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A Bacteriophage-Induced Inhibitor of a Host Enzyme

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DISCLAIMER

INTRODUCTION

Bacillus subtilis bacteriophage PBS2 is unique among viruses in that its DNA contains uracil instead of thymine (8,12). Our interest is to determine how and why PBS2 contains this unusual base in its DNA. New proteins induced after infection of *B. subtilis* by PBS2 phage include (see Fig. 1): dTMP 5'-phosphatase, which also has dUMP 5'-phosphatase activity (4,9); dCTP deaminase (7,13); dUMP kinase (4); DNA polymerase (8); RNA polymerase (1,11); an inhibitor of the host's deoxyribonuclease specific for uracil-containing DNA (14); and an inhibitor of the host's N-glycosidase specific for uracil-containing DNA (3). This paper describes the discovery of another PBS2 protein: a phage-induced inhibitor of the *B. subtilis* deoxyuridinetriphosphatase (dUTPase). The cellular dUTPase is thought to help exclude uracil from *B. subtilis* DNA, by hydrolyzing dUTP to dUMP plus PP_i (5). We believe that an important function for PBS2 phage is to inhibit the host's dUTPase, thereby allowing dUTP to accumulate for the synthesis of PBS2 uracil-containing DNA (see Fig. 1).

BACILLUS SUBTILIS DEOXYURIDINETRIPHOSPHATASE

Assays and Preparations

Two dUTPase assays were employed. Conditions optimal for the *B. subtilis* dUTPase activity were used in assay A: 120 mM TrisHCl buffer (pH 8.5), 50 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 2 mM [^{3}H]dUTP (about 20,000 cpm). Alternatively, conditions favorable to the PBS2 inhibitor's action on the *B. subtilis* dUTPase were used in assay B: 50 mM potassium phosphate buffer (pH 7.0), 0.3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 2 mM [^{3}H]dUTP. The labelled products (deoxyuridine and dUMP) were measured by anion exchange chromatography and scintillation counting (2). The

B. subtilis dUTPase and the PBS2-induced dUTPase inhibitor were partially purified (about 15-fold) from crude extracts by Sephadex G100 column chromatography using methods described previously (7,8,9).

Size

Gel filtration of the B. subtilis dUTPase on Sephadex in the presence of protein standards indicated a Stokes' radius of 30 A, corresponding to a molecular weight of 48,000 for a globular protein. This value was confirmed by sucrose density gradient centrifugation (see Fig. 2, middle panel), which indicated a sedimentation coefficient of 3.5 S. We do not know whether the dUTPase contains subunits.

Smaller forms of dUTPase have also been observed. They had Stokes' radii of 21 to 25 A and sedimentation coefficients of 2.1 to 2.3 S. These values indicate molecular weights in the range of 25,000 to 30,000. However, addition of the protease inhibitor, phenylmethanesulfonyl fluoride, during preparation of dUTPase from uninfected cells, substantially reduced or eliminated these smaller forms. Since these activities were not inactivated by direct addition of the protease inhibitor, we believe that the small forms of dUTPase represent artifacts arising from progressive proteolytic cleavage of the 48,000 dalton dUTPase. We have not studied these presumed artifacts further.

Specificity and pH Dependence

Of the various ribonucleotides and deoxyribonucleotides tested as possible substrates for the B. subtilis dUTPase, only dUTP was significantly hydrolyzed (see Table 1). The enzyme displayed simple Michaelis-Menten kinetics, with a K_M for dUTP of 2 μM in both assays A and B. The failure of the enzyme to hydrolyze dUDP does not appear to be due to a failure of binding, since dUDP at 2 mM is a good inhibitor when the substrate

dUTP is present at the low concentration of 1 μM. However, at 2 mM dUTP, none of these nucleotides was very effective as an inhibitor (Table 1).

The pH dependence of the B. subtilis dUTPase showed an activity optimum at pH 8.5 (Fig. 3). However, the curve was broad, with 50% of maximal activity still present at pH 6 and pH 10. The importance of this residual activity at high pH will be described below.

PHAGE PBS2 dUTPASE INHIBITOR

Phage-Induced Inhibition of dUTPase

We had predicted that PBS2 phage would have to inhibit or bypass the B. subtilis dUTPase activity after infection, in order to allow dUTP to accumulate for the synthesis of uracil-containing DNA by the PBS2 DNA polymerase (8). However, when assayed under conditions optimal for dUTPase activity (assay A), infected extracts were found to have the same level of dUTPase as uninfected extracts (Table 2). Yet our previous study (7) of the PBS2-induced dCTP deaminase indicated that the dUTP product was relatively stable during assays on crude extracts of infected cells. Therefore, we performed dUTPase assays under the low pH and magnesium ion concentration conditions (assay B) normally used in dCTP deaminase assays (7). We were then able to detect an 80 to 90% reduction in dUTPase activity after PBS2 phage infection (Table 2). This reduction in dUTPase activity was prevented by the addition of actinomycin D or chloramphenicol at the time of infection, suggesting that RNA and protein synthesis were required for expression of the dUTPase inhibition. Rifampin, which blocks host but not phage mRNA synthesis (11), had no effect (Table 2 and Fig. 4), indicating that the reduction in dUTPase activity after infection was a phage-controlled event.

The time course after infection of dUTPase inhibition as measured in assay B (Fig. 4) was similar to that of the appearance of the PBS2-induced dTMP 5'-phosphatase (Fig. 4), dCTP deaminase (13), DNA polymerase (8), RNA polymerase (11), and deoxyribonuclease inhibitor (14). Nonetheless, the dUTPase activity measured in assay A was essentially unchanged after PBS2 infection (Table 2). These results suggested that an inhibitor of dUTPase was induced after infection; this inhibitor appeared to be active under the low pH and magnesium ion conditions of assay B, but inactive under the high pH and magnesium ion concentration of assay A.

Properties of PBS2 dUTPase Inhibitor

Indeed, an excess of a dUTPase inhibitor was detected in infected extracts; mixtures of infected and uninfected extracts in assay B gave 50% less activity than that expected for the two extracts acting independently. The PBS2-induced inhibitor was partially purified on Sephadex by its action in reducing the dUTPase activity in an uninfected extract. Thus, the PBS2 inhibitor could be shown to have a Stokes' radius of 36.5 A, corresponding to a molecular weight of 83,000 for a globular protein. However, on sucrose gradients the inhibitor sedimented at about 2.8 S, suggesting a molecular weight of about 40,000 (Fig. 2, top panel). Therefore, the PBS2-induced inhibitor may exist as a dimer on our Sephadex column.

The inhibitor seems to act by forming a complex with the dUTPase. A mixture of B. subtilis dUTPase (3.5 S) and PBS2 inhibitor (2.8 S) gives a new peak of dUTPase (active in assay A, in which the inhibitor is not functional) sedimenting at 4.9 S (Fig. 2, bottom panel). The latter corresponds to an apparent molecular weight of about 91,000, which

we presume consists of a complex of the 48,000 dalton dUTPase and a 40,000 dalton monomeric inhibitor.

The inhibitor can be shown to act reversibly, since a mixture of B. subtilis dUTPase and PBS2 inhibitor, showing 80% inhibition in assay B, can be separated on a Sephadex column into active inhibitor and active enzyme (having the same K_M as originally) with over 60% recovery of each. The inhibitor has no detectable dUTPase nor dUMP 5'-phosphatase activity; it simply reduces the amount of dUMP produced from dUTP by the enzyme.

pH Dependence of PBS2 dUTPase Inhibitor in vitro and in vivo

The PBS2-induced inhibitor has an activity optimum in vitro at pH 6 to 7 (the pH used in assay B), and its action decreased to minimal levels as the pH was increased from pH 8.5 (as used in assay A) to pH 9.7 (see Fig. 5, top panel). This pH dependence of inhibition led us to speculate that increasing the pH of the culture medium might increase the intracellular pH in vivo, so that the PBS2 dUTPase inhibitor could not function. Since the B. subtilis dUTPase is most active at high pH (Fig. 3), the enzyme would be expected to remain functional, hydrolyzing dUTP to dUMP for dTTP synthesis (see Fig. 1). Hopefully, one might then observe a change from the synthesis at pH 7 of uracil-containing phage DNA to the synthesis at pH 9 of thymine-containing DNA.

Indeed, Fig. 5 (bottom panel) shows that raising the pH of the infected culture from pH 8.2 to 8.85 progressively increased the thymine/uracil ratio in newly synthesized DNA from less than 0.03 to over 1.0. These data support the proposal that infection at high pH prevents the PBS2 dUTPase inhibitor from functioning, thus allowing dUTP degradation and dTTP synthesis (see Fig. 1), so that thymine replaces some of the uracil in newly synthesized DNA.

The DNA synthesized during infection at high pH appears to be phage DNA, since it can be made (see Fig. 5) in the presence of a potent inhibitor (6,10) of B. subtilis DNA replication, 6-(p-hydroxyphenylazo)-uracil. Furthermore, this DNA has a [uracil plus thymine]/cytosine ratio close to that known for PBS2 DNA (2.6 uracil/cytosine; Ref. 8,12) and different from that known for B. subtilis DNA (1.5 thymine/cytosine; Ref. 12). The [uracil plus thymine]/cytosine ratios were 2.7, 2.7, 2.5, 2.4, and 1.9 when the pH of the medium at the time of infection was 8.2, 8.45, 8.55, 8.75, and 8.85, respectively. Other aspects of infection at pH 8.85 also seemed to be normal, including the growth rate of the cells, the eclipse of input phage, the time of onset of limited phage production, the induction of several PBS2 proteins [dTMP 5'-phosphatase, dCTP deaminase, dUTPase inhibitor, and DNA polymerase were tested], and the stability of intact phage.

However, increasing the pH of the medium led to a progressive decrease in the amount of DNA synthesis, in progeny phage production, and in lysis of the infected culture. The cause(s) of these effects of high pH remain to be determined, although the PBS2 DNA polymerase is known to function optimally at pH 8.5 to 9.0 in vitro (8). It is possible that incorporation of thymine into PBS2 DNA causes it to be replicated poorly, to be susceptible to degradation, or to be poorly packaged into virions. Yet it is known that the thymine-containing B. subtilis DNA is not extensively degraded and can be incorporated into transducing particles late in infection (14). By further characterizing DNA and protein synthesis during PBS2 infection at high pH, we hope to gain some insight into why this unusual virus normally has uracil instead of thymine in its DNA.

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TABLE 1. Substrate and Inhibitor Specificity of B.subtilis dUTPase

Compound added at 2 mM	Relative hydrolysis %	Percent Inhibition with	
		1 μM dUTP[b]	2 mM dUTP[c]
dUTP	100[a]	99	55
dUDP	< 2	99	15
dUMP	< 2	50	< 5
deoxyuridine	< 2	20	< 5
dTTP	< 5	50	< 5
dCTP	< 2	40	< 5
dATP	< 2	30	< 5
dGTP	< 5	10	< 5
UTP	< 5	20	< 5
PP_i	< 2	10	< 5
P_i		5	< 5

[a]Reaction mixtures contained the indicated substrate to be tested plus partially purified 48,000 dalton B. subtilis dUTPase (12 μg protein) sufficient to hydrolyze 160 nmol of [^{3}H]dUTP to dUMP in assay A (100 μl total volume) during incubation at 37 C. All possible hydrolysis products from each substrate were separated by paper chromatography in an isobutyrate solvent, eluted, and quantitated by ultraviolet absorption (9).

[b]Incubation mixtures contained B. subtilis dUTPase (0.06 μg protein) with 50 μg bovine serum albumin. In the absence of added inhibitor, the enzyme hydrolyzed 36 pmol of the 100 pmol [^{3}H]dUTP added in assay A.

[c]Incubation mixtures contained B. subtilis dUTPase (6 μg protein). In the absence of inhibitor, the enzyme hydrolyzed 42 nmol of the 200 nmol [^{3}H]dUTP added in assay A.

TABLE 2. Specific activity of dUTPase in uninfected and PBS2-infected B. subtilis cell extracts under two assay conditions.

Extract of treated cells[a]	dUTPase activity	
	Assay A	Assay B
Uninfected	2.4	0.48
Infected	2.5	0.09
Infected + actinomycin D	2.3	0.38
Infected + chloramphenicol	2.1	0.43
Infected + rifampin	2.1	0.10

[a]Some cultures were treated as indicated with actinomycin D (10 µg/ml), chloramphenicol (100 µg/ml), or rifampin (100 µg/ml) at the time of infection. Cells were harvested at 45 min after infection; extracts were prepared, dialyzed, and assayed for dUTPase specific activity (µmol per hour per milligram protein) using assay A (pH 8.5 in 50 mM $MgCl_2$) or assay B (pH 7.0 in 0.3 mM $MgCl_2$).

Legends to Figures:

FIG. 1. Proposed scheme for deoxyribonucleotide metabolism in B. subtilis as altered by PBS2 phage infection. Known PBS2-induced functions (heavy lines) are: 1) dTMP (dUMP) 5'-phosphatase; 2) dCTP deaminase; 3) dUMP kinase; 4) DNA polymerase; 5) inhibitor of host's deoxyribonuclease for uracil-containing DNA; 6) inhibitor of host's N-glycosidase for uracil-containing DNA; and 7) inhibitor of host's dUTPase.

FIG. 2. Sedimentation of B. subtilis dUTPase and PBS2 phage-induced dUTPase inhibitor on sucrose density gradients. Aliquots (100 µl) of partially purified dUTPase and dUTPase inhibitor (sufficient to cause an 80% inhibition of the dUTPase in assay B) were sedimented alone or mixed together through 4.8 ml-linear gradients (5 to 20%, w/v) of sucrose prepared in buffer containing the components of assay B. Centrifugation was at 4 C for 8 hours at 60,000 rpm in a Beckman SW65 rotor. Ten-drop fractions were collected from the bottom of the tube. Aliquots (10 µl) were assayed at 23 C for lactate dehydrogenase (o, vertical line marks the peaks; 20 µg of enzyme were added as a 6.5 S internal standard). Assay A was used to measure dUTPase activity (●) in 50 µl aliquots incubated for 4 hours (middle and bottom panels). To detect the presence of dUTPase inhibitor (upper panel), 50 µl aliquots were incubated for 2 hours in assay B with 35 µg of uninfected B. subtilis extract containing dUTPase (●).

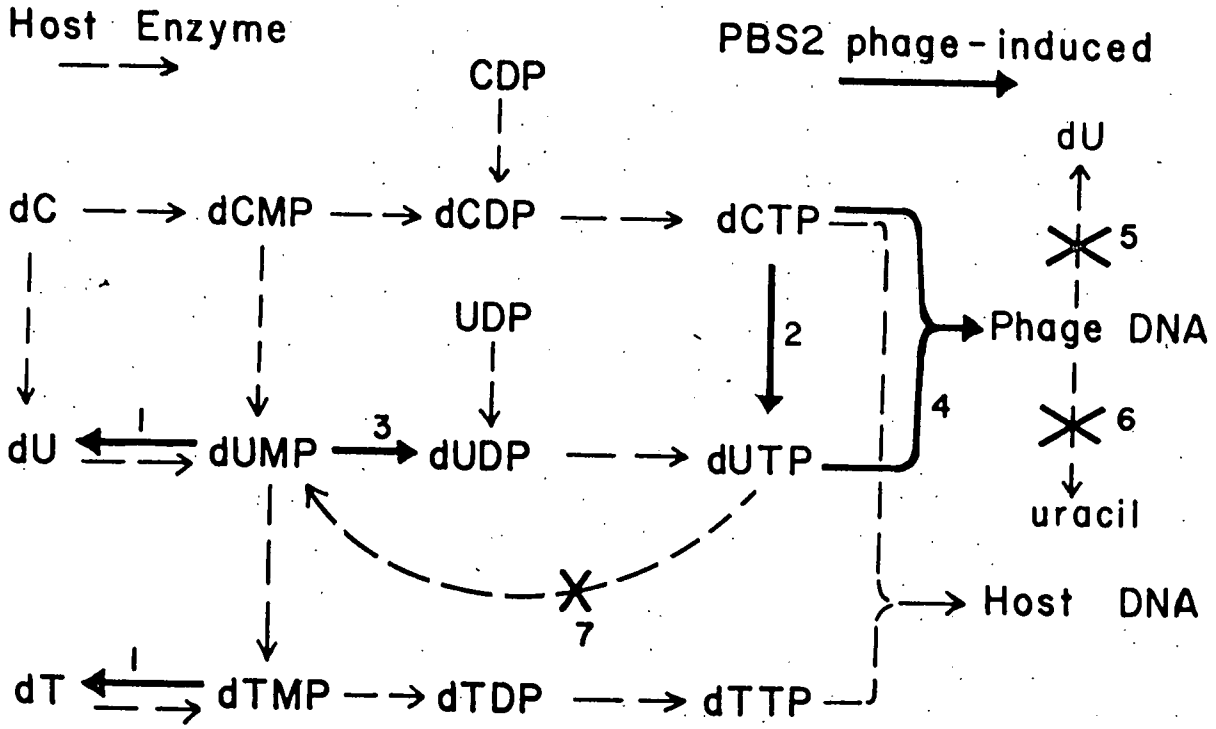
FIG. 3. Dependence on pH of B. subtilis dUTPase activity. Various 100 mM buffers were employed to give the indicated pH values in assay A at 37 C. An activity of "100" corresponds to 65 nmol dUMP produced in 45 min by 8 µg partially purified dUTPase.

FIG. 4. Time course after PBS2 infection of dUTPase and dTMP 5'-phosphatase activities. Cells were grown, infected at 37 C in the

presence of rifampin (25 µg/ml), harvested at the indicated times, resuspended in buffer containing chloramphenicol (100 µg/ml), and disrupted to make extracts (see Ref. 8). Dialyzed extracts were assayed for dUTPase (µmoles of deoxyuridine and dUMP produced per hour per milligram protein in assay B) or for the phage-induced dTMP 5'-phosphatase (µmoles P_i produced in 15 min per milligram protein). Lysis of the culture and release of progeny phage began at 50 to 60 min after infection.

FIG. 5. Upper panel: Effect of assay pH on the in vitro activity of the PBS2-induced dUTPase inhibitor on the B. subtilis dUTPase. Partially purified dUTPase (13 µg protein) was incubated with or without PBS2 inhibitor (5 µg protein) for 60 min in assay B. Various 100 mM buffers were employed to alter the pH of the assay mixture. The percent inhibition of dUTPase (maximum activity was 29 nmol dUMP produced in TrisHCl at pH 8.4) by PBS2 inhibitor was calculated from assays at each pH. Lower panel: Effect of media pH on the in vivo activity of the PBS2-induced dUTPase inhibitor as reflected by phage DNA composition. Cultures of B. subtilis in Penassay broth (Difco) containing 50 mM TrisHCl were adjusted to pH 7.0, 8.5, 8.7, 8.9, 9.1, and 9.3. After growth of the culture at 37 C to A_{660} (1 cm) = 1.0, the cultures' pH values had decreased to pH 6.75, 8.2, 8.45, 8.55, 8.75, and 8.85, respectively. All cultures were infected with PBS2 at an MOI of 7.5 phage per cell. 6-(p-hydroxyphenylazo)-uracil was added at 40 µM, sufficient to block any residual host DNA synthesis (6,10). By 3 hours after infection, all cultures had lysed ($A_{660} < 0.18$), except for the pH 8.75 culture (A_{660} = 0.47) and the pH 8.85 culture ($A_{660} = 1.10$). Lysozyme was added at 100 µg/ml to complete the lysis, and culture lysates were titered for PBS2 phage production (●, 100% = 7×10^9/ml). Culture pH values at lysis had decreased slightly to pH 6.8, 8.15, 8.4, 8.5, 8.6, and 8.7, respectively. At

10 min after infection, 0.5 ml-aliquots had been removed for labelling for 30 min with 0.5 µM [6-^{3}H]uracil at 10 μC_i/ml; a 10 µl-aliquot was spotted on a paper disc and processed (6) to measure DNA synthesis (o, 100% = 8,000 cpm). The remainder of the labelled culture was treated with trichloroacetic acid and processed to isolate the DNA (10), aliquots of which (7,000 to 30,000 cpm) were hydrolyzed in formic acid to determine (8) the ratio of thymine to uracil in the newly synthesized DNA (▲). Values are plotted against the pH of the culture at the time of infection.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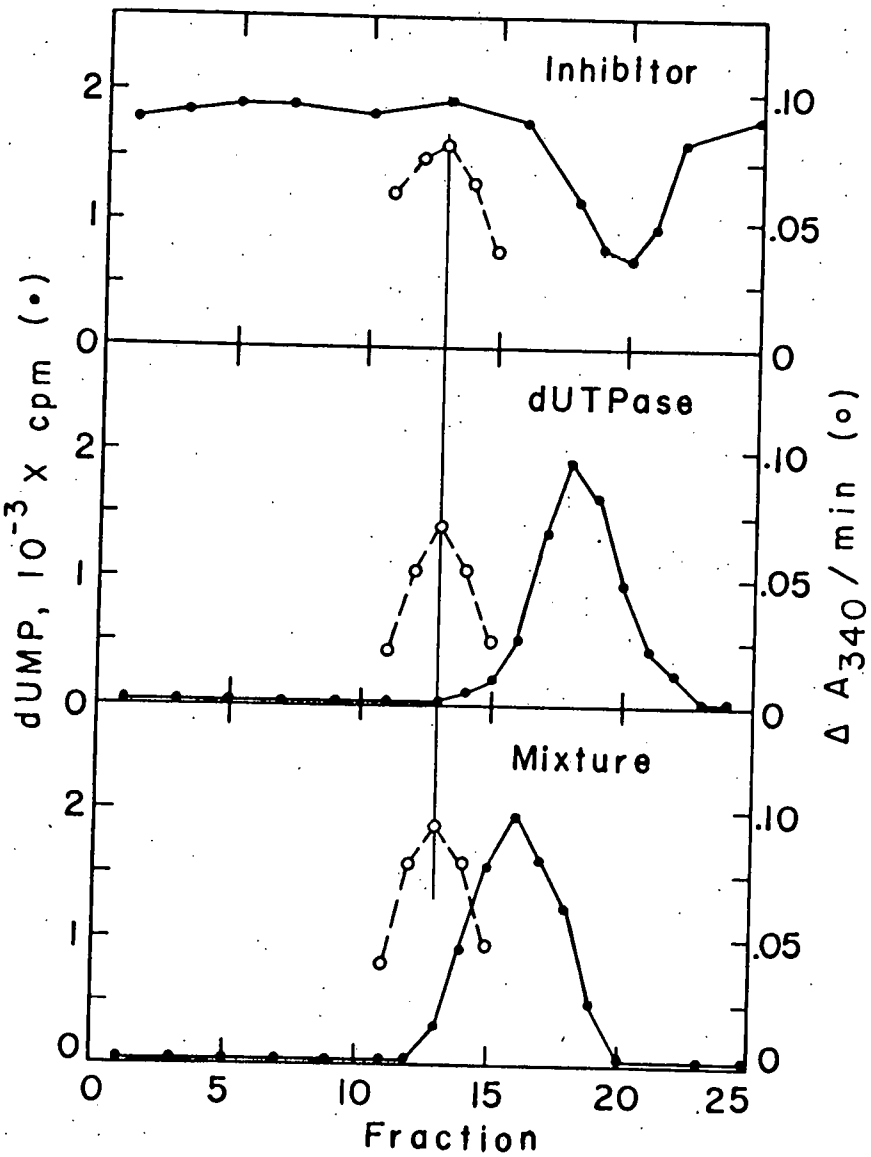
dCMP
dCDP
dCTP
dU
5
UDP
2
Phage DNA
dU
1
dUMP
3
dUDP
dUTP
4
6
uracil
7
Host DNA
dT
1
dTMP
dTDP
dTTP

Fig. 2


Inhibitor
dUTPase
Mixture
dUMP, 10^{-3} x cpm (•)
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.10
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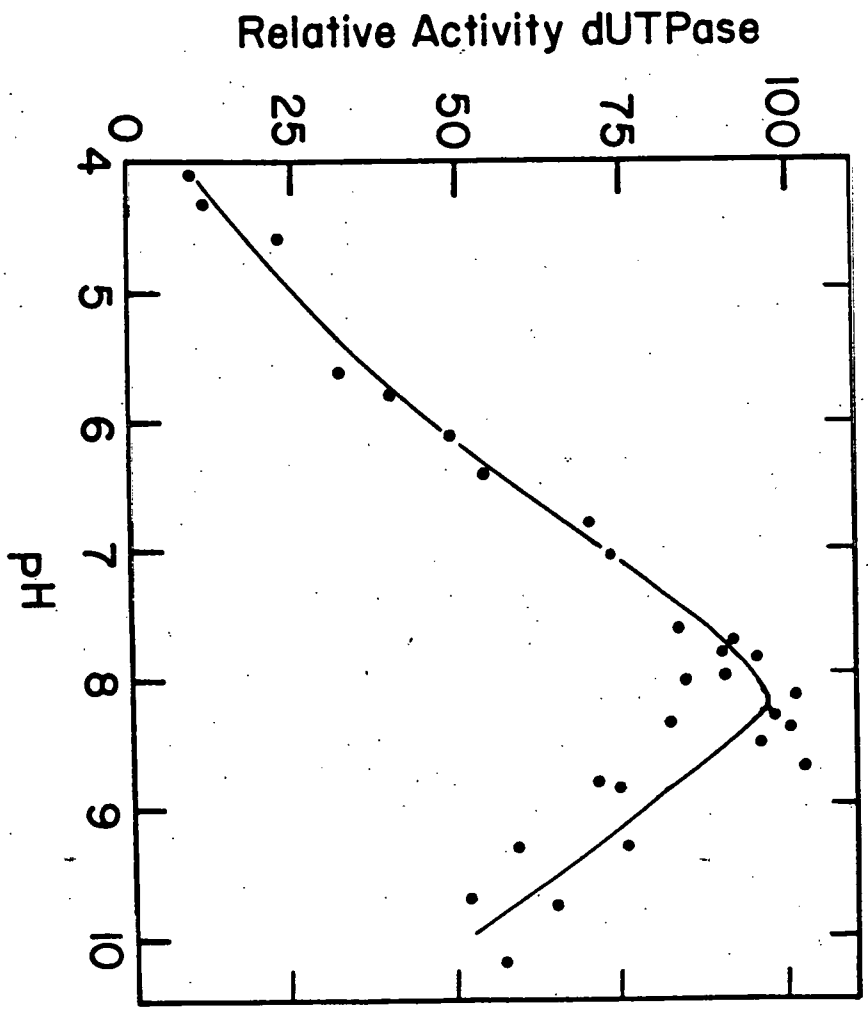
5
10
15
20
25
Fraction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75
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pH
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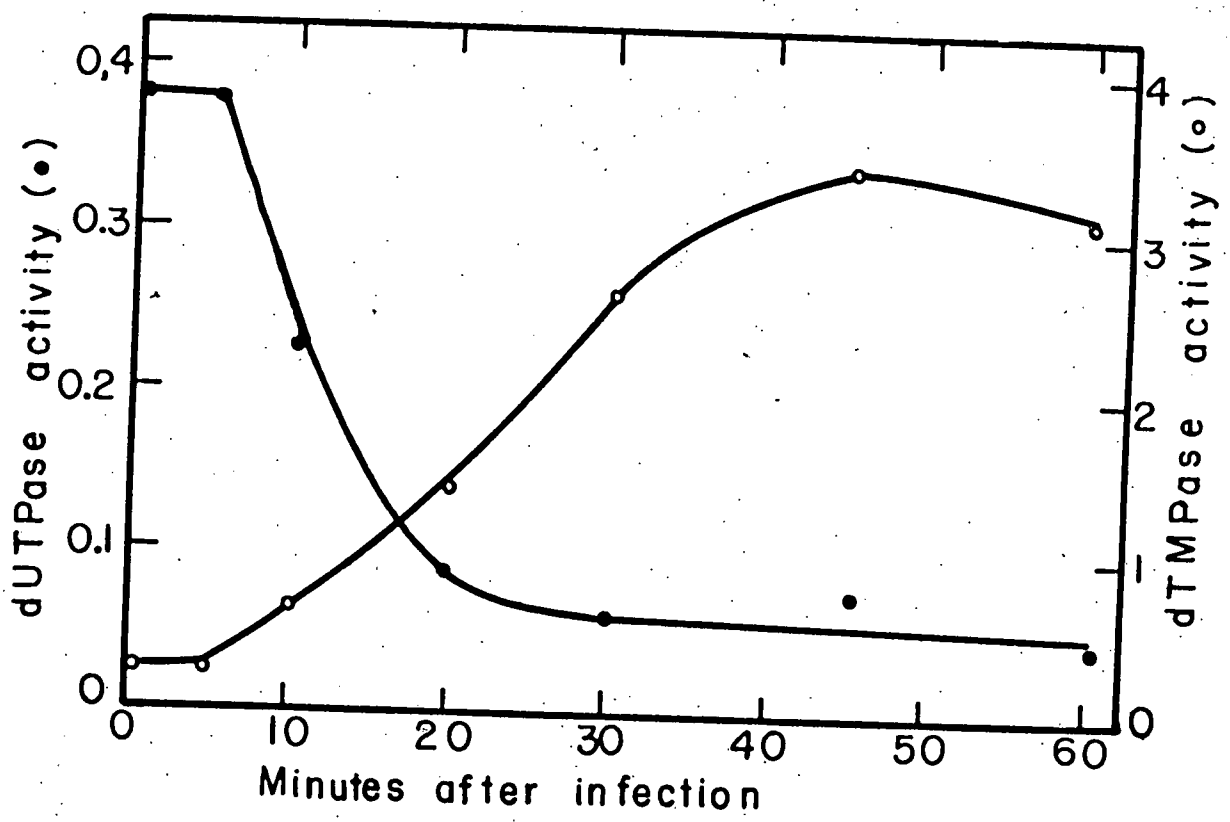
5
6
7
8
9
10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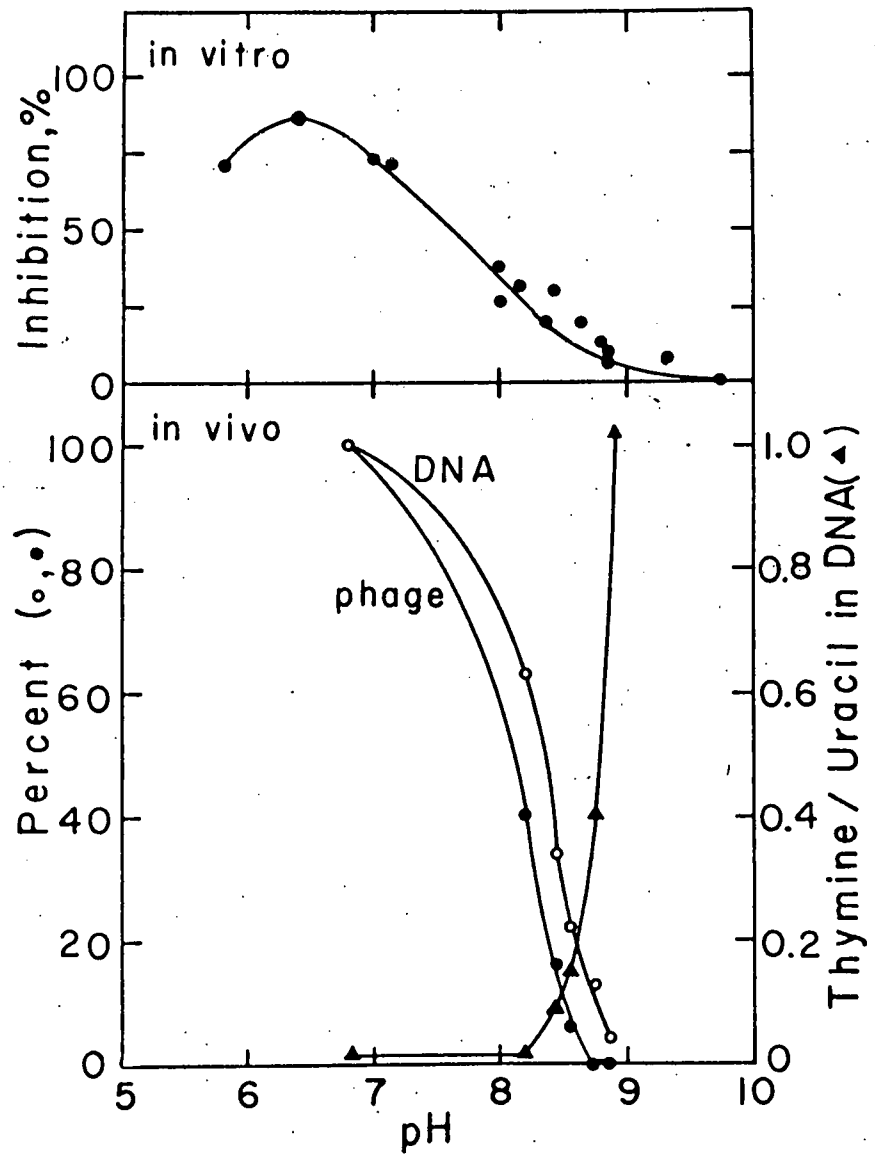
0.3
0.2
0.1
0
dUTPase activity (•)
4
3
2
1
0
dTMPase activity (○)
0
10
20
30
40
50
60
Minutes after infection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in vivo
DNA
phage
Percent (○, ●)
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80
60
40
20
0
Thymine / Uracil in DNA(▲)
1.0
0.8
0.6
0.4
0.2
0
5
6
7
8
9
10
pH