Volume 2 number 7 July 1975 Nucleic Acids Research

The metabolism of N^4-hydroxycytidine - a mutagen for Salmonella typhimurium

Ewa Popowska and Celina Janion

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warsaw, Rakowiecka 36, Poland

Received 19 May 1975

ABSTRACT

Salmonella typhimurium cells were grown in the presence of [^{14}C]-N^4-hydroxycytidine (N^4OHcyd), a mutagenic nucleoside, and labelling in DNA and RNA digest was traced. The results show that this analogue is incorporated into RNA at a level of 40 µg/100 mg, and into DNA with a yield at least 100-times smaller. Some N^4OHcyd was rapidly metabolized and labelling was found in all ribo- and deoxyribo-nucleosides.

INTRODUCTION

Preliminary experiments on the incorporation of radioactive N^4-hydroxycytidine (N^4OHcyd)[x] into growing cells of S.typhimurium have shown that this mutagenic nucleoside is actively taken up by bacteria, and that the major part of labelling is associated with the acid-insoluble fraction[1]. In the present study, after isolation and hydrolysis of nucleic acids, the metabolic fate of N^4OHcyd in the aspect of its mutagenic properties was investigated.

MATERIALS AND METHODS

Chemicals. Uniformly labelled [^{14}C]-cytidine (sp.act. 220 mCi/mM) and 2-[^{14}C]-uridine (sp.act. 118 mCi/mM) were purchased from The Czechoslovak Institute of Radioisotopes (Prague). [^{14}C]-N^4OHcyd was obtained by the reaction of labelled cytidine with 1.6 M HA, and after acid conversion and neutralization it was purified on a Sephadex G-10 column and eluted with water. For testing the effectiveness of every step of the reaction, an analogous reaction with cold cytidine was carried out as

[x] Abbreviations used are: N^4OHcyd, N^4-hydroxycytidine; HA, hydroxylamine; 2AP, 2-aminopurine; EDTA, ethylenediaminetetraacetate.

control.

Bovine pancreas RN-ase and crystalline lysozyme were obtained from Reanal (Budapest, Hungary); crystalline DN-ase I - from Sigma Chemical Co. (St. Louis, Mo): venom phosphodiesterase and bacterial alkaline phosphatase (BAPF) - from Worthington Biochemical Corp. (Freehold, N.J.); pronase - from Koch-Light Lab. Ltd. (Colnbrook, England) and sarkosyl - from Geigy Industrial Chemicals (Ardsley N.Y.).

Bacterial strains. The following strains were used: - (i) S.typhimurium JL 1045 (pyrG,cod,cdd), cytidine-dependent, with blocked cytosine and cytidine deaminases and CTP synthetase (formerly called DP-45)[2]. The cytidine requirement cannot be replaced by N^4OHcyd. - (ii) Spontaneous mutant of the above strain - JL 1045 hxc. This strain can grow slowly when N^4OHcyd replaces cytidine in the medium. - (iii) PyrG $\longrightarrow$ $pyrG^+$ revertant of JL 1045 induced by N^4OHcyd. This strain does not require cytidine, and N^4OHcyd strongly inhibits its growth.

Growth medium. Nutrient broth (Difco), or glucose-salt medium containing per litre: 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g citric acid$\cdot H_2O$, 10 g $K_2HPO_4 \cdot H_2O$, 10 g glucose enriched with 4 g vitamin-free casamino acids (Difco), were used for growth of all bacterial strains and - when necessary - supplemented with cytidine (20 µg/ml).

Isolation of nucleic acids. Nucleic acids were isolated by caesium chloride density gradient centrifugation of the lysed cells[3]. In general, the procedure was as follows: bacteria were night-cultivated in nutrient broth, harvested and suspended in salt-glucose medium (enriched with cytidine when cytidine-dependent strains were used), till its absorption at 600 nm was 0.2-0.4 OD ($2-4 \times 10^8$ cells/ml). After one cell division (1-1.5 hrs), to 4.5 ml of the above bacterial culture $[^{14}C]$-N^4OHcyd was added and growth was continued for the next 1-2 divisions. Then the cells were harvested by centrifugation, washed three times with Tris-EDTA buffer 0.01 M, pH 8.0, suspended in 2 ml of the same buffer and lysed with 1.5 mg lysozyme (30 min at 37°C), sarkosyl - final conc. 1% (10 min at 56°C) and pronase - final conc. 1 mg/ml (2 hrs at 37°C).

Pronase was preincubated for 4 hrs at 37° and for 2.0 min at 80°C[4]. Subsequently the volume was made up with Tris-EDTA buffer to 3.5 ml, and 4.5500 g CsCl (density 1.713 g/ml) were added. 4 ml of the above solution were transfered to a centrifuge tube, overlaid with mineral oil and centrifuged at 39,000 rev/min and 20°C for 45 hours. Fractions of 20 drops (240-260 µl) were collected from the bottom of the tube, and rRNA, tRNA and DNA were separated. The major part of rRNA sedimented at the bottom of the tube, and tRNA and DNA - between fractions 2-5 and 7-9, respectively. Before enzyme digestion nucleic acids were dialyzed and flash-evaporated. The above described bacterial culture afforded 2-3 OD_{260} units of DNA and 8-10 OD_{260} units of RNA.

Hydrolysis of nucleic acids. Nucleic acids were hydrolyzed by successive treatment with DN-ase (or RN-ase), phosphodiesterase and alkaline phosphatase, according to Duch and Laskowski[5]. The resulting deoxyribonucleosides, with cold carriers added, were separated on Bio-gel P-2 by elution with 2 mM ammonium carbonate, pH 10.2, alone or with an addition of sodium tetraborate (1 mM), according to Piperno and Bernardi[6]. The addition of sodium tetraborate much improved the resolution of all five nucleosides tested (not shown in this paper). Then the fractions were purified by paper chromatography. Ribosides were separated only by paper chromatography, and the N^4OHcyd spots were - for sake of purity - eluted and rechromatographed in the same system. The spots were cut out and the radioactivity determined by liquid scintilation counting. The amounts of N^4OHcyd were calculated on the basis of its radioactivity; the results were expressed as N^4OHcyd incorporated or converted into the corresponding nucleoside per 1 mg DNA or RNA. It was accepted that 1 mg DNA = 21 OD_{260} units[7] and 1 mg RNA = 25 OD_{260} units[8].

Chromatographic systems. All nucleosides were well separated in Lane system[9] on Whatman paper No. 1 previously soaked with sat. $(NH_4)_2SO_4$:H_2O (1:9), and after drying developed (ascending) in 96% ethanol:H_2O (8:2) solvent. The following Rf values were obtained for ribosides: Urd - 0.62; N^4OHcyd - 0.55; Ado - 0.46; Guo - 0.32; Cyd - 0.24; for deoxyribosides: dThd - 0.89;

dN^4OHcyd - 0.79; dAdo - 0.64; dGuo - 0.54; dCyd - 0.41.

The same system was used for the separation of adenine (Rf 0.37) from deoxyadenosine (Rf 0.64) and deoxyribose (Rf 0.79). For the resolution of deoxyguanosine from guanine and deoxyribose, isoamyl alcohol: 5% Na_2HPO_4 (1:1) was used (Rf - 0.59; 0.39 and 0.84, respectively). Thymine was separated from thymidine and deoxyribose 1 -phosphate in the upper phase of a mixture of ethyl acetate:H_2O; formic acid (60:35:5), giving Rf values, 0.80, 0.49 and 0.18, respectively.

RESULTS.

Results of column chromatography of the DNA digest and the pattern of nucleoside labelling after growth of JL 1045 cells in the presence of $[^{14}C]$-N^4OHcyd, are given in Fig. 1.

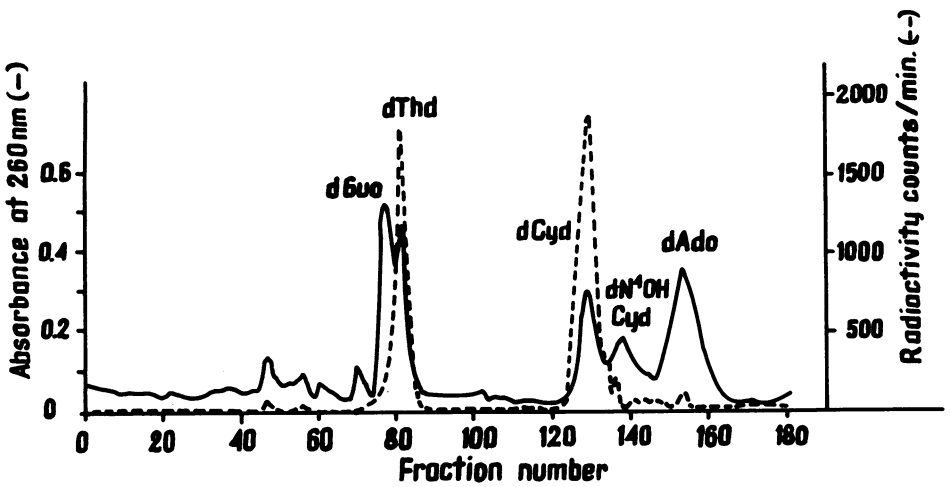

Fig. 1. Chromatography of DNA digest (Test No. 1) on Bio-gel P-2 column (110 cm x 0.9 cm); DNA was isolated from S.typhimurium JL 1045 cells growing in the presence of labelled N^4OHcyd. 0.7 A_{260} units of labelled DNA were digested, applied to a column with 3.1 A_{260} units of a mixture of cold deoxynucleosides and eluted with 2 mM ammonium carbonate, pH 10.2[6]. Fractions of 0.4 ml were collected. Solid line represents the absorbance, and broken line - the radioactivity.

The fractions of DNA digest collected from a Bio-gel column were further purified by paper chromatography, and the quantities of $[^{14}C]$-N^4OHcyd incorporated or converted into the corresponding nucleosides were calculated (see Methods). The results are shown in Table 1.

TABLE 1

The distribution of labelling in DNA digest of S.typhimurium strains growing in the presence of [^{14}C]-N^4-hydroxycytidine.

No. of test	Bacterial strain	Content of N^{4}OHcyd in medium		Radioactivity recovered in DNA digest (expressed as ng of N^{4}OHcyd converted to deoxynucleosides per mg of DNA)					Number of bacterial divisions
		Amount (nmole/ml)	Specific activity (μCi/nM)	dCyd	dThd	dGuo	dAdo	dN^{4}OHcyd	
1[x]	IL-1045	1.6	0.22	492.6	184.8	37.8	18.4	0	2.5
2	IL-1045	1.1	0.22	155.4	95.5		2.9	0	2.3
3	IL-1045	62.5	0.0063	381.1	128.2	25.6	25.6	0	1.6
4	IL-1045	58.0	0.0048	615.5	201.9	125.0	38.4	0	1
5[xx]	IL-1045	385.0	0.019	2016.6	1394.0	221.1	157.9	0	1
6	IL-1045 hxc	20.4	0.015	8102.2	1663.5	120.1	27.7	0	2.2
7	PyrG→pyrG$^+$ revertant	1.3	0.22	195.3	92.9	52.7	39.9	0	2.3

[x]In test No. 1 the conditions of bacterial growth differ from those given in Methods: 10 μg of cytidine per 1 ml of growth medium was added three times - at zero time, after 1.5 hours and after 3.5 hours. The total duration of growth was 5 hours.

[xx]In test No. 5 dithiothreitol was added to 50 mM concentration.

The distribution of radioactivity is as follows: the most appears in the deoxycytidine fraction, and less - in the thymidine fraction. Still smaller amounts of radioactivity are present in purine deoxynucleosides, while no radioactivity is detected in the deoxy-N^4-hydroxycytidine fraction.

As mentioned above, in this bacterial strain the only pathway for the synthesis of cytidine compounds: UTP ⟶ CTP, catalyzed by CTP synthetase, is blocked. Hence it was unexpected to find the major part of radioactivity in the deoxycytidine fraction.

In order to check whether blocking of CTP synthetase is complete, a similar experiment, using [^{14}C]-uridine instead of [^{14}C]-N^{4}OHcyd, was performed. The result is presented in Fig. 2. It is seen that practically the whole radioactivity is bound with the thymidine fraction, and no labelling is present in deoxycytidine. This testifies to the absence of the CTP synthetase activity in this bacterial strain.

Studies of the pathway by which N^{4}OHcyd is transformed into cytidine permitted us to demonstrate the presence of a new enzyme system capable of cytidine formation by direct reduction of N^{4}OHcyd to Cyd. This will be the subject of a separate report which will be publish elsewhere[10].

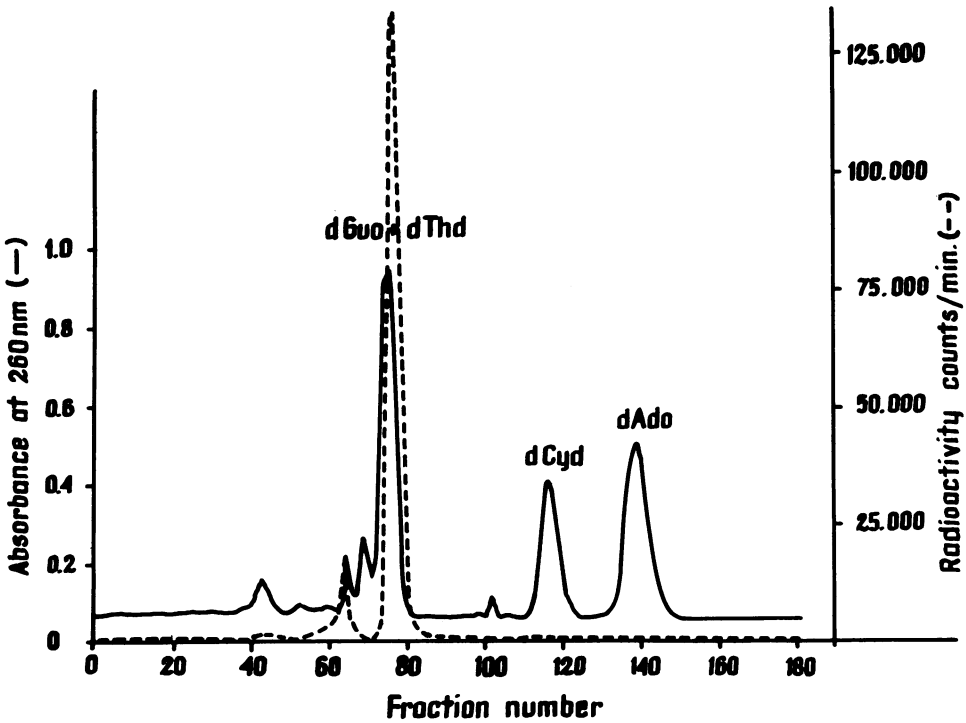

Fig. 2. Chromatography of DNA digest on Bio-gel P-2 column; DNA was isolated from S.typhimurium JL 1045 cells growing in the presence of labelled uridine. 2.4 A_{260} units of labelled DNA were digested and applied to a column with 3.3 A_{260} units of a mixture of cold deoxynucleosides. Descriptions as in legend to Fig. 1.

Mild acid hydrolysis of purine nucleosides, followed by paper chromatography, revealed that only the sugar moiety of uniformly labelled $[^{14}C]$-N^4OHcyd is utilized for purine nucleoside formation. This can be explained by the joint action of pyrimidine and purine phosphorylases: 1-phosphate deoxyribose (or ribose) obtained by phosphorolysis of thymidine (or uridine) can serve as a source of the sugar moiety for the synthesis of purine nucleosides.

In case of labelled thymidine subjected to enzymatic phosphorolysis with toluinized S.typhimurium cells and followed by chromatography, it was found that labelling is present in both components formed, i.e. thymine and deoxyribose 1 -phosphate.

The above results clearly show that N^4OHcyd is involved

in different metabolic processes. It can be reduced to cytidine, deaminated directly or via cytidine to uridine. In other experiments on labelled cytidine and toluinized bacterial cells it has been shown that the bacterial strains used exhibit, in fact, some residual activity of cytidine deaminase[10]. The ribonucleosides are subsequently converted into deoxyribo derivatives[11].

The probability was considered that the rapid metabolism of N^4OHcyd is the main handicap preventing substantial incorporation of N^4OHcyd into DNA. In a series of experiments we have tried to saturate the metabolizing enzymes by increasing the N^4OHcyd concentration in the incubation medium (tests No. 3-5). The amount of N^4OHcyd in the last experiment of this series (No. 5) was the same as used for evoking a mutagenic effect (100 µg/ml).

These attempts failed to bring about the expected effect and did not lead to the incorporation of N^4OHcyd into DNA at a detectable level.

In Table 1 the metabolic data of N^4OHcyd for two other S.typhimurium strains: JL 1045 hxc and JL 1045 pyrG → $pyrG^+$ revertant are included. As it was expected, JL 1045 hxc strain in which cytidine requirement can be satisfied with N^4OHcyd, converts more N^4OHcyd into dCyd than the other strains.

TABLE 2

The distribution of labelling in RNA digest of S.typhimurium strains growing in the presence $[^{14}C]$-N^4-hydroxycytidine.

No. of test	Bacterial strain	Content of N^4OHcyd in medium		Radioactivity recovered in RNA digest (expressed as ng of N^4OHcyd converted or incorporated to nucleosides per mg of RNA)				
		Amount (nmole/ml)	Specific activity (µCi/nM)	Cyd	Urd	Guo	Ado	N^4OHcyd
3	IL 1045	62.5	0.0063	645.6	148.3	43.6	61.1	366.5
4	IL 1045	58.0	0.0048	1797.6	309.2	-	194.6	675.5
5[x]	IL 1045	385.0	0.019	1757.2	367.3	263.2	205.4	134.2
6	IL 1045 hxc	20.4	0.015	12909.6	458.4	-	100.8	399.7
7	PyrG → $pyrG^+$ revertant	1.3	0.22	233.1	30.3	35.5	26.6	425.5

[x] In the test No. 5 dithiothreitol was added to 50 mM concentration.

The distribution of labelling in pyrG → pyrG$^+$ revertant requiring no cytidine for growth does not deviate to any greater extent from that of its parent strain (cf. test No. 7 and No. 2).

In Table 2 similar data regarding the distribution of radioactivity in RNA digest are presented. The numeration of the tests corresponds to that used in Table 1. We found no pronounced differences in the pattern of nucleoside labelling between tRNA and rRNA; the results given in Table 2 concern both nucleic acids taken jointly.

The most striking difference between the results shown in Table 1 and Table 2 lies in the fact that, in contrast to DNA, a substantial amount of N^4OHcyd is incorporated into RNA. It appears that there is no correlation between the concentration of N^4OHcyd in the medium and the degree of its incorporation. In spite of the wide range of the N^4OHcyd concentrations used, the degree of its incorporation persists at the same level. The fluctuations in the amounts of N^4OHcyd incorporated, as observed in some experiments, can be a reflection of accidental differences in the bacterial growth.

The radioactivity found in the remaining nucleosides is distributed similarly as in the DNA digest.

DISCUSSION

Wacker, Kirschfeld and Träger have reported that 2-aminopurine (2AP), another mutagenic analogue, is incorporated into E.coli nucleic acids in amount of 0.13 µg and 0.03 µg per 100 mg RNA or DNA, respectively[12]. If we assume that the mean N^4OHcyd incorporation into S.typhimurium RNA is 40 µg/100 mg, and after correction for the ribose content - 24 µg/100 mg, then this incorporation is about 180 times higher than that of 2AP into E.coli RNA. The sensitivity of our methods allows the detection of labelled base when its incorporation level is higher than 1/100 of the value found for RNA (>0.24 µg/100 mg). Thus it can be concluded that the level of N^4OHcyd incorporation into DNA is below this value and probably it lies near to the value reported by Wacker et al. for the incorporation of 2AP into DNA.

There are two possibilities of explaining the low incorporation of N^4OHcyd into DNA: 1) Ribonucleoside diphosphate reductase exhibits a low activity against N^4OHcyd derivative and 2) N^4OHcyd acts mainly as uridine analogue. The similar level of N^4OHcyd incorporation into cytidine--independent and cytidine-dependent strains appears to be consistent with the latter assumption. Unfortunately S.typhimurium cells do not contain deoxycytidine kinase[13], and the above suggestions cannot be verified by testing the correlation between the incorporation and mutagenic activity of deoxy-N^4OHcyd. Perhaps the use of RNA-containing bacteriophages would allow elucidation of this problems.

ACKNOWLEDGEMENTS

This work was supported by the Polish Academy of Sciences (Project 09.3.1.) and in part by the Agriculture Research Service, U.S. Department of Agriculture.

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