CULTURE CONDITION-DEPENDENT CHANGES OF RIBOTHYMIDINE IN BACTERIAL TRANSFER RNA

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ABSTRACT

Transfer RNA of the non-symbiotic nitrogen-fixing bacterium, *Azotobacter vinelandii* when grown under nitrogen fixing conditions contains the modified nucleoside ribothymidine to the extent of one nucleoside per tRNA molecule. On the other hand, the tRNA synthesised by the bacterium in the presence of ammonium salt, *i.e.* in the absence of nitrogen fixation, does not contain ribothymidine. The tRNA from *Rhizobium meliloti* which fixes nitrogen symbiotically also lacks ribothymidine when grown in the presence of ammonium salt. This is the first report of a change in the content of ribothymidine in the tRNA of an organism dependent upon culture conditions. The possible causes for such a change are discussed.

INTRODUCTION

RIBOTHYMIDINE is one of the unique modified nucleosides occurring in most tRNAs except the initiator methionine tRNA[1]. It occurs at the 54th position from the 5′ end of the tRNA in the sequence —GTΨC—. It is believed to play some role in protein synthesis as the sequence —TΨCG— is involved in ribosome binding[2-5]. It has been demonstrated that mammalian phenylalanine tRNA functions more efficiently in protein synthesis if it contains ribothymidine[6]. On the contrary, glycine tRNA from wheat has been shown to function better in protein synthesis when it lacks ribothymidine[7]. Isoleucine tRNA from *Mycoplasma* (sp.) Kid does not contain ribothymidine. Still it can support protein synthesis *in vitro* in *Escherichia coli* system[8]. It is clear from all these studies that ribothymidine has a subtle role to play in protein synthesis, although its exact function is not known.

Changes in modified nucleosides dependent upon growth conditions, phases of growth, differentiation, tumour production, and viral infection have been observed[9]. Biosynthesis of 2′-O-methylguanosine and thiolation of ribothymidine in the tRNA of the extreme thermophiles like *Thermus thermophilus* are thermally induced[10 11]. The ribothymidine content of the tRNA of *Dictyostelium discoideum* decreases two-fold during development and differentiation[12]. On the contrary, in *Acetabularia mediterranea* the proportion of ribothymidine increases rapidly by almost four-fold during development[13]. Recent work in our laboratory has shown that drastic modifications of 2-thiouridine derivatives occur in the tRNA of *Agrobacterium tumefaciens* during different phases of growth of the organism[14 15].

In the present studies it has been observed that total tRNA isolated from *A. vinelandii* grown in the absence of ammonium salt contains the normal content of ribothymidine while that isolated from the bacteria grown in the presence of ammonium ions lacks ribothymidine. Interestingly, tRNA from *R. meliloti* grown in the presence of ammonium salt also lacks ribothymidine. The possible causes for such a change are discussed.

MATERIALS AND METHODS

A. vinelandii (OP) Wisconsin, was a gift from the late Dr. V.N. Vasantharajan, Microbiology and Cell Biology Laboratory of this Institute. *R. meliloti* 41 was a gift from Dr. Tibot Sik of the Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences, Hungary. Ribonuclease T_1 (RNase T_2) was from Sigma Chemical Company, St. Louis, USA. Deoxyribonuclease I (DNase I) was from Worthington Biochemical Corporation, New Jersey, USA. Carrier-free (^{32}p)-orthophosphate was from Bhabha Atomic Research Centre, Bombay, India. Thin layer microcrystalline cellulose plates with plastic support were from Macherey-Nagel, Germany.

A. vinelandii was grown in Burk's modified nitrogen-free medium[16]. For growth in the presence of ammonium salt, 2.8 mg N/ml equivalent of ammonium chloride was added to the above medium. *R. meliloti* was grown in the medium described by Kondorsi *et al*[17]. Ammonium ion concentration in this case was 0.7 mg N/ml equivalent. The concentration of ammonium salt used in the present studies has been shown to be sufficient to completely repress the nitrogen fixing (nif) genes in the case of *A. vinelandii*[18]. *Rhizobium* species grown under free-living conditions do not fix nitrogen. Five per cent of the respective log phase cultures were transferred to low phosphate medium containing all the normal ingredients with only one-tenth of the amount of phosphate in addition to 100 mM Tris HCl pH 7.0, and 5 mCi of ^{32}p-orthophosphate per 100 ml medium. The cells were

grown as shake cultures at 30° C to late log phase and harvested.

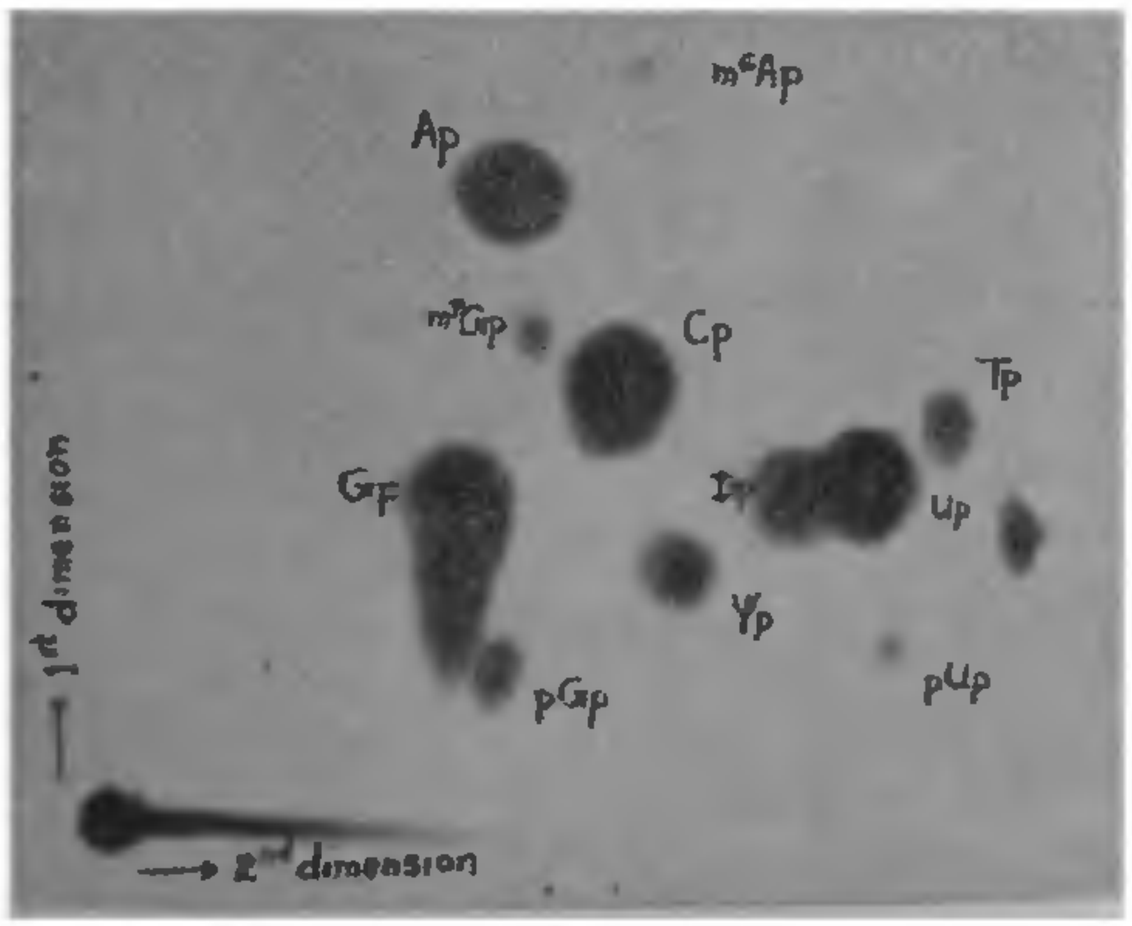
Total RNA was isolated by extraction with phenol and the tRNA was purified from ribosomal RNA by DEAE cellulose column chromatography[19]. The crude total tRNA thus obtained was subjected to high salt precipitation to remove ribosomal RNA contamination[20]. The tRNA was further treated with DNase I, followed by cetyl trimethylammonium bromide precipitation[21]. The pure total tRNA thus obtained was deacylated[19] and then digested with RNase T_2 to get the nucleotides.

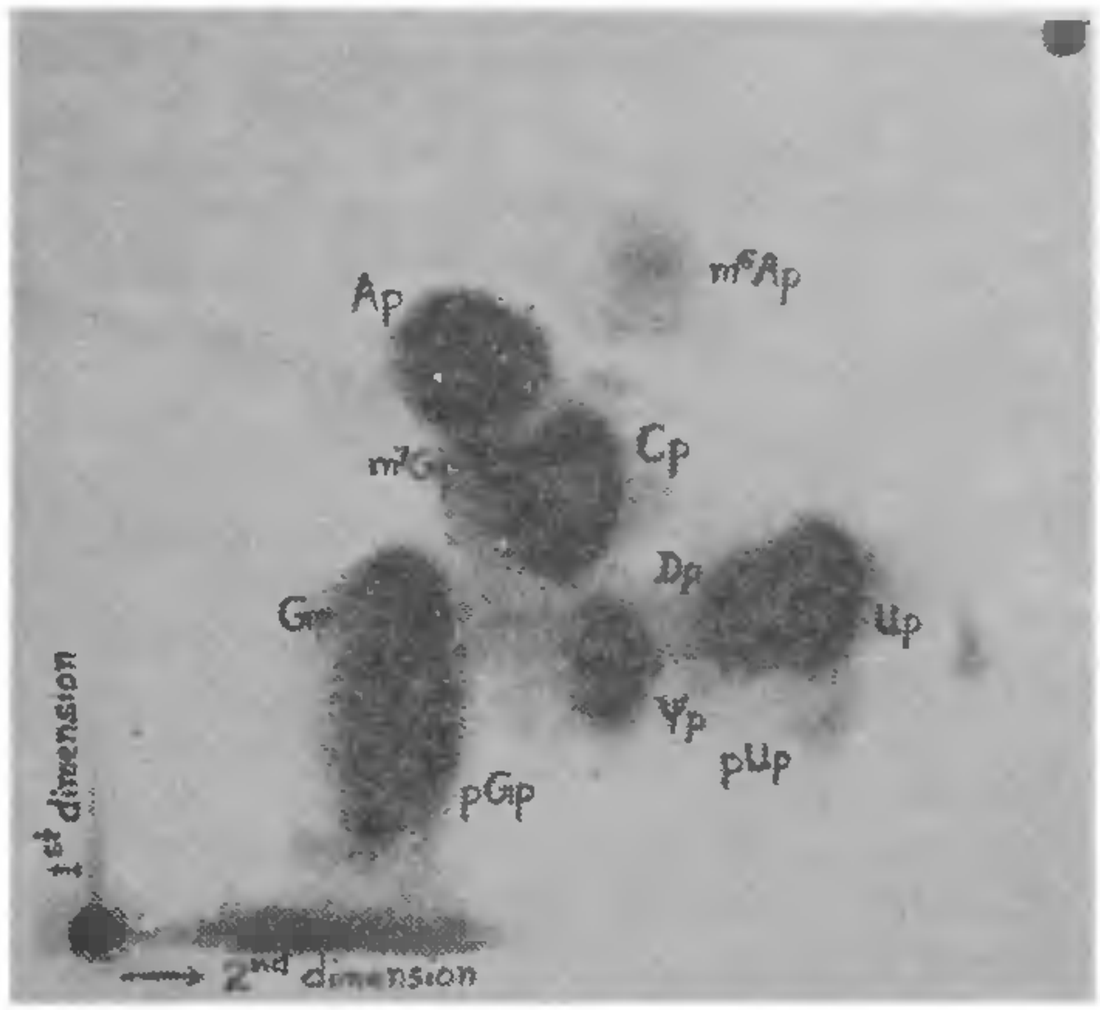
Two-dimensional thin layer chromatography of the nucleotides was carried out on cellulose plates of size 20 × 20 cm with 0.1 mm thickness using the solvent systems, isobutyric acid:0.5 M ammonium hydroxide, 5:3 (v/v) in the first dimension and isopropanol: concentrated HCl:water, 70:15:15 (v/v/v) in the second dimension[22]. Descending chromatography of the nucleotides was done on Whatman No. 1 paper in the solvent system isopropanol:water:liquor ammonia, 70:30:1 (v/v/v)[23]. Electrophoresis of the nucleotides was carried out on Whatman No. 3 paper in acetic acid-pyridine buffer, pH 3.5 (5% acetic acid containing 5 mM disodium EDTA adjusted to pH 3.5 with pyridine) at 60 V/cm using xylene cyanol and acid fuchsin as dye markers[24].

The radioactive spots due to nucleotides were detected by autoradiography. Radioactivity was measured in a liquid scintillation counter.

Results

The two-dimensional thin layer chromatogram of the RNase T_2 digest of the total tRNA isolated from *A. vinelandii* cells grown under nitrogen fixing conditions in the presence of ^{32}p-orthophosphate showed a spot due to ribothymidylic acid (Tp) slightly above to the right of uridylic acid (Up) (figure 1a). This





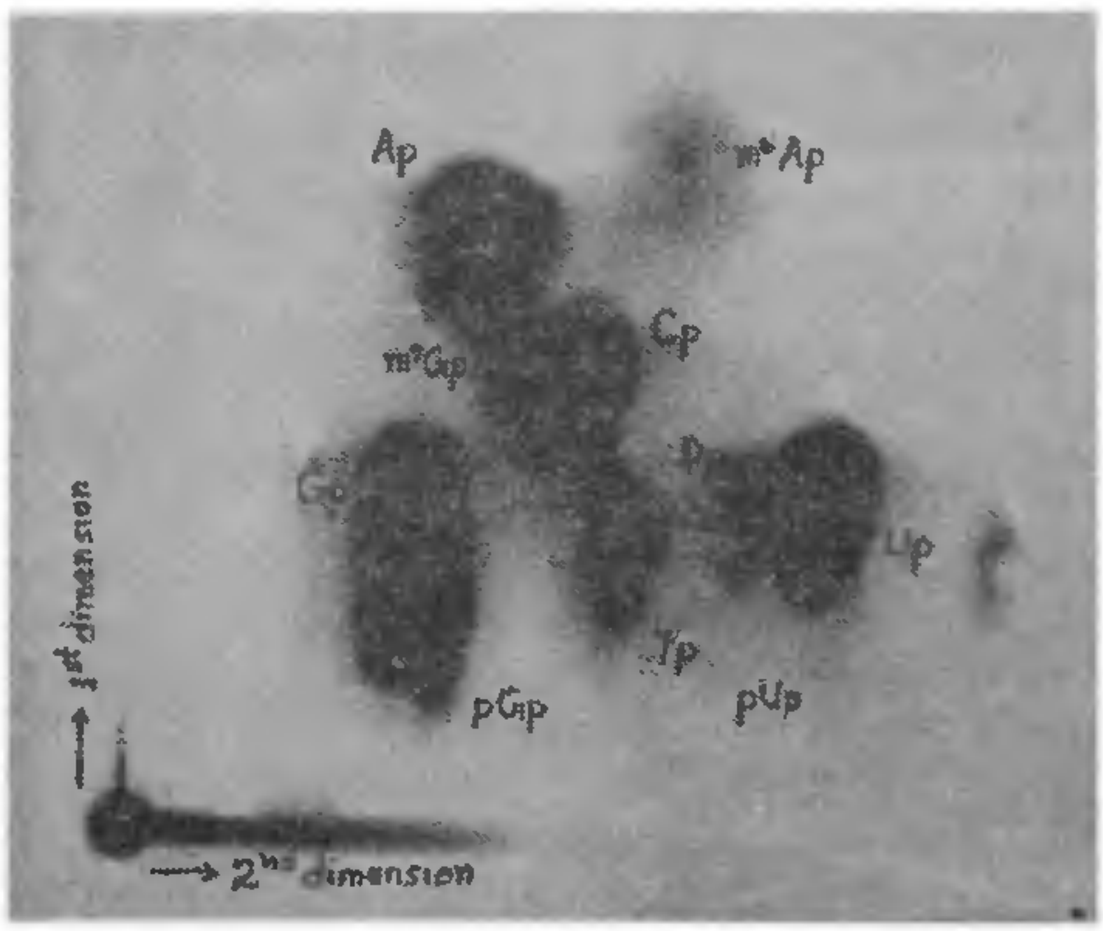


Figure 1. Two-dimensional thin layer chromatogram of RNase T_2 digest of tRNA samples.

RNase T_2 digests of ^{32}p-labelled tRNA samples were subjected to two dimensional thin layer chromatography on cellulose plates as indicated in the text. Samples from (a) *A. vinelandii* cells grown in the absence of ammonium salt, (b) *A. vinelandii* cells grown in the presence of ammonium salt, (c) *R. meliloti* cells grown in the presence of ammonium.

amounted to about 1.4% of the total radioactivity recovered from the spots due to the nucleotides listed in table 1. The molar per cent of Up as analysed by the above method was found to be 18.70 (table 1). Hence the Tp content is 7.6% of Up. The particular spot due to Tp was absent in the case of tRNA isolated from *A. vinelandii* and *R. meliloti* cells grown in the presence of ammonium salt (figures 1b and 1c). In order to confirm the absence of Tp in these samples, they were further analysed by the following procedure.

TABLE 1

Nucleotide composition of total tRNA, from A. vinelandii grown in the absence of ammonium salt, determined by two-dimensional thin layer chromatography

Nucleotide	Radio-activity in cpm	Molar % of nu-cleotides
Adenylic acid (Ap)	42595	19.96
Cytidylic acid (Cp)	55669	26.09
Guanylic acid (Gp)	55718	26.11
Uridylic acid (Up)	39929	18.71
5′ phospho Guanylic acid (pGp)	3239	0.76
5′ phospho Uridylic acid (pUp)	866	0.20
Ribothymidylic acid (Tp)	3061	1.43
Dihydrouridylic acid (Dp)	5210	2.44
Pseudouridylic acid (Ψp)	5266	2.45
7-methyl Guanylic acid (m^7Gp)	1426	0.67
6-methyl Adenylic acid (m^6Ap)	394	0.18

^{32}p-labelled total tRNA from *A. vinelandii* cells grown in the absence of ammonium salt was digested with RNase T_2 and subjected to two-dimensional thin layer cellulose chromatography[22]. After chromatography each of the spots was cut out from the plate, counted and the molar per cent calculated.

The ^{32}p-tRNA digest was subjected to paper electrophoresis at pH 3.5. The electrophoretic pattern is shown in (figure 2). At this pH Tp moves with an R_{Up} value of 0.98. In addition, Dp and Ψp also comigrate with Tp and Up[24]. The Up region was cut out from the paper, the radioactivity eluted from the paper with water[25] and it was subjected to descending paper chromatography in the solvent system, isopropanol: water: liquor ammonia, 70:30:1 (v/v/v). In this system Tp moves[23] with an R_{Up} value of 1.7. The spot moving fastest on the chromatogram had an R_{Up} value of 1.6 (figure 3). This showed the presence of Tp in the tRNA digest of *A. vinelandii* cells grown in the absence of ammonium salt. The Tp and Up spots were cut out from the paper and counted. The percentage of Tp with respect to Up was about 7.3 representing 1.36% of the total nucleotides, the molar per cent of Up in the tRNA being 18.7 (table 2). The molar percent of Tp determined by both methods thus agreed

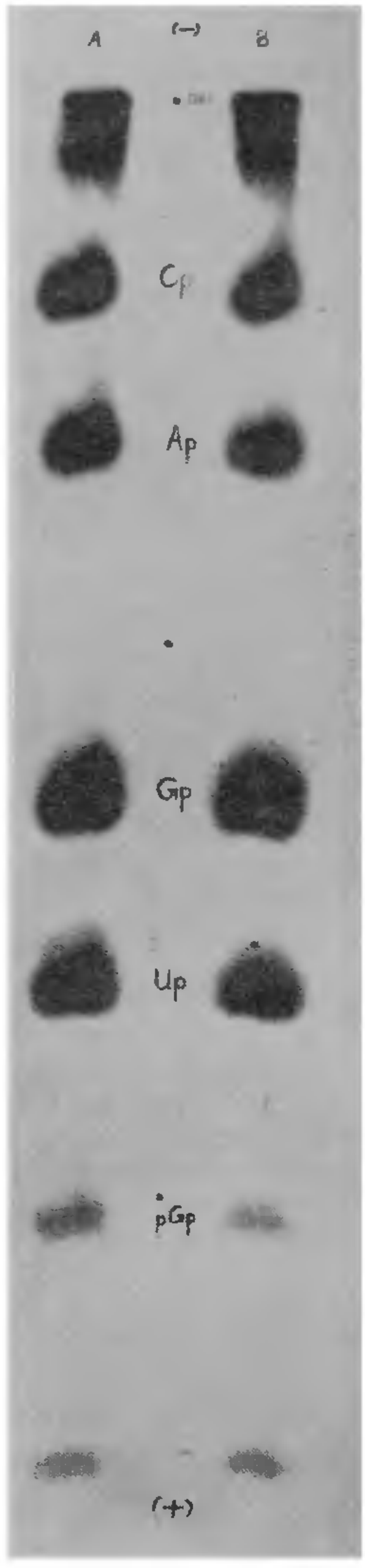


Figure 2. Electrophoretic separation of nucleotides at pH 3.5.

RNase T_2 digest of ^{32}p-labelled tRNA samples were separately subjected to electrophoresis at pH 3.5 at 60 V/cm on Whatman No. 3 paper as indicated in the text. After drying the paper was put for autoradiography. Sample from (A) ammonium-free medium. (B) ammonium containing medium.

within experimental error. The Tp content amounted to one residue per tRNA molecule, taking 77 as the average number of nucleotides per chain of tRNA. A similar spot was absent in the case of the tRNA digests from *A. vinelandii* and *R. meliloti* cells grown in the presence of ammonium salt (figure 3.).

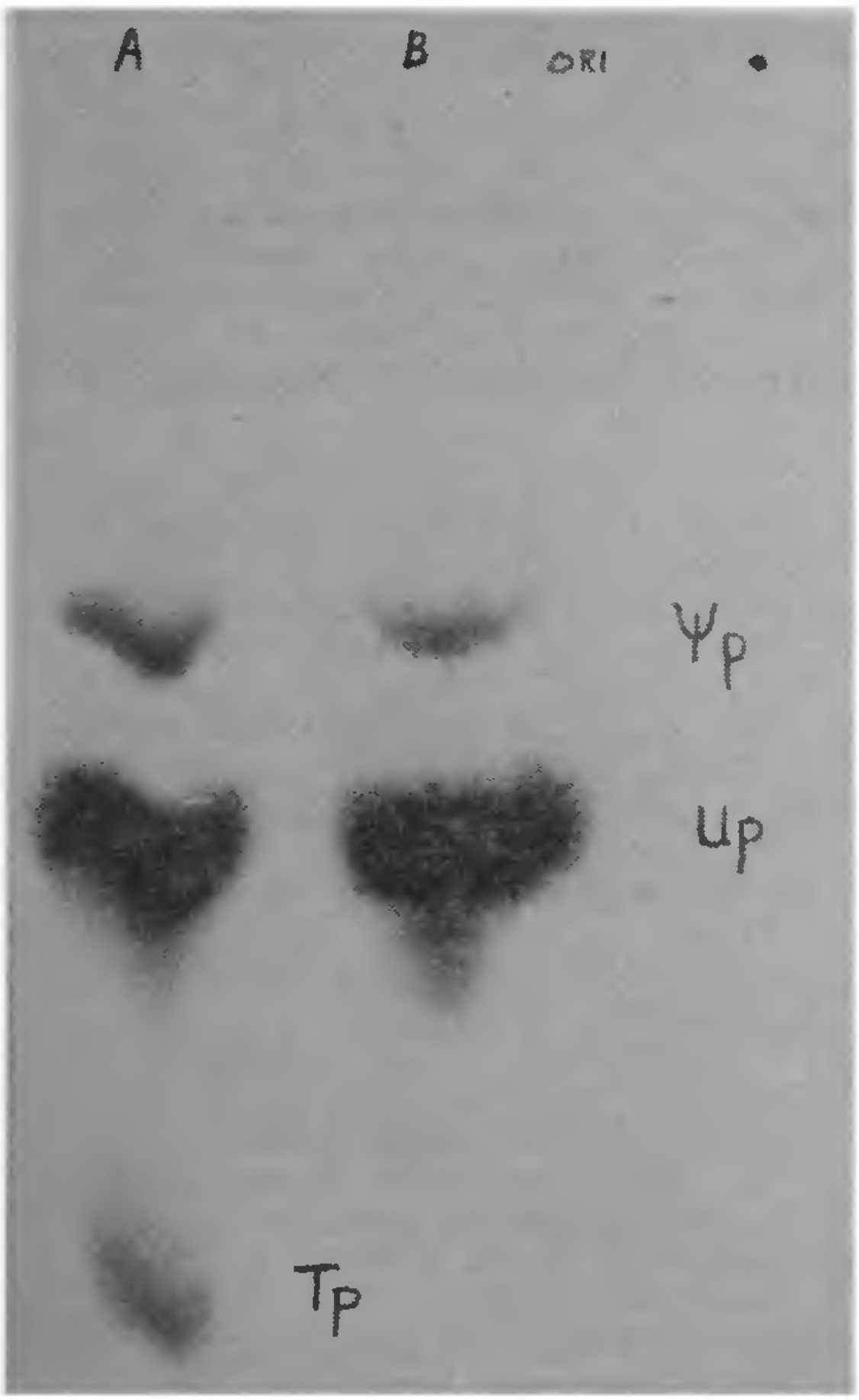


Figure 3. Paper Chromatography of Up region of electrophoresed sample.

Radioactivity from the Up spot region (figure 2) was eluted from the paper and subjected to descending paper chromatography as given in the text. (A) Sample from *A. vinelandii* cells grown in the absence of ammonium salt. (B) Sample from *A. vinelandii* cells grown in the presence of ammonium salt.

Discussion

The experimental results described here show the absence of ribothymidine in the tRNA from both *A. vinelandii* and *R. meliloti* cells grown in the presence of ammonium salt. The spot corresponding to Tp is absent in the two-dimensional thin layer as well as paper chromatograms (figures 1*b* and c). When *A. vinelandii* is grown under nitrogen fixing-conditions, its tRNA contains Tp as revealed by both the chromatographic methods (figures 1a and 3). Since *R. meliloti* does not grow in the absence of fixed nitorgen, a similar experiment could not be conducted in this bacterium. These results have been obtained with independent samples prepared at different times and they are highly reproducible. The relative proportion of ribothymidine in the total tRNA in the latter case has been calculated to be about 1.4% with reference to total nucleosides, representing one ribothymidine residue per tRNA molecule (tables 1 and 2). The absence of ribothymidine in the former case shows that the uridine moiety at the 54th position from the 5′ end of the tRNA is either not methylated to form ribothymidine or it is modified to some nucleoside other than ribothymidine. Presence of 2-thioribothymidine has been observed in the case of tRNA from thermophilic organisms[11]. But the presence of 2-thioribothymidine is ruled out in the present case since no spot corresponding to 2-thioribothymidylate which appears slightly above to the right of uridylate on the two-dimensional thin layer chromatogram has been observed. If Up at the 54th position remains unmodified, then it will merge with the major Up spot and cannot be detected by the two methods employed. Another possible modification is ribose methylation of uridine or ribothymidine to form 2′-O-methyluridine or 2′-O- methylribothymidine[26]. In such a case, RNase T_2 hydrolysis of tRNA will yield UmpΨp or TmpΨp. But no additional spot is observed on the two-dimensional thin layer chromatogram of the tRNA hydrolysate from cells grown in the presence of ammonium salt (figures 1b and c). However, if it is merged with any of the major nucleotides it cannot be detected by the present methods. Experiments are in progress to find which of the possibilities is involved in the present case.

It is interesting that methylation of uridine to ribothymidine occurs under nitrogen-fixing conditions in *A. vinelandii* and that ribothymidine is totally absent in the tRNA when the organism is grown under conditions when nitrogen fixing genes are repressed[27]. A possible reason for the absence of ribothymidine in the tRNA formed in the presence of ammonium may be that the particular modifying enzyme which synthesises ribothymidine is inhibited by ammonium ions. Another possibility is that an unknown metabolite of ammonium ion might be inhibiting the enzyme or acting as a repressor to the gene coding for the protein. A third possibility is that the gene coding for the modifying enzyme might be in coordinate expression and repression with the genes in the nitrogenase operon. Hence when ammonium ions repress the nif operon the gene for the modification enzyme also gets repressed and *vice versa*. If so, the significance of this is

TABLE 2

Molar per cent of Ribothymidylate (Tp) determined by descending paper chromatography

Expt. No.	Uridylic acid (Up) cpm	Ribothymidylic acid (Tp) cpm	Molar % of Tp with respect to Up	Molar % of Tp with respect to total nucleotides
1.	5879	425	7.23	1.35
2.	3426	251	7.32	1.37

^{32}p-labelled total tRNA from *A. vinelandii* cells grown in the absence of ammonium salt was digested with RNase T_2 and subjected to paper electrophores is at pH 3.5. The radioactivity in the spot corresponding to Up (as revealed by autoradiography) was eluted from the paper and subjected to descending paper chromatography [23]. After autoradiography the spots corresponding to Up and Tp were cut out and counted. The relative proportion of Tp in the total nucleotides was calculated taking molar percentage of Up to be 18.7 (see table 1).

not known presently. In this connection it is of great interest that the relative proportion of certain thionucleosides in the tRNA from *A. vinelandii* vary eight- to ten-fold when grown under the two conditions namely, nitrogen-fixing condition and in the presence of ammonium salt which represses the nif genes (manuscript in preparation). Further work on these aspects are under active investigation.

Note added in proof

Essentially the same results were obtained when *A. vinelandii* cells were grown in the presence of ammonium salt equivalent to 0.49 mg N/ml instead of 2.8 mg N/ml. A concentration of 0.4 mg N/ml has been reported to be sufficient to completely repress nitrogenase genes[18].

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THE SITE OF SEX PHEROMONE PRODUCTION IN THE FRESHWATER PRAWN, *MACROBRACHIUM KISTNENSIS*

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ABSTRACT

The sex pheromone is produced in the sternal glands of the mature females. The sex pheromone appears to be a mucopolysaccharide. The pheromone is detected by the chemoreceptors of the inner flagellum of the antennules of male prawn.

INTRODUCTION

A pheromone is defined as a chemical communicant released by an organism that influences the behaviour of the other organism of the same species[1]. Sexual recognition and attraction which precede the mating behaviour is stimulated by sex pheromone[2]. The sex pheromones of insects have been studied extensively[3]. Little information is available on the sex pheromones in freshwater crustaceans. The present study was aimed to study the site of production of sex pheromone and also its chemical nature in the freshwater prawn, *Macrobrachium kistnensis*.

MATERIALS AND METHODS

The prawns *M. kistnensis* were collected from Kham river near Aurangabad and kept in aerated aquaria and were fed daily on branned wheat and pieces of dead prawns. The water was changed daily. The prawns having carapace length of 18–22 cm were chosen for the experiment.

OBSERVATIONS

Kamiguchi[4] reported that the sex pheromone in the freshwater prawn, *Palaemon paucidens* is produced by the sternal glands. In *M. kistnensis* there are seven sternal glands out of which 3 are paired, and situated at the bases of the coxopodite of third, fourth and fifth pair of periopods. A single sternal gland lies on the ventral side of thoracic region at the level of fifth periopod (figure 1 and 2b). During the breeding season they are white in colour and become milky white just before the parturial moult. One hour after parturial moult they are hardly distinguishable.

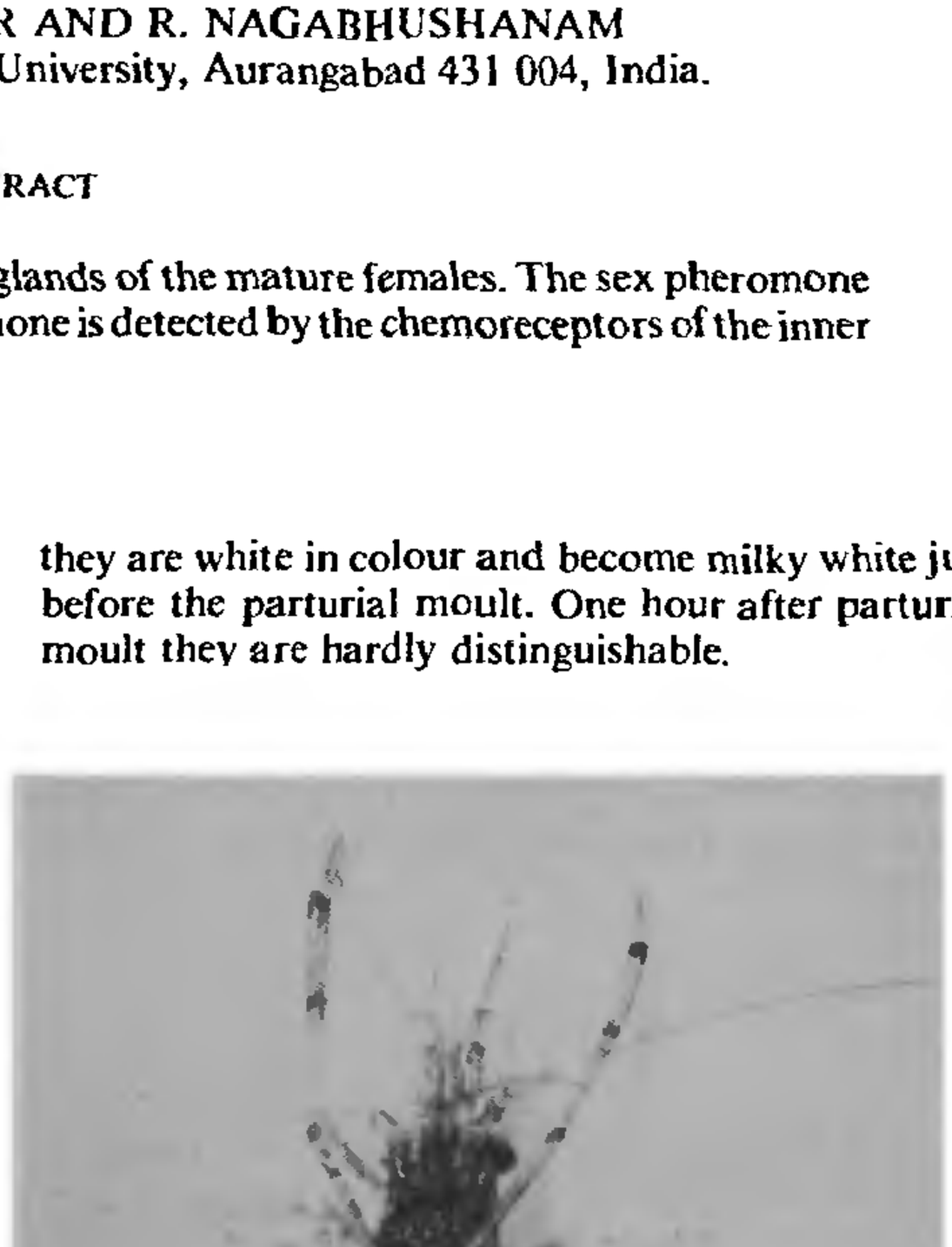

Figure 1. The ventral view of the female prawns showing sternal glands (white in colour). SG = Sternal gland.