THIONUCLEOTIDE FORMATION IN *ESCHERICHIA COLI* TRANSFER RIBONUCLEIC ACID IN THE PRESENCE OF 5-FLUOROURACIL

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1. Introduction

It is well established that *E. coli* tRNA possesses sulphur-containing nucleotides [1–3]. Such nucleotides are thought to arise by post-transcriptional enzymic modification of common nucleotides in the polynucleotide chain [4, 5]. Because little is known about the specificity of the modification enzymes, it was decided to investigate thionucleotide formation under conditions where the synthesis of ribothymidylate and pseudouridylate in the polynucleotide chain was prevented by the replacement of uracil by 5-fluorouracil (FU) in the growth medium of a uracil-requiring strain of *E. coli* [6]. Contrary to other reports [7], it is suggested that synthesis of 4-thioFUMP does occur, but the capacity of the cells to form 4-thioFUMP disulphides is affected.

2. Materials and methods

Escherichia coli (strain B148) was grown at 37° in a medium containing KH_2PO_4 (3 g/l), K_2HPO (7 g/l), sodium citrate (0.5 g/l), $MgSO_4$ (0.1 g/l), $(NH_4)_2SO_4$ (1 g/l), uracil (25 mg/l) and glucose (10 g/l). Cells in midlogarithmic growth were harvested by centrifugation and resuspended in a fresh growth medium similar to that given above except for (i) the omission of uracil, and (ii) the reduction of the level of sulphate ions to 1% of that given by substitution of the appropriate chloride. After allowing 30 min for the utilization of residual uracil, 5-fluorouracil (32 mg/ml) and thymidine (50 mg/ml) were added. Following a further 30 min period allowed for equilibration, radioactive sulphur (as $^{35}SO_4$) was added (1.0 μc/ml). The incubation was continued for 2 hr, during which time the cell density doubled once, and the cells were then harvested by centrifugation.

The cells were resuspended in bentonite–pyrophosphate–polyvinylsulphate–EDTA (composition described by Rammler, Okabayashi and Delk [8]), shaken with isoamylalcohol (10%), extracted with phenol at 50° and the aqueous phase precipitated with three volumes of ethanol at −20°. The precipitate was washed with 75% ethanol, dissolved in 0.5 M Tris-HCl buffer pH 9 and incubated at 37° for 1 hr to remove amino acids bound to the tRNA, and finally neutralized and reprecipitated with ethanol. Transfer RNA was separated from other nucleic acid species by gel filtration through a column of Sephadex G100 (215 cm × 1.8 cm dia), using 0.015 M sodium citrate and 0.15 M sodium chloride as eluent, 4 ml fractions being collected. The tRNA (fractions 65 to 85) which was well resolved from 55 RNA (fractions 52 to 61), was precipitated with three volumes of ethanol, redissolved in distilled water and dialysed against distilled water.

The tRNA was hydrolysed with 0.33 N potassium hydroxide for 18 hr at 37°, neutralized with perchloric acid and reduced in volume by rotary evaporation. Conversion of the resultant nucleotides to nucleosides was obtained with *E. coli* alkaline phosphatase in 0.1 M Tris-HCl at pH 8.5.

Separation of the nucleotides and of the nucleosides was achieved by a paper chromatographic technique which employed Whatman No. 1 paper impregnated with ammonium sulphate and 75% ethanol as solvent [9]. After 18 hr chromatography, the nucleotides (or nucleosides) on the dried chromatogram were visualized with ultraviolet light. The chromatogram

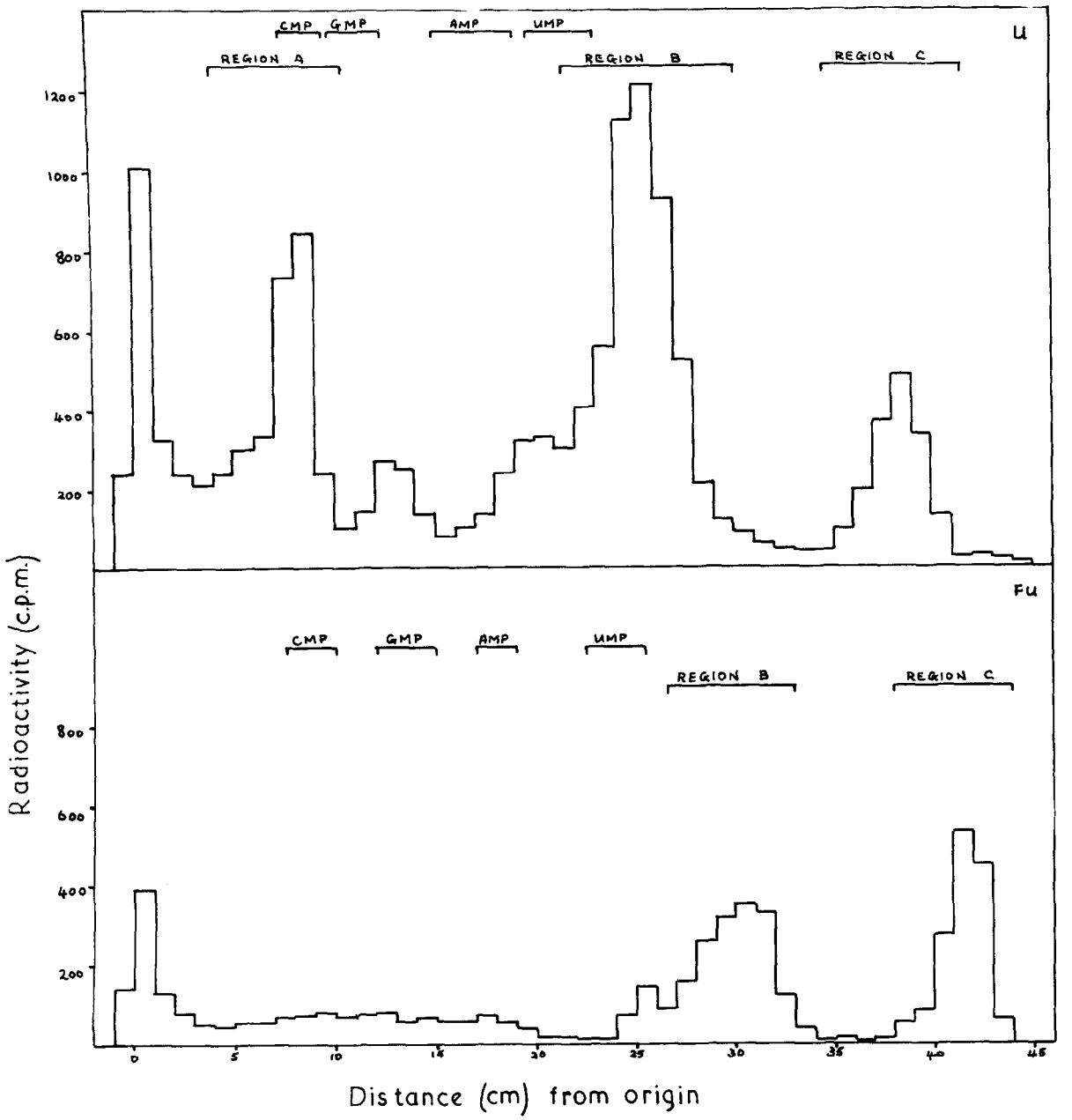

Fig. 1. Incorporation of ^{35}S into tRNA by *E. coli* in the presence of uracil and fluorouracil. Separation of the resultant nucleotides following alkaline hydrolysis.

was then cut into 1 cm strips which were counted using a Packard liquid scintillation counter.

Authentic 4-thiouridine was obtained by reduction of uridine-4-disulphide (Sigma Chemical Co.) with 0.05 M scdium thiosulphate.

3. Results and discussion

Paper chromatography (fig. 1) of the nucleotides obtained in the alkaline hydrolyzate of the tRNAs showed that one major radioactive peak (region A) which was given by the control cell tRNA hydrolyzate was absent in the tRNA hydrolyzate from the FU-treated cells. Kaiser [7] has reported that when treated with FU tRNA from *E. coli* did not contain 4-thio UMP. The possibility that region A (absent in the FU-treated tRNA) was 4-thioUMP was therefore investigated. The nucleotides obtained by KOH hydrolysis were converted into nucleosides and chromatographed together with authentic 4-thiouridine (fig. 2). It appears that region B is 4-thiouridine and not region A. Since separation of the nucleosides is similar to that of the nucleotides it is reasonable to conclude that region B, and not region A (fig. 1) is 4-thioUMP. This was confirmed by elution of regions A and B from the original chromatogram and their rechromatography in solvent systems for which data on the mobility of 4-thioUMP have been published (table 1). The appearance of radioactivity in regions A and B when the cells were incubated in the presence of FU is taken to imply that such cells can synthesize 4-thio derivatives of 5-fluorouridylate residues. This contradicts directly Kaiser's finding [7] for which one possible explanation is offered. Kaiser estimated the 4-thioUMP content of FU-containing tRNA by titration with *p*-chloromercuribenzoate which assumes that the reactivity of the sulphydryl group of 4-thioFUMP is similar to that of 4-thioUMP. That this may not be the case is suggested by a finding in table 1. Lipsett [9] has shown that 4-thioUMP disulphide breakdown on alkaline hydrolysis to give a certain 'compound T' which when chromatographed in solvent 1 in table 1 gave a R_{UMP} of 1.07. An identical mobility was shown by region A in the present work. It is reasonable to conclude, therefore, that region A is derived from 4-thioUMP disulphide on alkaline hydrolysis and may be identical to Lipsett's 'compound T'. The absence of region A ('compound T') in the hydrolyzate of the FU-containing tRNA may be explained by either the absence of disulphide formation, or the instability of a compound analogous to 'compound T' derived from 4-thioFUMP disulphide. The former possibility could result from either the absence of necessary tertiary structure normally provided by pseudouridine and ribothymidine in the tRNA, or steric hindrance by the adjacent fluorine ligand.

Regions C (fig. 1) and C (fig. 2) are thought to be the respective 2-methylthioisopentenyl adenine nucleotide and nucleoside. Region C was absent when a methionine strain of *E. coli* was labelled with ^{35}S in (a) the absence of methionine, and (b) in the presence of ethionine; whereas region C was present when methionine was supplied, which suggests that a methylation step was required for region C formation.

Table 1
Rechromatography of regions A and B.

Conditions	Mobility of			
	Region A	Region B	4-thioUMP	Reference
1	R_{UMP} = 1.07	R_{UMP} = 0.40	R_{UMP} = 0.40	Lipsett [10]
2	R_{UMP} = 0.88	R_{UMP} = 0.63 R_{UMP} = 0.70	R_{UMP} = 0.65 R_{UMP} = 0.73	Lipsett [10]
3	R_f = 0.61	R_f = 0.20	R_f = 0.19	Scheit [11]

Conditions of chromatography:
1 = 0.2 M NH_4HCO_3 on DEAE-cellulose paper
2 = saturated $(NH_4)_2SO_4$/2-propanol/1 M sodium acetate (80/2/18) on Whatman No. 1 paper
3 = ethanol/1 M ammonium acetate (7/3) on Whatman No. 1 paper.

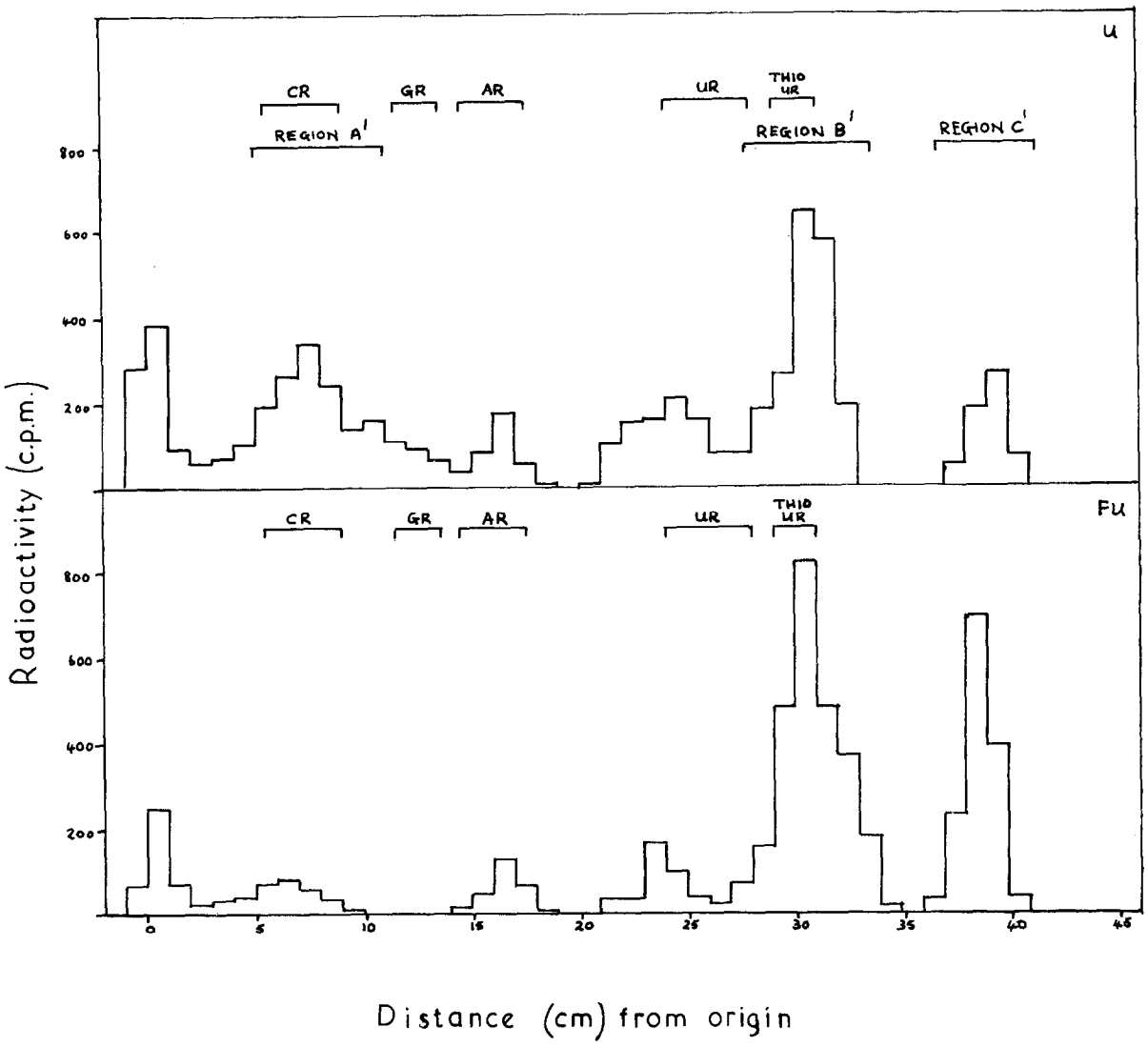

Fig. 2. Incorporation of ^{35}S into tRNA by *E. coli* in the presence of uracil and fluorouracil. Separation of the resultant nucleosides following alkaline hydrolysis and alkaline phosphatase treatment.

The nature of the radioactive sulphur incorporated into region C appeared to differ from that in region B (4-thioUMP) and region A because no loss of radioactivity occurred from region C on treatment of the tRNA with cyanogen bromide at 100° [12] whereas a marked loss of radioactivity occurred from both regions A and B on similar treatment. Cyanogen bromide is known to convert 4-thioUMP to UMP [12]. When paper chromatographed using 2-propanol/ammonia/water (7/1/2) as solvent, region C gave an R_f of 0.91 which corresponds to the published R_f of 2-methylthioisopentenyl adenosine [13]. FU treatment of the cells did not inhibit but appeared to stimulate the synthesis of the 2-methylthioisopentenyl adenylate (region C) as judged from the amount of radioactivity in region C (i) as a proportion of the total radioactivity in the hydrolyzate, and (ii) relative to the radioactivity in region B, as

compared to the uracil-treated control cell tRNA. Alternatively this result could be explained by suggesting that synthesis of 4-thioFUMP was depressed as compared 4-thioUMP synthesis. Kaiser's [7] findings would obviously support such a suggestion.

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