The isolation of 5-methylcytidine from RNA

Studies in recent years have indicated the presence in RNA from a number of sources of several minor components in addition to the four main nucleotides. These include pseudouridine[1–4], thymine[5], methylated adenines[5,6], methylated guanines[6,7], 2-methylribose[8] and 5-methylcytosine[9,10]. A compound which appeared to be the ribonucleotide of the latter was isolated from the RNA of *Escherichia coli* K 12 by AMOS AND KORN[9]. The present studies indicate that 5-methylcytosine is present in RNA of animal, plant and bacterial origin combined in 3′–5′ phosphodiester linkages. As 5-methylcytosine is a known constituent of DNA[11] it has been isolated as the riboside and identified by comparison with a synthetic sample as well as by conversion to 5-methylcytosine and thymine riboside.

The preparation of RNA from wheat germ, rat-liver microsomes and *Aerobacter aerogenes* has already been described[5]. Samples of rat-liver soluble RNA (s-RNA[12,13]) were obtained from Dr. M. B. HOAGLAND. A sample of RNA from pig liver which probably represented mainly s-RNA was obtained by isolation of the RNA according to KIRBY[14] and fractionation using 2 *M* NaCl and ethanol[15]. Most of the RNA samples were hydrolysed with 1 *N* KOH at 30° for 18 h, the alkali neutralised with $HClO_4$ and the nucleotides isolated by chromatography in isopropanol–water–NH_3[16]. The adenylic and cytidylic acids were separated from the uridylic and methylated guanylic acids by electrophoresis at pH 2.6[7] and rechromatographed in isopropanol–water–NH_3 to remove phosphate. The nucleotides were treated with prostatic phosphomonoesterase[17] under the conditions previously used[5], and the resulting nucleosides were separated by chromatography in isopropanol–water–HCl[11]. The 5-methylcytidine was separated from the cytidine by chromatography of the leading half of the cytidine band in *n*-butanol–water–NH_3[18] for 36–72 h. Double spotting of the cytidine and 5-methylcytidine occurred in the butanol solvent but could be prevented if the material eluted from the HCl chromatogram was first chromatographed in isopropanol–water–NH_3. It was found that satisfactory results could be obtained if the chromatogram was run for 3–6 h in the isopropanol–water–NH_3, then dried and the same paper chromatographed in the *n*-butanol.

As in previous studies[5,7] the riboside was identified by comparison with a known sample of 5-methylcytidine. Attempts to synthesize this riboside using inosine, 5-methylcytosine and the nucleoside phosphorylase previously used[5,7] were unsuccessful but I was fortunately able to obtain a sample of the ribofuranoside recently synthesized by FOX *et al.*[19]. In four solvent systems the nucleoside from RNA had the same R_F as the synthetic riboside (Table I) and its spectra at pH 1 and 13 corresponded closely to those obtained by FOX *et al.* at pH 0 and 14 respectively (Fig. 1). On heating my nucleoside with 72 % $HClO_4$ for 2 h at 100° I obtained a compound identified chromatographically in four solvents (Table I) and spectroscopically as 5-methylcytosine. AMOS AND KORN[9] reported that under similar conditions their nucleotide was converted to thymine, but no detectable amount of this compound was formed in my experiments. As expected on treatment with HNO_2 the nucleoside was converted to a compound identified chromatographically and spectroscopically as thymine riboside[5].

Abbreviations: RNA, ribonucleic acid; s-RNA, soluble RNA; DNA, deoxyribonucleic acid.

TABLE I

R_F VALUES OF 5-METHYLCYTOSINE, 5-METHYLCYTIDINE AND OTHER RIBOSIDES IN FOUR SOLVENT SYSTEMS

	isopropanol –water–HCl	*isopropanol –water–NH_3*	*n-butanol –water–HCOOH*[18]	*n-butanol –water–NH_3*
5-Methylcytosine	0.60	0.59	0.25	0.30
5-Methylcytidine	0.57	0.57	0.12	0.12
Cytidine	0.55	0.54	0.10	0.10
Adenosine	0.41	0.59	0.17	0.18

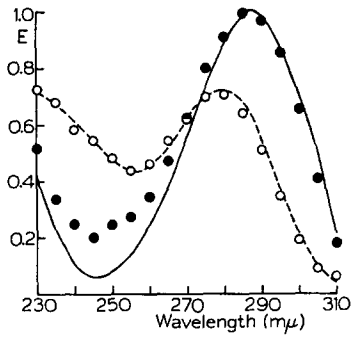

Fig. 1. U.v.-absorption spectra of 5-methylcytidine from Fox *et al.* at pH 0 (———) and at pH 14 (— — —). Points are for a sample isolated from rat-liver soluble RNA at pH 1 (●) and pH 13 (○). Spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for E of 1.0 at 287 mμ.

The R_F values of the nucleoside in the butanol solvents agreed with the presence of ribose rather than deoxyribose as the deoxyriboside would have a higher R_F in these solvents[20]. The presence of ribose was also indicated by the formation of a negatively-charged borate complex: both the synthetic and the nucleoside from RNA moving with cytidine on paper electrophoresis in borate, pH 9.2. In addition, 5-methylcytosine was identified as the major u.v.-absorbing product when the nucleoside was treated successively with periodate and alkali[21,5].

5-Methylcytidine was also isolated from a sample of pig-liver RNA which was hydrolysed with snake venom. The RNA (4 mg) was incubated at 37° in glycine buffer, pH 9, with 1 mg Russell viper venom for 6 h, chloroform being added to prevent bacterial contamination. The products were chromatographed in isopropanol–water–NH_3 and the band containing adenosine, cytidine and uridine was subjected to paper electrophoresis in phosphate, pH 2.6. The fastest moving band of cytidine and adenosine was chromatographed in isopropanol–water–HCl and the 5-methylcytidine isolated as described above. It was identified by its R_F values in the four solvents, its spectra and by conversion to 5-methylcytosine. As hydrolysis with snake venom would involve formation of the 5′ nucleotides[22] while alkali cleaves RNA to the 2′ and 3′ phosphates[23] it seems that the 5-methylcytidine residues must be present in the polynucleotides mainly in 3′–5′ phosphodiester linkages.

The 5-methylcytidine was estimated spectrophotometrically using a value of ε of $12.5 \cdot 10^3$ at 287 mμ in 0.1 *N* HCl[19]. As observed with the other additional com-

ponents[24] the highest proportion was detected in rat-liver s-RNA (10 moles/100 moles uridine) and a similar proportion (6 moles/100 moles) was found in the pig-liver RNA. A smaller proportion was found in rat-liver microsome RNA (0.4 mole/100 moles uridine) and even smaller proportions in RNA from wheat germ (0.2 mole/100 moles uridine) and *A. aerogenes* (0.15 mole/100 moles uridine).

It has been suggested[9] that the thymine we previously detected in RNA[5] arose from 5-methylcytidine residues during the alkaline hydrolysis. The detection of 5-methylcytosine but not thymine[5,10] in both the rat-liver fractions after alkaline hydrolysis seems to rule out this possibility. It is noticeable, however, that the sources such as wheat germ and *A. aerogenes*, which contain comparatively high proportions of thymine, contain little 5-methylcytosine. A small proportion of thymine was detected in the pig-liver RNA hydrolysed with venom or alkali and the proportion seemed variable in different preparations. Further investigations will be necessary to establish if the thymine arises from 5-methylcytosine residues, but it seems quite possible that the tissues used may contain enzymes capable of deaminating the 5-methylcytidine residues either before or during the isolation of the RNA.

I wish to thank Dr. J. J. FOX and his colleagues for the sample of 5-methylcytidine, Dr. M. B. HOAGLAND for the samples of rat-liver RNA and I am grateful to Dr. J. D. SMITH for much valuable discussion on this work.

Agricultural Research, Council Virus Research Unit, Huntingdon Road, Cambridge (Great Britain) D. B. DUNN

1 W. E. COHN, *Federation Proc.*, 16 (1957) 166.
2 F. F. DAVIS AND F. W. ALLEN, *J. Biol. Chem.*, 227 (1957) 907.
3 C. T. YU AND F. W. ALLEN, *Biochim. Biophys. Acta*, 32 (1959) 393.
4 W. E. COHN, *Biochim. Biophys. Acta*, 32 (1959) 569.
5 J. W. LITTLEFIELD AND D. B. DUNN, *Biochem. J.* 70 (1958) 642.
6 M. ADLER, B. WEISSMANN AND A. B. GUTMAN, *J. Biol. Chem.*, 230 (1958) 717.
7 J. D. SMITH AND D. B. DUNN, *Biochem. J.*, 72 (1959) 294.
8 J. D. SMITH AND D. B. DUNN, *Biochim. Biophys. Acta*, 31 (1959) 573.
9 H. AMOS AND M. KORN, *Biochim. Biophys. Acta*, 29 (1958) 444.
10 D. B. DUNN AND J. D. SMITH, *Proc. 4th. Intern. Congr. Biochem.*, Vol. 7, 1959, p. 72.
11 G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
12 P. C. ZAMECNIK, M. L. STEPHENSON AND L. I. HECHT, *Proc. Natl. Acad. Sci., U.S.* 44 (1958) 73.
13 M. B. HOAGLAND, M. L. STEPHENSON, J. F. SCOTT, L. I. HECHT AND P. C. ZAMECNIK, *J. Biol. Chem.*, 231 (1958) 241.
14 K. S. KIRBY, *Biochem. J.*, 64 (1956) 405.
15 D. B. DUNN AND J. D. SMITH, unpublished.
16 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
17 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 558.
18 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
19 J. J. FOX, D. V. PRAAG, I. WEMPEN, I. L. DOERR, L. CHEONG, J. E. KNOLL, M. L. EIDINOFF, A. BENDICH AND G. B. BROWN, *J. Am. Chem. Soc.*, 81 (1959) 178.
20 J. G. BUCHANAN, *Nature*, 168 (1951) 1091.
21 P. R. WHITFELD, *Biochem. J.*, 58 (1954) 390.
22 W. E. COHN AND E. VOLKIN, *Arch. Biochem. Biophys.*, 35 (1952) 465.
23 D. M. BROWN AND A. R. TODD, *J. Chem. Soc.*, (1952) 52.
24 D. B. DUNN, *Biochim. Biophys. Acta*, 34 (1959) 286.

Received May 25th, 1959