N^4-HYDROXYCYTIDINE - A NEW MUTAGEN OF A BASE ANALOGUE TYPE.

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Summary - N^4-hydroxycytidine (N^4-OHcyd)[x] is incorporated into nucleic acids of a cytidine-requiring strain of S.typhimurium 1045 and can act mutagenically. The reversion frequency of $pyrG^- \longrightarrow pyrG^+$ is 10-20 fold higher than the spontaneous background. N^4OHcyd-induced revertants show a strong inhibitory effect in the presence of N^4OHcyd. The influence of N^4OHcyd on cytidine metabolism is discussed.

Hydroxylamine (HA) and lastly its N and O-methylated analogues belong to the most widely studied chemical mutagens (1,2). The chemical basis of its mutagenic action - the reaction with the cytosine nucleus (3-6), as well as its mutagenic consequences, G:C to A:T transitions (7-9) are well documented. There is fairly convincing evidence that N^4-hydroxy-5-methylhydroxycytosine, the only product formed in DNA T-even phages (5,6), and N^4-hydroxycytosine residues formed in normal cytosine containing DNA, are responsible for hydroxylamine mutagenesis (6,10-12).

In this report we present evidence that N^4-hydroxycytidine, when incorporated into bacteria, can act as a mutagen.

Materials and Methods.

Bacterial strains: Cytidine-dependent S.typhimurium JL 1045 ($pyrG^- cod^- cdd^-$) deficient in CTP synthetase, cytosine and cytidine deaminases (formerly called DP-45 (13)), and parent strain S.typhimurium DL-38 ($cod^- cdd^-$) were obtained thanks the kindness of prof. J.Neuhard.

Medium: 1) Glucose-salt - casamino acids medium containing per litre: 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g citric acid$\cdot H_2O$, 10 g K_2HPO_4, 3.5 g $NaNH_4HPO_4 \cdot 4H_2O$ + 20 g glucose + 4 g vitamino free casamino acids (enMM), 2) Enriched

[x] Abbreviation used are: HA, hydroxylamine; N^4OHcyd, N^4-hydroxycytidine; N^4OMecyd, N^4-methoxycytidine.

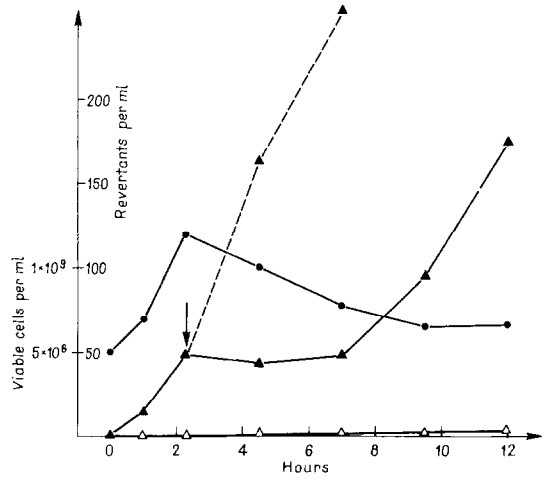

Fig. 1. The influence of N^4-hydroxycytidine (100 µg/ml) on mutagenesis of S.typhimurium JL 1045 (see Methods for details). All viable counts: (o—o); $pyrG^- \longrightarrow pyrG^+$ N^4OHcyd-induced revertants: (▲—▲); $pyrG^- \longrightarrow pyrG^+$ spontaneous revertants: (△—△). Revertant growth after washing and suspension in fresh medium (enMM + cyd without N^4OHcyd) at indicated time intervals is given by the broken line.

medium with 20 µg/ml of cytidine (enMM + cyd). 3) Nutritient broth (Difco) - 1.5% agar for viable counts (NB). 4) enMM - 1.5% agar for revertant counts.

N^4-hydroxycytidine (N^4OHcyd) was obtained by reaction of cyd with HA and, after acid conversion and Sephadex G-10 filtration, crystallised.

Mutagenization with N^4-hydroxycytidine S.typhimurium JL 1045, about $5x10^8$ cells/ml, was grown on enMM + cyd (control) or on enMM + cyd + 100 µg/ml N^4OHcyd (mutagenization) and, after appropriate time intervals, aliquots were taken and plated for viable and $pyrG^- \longrightarrow pyrG^+$ revertants counts.

Results.

The spot test analysis (14) of S.typhimurium with N^4OHcyd centered on the plate revealed the presence of a ring of revertants ($pyrG^+$) able to grow without cytidine. On the control plate no or few spontaneous revertants were seen. There was no effect when the following compounds were tested: N^4-methoxycytidine, 5-bromodeoxyuridine, 2-aminopurine riboside.

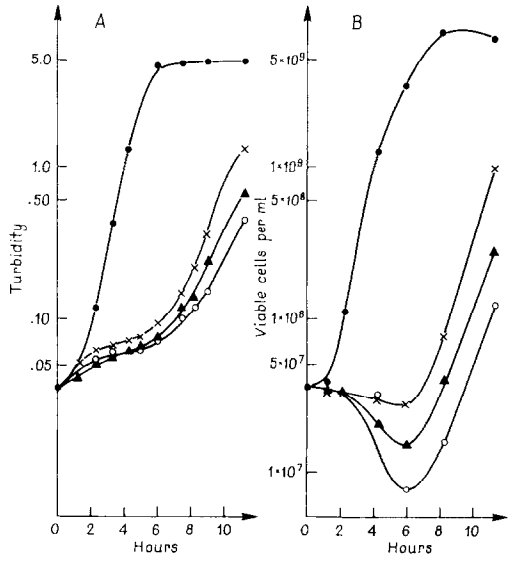

Fig. 2. The influence of N^4OHcyd on N^4OHcyd-induced pyrG$^-$ ⟶ pyrG$^+$ revertants of S.typhimurium JL 1045. No N^4OHcyd: (●—●); with 10 µg/ml: (×—×); with 50 µg/ml: (▲—▲); and with 100 µg/ml of N^4OHcyd: (○—○); A) - optical density at λ = 600 nm; B) - viable counts.

A more detailed study of the growth kinetics of this bacterial strain, and appearance of pyrG$^-$ ⟶ pyrG$^+$ after N^4OHcyd mutagenization, were undertaken in liquid medium. One of the experiments is shown in Fig. 1. The amount of cytidine in the medium was sufficient for 1-2 divisions. It can be seen that after two hours, when bacterial growth has stopped, no new revertants are visible. It is well known that mutagens of the base analogue type act on dividing cells. But it was puzzling that between about 2-7 hours no revertant growth due to normal bacterial propagation can be seen. When after two hours of growth the bacterial population was harvested, washed and suspended in fresh enMM + cyd medium, growth proceeded rapidly (broken line in Fig. 1).

The inhibitory effect of N^4OHcyd on revertant growth is clearer when pure colonies of revertants were cultivated in the presence or absence of N^4OHcyd. The optical density and, simultaneously, viable counts, were checked (Fig. 2). As may be seen, this effect is dose dep-

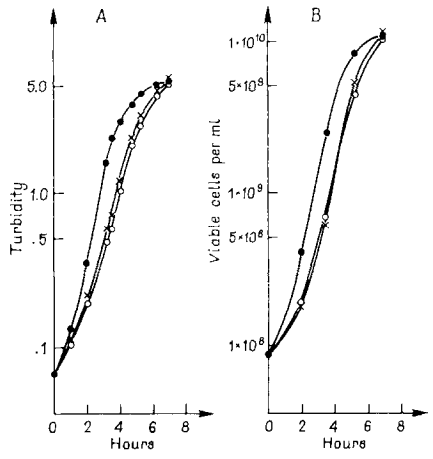

Fig. 3. The influence of N^4-hydroxycytidine on S.typhimurium DL 38. Without N^4OHcyd: (●——●); with 10 µg/ml of N^4OHcyd: (×——×); with 100 µg/ml of N^4OHcyd: (○——○).

endent. At a concentration of 10 µg/ml N^4OHcyd there is some growth inhibition, whereas at 50 µg/ml and 100 µg/ml there is a pronounced lethal effect. Both effects, the growth inhibition and lethality, are overcome after 6 hours incubation. The inhibition is almost fully reversed when cytidine is present at the same concentration as N^4-hydroxycytidine. Of the other cytosine analogues tested: N^4-methoxycytidine, N^4-hydroxydeoxycytidine, 2'-O-methylcytidine, all at concentrations of 100 µg/ml have no effect on revertant growth.

The JL 1045 strain was originally obtained by treatment with nitrosoguanidine of the S.typhimurium DL-38 strain (13); DL-38 susceptibility to N^4OHcyd was then tested (Fig. 3). It can be seen that this strain responds to N^4OHcyd in a different way than $pyrG^- \longrightarrow pyrG^+$ revertants of DL-1045. There is no difference in the rate of growth and viable counts between doses of 10 µg and 100 µg/ml N^4OHcyd. There is no visible lethal effect. The only effect is some delay in cell division which persists about one hour.

It is reasonable to suppose that N^4OHcyd-induced reversions are not true back-mutations, or that the JL 1045 strain originates from

DL 38 strain by two independent mutagenic hits. But these conclusions require further experimental support.

The reversion frequency induced by N^4OHcyd was $2\text{-}4 \times 10^{-8}$; the spontaneous reversion frequency was $0.1\text{-}2 \times 10^{-9}$.

It is worth mentioning that N^4OHcyd at a dose of 100 µg/ml after a short period of slight inhibition, stimulates bacterial growth of the mutagenised DL 1045 strain. The stimulatory effect is more evident when the cytidine in the medium is exhausted. After this time the viability of cells decreases more slowly in the presence of N^4OHcyd.

Reconstruction experiments. The fact that $pyrG^- \longrightarrow pyrG^+$ revertants obtained through N^4OHcyd-induced mutation are sensitive to its presence excluded the posibility that selection, and not mutation, is responsible for the appearance of revertants. Noneless a reconstruction experiment, which is regarded as the best control for detection of selection, was undertaken (Fig. 4).

Preserving the same experimental conditions, a large population (4×10^8/ml) of S.typhimurium JL 1045 cytidine-dependent streptomycin sensitive cells ($pyrG^- str^s$) were mixed with a small number (150/ml) of N^4OHcyd revertants, streptomycin resistant ($pyrG^+ str^r$). At intervals of 30-40 minutes, appriopriate samples were taken and plated on three types of plates: i) NB for viable counts, ii) enMM + 200 µg streptomycin for $pyrG^+ str^r$ counts and iii) enMM for $pyrG^+ str^s$ and $pyrG^+ str^r$ counts.

The growth rate of $pyrG^+ str^r$ cells did not exceed that of $pyrG^+ str^s$ cells, excluding the possibility of selection.

The incorporation of N^4-hydroxycytidine into bacterial cells.

The preliminary result of incubation of S.typhimurium JL 1045 cells with N^4OHcyd at a concentration of 0.3 µg/ml (0.3 µCi) showed that the radioactivity is actively taken up by the cells (Table I). When growth of bacteria due to lack of cytosine was stopped, the uptake of N^4OHcyd ceased. The amount of radioactivity inside the cells was

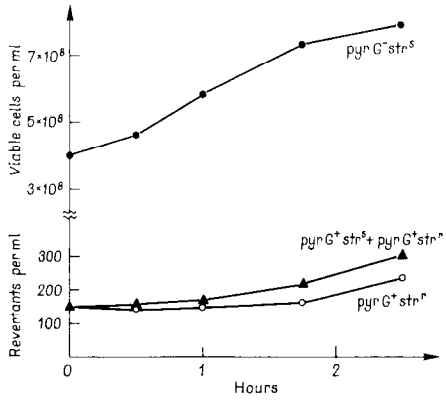

Fig. 4. Reconstruction experiments in the presence of N^4OHcyd 100 µg/ml. Viable counts of cytidine-dependent streptomycin sensitive cells ($pyrG^-str^S$): (●—●); cytidine-independent streptomycin resistant cells ($pyrG^+str^R$): (○—○); cytidine-independent streptomycin sensitive and resistant cells ($pyrG^+str^S$ + $pyrG^+str^R$): (▲—▲), (see text for details).

more or less evenly distributed between acid soluble and acid insoluble material. RNase and DNase treatment showed that considerable amounts of radioactivity were bound by DNA. The isolation of DNA and identification of N^4OHcyd are planned.

Discussion.

The use of a bacterial strain lacking cytosine and cytidine deaminase was a necessity, for N^4-hydroxycytidine and its base are slowly deaminated by these enzymes (16, Popowska and Janion, unpublished). The facility with which N^4OHcyd is incorporated into RNA and DNA testify that all enzymes on the cytidine pathway can use N^4OHcyd and intermediates as substrates.

Lieberman has shown that hydroxylamine can replace ammonia in enzymic amination of UTP to CTP and N^4OH-CTP is formed (17). The most probable explanation of the sensitivity of induced revertants towards N^4OHcyd is that this compound, probably at the triphosphate level, is disturbing the metabolic pathway(s) of CTP formation. The reversibility of this effect by cytidine seems to support this conclusion. The more pronounced effect of N^4OHcyd on N^4OHcyd-induced revertants than on the

Table I. Incorporation of N^4-hydroxycytidine into S.typhimurium JL 1045[x]

Time (in hours)	Radioactivity (imp/min)	
	whole cells	acid insoluble fraction
0	4 000	-
1	48 100	34 600
2	317 000	124 600
3	244 300	130 500
7.5	232 800	-

[x] according to Ref. 15.

parent strain, can be due to incomplete recovered activity of the mutated enzyme. Apparent mutagenic activity of N^4OHcyd supports the previous presumption that formation of this compound is responsible for hydroxylamine mutagenesis. It is surprising, however, that no $pyrG^-$ $\longrightarrow$ $pyrG^+$ reversions are observed with N^4-O-methoxycytidine - the compound formed by O-methylhydroxylamine treatment of cytidine. N^4-OMecyd when given to the liquid medium even inhibits the growth of the cytidine-dependent strain. Among the several reasons explaining this lack of mutagenic activity at least two seem most probable: N^4OMecyd derivatives are not substrates for all enzymes on the metabolic pathway of cytidine utilization, or the presence of N^4OMecyd leads to different mutagenic events than N^4OHcyd.

Further work on mutagenic activity of N^4hydroxycytidine and N^4-methoxycytidine are under investigation.

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