MULTIPLE SPECIES OF THIONUCLEOSIDES IN RAGI (*ELEUSINE CORACANA*) tRNA

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ABSTRACT

Presence of multiple species of thionucleosides in ragi tRNA has been shown by the incorporation of ^{35}S-label into tRNA. The four major thionucleosides have been identified as 5-methylamino-methyl-2-thiouridine, 2-methylthioribosyl-cis-zeatin, 5-methyl-2-thiouridine and N[N-(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl) carbamoyl] threonine. This is the first demonstration of the presence of 5-methylaminomethyl-2-thiouridine, 5-methyl-2-thiouridine and N[N-(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl) carbamoyl] threonine in a plant tRNA.

INTRODUCTION

TRANSFER RNA contains various classes of modified nucleosides of which the thionucleosides are unique themselves. These are derivatives of either uridine or adenosine with the exception of 2-thiocytidine. Derivatives of 2-thiouridine and 2-methylthio adenosine, when present, occur at the 5′ end of the anticodon and next to the 3′ end of the anticodon respectively[1]. These nucleosides have been demonstrated to enhance the specificity of codon recognition[2] and the ability for aminoacylation[3] of tRNA. However, 5-methyl-2-thiouridine (m^5s^2U) has been shown to replace ribothymidine at position 54 in *Thermus thermophilus* tRNA[4]. Although the distribution of thionucleosides is widespread among prokaryotes, they seem to be present only occasionally in the eukaryotes. Methylthioisopentenyl adenosine (ms^2i^6A) and methylthioribosylzeatin (ms^2io^6A) are the only thiolated nucleosides reported to occur in plant tRNA[5]. tRNAs from yeast, rat liver, and rabbit liver are reported to contain thionucleosides[6]. These data come from the nucleotide sequence analyses. No systematic study on the thionucleosides of eukaryotic tRNA is yet available, less so of plant tRNAs. In this paper we report the presence of multiple species of thionucleosides and identification of the four major thionucleosides in the tRNA of ragi seedlings. Three of these thionucleosides are being reported for the first time in a plant tRNA.

MATERIALS AND METHODS

Ribonuclease T2 was obtained from Sigma Chemical Co., USA. Bacterial alkaline phosphatase was from Worthington Biochemical Corporation, USA, and plastic backed cellulose and silica thin layers from Macherey-Nagel, Germany. ^{35}S-Labelled H_2SO_4 was from Bhabha Atomic Research Centre, Bombay, India. 2-Methylthioisopentenyl-adenosine and *cis*- and *trans*-isomers of 2-methylthioribosyl-zeatin were a kind gift from Professor J Corse, United States Department of Agriculture.

Surface sterilized ragi seeds (1g) were germinated in the dark at 30° for 24 hr in distilled water under sterile conditions. The germinated seeds were then grown in the dark in the presence of neutralised carrier-free ^{35}S-H_2SO_4 for a further 72 hr period. Chloramphenicol (50 μg/ml) was added during the culture to maintain asceptic conditions. tRNA was extracted from the labelled seedlings by the phenol-SDS method and DEAE-cellulose chromatography. Contaminating polysaccharides were removed by extraction of RNA into 2-methoxyethanol and precipitation by acetyl trimethyl ammonium bromide[7], followed by elution of tRNA from a 8% polyacrylamide gel. The purified tRNA was deacylated at pH 8.8 (Tris, 0.5 M) for 3 hr at 37°C[8]. *E. coli* and *A. tumefaciens* were grown in minimal media containing ^{35}S-H_2SO_4, and tRNAs were prepared by SDS-phenol extraction and DEAE-cellulose chromatography. They were deacylated as described above. The tRNAs were digested to nucleotides by treatment with RNase T2 at pH 4.5 (ammonium acetate, 20 mM) for 16 hr at 37°. The thionucleotides were dephosphorylated by incubation with bacterial alkaline phosphatase at pH 8.8 (Tris, 100 mM) for 3 hr at 37°. ms^2t^6Ap was converted to ms^2Ap by treatment with 0.1 N NaOH at 100° for 1 hr in a sealed tube[9]. High voltage electrophoresis of thionucleotides on Whatman 3 mm paper was run at pH 3.5 (pyridine-acetate), at about 75 V/cm. Two dimensional thin layer chromatography on cellulose was according to Nishimura[10], in the following solvent systems: (I). Isobutyric acid: 0.5 M NH_3, 5:3 (v/v), (II). Isopropanol:HCl:H_2O, 70:15:15 (v/v/v).

RESULTS

The ^{35}S-labelled tRNA isolated as described was free of radioactive polysaccharide contamination as judged by gel filtration before and after ribonuclease digestion (data not shown). The labelled tRNA was hydrolyzed by RNase T2 and the thionucleotides were separated by electrophoresis on Whatman 3 mm paper. The autoradiogram showed the presence of four major thionucleotides and a few minor species (figure 1a). The major species were numbered 1 through 4 and their relative proportions were determined by cutting the spots and counting for radioactivity (table 1). Spot No. 1 varied in its proportion from preparation to preparation. The

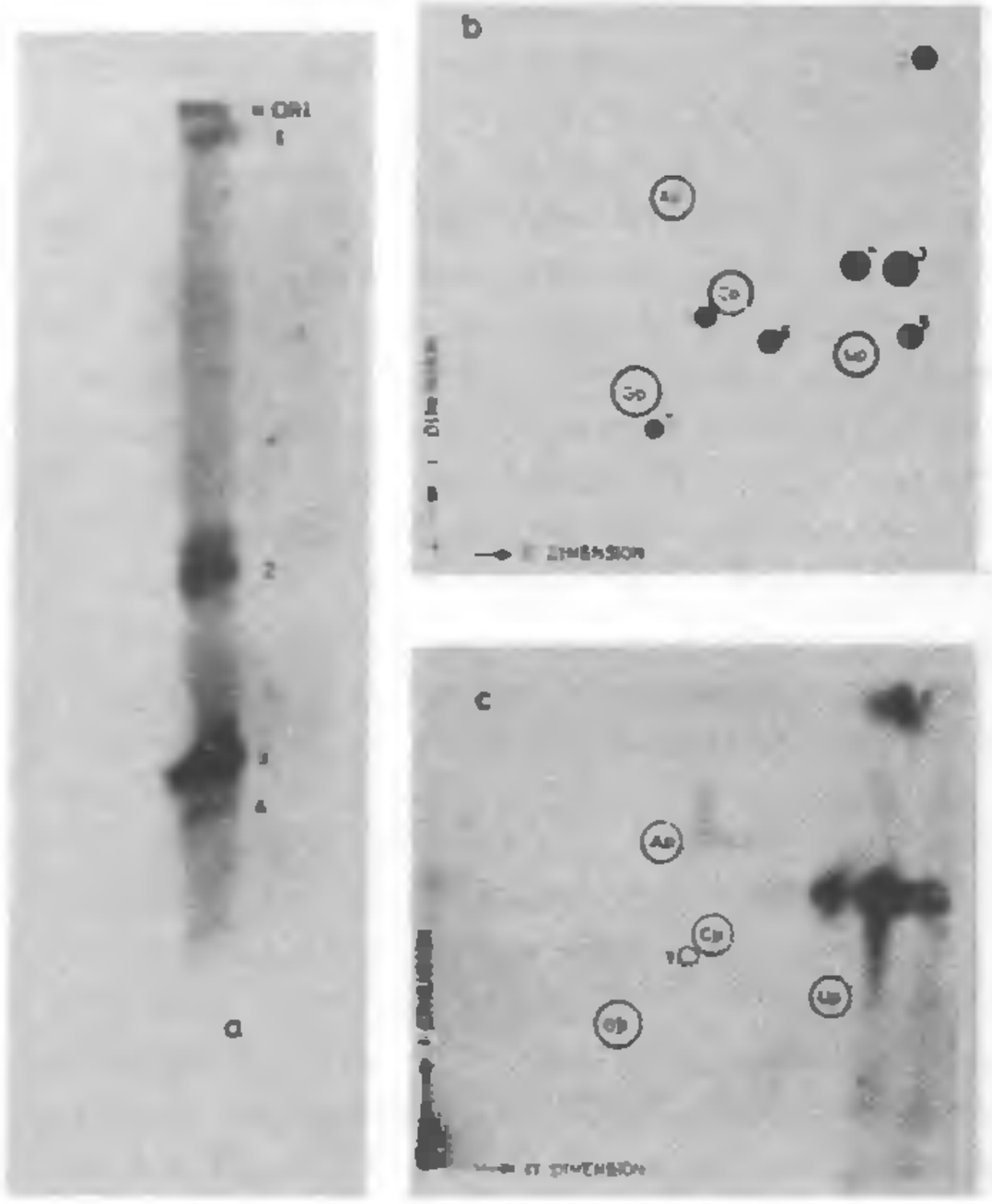

Figure 1. Resolution of thionucleotides from ragi tRNA. (a) Autoradiogram showing high voltage electrophoretic separation of thionucleotides from the RNase T_2 digest of ^{35}S-labelled ragi tRNA. (b). A schematic representation of the relative positions of thionucleotides on a two-dimensional cellulose thin layer chromatogram (Ref. 10). 1,mnm^5s^2Up; 2,ms^2io^6Ap; 3,m^5s^2Up; 4,ms^2t^6Ap; 5,s^4Up; 6,s^2Cp and 7,$cmnm^5s^2Up$. (c). Autoradiogram showing two-dimensional thin layer chromatographic separation of thionucleotides from the RNase T_2 digest of ragi tRNA.

Table 1 *Relative proportions of Thionucleotides in Ragi tRNA*

Spot No.	% radioactivity
1	20.0
2	25.8
3	28.8
4	10.7

Relative proportions were determined by cutting the spots in figure 1a and counting for radioactivity. The four major spots accounted for 85.3% of the total.

RNase T2 digest was also resolved by two dimensional thin layer chromatography on cellulose (figure 1c), and the thionucleotides were identified by analysis of the spots.

Identification of thionucleosides: The electrophoretic mobilities of spots 1 and 2 suggested their identities as 3′ phosphates of 5-methylaminomethyl-2-thiouridine (mnm^5s^2Up) and 2-methylthioribosyl zeatin (ms^2io^6Ap) respectively[11]. These identities were established as follows: (a) Standard mnm^5s^2Up was prepared by growing *E. coli* in ^{35}S-sulphate containing media. ms^2i^6Ap and ms^2io^6Ap were prepared from ^{35}S-labelled *A. tumefaciens* cells. The RNase T2 digest of ^{35}S-ragi tRNA was coelectrophoresed with these standard thionucleotides. Spots 1 and 2 had the same electrophoretic mobilities as those of standard mnm^2s^2Up and ms^2io^6Ap respectively (figure 2). (b) Spots 1 and 2 were eluted, converted to nucleosides and chromatographed on cellulose and silica thin layer with mnm^5s^2U from *E. coli* and authentic samples of ms^2i^6A and *cis*- and *trans*-isomers of ms^2io^6A as markers. Spot 1 migrated with the same R_f as that of mnm^5s^2U and spot 2 had the R_f of *cis*-ms^2io^6A (table 2). This was consistent with the presence of the corresponding ms^2io^6Ap species in the two dimensional thin layer chromatogram (figure 1c). Small amounts of ms^2i^6Ap also could be seen just above spot 2 (figure 1a). Since ms^2i^6Ap and ms^2io^6Ap are closely related and the former is present only in small amounts it is not treated separately.

Spot No. 3 was presumed to be a thiouridine derivative because of its electrophoretic mobility near Up and because it contained a free thiol group (figure 3, See Ref. 12). Its identity was established as m^5s^2Up by its conversion to nucleoside and chromatography in three different solvent systems. In all the systems the thionucleoside had the identical R_f values of those for authentic m^5s^2U (table 2). Presence of a spot at the

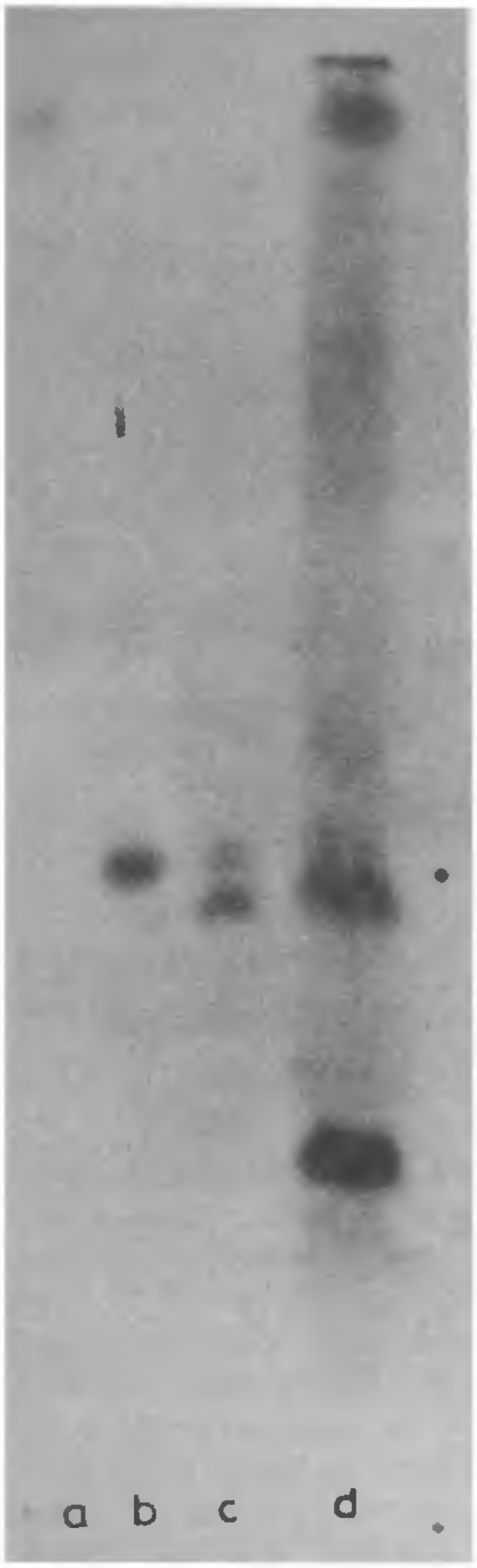

Figure 2. Autoradiogram showing the electrophoretic mobilities of mnm^5s^2Up (lane a), ms^2i^6Ap (lane b), and ms^2io^6Ap (lane c). The RNase T_2 digest of ragi tRNA was coelectrophoresed (lane d).

position indicated from m^5s^2Up in the two dimensional thin layer chromatogram[10] further supported this conclusion. CNBr-resistance of spot No. 4 (figure 3) suggested that it contained a methylthio group. So far only three methylthiolated nucleosides have been reported: ms^2i^6A, ms^2io^6A and N-[N-(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl) carbamoyl] threonine, (ms^2t^6A). Because of the higher electrophoretic mobility, spot No. 4 was assumed to be ms^2t^6Ap. Treatment with alkali converts ms^2t^6Ap to ms^2Ap and threonine[9]. When spot No. 4 was treated with alkali, electrophoresed and the paper cut and counted for radioactivity, it was seen that the major radioactive species migrated a little ahead of Ap which we believe is ms^2Ap (figure 4). Because of the low level of radioactivity, further characterization of spot No. 4 was not done and we tentatively conclude that spot 4 represents ms^2t^6Ap. Further evidence came from the presence of a species at the position characteristic for ms^2t^6Ap in the two dimensional chromatographic analysis (figure 1c).

Table 2 *R_f values of Thionucleosides from Ragi tRNA*

Sample	Solvent systems A	B	C	D	Identity
Spot No. 1		0.49			mnm^5s^2U
mnm^5s^2U (From *E. coli*)		0.49			
Spot No. 2				0.50	cis-ms^2io^6A
Authentic ms^2io^6A-cis				0.50	
authentic ms^2io^6A-trans				0.39	
authentic ms^2i^6A				0.71	
Spot No. 3	0.66	0.55	0.75		m^5s^2U
authentic m^5s^2U	0.66	0.55	0.75		

Spots 1, 2 and 3 (figure 1a) were converted to nucleosides. Chromatographic separations with thionucleoside standards were done in solvents A, B and C using cellulose thin layers, and in solvent D using silica thin layer. Solvent systems: *A*, Butanol : Acetic acid : Water, 5 : 3 : 2 (v/v/v); *B*, Isopropanol : Conc. NH_3 : Water, 7 : 1 : 2 (v/v/v); *C*, 95% Ethanol : Water, 4 : 1 (v/v) and *D*, Chloroform : Methanol, 9 : 1 (v/v).

DISCUSSION

Thionucleosides are not so much a feature of eukaryotic tRNA as of prokaryotic tRNA. Only about 10 or so of the 150-odd eukaryotic tRNA sequences contain a thiolated nucleoside. ms^2i^6A and ms^2io^6A are the only thiolated nucleosides known to occur in plant tRNAs. Here we have identified the presence of multiple species of thionucleosides in ragi tRNA and characterized among them the four major ones.

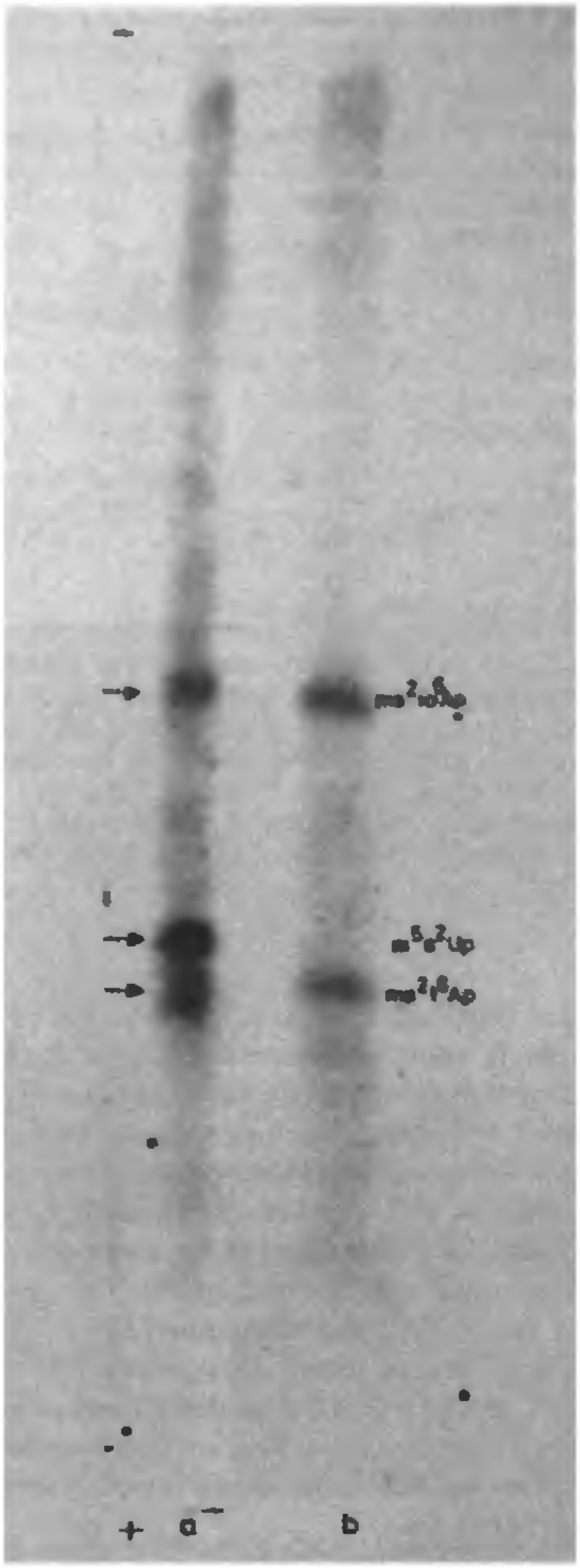

Figure 3. Effect of CNBr treatment on thionucleotides of ragi. RNase T_2 digest of ragi ^{35}S-labelled tRNA was treated with CNBr (Ref. 12) and subjected to high voltage electrophoresis on Whatman 3 MM paper. (a) Control, (b) Treated.

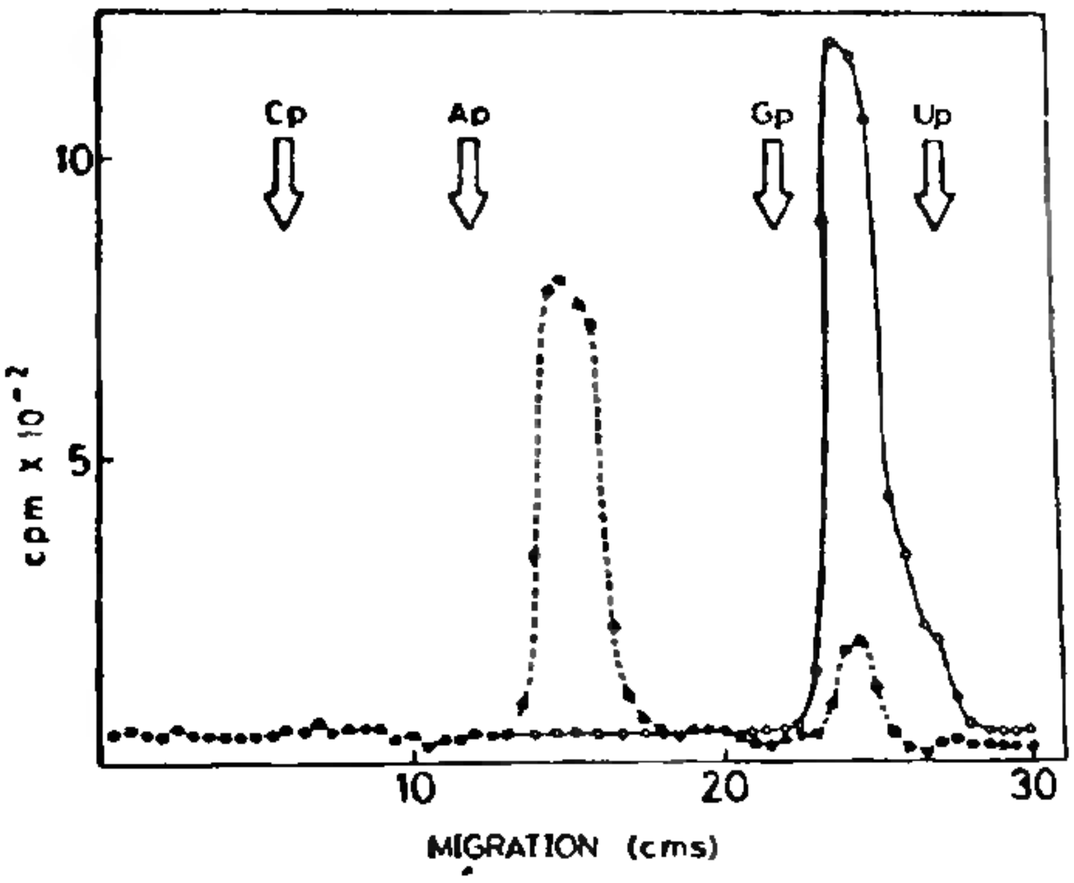

Figure 4. Analysis of thionucleotide spot No. 4. The thionucleotide was eluted from the paper, treated with 0.1 M NaOH at 100°C for 1 h, neutralized and reelectrophoresed on Whatman 3 MM paper. The paper was cut into 0.5 cm strips and counted for radioactivity. Solid line, no treatment; broken line, alkali traatment. Cp, Ap, Gp and Up are cold nucleotide markers.

Cytokinin-active ribonucleosides have long been known to occur in the tRNA of bacteria, animals and plants. Although all the four cytokinin-active ribonucleosides (i^6A, io^6A, ms^2i^6A and ms^2io^6A) have been shown to be present in plant tRNA (5), zeatin riboside (io^6A) is the one that is most frequently present. On the contrary, in ragi tRNA it appears that the major cytokinin-active ribonucleoside is the thiomethylated derivative of io^6A (cis-ms^2io^6A). This view is borne from the fact that io^6A could not be detected in ^{32}p-labelled ragi tRNA hydrolysates (data not presented). Zeatin riboside is mostly present as its *cis*-isomer in tRNA[13]. However in Pisum, tRNA from leaf contained both *cis*- and *trans*-isomers[13], whereas tRNA from roots contained only the *cis*-isomer[14]. The thiomethylated derivative of zeatin riboside in ragi tRNA is found to be in *cis*-isomeric form.

mnm^5s^2U has so far not been reported in any eukaryotic tRNA. In prokaryotes it is widely distributed and occurs at the 5′ end of the anticodon[6,15]. It is interesting that this prokaryote specific thionucleoside is present in ragi tRNA. Its presence cannot be due to contamination by bacteria as the seeds were grown under asceptic conditions and chloramphenicol was included in the growth medium. It is possible that this is present in the plastid tRNA. Also mnm^5s^2U in ragi tRNA varies in its proportion from preparation to preparation. Its presence in very low amounts in figure 1c as opposed to figure 1a is an illustration of this

variation. Many modified nucleosides vary in their content upon perturbation of the environment in which the organism grows[16]. Also Chackalaparampil and Cherayil[11] have envisaged a precursor-product relationship between m^5s^2U and mnm^5s^2U. Thus, the variation in the proportions of mnm^5s^2U may have some important implications with respect to the functioning of tRNA.

ms^2t^6A so far has been reported only in the $tRNA^{Lys}$ of *B. subtilis* and rabbit liver, where it occurs at the 37th position[6] m^5s^2U is found in the tRNAs of *T. thermophilus* and rat liver. Although most 2-thiouridine derivatives occur at the 5′ end of the anticodon, m^5s^2U replaces ribothymidine at position 54 in *T. thermophilus*[4]. It will be interesting to see where and in which ragi tRNA m^5s^2U is present. Amino-acylation experiments using total tRNA and ^{14}C-lysine showed that lysine acceptor activity decreases significantly after CNBr treatment of the tRNA (data not presented). The fact that CNBr removes free thiol groups[12] and that the nucleosides at the anticodon loop are involved in the recognition of tRNA by cognate aminoacyl tRNA-synthetases make us believe that either m^5s^2U or mnm^5s^2U is present in the anticodon loop of ragi $tRNA^{Lys}$.

ACKNOWLEDGEMENT

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ANNOUNCEMENT

WORLD ENVIRONMENT DAY

Bharathiar University, Coimbatore will be celebrating the World Environment Day in August 1984 in collaboration with the Department of Environment, New Delhi. The aim of the programme will be to create public awarness on environmental aspects like environmental hygiene, conservation and management of environment, impact of population explosion, towards clean environment etc.

For details please write to: Dr R. Manavalaramanujam, Staff-in-Charge, World Environment Day Celebration, Department of Zoology, Bharathiar University, Coimbatore 641 046.