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ACTION OF INHIBITORS OF MACROMOLECULE FORMATION ON DUPLICATION OF *B. SUBTILIS* PHAGE 2C-DNA

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Abstract—The action exerted by inhibitors of DNA, RNA and protein synthesis on the replication of *B. subtilis* phage 2C and on the duplication of its DNA was investigated. Virus yield was reduced by all the antibiotics tested, the most active drugs being actinomycin, rifampicin and the mixture of the two virginiamycin components VM and VS.* The effect of these inhibitors on virion formation was irreversible, whereas that of chloramphenicol, nalidixic acid, mitomycin and of VM and VS separately was fully reversible. The phage genome, containing hydroxymethyluracil instead of thymine, was labeled by adding [^{3}H]uracil to infected cells. Hence, this precursor was used to monitor the action of inhibitors on viral DNA replication. Actinomycin prevented completely viral DNA formation at any moment of the viral cycle, whereas the effect of rifampicin was restricted to the eclipse phase. Duplicational and translational inhibitors (mitomycin, nalidixic acid, chloramphenicol, VM and VS), were partly inhibitory during the entire replication cycle, the extent of inhibition of DNA synthesis being inversely related to the time elapsed from infection. The implication of these findings on the pattern of macromolecule formation during the viral cycle is discussed.

Macromolecule metabolism in *E. coli* infected with phage T4 has been largely clarified by the addition of inhibitors at different moments of the viral cycle. In the T4-*E. coli* system, chloramphenicol proved able to block completely viral DNA formation when added at the beginning of the eclipse phase, but was without effect at the end of it [1, 2]. Moreover, this antibiotic prevented the shut-off of cellular RNA synthesis, resulting from the attachment of T4 particles [3–5]. On the other hand, both transcriptional and translational inhibitors were found to hinder the lytic development in lysogenic cyanophyces: irreversibly when present during induction, and reversibly when added during the multiplication phase [6].

Replication of phage 2C in *B. subtilis* has several unique traits. The virions harbor a molecule of double-stranded bimodal DNA of 10^8 daltons, in which hydroxymethyluracil (HMU) replaces completely thymine [7–9]. The DNA of the infecting particles duplicates semiconservatively and then undergoes genetic recombination with newly formed molecules [11, 12]. The size of the pieces transferred by recombination is comparable to that of the Okazaki pieces which are polymerized in the discontinuous duplication of 2C-DNA *in vivo* and *in vitro* [13, 14].

In the present work, known inhibitors of DNA, RNA and protein synthesis in bacteria have been added at different moments of the replication cycle to cultures of 2C-infected *B. subtilis* and the formation of viral DNA has been analysed. The purpose of the work is to contribute, by use of inhibitors, to clarify the role of transcriptional and translational events in the processes of synthesis of the viral genome.

* Abbreviations: Virginiamycin M (VM); Virginiamycin S (VS).

MATERIALS AND METHODS

Virus 2C was multiplied in the 168/2 (leu$^-$ tryp$^-$) strain of *B. subtilis*. Composition of synthetic YS medium, and techniques for concentration and titration of virus 2C, have been previously described [15].

Evaluation of viral DNA from [^{3}H]uracil-labeled virus-infected cells was carried out by taking samples as indicated in the legends to the figures and hydrolyzing total intracellular nucleic acid with 0.5 M KOH (14 hr at 37°): the hydrolysate was neutralized on ice, treated with 0.5 M trichloracetic acid and filtered through micropore membranes, which were dried and counted [15].

Intracellular DNA was extracted with phenol [13] and anlysed by CsCl isopycnic centrifugation (Spinco centrifugation; R40 angular rotor; 68 hr; 17° C). Fractions were collected from the bottom of the centrifuge tube on Whatman filter papers, treated with 0.5 M KOH (14 hr at 37° C) and 0.5 M trichloroacetic acid and the radioactivity was determined [12, 13].

[6-^{3}H]Uracil (28 Ci/mmole) was obtained from IRE (Mol, Belgium). L-Broth, Casamino Acids (acid hydrolysate of casein), and yeast extract were purchased from Difco Laboratories (Detroit, Mich., U.S.A.); and EDTA, trichloroacetic acid, and sodium dodecyl sulfate (SDS) from BDH (Poole, England). Purification, crystallisation and structure

Table 1. Inhibition of virus particle formation by increasing concentrations of different antibiotics

Inhibitor	Virus titer ($\times 10^7$ pfu/ml)†‡ in the presence of					
(μg/ml)*	0.1	0·5	5.0	10	25	50
None	1000					
Mitomycin	238.8	268.7	0.22	0.21		
Nalidixic acid			955.6	1177.8	1.40	0.78
Actinomycin	0.127	0.051	0.023	0.0091		
Rifampicin	4.53	0.62	0.0096	0.0056		
Chloramphenicol	511.1	511.1	1.36	0.027		
VM		765.7	38.8	4.5		
VS		551.0	0.087	0.029		
VM + VS	5.82					

* Inhibitors present during the entire cycle.
‡ All samples lysed 2 hr after infection.
† Virus titers normalized to a control value = 10^{10} pfu/ml.

of the 2 virginiamycin components VM and VS was previously reported [15]. The other antibiotics were obtained from Serva (Heidelberg, West Germany).

RESULTS

Virus yield in the presence of different inhibitors

The inhibitors used in the present work were tested on the 2C-*B. subtilis* system under 2 experimental conditions: (a) increasing amounts of antibiotics were present during the entire replication cycle, and (b) inhibitors were added at different moments of the replication cycle, and removed after 10 min, thereafter the viral cycle being allowed to proceed to completion in the absence of antibiotics. In all cases, cultures were treated with chloroform and lysozyme, and viral particles were titrated in the lysates.

As shown in Table 1, all the antibiotics tested produced different levels of inhibition of virus 2C replication. Titers are given, calculated relative to the control without inhibitor in each experiment, made equal to 10^{10} pfu/ml (the control titers were in all experiments close to this value).

A possible reversibility of the antibiotic action was tested as shown in Table 2. The inhibitory action of actinomycin and of the VM + VS mixture was very pronounced and irreversible, during the entire viral cycle, that of rifampicin and mytomicin was irreversible during the eclipse phase but decreased as the cycle progressed, that of nalidixic acid, chloramphenicol, VM and VS was reversible at any time including the early latent period.

Uracil as a precursor of viral DNA in phage infected bacteria

Because of the presence of the abnormal base (hydroxymethyluracil) in phage DNA, uracil was used as labeled precursor. Total DNA synthesis was measured as a function of time after infection: *B. subtilis* growing exponentially (5×10^7 cells/ml) were infected with virus 2C (MOI = 30). Five min after infection, [3H]uracil was added (20 μCi/ml, 28 Ci/mM), aliquots were taken and treated as described under Materials and Methods. Figure 1(A) shows incorporation of [3H]uracil into DNA as a function of time after infection. It can be seen that DNA synthesis starts 10 min after infection.

In order to show that viral DNA was being labeled under these conditions [^{3}H]uracil was added 15 min after infection. The culture was quenched 5 min later with 1 × SSC, DNA was phenol extracted and

Table 2. Reversibility of the action of inhibitors on virion formation

Inhibitor	(μg/ml)*	Virus titer ($\times 10^7$ pfu/ml)*† Time of contact 0–10	5–15	10–20	15–25
			min after infection‡		
None		1000	1000	1000	1000
Mitomycin	5	145	226	516	532
Nalidixic acid	50	1265	984	893	326
Actinomycin	0.1	1226	1016	516	839
	1	<0.01	<0.01	<0.01	<0.01
Rifampicin	0.5	0.44	2.42	5.1	28.3
Chloramphenicol	10	1441	790	964	630
VM	5	578	723	651	481
VS	5	871	1290	1113	758
VM + VS	0.1	548	1065	694	521
	1	<0.01	<0.01	<0.01	<0.01

* Inhibitors added at indicated times and present for a 10-min period.
† Samples incubated for 2 hr in the absence of inhibitors and then lysed.
‡ Virus titers normalized to a control value = 10^{10} pfu/ml.

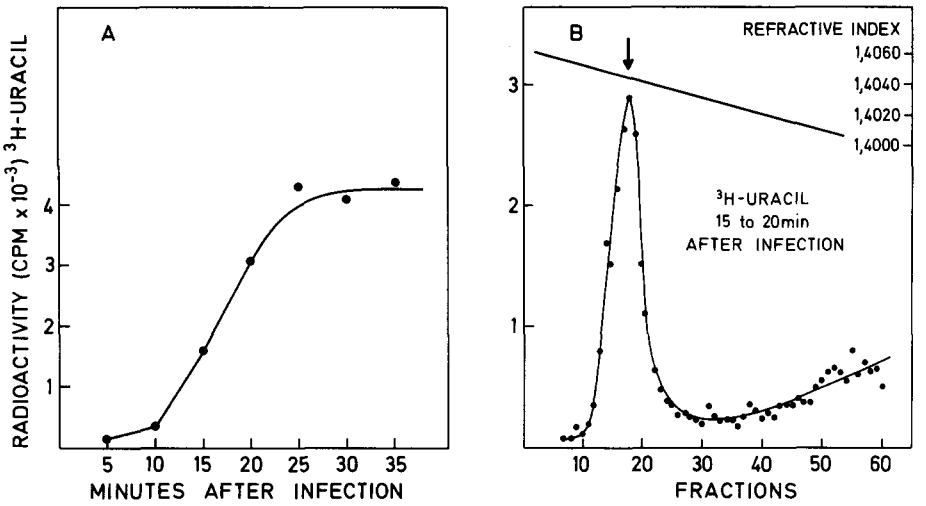

Fig. 1. (A) DNA synthesis as a function of time after infection. [^{3}H]Uracil (20 μCi/ml; 28 Ci/mM) was added to virus infected bacteria (1.5×10^8 pfu/5×10^7 cells/ml) 5 min after infection. Aliquots were taken thereafter and the amount of radioactivity incorporated into DNA was determined (see Materials and Methods). (B) Isopycnic CsCl centrifugation of [^{3}H]uracil-labeled DNA. [^{3}H]Uracil (20 μCi/ml; 28 Ci/mM) was added to virus infected bacteria (1.5×10^8 pfu/5×10^7 cells/ml) 15 min after infection. After 5 min in contact with labeled precursor, 1 × SSC was added, infected bacteria were pelleted, treated with lysozyme (100 μg/ml) and DNA was extracted with phenol. After addition of ^{32}P-labeled viral DNA [12] as reference, DNA was centrifuged in a CsCl gradient and analysed as described under Materials and Methods. The arrow indicates the buoyant density of ^{32}P-labeled reference viral DNA.

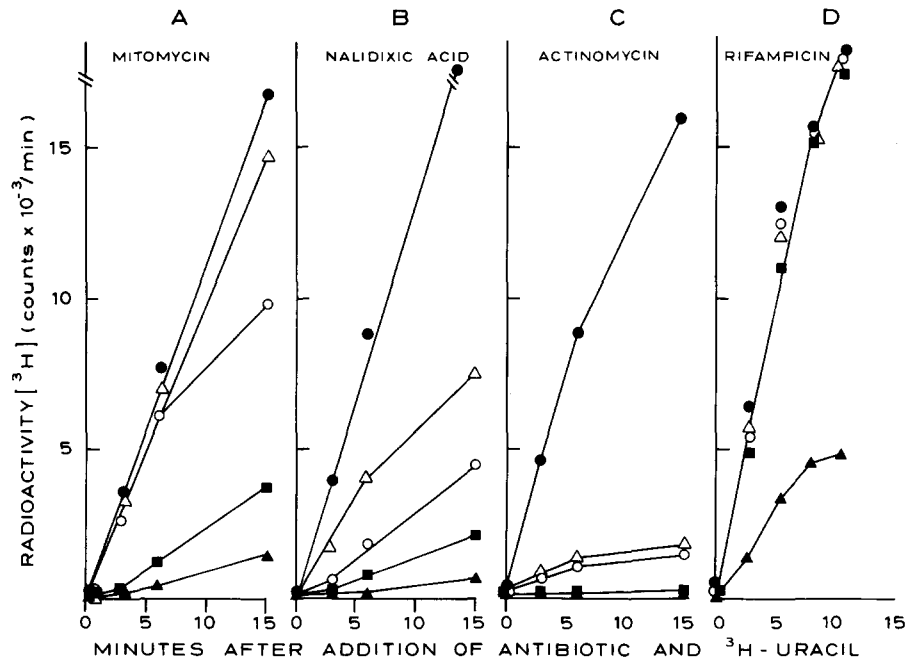

Fig. 2. Action of duplicational and transcriptional inhibitors on viral DNA synthesis. To samples of virus-infected bacteria (1.5×10^8 pfu/5×10^7 cells/ml) [^{3}H]uracil (20 μCi/ml) and one of the following inhibitors: mitomycin (5 μg/ml, A), nalidixic acid (50 μg/ml, B), actinomycin (25 μg/ml, C) or rifampicin (20 μg/ml, D), were added 5 (—▲—), 10 (—■—), 15 (—○—) and 20 (—△—) min after infection. Controls (—●—) were labeled in the absence of inhibitors. For the sake of clarity, only the control curve where the label is added 15 min after infection is shown. (All control curves had similar slopes; the 5 min control curve is shown in Fig. 1(A). Aliquots of the cultures were withdrawn at different times, incubated at 37° with 0.5 N KOH for 16 hr, treated at 4° with 0.5 N trichloroacetic acid for 30 min, filtered on micropore filters and radioactivity in DNA was counted.

analysed by CsCl isopycnic centrifugation. The [^{3}H]uracil-labeled DNA had a buoyant density identical to viral ^{32}P-labeled DNA added as reference and indicated by an arrow in Fig. 1(B). Under these conditions, no label was incorporated into cellular DNA, which has a much lower buoyant density (1.706 g cm^{-3}; refr. ind.: 1.4010) [14].

Inhibition of viral DNA formation

In the experiments described in this section, [^{3}H]uracil was used to trace specifically viral DNA formation. The labeled precursor and a given inhibitor were added to cultures of 2C-infected *B. subtilis* at different moments of the duplication cycle, and the radioactivity incorporated into viral DNA was measured. Higher concentrations of inhibitors were used in the experiments reported here than in the experiments of the previous section (Tables 1 and 2), virus production being more sensitive to inhibitors than DNA synthesis. An almost complete block of viral DNA formation was produced at any moment of the cycle by the addition of actinomycin D (Fig. 2(C)), whereas the inhibition produced by rifampicin (Fig. 2(D)) was restricted to the eclipse phase (the drug was ineffective during the 2d part of the latent period and the maturation phase). The inhibitory action of mytomycin (Fig. 2(A)), nalidixic acid (Fig. 2(B)), chloramphenicol (Fig. 3(A)), and virginiamycin (Figs 3(B)–(D)) was quite pronounced during the eclipse phase, but decreased as the replication cycle progressed.

Further work was focused on chloramphenicol and rifampicin, because of their capacity of completely suppressing phage synthesis only when added at the moment of infection. The reversibility of these antibiotics on 2C-DNA formation was assessed by labeling the infected host cells after a transient contact with the antibiotics (Fig. 4). Indeed, the inhibitory action of chloramphenicol was reversed by removal of the drug at any time, while that of rifampicin, though restricted to the eclipse phase, was irreversible. Similar results (not shown) were obtained when administering to virus-infected cells short pulses of [^{3}H]uracil plus the inhibitor.

DISCUSSION

Multiplcation of virus 2C in *B. subtilis* is inhibited only by quite high concentrations of nalidixic acid (Table 1), and this effect is fully reversible (Table 2). Accordingly, high doses of this drug are required for preventing the formation of 2C-DNA. A similar phenomenon has been already reported: nalidixic acid has little inhibitory effect on SPO1 DNA synthesis at concentrations completely preventing DNA formation in his host *B. subtilis* [17]. That this is not a general phenomenon, however, is indicated by the observation that replication of *E. coli* phages was strongly inhibited by this drug, although the replication cycle of the T-odd phages was more sensitive than that of viruses of the T-even group [18]. Since the DNA of SPO1 contains hydroxymethyluracil and that of T-phages has thymine, it was suggested that duplication of chromosomes containing unusual bases is refractory to nalidixic acid [17]. Such inference proved wrong when the sensitivity to the drug of several *E. coli* and *B. subtilis* phages was confirmed, and no correlation between the resistance of phage growth to nalidixic acid, and the presence of unusual bases in DNA, was found [18]. The conclusion is that, unlike enterobacterial viruses, some *B. subtilis* phages are insensitive to nalidixic acid. The target for nalidixic acid has been shown to be DNA gyrase [19]. It can be suggested, therefore, that some unique features of the replication forks of the DNA of 2C and few related phages, prevent inhibition by nalidixic acid.

Data in Table 1 and Fig. 2 indicate that formation

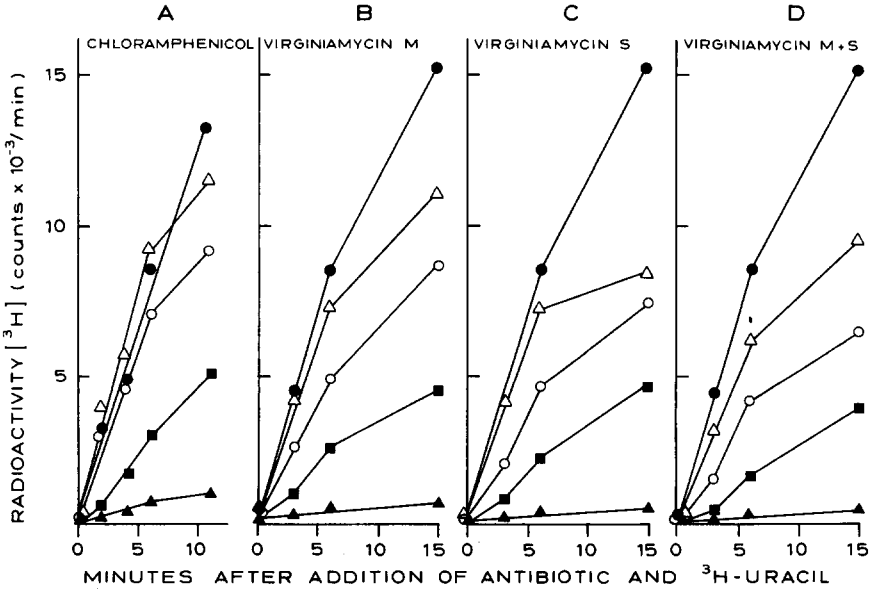

Fig. 3. Action of translational inhibitors on viral DNA synthesis. Experimental conditions as in the legend to Fig. 2. Inhibitors used: chloramphenicol (50 μg/ml, A), VM (50 μg/ml, B); VS (50 μg/ml, C): VM + VS (5 μg/ml, D).

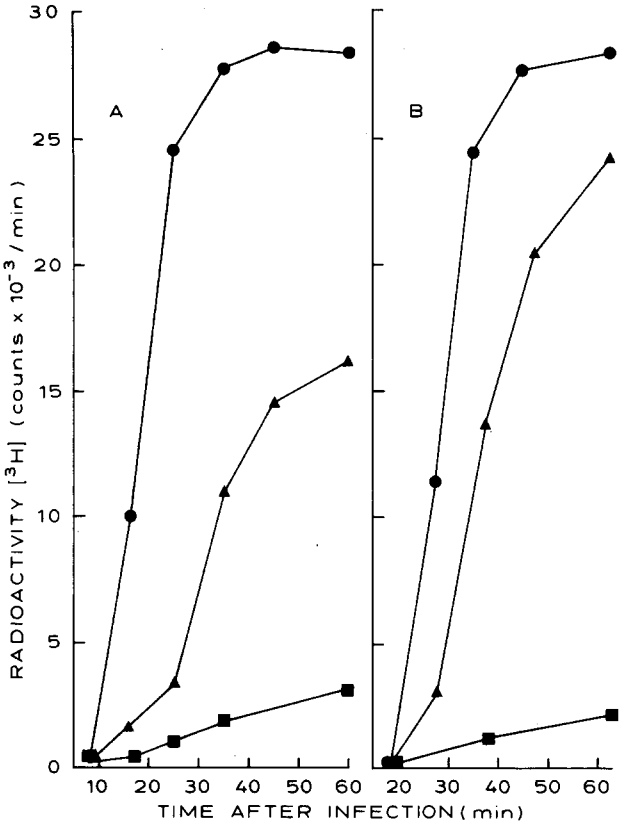

Fig. 4. Reversibility of the inhibition by chloramphenicol and rifampicin of viral DNA synthesis. Aliquots of virus-infected bacteria (1.5×10^8 pfu/5×10^7 cells/ml) were incubated for 8 min with either chloramphenicol (20 μg/ml, —▲—) or rifampicin (5 μg/ml, —■—), or none (control, —●—). Inhibitors were added at the time of infection (A) or 8 min later (B). Harvested bacteria were labeled with [^{3}H]uracil (20 μgCi/ml) in antibiotic-free medium. Samples were withdrawn and radioactivity in viral DNA was measured (cf. legend to Fig. 2).

of 2C virion and DNA is tenfold to twentyfold more sensitive to mitomycin than to nalidixic acid. This result can be accounted for by the observation that mitomycins, as bi-alkylating agents, crosslink to double-stranded DNA, as shown by the resistance to heat-denaturation of the DNA–mitomycin complex [20]. This also explains the reduced reversibility of mitomycin action, as compared to that of nalidixic acid (Table 2). The duplication of several other DNA phages was already found to be inhibited by mitomycin [21].

Multiplication of 2C proved highly sensitive to the transcriptional inhibitors actinomycin and rifampicin (Table 1), and an irreversible inhibition was found with both drugs (Table 2). Yet, actinomycin inhibited 2C-DNA formation during the entire replication cycle, while rifampicin was effective during the early eclipse phase only (Fig. 2). Such dissimilarity can be accounted for by the different mechanisms of action of the 2 drugs. Actinomycin binds to single deoxyguanines in double-stranded DNA molecules [22, 23]. A possible interference of actinomycin not only with transcription but also with duplication is conceivable, since the drug binds to DNA which is the template for both polymerisation processes. Rifampicin, on the other hand, interfers directly with RNA polymerase, and apparently does not link to DNA [24]. One may expect, therefore, that rifampicin acts as a true transcriptional inhibitor and that its interference with DNA duplication is indirect, i.e. by preventing the synthesis of a viral DNA polymerase (the drug is no longer active when the formation of the enzyme has occurred): this is precisely what was observed (cf. Fig. 2). The T7-*E. coli* system was also reported to be sensitive to rifampicin during the early eclipse phase and to become resistant after 5 min [25], in coincidence with the appearance of a specific viral RNA polymerase: indeed, the purified enzyme proved resistant to the drug *in vitro* [26].

Chloramphenicol and virginiamycins, inhibitors of the 50S ribosomal subunit, block reversibly 2C multiplication, while an irreversible effect is produced by a mixture of VM + VS (Table 2). Similar synergistic effect of the 2 virginiamycin components was previously observed with bacteria [10], blue-green algae [27], and virus-infected protists [15, 16, 6]. Moreover, our data show that all translational inhibitors tested inhibit 2C-DNA formation early in infection, but fail to do so at later stages of viral development (Fig. 3). Similar findings were reported for enterobacteria infected with T-even phage, and correlated with a block of viral DNA polymerase formation: the appearance of early proteins renders

DNA synthesis refractive to protein synthesis inhibitors ([28], for review).

This and a previous study on viral DNA synthesis in permeabilized infected bacteria [14] are preliminary to a direct study of virus-specific macromolecules, isolated from infected cells, and involved in DNA duplication and recombination.

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