Incorporation of 5-Substituted Uracil Derivatives into Nucleic Acids

THE ISOLATION AND γ-RADIATION-SENSITIVITY OF BACTERIOPHAGE T3 CONTAINING THE THYMINE ANALOGUE 5-VINYLURACIL

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Bacteriophage T3 was produced in a form that contained 32% of its normal DNA thymine residues replaced with 5-vinyluracil residues by infecting a thymine-requiring strain of *Escherichia coli* with phage T3 in a medium containing 5-vinyluracil. When 2'-deoxy-5-vinyluridine was added to the medium instead, no incorporation was observed into the phage DNA, and the presence of the deoxyribonucleoside severely decreased the number of viable phage particles produced. The analogue-containing phage, although initially viable, rapidly lost viability when stored, but it was no more sensitive than was normal phage T3 to the effect of γ-radiation.

For some while we have been interested in the synthesis of some 5-substituted uracil derivatives that could be regarded as thymine analogues and hence potentially capable of being incorporated into DNA and that would then cause the DNA to be sensitive to radiation.

We have already shown that 5-vinyluracil can be incorporated into the DNA of *Escherichia coli* (Chelton *et al.*, 1973) and a *Mycoplasma* species (Jones & Walker, 1975). We have reported results obtained from the γ-irradiation of the *Mycoplasma* cells containing the analogue (Chelton *et al.*, 1979) and it was shown that the cells were 3 times as sensitive to irradiation as normal cells. However, the incorporation of analogue into *E. coli* cells was not very satisfactory, as, under the conditions required to obtain significant incorporation (10% thymine replacement), the concentration of analogue required in the growth medium was so high that less than 10% of the normal cell viability was achieved. Thus we could never be sure that any increase in radiation-sensitivity of these remaining viable cells was due to the presence of the analogue in the DNA or to the fact that one was looking at the response of a non-representative cell sample that had managed to survive the effects of the near-toxic growth conditions. Incorporation of 5-vinyluracil into *Mycoplasma mycoides capri* was more satisfactory in that, under the conditions used, little cell toxicity was observed; however, to achieve this, the concentrated growth medium had to be used, and thus the incorporation of analogue into the cells was low

(≪5% thymine replacement) and it required a massive concentration (1 mM) of analogue in the growth medium.

The present communication describes attempts to incorporate 5-vinyluracil into the DNA of bacteriophage T3.

Experimental

General

NaB³H₄, [2-¹⁴C]thymine and [*Me*-³H]thymidine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 5-Bromo[6-³H]uracil was obtained from New England Nuclear, Winchester, Hants., U.K. 5-[1-³H]Vinyluracil was prepared as previously described (Evans *et al.*, 1973) to give a product of specific radioactivity 30 mCi/mmol. 2'-Deoxy-5-vinyluridine was prepared essentially by the method of Sharma & Bobek (1975), although HgBr₂ (0.1 mol.equiv.) and molecular sieve were used as catalysts. 2'-Deoxy-5-[2-³H]vinyluridine (2.6 mCi/mmol) was prepared in a similar manner starting from 5-[*Me*-³H]acetyluracil prepared as previously described (Evans *et al.*, 1973), from which 5-[2-³H]vinyluracil was prepared and then condensed with a suitably protected sugar derivative to give the labelled deoxynucleoside.

Radioactivities of solutions of ³H- and ¹⁴C-labelled compounds were counted as previously described (Chelton *et al.*, 1973).

Organism

The thymidine-requiring strain of *E. coli* CR34 was obtained from Professor D. Shugar. The coliphage T3 (N.C.I.B. 10090) was used.

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I

Medium and growth conditions

A modified M9 medium was used for the growth of *E. coli* CR34 as previously described (Chelton *et al.*, 1973), except that arginine, tryptophan and methionine solutions were omitted and replaced with casamino acids (1.0%). Thymine (1.0 μg/ml) was also added. The inocula used were as previously described (Chelton *et al.*, 1973), and the cells were incubated at 37°C with aeration at 7 litres/min. After 5 h, the A_{750} had reached 0.35 and the cells were harvested by centrifugation at 25 000 g for 5 min at 4°C. The supernatant was decanted off and the cells were carefully resuspended in growth medium lacking thymine (200 ml). The cells were thus washed twice and finally resuspended in medium lacking thymine (800 ml) in a 2-litre flask at 37°C, and aeration was continued at 7 litres/min for 5 min. Analogue solution (approx. 10 ml of sterile solution to give the final concentration given in Table 1) was then added, followed by phage T3 (1 ml; titre 1.0×10^9 plaque-forming units/ml), and aeration was continued. Lysis was complete in 1 h, at which stage samples could be removed for phage assay. To the remaining solution was added ribonuclease (8 mg) and deoxyribonuclease (1.6 mg), and, after incubation at room temperature for 2 h, the lysate was left at 4°C overnight.

Isolation of bacteriophage particles

The above lysate was centrifuged at 25 000 g for 5 min, filtered and the supernatant centrifuged at 78 000 g for 3 h. The supernatant was removed, the phage pellet was dissolved in 0.5 M-NaCl/1 mM-MgCl$_2$ (10 ml) and re-centrifuged at 10 000 g for 5 min, and the supernatant was centrifuged at 500 000 g for 1 h. The resulting phage pellet was then used for the isolation of DNA.

Isolation and analysis of DNA

The DNA was isolated by using phenol and *p*-aminosalicylate solutions as previously described (Jones & Walker, 1963), except that no fractionation of DNA from RNA was of course necessary. The DNA was always obtained as fibrous material that could be removed on a glass rod, dissolved in water, dialysed and stored frozen at −20°C. The base-analogue content of the DNA was calculated from the specific radioactivity of the DNA so isolated, and that this radioactivity was only present in the expected residues was confirmed by analysing the DNA as previously described (Chelton *et al.*, 1973).

Conditions of radiation

γ-Radiation from a ^{60}Co source was used and the conditions used were as previously described (Chelton *et al.*, 1979). The phage assay on irradiated and suitable controls was determined in the standard way (Lark *et al.*, 1963).

Results and Discussion

The amount of base analogue incorporated into phage-T3 DNA is shown in Table 1. The results show quite clearly that, under the conditions used in the control experiments, the specific radioactivity of the thymidine and thymine in the medium was the same as that found in the isolated DNA; that is no other significant competing source of unlabelled thymine was available for the phage, and thus when 5-bromouracil was added, within experimental error, all of the thymine in the phage DNA could be replaced with this analogue and the number of viable phage particles produced was unaffected. The results also show that the deoxyribonucleoside 2'-deoxy-5-

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Table 1. *Base composition of bacteriophage T3*

Phage T3 was replicated in *E. coli* CR34 cells with the radioactive base or nucleoside indicated in the medium. The number of viable particles was determined and the DNA was isolated from the virus pellet obtained from 800 ml of medium. The analogue content of the DNA as calculated from the specific radioactivity of the analogue is given as the percentage of the total thymine content of normal phage-T3 DNA.

Analogue	Concn. of analogue in the medium (μM)	Viable phage (plaque-forming units/ ml of culture)	DNA isolated (A_{260}/ml of culture)	Analogue content (% thymidine replacement)
Thymidine	100	3.0×10^9	0.0050	100*
Thymine	100	3.4×10^9	0.0036	100*
5-Bromouracil	100	2.5×10^9	0.0032	100
2'-Deoxy-5-vinyluridine	25	1.5×10^9	0.0020	<5
2'-Deoxy-5-vinyluridine	100	0.5×10^9	0.0010	<5
5-Vinyluracil	100	2.1×10^9	0.0025	32

* This value signifies that the specific radioactivity of the thymine (thymidine) in the culture medium was identical with that in the DNA, indicating that no other significant source of thymine (thymidine) had been used for phage DNA synthesis.

vinyluridine is not incorporated into phage-T3 DNA and that the analogue is rather toxic, with only 25% of the normal number of phage particles being produced. Any decrease in the concentration of the deoxyribonucleoside in the medium in order to lessen its toxic effect did not result in any significant incorporation of the analogue into the phage DNA. This result is perhaps not too surprising, as the concentration required to inhibit the growth of some other DNA viruses (ID_{50}) of 2'-deoxy-5-vinyluridine is $0.3 \mu M$ for herpes virus type 1 and $3 \mu M$ for vaccinia virus, compared with the concentration of $100 \mu M$ used here (De Clercq et al., 1979a,b; E. De Clercq & R. T. Walker, unpublished work). However, the base 5-vinyluracil can be incorporated into the DNA of T3 phage such that more than 30% of the thymine residues are replaced by 5-vinyluracil, as shown by the recovery of all the radioactivity in the DNA as 2'-deoxy-5-vinyluridine on enzymic digestion and chromatographic separation of the nucleosides so formed. No decrease in the virus yield is seen, but, as we do not get complete replacement of the thymine by 5-vinyluracil, it is clear that some barrier to this analogue incorporation exists when compared with that of 5-bromouracil. However, this is a much higher incorporation of 5-vinyluracil into DNA than that which we have previously achieved in other systems, and its presence does not apparently affect the initial capability of the virus to replicate. It should of course be remembered that the *E. coli* used is a thymine-requiring strain, and thus only the salvage pathway is available for the synthesis of thymine (or thymine-analogue) residues in the DNA of the bacterium and phage.

It is difficult to rationalize these incorporation results when taken together with the fact that 5-vinyluracil, even at very high ($1000 \mu M$) concentration, has no effect on the number of viable T3-phage particles produced, whereas a relatively low ($25 \mu M$) concentration of the deoxyribonucleoside exerts a considerable effect (Table 1). It seems most unlikely that 5-vinyluracil is incorporated into the DNA other than via the normal thymine salvage pathway, the first product of which is the deoxynucleoside, and the explanation is likely to be connected with the different mechanisms and ease of transport of the base relative to the deoxyribonucleoside into the cell.

The results of the γ-irradiation are shown in Fig. 1. The effects of radiation in broth are due primarily to the direct deposition of energy within the phage particle (Watson, 1950). However, no significant difference in viability was found when irradiation was performed in buffer (results not shown), and, as the sensitivity of DNA in most cells seems to be of the same order of magnitude as that of DNA within a phage suspended in broth rather than in the artificial pure ionic environment of buffer (Freifelder
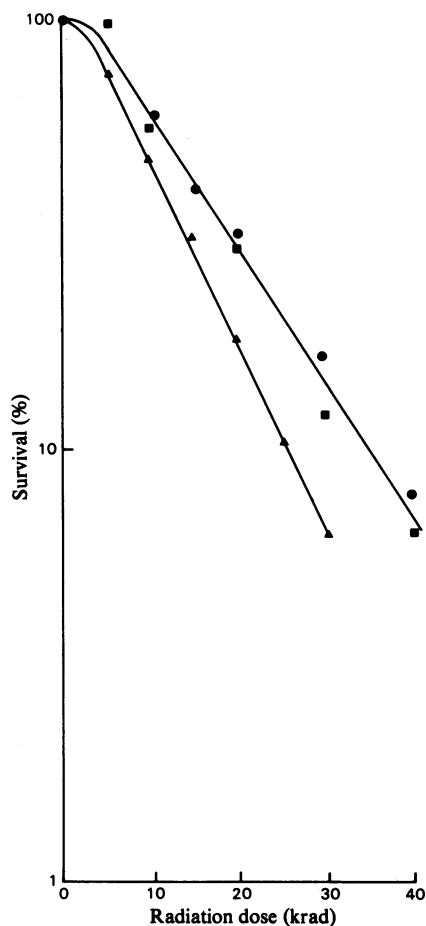

Fig. 1. *γ-Irradiation of bacteriophage T3*
Survival curves are shown for control (■), 5-vinyluracil-substituted (●) and 5-bromouracil-substituted (▲) phage T3 after exposure to ^{60}Co γ-radiation while suspended in broth.

& Freifelder, 1966), it is considered that these results should be relevant.

It can be seen that there is no significant difference in viability between the normal and 5-vinyluracil-containing phage particles, whereas the 5-bromo-uracil-containing phage showed a degree of sensitization of 1.4, which corresponds well with results previously reported (Freifelder & Freifelder, 1966; Myers et al., 1977). Previously we have shown that a *Mycoplasma* species with DNA containing a small percentage of 5-vinyluracil (≤5% replacement of thymine residues) was 3 times as sensitive to γ-radiation as cells not containing the analogue (Chelton et al., 1979). In that system, one necessarily has to irradiate not only the genetic material but the entire cell, including the components of the DNA-replication process, and also the cell has been exposed to high concentrations of the analogue in

the growth medium. Under these conditions radiation-sensitivity is seen, but it is not clear whether this is due to the presence of the analogue in the DNA or to some other effect caused perhaps by the irradiation or suboptimal growth conditions. In the phage experiments, only the phage has been exposed to the analogue and the irradiation. The subsequent replication is performed in a normal untreated host *E. coli*. Under these conditions the presence of significant amounts of analogue in the phage DNA initially seems to cause neither immediate loss of viability nor an increase in sensitivity to γ-radiation.

However, the 5-vinyluracil-containing phage rapidly loses viability when kept (linear survival curve with 90% loss of viability in 30 days). Presumably the presence of one inter-strand cross-link per DNA molecule would be sufficient to cause a loss of viability. A similar toxicity caused by incorporation of 2'-deoxy-5-hydroxymethyluridine into DNA has been postulated as being caused by the production of a base–protein cross-link after production of a stabilized cation, and a similar situation could occur here (Matthes *et al.*, 1979). It is not absolutely clear from the experiments reported in the present paper and those described previously (Chelton *et al.*, 1979) whether the presence of 5-vinyluracil in the DNA of a higher organism is likely to cause an increase in the susceptibility of the organism to γ-irradiation nor not, but it does appear possible that, if sufficient 5-vinyluracil can be incorporated into DNA, this in itself may be sufficient to prevent replication.

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