preparation from control plants germinated and grown without 4-thiouridine lacked this peak (fig.1B). Both diluted RNA preparations had absorption profiles characteristic of RNA, with an absorption minimum and a maximum at 232 and 258 nm, respectively (fig.1C).

Since 4-thiouridine and 4-thiouridylate have maximum absorptions at 331 nm [4–6], as compared to the usual maximum near 260 nm, the presence of 4-thiouridine residues in the RNA can be monitored by this characteristic absorption maximum. 4-Thiouridine in *Escherichia coli* tRNAs reacts with hydrogen peroxide [5,6] or hydroxylamine [14] to give uridine of N^4-hydroxycytidine, respectively. In addition, the 4-thiouridine residues of tRNA are transformed in the presence of periodate and [^{14}C]methylamine (at pH 10.4) to labeled N^4-methylcytidine residues [11]. These chemical modifications occur selectively at the 4-thiouridine residues in RNA [5,6,11,14].

The effect of selective chemical modification by hydrogen peroxide (pt A) and hydroxylamine (pt B) on the absorption profile between 300 and 400 nm of the RNA preparation from the 4-thiouridine-cultured radish cotyledons is shown in fig. 2. The characteristic peak at 331 nm of the RNA disappeared with time due to selective chemical modification, evidence that 4-thiouridine is present in the RNA preparation. A residual absorption at 331 nm after the modification was attributed to the tail from the UV absorption of the concentrated RNA, because the control RNA containing no 4-thiouridine residues showed a similar absorption curve (fig.1B).

Thus, the contents of 4-thiouridine in RNA can be determined spectrophotometrically by the difference in A_{331} before and after the chemical modification of RNA with hydrogen peroxide. By this spectrophotometric analysis, we estimated that the RNA from 0.5 mM 4-thiouridine-cultured radish cotyledons contains 7–10 nmol 4-thiouridine/mg RNA (table 1). More-

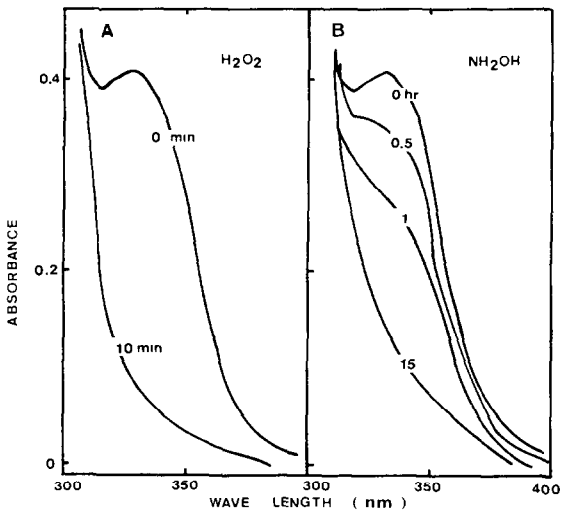

Fig.2. Effect of selective chemical modification on the absorption spectra of the RNA from 4-thiouridine-cultured radish cotyledons: (A) treated with 0.176 M hydrogen peroxide in 10 mM Tris–HCl (pH 8.0), (B) treated with 0.5 M hydroxylamine in 10 mM potassium phosphate (pH 7.0).

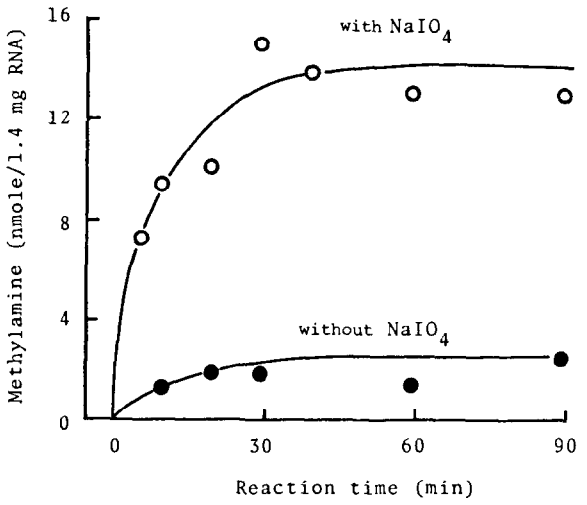

Fig.3. Kinetics of the incorporation of ^{14}C from [^{14}C]methylamine into RNA from 4-thiouridine-cultured radish cotyledons. RNA (1.4 mg) containing 12.1 nmol 4-thiouridine residues (determined spectrophotometrically as in the text) was incubated with [^{14}C]methylamine with or without $NaIO_4$ as in section 2. The assay was for trichloroacetic acid-insoluble radioactivity.

over, incubation of the RNA from 4-thiouridine-cultured radish cotyledons in [^{14}C]methylamine [11] resulted in the periodate-dependent incorporation of ^{14}C from [^{14}C]methylamine into the trichloroacetic acid-insoluble fraction (fig.3). These results also demonstrate the presence of 4-thiouridine in the RNA.

Table 1
Properties of 4-thiouridine-containing RNA

	Total RNA[a]	Chloroplast RNA[b]
4-Thiouridine in RNA (nmol/mg RNA)[c]	7.28	3.75
Hg-cellulose-bound RNA		
(% total amount)	3.94	3.60
(A_{331}/A_{260} × 100)	5.72	3.38
(mol% 4-thiouridine)[d]	2.2	1.3

[a] RNA prepared from 0.5 mM 4-thiouridine-cultured, 4-day-old etiolated radish cotyledons
[b] 4-Thiouridine-cultured, 4-day-old radish seedlings were illuminated for 24 h (2500 lux). RNA was prepared from chloroplasts, which were isolated from greening radish cotyledons and purified by sucrose density gradient centrifugation as in [9]
[c] Determined spectrophotometrically as in the text
[d] Calculated from the A_{331}/A_{260} × 100 ratio using the equation in [15]

The incorporation of ^{14}C reached a plateu when 8.6 nmol ^{14}C/mg RNA was bound. This value was in fair agreement with that obtained by the spectrophotometric method above.

3.2. *Separation of the 4-thiouridine-containing RNA by affinity chromatography on mercurated cellulose*

The radish RNA from 0.5 mM 4-thiouridine-cultured etiolated cotyledons was applied to Hg-cellulose (fig.4). After washing out the unbound RNA, the bound RNA was eluted with 2-mercaptoethanol. Some 3–4% of the RNA bound to the mercurated cellulose. After dialysis to remove 2-mercaptoethanol, the bound RNA gave an A_{331}/A_{260} × 100 ratio of 5.72. This value corresponds to 2.2 mol% of 4-thiouridine in the bound RNA (table 1), assuming that the A_{331}/A_{260} × 100 ratio of 2.03 for *E. coli* tRNAs equals 0.875 mol% of the 4-thiouridine residue [15].

The properties of chloroplast RNA from isolated-chloroplasts of 4-thiouridine-cultured radish cotyledons illuminated at 2500 lux for 24 h are shown in table 1. Chloroplast RNA also contained 4-thiouridine residues of ~4 nmol/mg RNA. Some of the RNA bound to the Hg-cellulose and was eluted with mer-

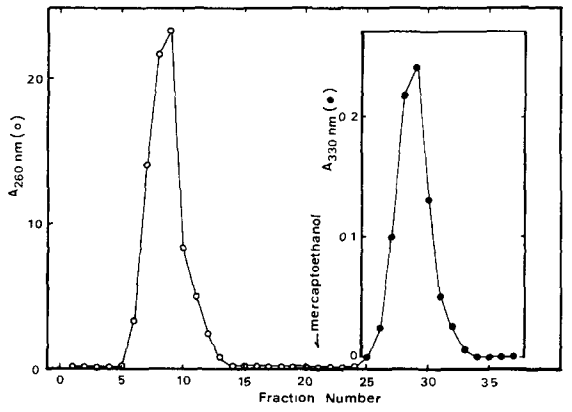

Fig.4. Affinity chromatography of RNA on mercurated cellulose. RNA prepared from 0.5 mM 4-thiouridine-cultured radish cotyledons was applied to an Hg-cellulose column (0.9 × 5 cm) equilibrated with 0.5 M KCl containing 4 mM Tris–HCl (pH 7.4) and 4 mM EDTA. Non-bound RNA was eluted with KCl solution. Bound RNA was eluted with 50 mM 2-mercaptoethanol in 4 mM Tris–HCl (pH 7.4). Elution of the bound RNA was monitored at A_{330}.

captoethanol. The mol% of 4-thiouridine in the bound chloroplast RNA was estimated to be 1.3.

The total RNA preparation from 0.5 mM 4-thiouridine-cultured, etiolated radish cotyledons was fractionated into poly(A)-, high M_r, and low M_r RNAs by the methods in [3]. The low M_r and high M_r RNAs showed distinct peaks at 331 nm, and their 4-thiouridine residues were spectrophotometrically calculated to be 19.7 and 6.5 nmol/mg RNA, respectively (table 2). A part of each fractionated RNA bound to the Hg-cellulose, and was eluted with 2-mercaptoethanol, indicating the presence of 4-thiouridine in each RNA species (table 2). Thus, we concluded that the 4-thiouridine absorbed by radish seeds is incorporated into mRNA, rRNA, and into chloroplast RNA.

Table 2
Properties of fractionated RNA species from 0.5 mM 4-thiouridine-cultured radish cotyledons

RNA species	4-thiouridine (nmol/mg RNA)[a]	Hg-cellulose-bound (% total amount)
Poly (A)	–	12.3
High M_r	6.50	1.95
Low M_r	19.7	6.51

[a] Determined spectrophotometrically as in the text

4. Discussion

At least 3 sulfur-containing analogues of nucleic acid precursors, 6-thioguanosine [3], 2-thiouridine [1,2] and 4-thiouridine [3], are incorporated into the RNA in animal cells. The incorporation of these analogues into RNA was demonstrated mainly by the retention of the RNA on a mercurial support. We demonstrated more clearly the incorporation of 4-thiouridine into the RNA of germinating radish seeds by 4 different methods. Three methods: (1) the absorption maximum of isolated RNA at 331 nm (fig.1), (2) the selective chemical modification of the RNA (fig.2), and (3) the incorporation of radiocarbon into the RNA from [^{14}C]methylamine in the presence of periodate (fig.3), provided the conclusive proof of the presence of 4-thiouridine residues in bacterial tRNAs [4–11]. The fourth method was the retention of the RNA on Hg-cellulose (fig.4).

Poly(4-thiouridylic acid) can act as mRNA for the synthesis of polyphenylalanine [16]. Following treatment of baby hamster kidney cells with 4-thiouridine, the newly synthesized RNA containing 4-thiouridine occupied >60% of the total RNA (calculated from the results in [3]). However, the incorporation of 4-thiouridine into RNA does not result in the inhibition of protein synthesis, or in the production of abnormal proteins [3]. The tRNAs of *E. coli*, strain B synthesized in the presence of 5-fluorouracil have up to a 100% replacement of the uridine residues by 5-fluorouracil, but the incorporation of 5-fluorouracil into tRNA does not affect its ability to accept amino acids [17]. From these results and from the relatively low content (3–5% of the total RNA) of 4-thiouridine-containing RNA (table 1), the inhibition of chloroplast development by the 4-thiouridine culture [8–10] might not be fully accounted for by the presence of 4-thiouridine-containing RNAs. However, we observed [8] a reduced RNA synthesis at an early stage of germination of the 4-thiouridine-cultured radish cotyledons. Thus, 4-thiouridine, or one of its anabolized derivatives such as 4-thiouridine-5′-triphosphate, probably inhibits RNA synthesis thereby causing the inhibition of chloroplast development.

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