THE DETERMINATION OF 5-AZAPYRIMIDINES AND THEIR DERIVATIVES IN BACTERIAL RNA

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1. Introduction

5-Azapyrimidine nucleoside analogues inhibit protein synthesis in *E. coli* [1, 2]. 5-Azacytidine is a much weaker inhibitor than 5-azauridine, provided deamination [3] of the former is prevented by using an organism deficient in cytidine deaminase [4, 5]. In aqueous media the 5-azapyrimidine nucleosides are unstable, forming ribosides of N-amidinourea or of N-formylbiuret and biuret [6]. The inhibitory effects of 5-azapyrimidine may therefore be due in part to ring-opening after their incorporation into RNA [7]. In order to evaluate the extent and biological significance of the ring-fission reaction an attempt was undertaken to determine the form in which the incorporated 5-azapyrimidine actually occur in RNA. The results show that a variable portion of labeled 5-azacytidine is incorporated primarily as 5-azauracil by wild-type bacteria, while strains deficient in cytidine deaminase contain exclusively 5-azacytosine and its open-ring derivative in their RNA. Most of the incorporated 5-azacytosine may be recovered intact, either in the form of nucleotide or nucleoside, whereas 5-azauracil is completely degraded to open-ring form.

2. Materials and methods

5-[4-^{14}C] Azacytidine was prepared in this Institute by Ing. J. Kopecký and had a specific radioactivity of 33.7 mCi/mmole. In S_2 (see below) this preparation gave a single symmetrical radioactive peak (R_F = 0.52) proving its radiochemical purity.

5-Azapyrimidines and related compounds (table 1) were chromatographed on Whatman 3 MM paper, using acidic solvents (in which the 5-azapyrimidines have been found stable) namely S_1, n-butanol–acetic acid–water (5:2:3, v/v); S_2, isobutyric acid–water–aqueous ammonia (66:33:1.5, v/v); S_3, isopropanol–HCl–water (170:41:39, v/v). The substances on the chromatograms were detected as described [7]. Radioactive compounds were detected by scanning the chromatograms with a Geiger-Müller counter, fitted with an automatic radioactivity recorder.

The strain *E. coli* OK 408, deficient in cytidine deaminase, was isolated by Karlström [8]. *E. coli* B was used as cytidine-deaminase plus strain. The conditions of labeling with 5-azacytidine were similar to those previously described [2]. RNA was isolated according to Salser, Gesteland and Bolle [9], extracting, however, the RNA at room temp. rather than at 64°. Enzymatic hydrolysis of RNA with pancreatic RNase, spleen phosphodiesterase and prostate phosphomonoesterase was performed similarly as described [2], except that a more active preparation of spleen phosphodiesterase was used.

3. Results

The facilitate the identification of 5-azapyrimidines and their derivatives, substances likely to occur in the hydrolysates were chromatographed and their R_F-values have been listed in table 1.

Conventional acid hydrolysis [10] with 1 N HCl at 100°, 1 hr, of standard 5-azacytidine or labeled RNA produced mainly II (see table 1) with minor amounts of I and a trace of an unidentified product.

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Table 1
Chromatographic mobilities of some 5-azapyrimidines and their breakdown products in different solvents.

Compound	R_F-values		
	S_1	S_2	S_3
5-Azacytosine (I)	0.11	0.61	0.37
5-Azauracil	0.23	0.35	0.38
5-Azacytidine (III)	0.065	0.52	0.45
5-Azauridine	0.12	0.29	0.43
Guanidine	0.34	0.75	0.67
Amidinourea (II)	0.32	0.75	0.60
N-Amidino-N'-β-D-ribofuranosylurea (IV)	0.10	0.73	0.55
Biuret	0.34	0.69	0.75
N-Formylbiuret	0.33	0.71	not determined
1-β-D-Ribofuranosyl-biuret (V)	0.08	0.48	0.68

Table 2
The dephosphorylation of products of acid hydrolysis of RNA labeled with 5-azacytidine.

Compound	First chromatography		Second chromatography		
	Solvent	R_F	Solvent	no PMP R_F	with PMP R_F
VI + VII	S_3	0.53	S_2	0.27, 0.37	0.50, 0.67
VI + VII	S_3	0.53	S_1	0.00	0.065, 0.11
VI	S_2	0.27	S_2	0.27	0.50
VII	S_2	0.37	S_2	0.37	0.67
VIII	S_3	0.73	S_2	0.21	0.49
VIII	S_3	0.73	S_3	0.73	0.68
VIII[a]	S_2	0.21	S_2	0.21	0.49

Compounds VI, VII and VIII, obtained by chromatographic fractionation of the hydrolysate, were eluted and rechromatographed after incubation, at pH 5.8, with or without prostate phosphomonoesterase (PMP).

[a] Contaminated with some VI.

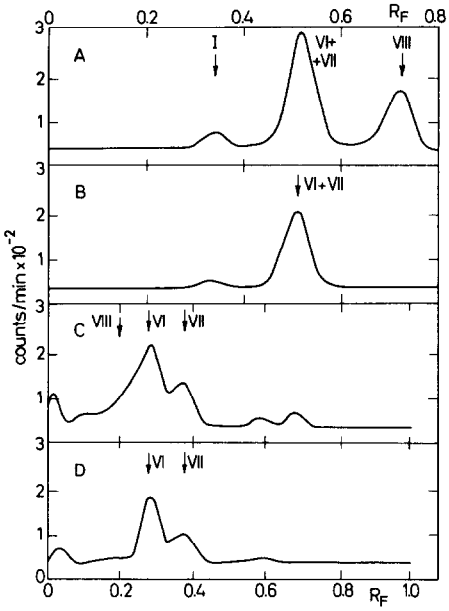

Fig. 1. Chromatographic analysis of cold-acid hydrolysate of RNA labeled with 5-azacytidine. RNA was isolated from *E. coli* B (A, C) or from strain OK 408 (B, D), and hydrolysed with 1 N HCl at 45°, 90 min. The hydrolysate was chromatographed in S_3 (A, B) or in S_2 (C, D).

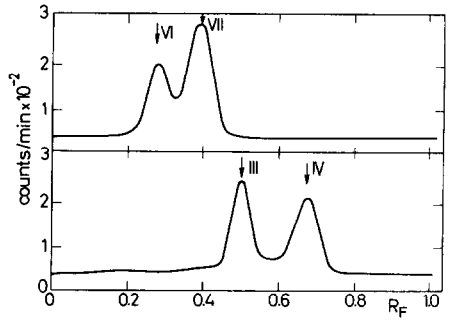

Fig. 2. The dephosphorylation of products* of cold-acid hydrolysis of RNA labeled with 5-azacytidine. The radioactive zone (R_F = 0.53) was eluted from the chromatogram shown on fig. 1A and rechromatographed in S_2 after incubation, at pH 5.8, without (A) or with (B) prostate phosphomonoesterase.

* The ratios of the heights of peaks VI and VII, or III and IV, do not truly indicate the ratio of these compounds in the original hydrolysate, since partial conversion of VI to VII, or III to IV, might have continued during elution and subsequent incubation.

Cold-acid hydrolysis (1 N HCl, 45°, 90 min) was found sufficient for complete release of 5-azapyrimidines from polynucleotides; 68% of standard 5-azacytidine remained intact under these conditions, the rest being converted to I; 5-azapyrimidine ring appears to be stable under these conditions. Labeled RNA from strain OK 408 (fig. 1B, D) produced radioactive substances which chromatographed as a single zone in S_3 (accompanied by some I), but were resolved into two components (VI and VII) in S_2. RNA from *E. coli* B (fig. 1A, C) gave, in addition to VI and VII, another radioactive substance (VIII). In S_1 all these compounds except I had zero chromatographic mobility. Enzymatic hydrolysis with RNase and spleen phosphodiesterase produced a similar chromatographic pattern except for the complete absence of I. The positions of these compounds on the chromatograms were displaced by treating the eluates with phosphomonoesterase (fig. 2). Identical products were obtained by hydrolysis of RNA to nucleosides using RNase, spleen phosphodiesterase and prostate phosphomonoesterase. The dephosphorylated products were identified on the basis of their chromatographic mobilities as indicated in table 2. We see that, using cold-acid or enzymic hydrolysis of nucleotides, about two-thirds of 5-azacytosine incorporated was recovered as 5-azacytidine-2′ (or 3′)-phosphate (VI), the rest being present in the form of N-amidino-N′-β-D-ribofuranosylurea-2′ (or 3′)-phosphate (VII). 1-β-D-Ribofuranosylbiuret-2′ (or 3′)-phosphate (VIII), a derivative of 5-azauridine, was found in wild-type RNA only. Deamination on the nucleotide level by cytidylate deaminase [11], converting a part of cytidine label to RNA-uracil in cytidine-deaminase-less strains [12], evidently does not operate with the 5-aza-analogue *in vivo*. Intact 5-azauridine was never detected among the nucleosides; knowing that this compound may be chromatographed in S_2 and S_3 without decomposition, we may exclude the possibility that its degradation could have occurred during chromatography.

4. Discussion

Considering the fact that a predominant part of incorporated 5-azacytosine survives the relatively long period of labeling and the time necessary for isolating the RNA, it appears that the extent of ring-opening of this base during functional life-time of bacterial mRNA is small. Inhibition of synthesis of inducible β-galactosidase in cytidine-deaminase-less *E. coli* [5] probably is due to the presence of intact 5-azacytosine in mRNA. On the other hand almost complete arrest of total protein synthesis, shown to be connected with uptake of 5-azauridine [4], is probably a consequence of partial depyrimidination of mRNA, caused by incorporation of 5-azauridine and its rapid subsequent breakdown to open-ring form.

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