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SYNTHESIS OF DNA CONTAINING URACIL

DURING

BACTERIOPHAGE INFECTION OF *Bacillus subtilis*

TECHNICAL PROGRESS REPORT (6th year)

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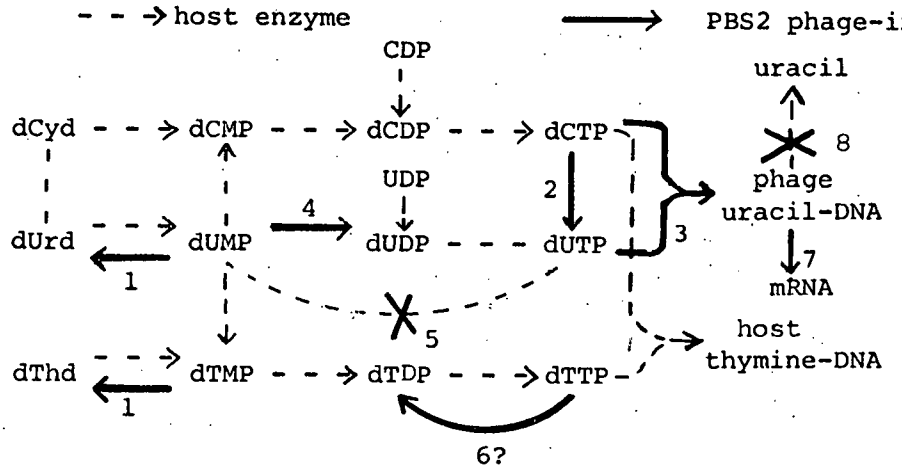
Title: Synthesis of DNA Containing Uracil during Bacteriophage Infection of Bacillus subtilis

Abstract

We have studied the synthesis of proteins and nucleic acids to understand how the bacteriophage PBS2 can make uracil-containing DNA in a cell which normally makes thymine-containing Bacillus subtilis DNA. We showed that the earliest known PBS2-induction of a protein is the inhibitor of the B. subtilis N-glycosidase (or uracil-DNA nuclease). This enzyme cleaves uracil from PBS2 DNA, and it is inhibited before any other known PBS2 protein appears in infected cells. The PBS2 dUMP kinase, which probably provides dUTP for uracil-DNA synthesis, has been partially characterized. Purification and induction in polymerase I- and III-deficient host cells indicate that the PBS2 DNA polymerase is a new enzyme. However, infection of temperature-sensitive polymerase III mutants suggests that the host's DNA polymerase III may play some role in PBS2 replication, at least under certain conditions. Two-dimensional thin-layer chromatography methods have been developed to quantitate the deoxyribonucleoside triphosphate pools before and after PBS2 infection.

Introduction

Bacteriophage PBS2 contains DNA with uracil instead of thymine, which is found in most DNAs (1,2). Based on the known Bacillus subtilis enzymes and by analogy to the well-studied T4 phage infection of Escherichia coli (3), we have proposed the following scheme for the modification of B. subtilis deoxyribonucleotide metabolism after PBS2 infection:



As indicated in the above scheme in solid lines, the infection of B. subtilis by phage PBS2 results in the induction of proteins believed to allow the phage to accumulate dUTP in a cell which normally makes dTTP for DNA synthesis. Those PBS2 proteins include:

PBS2 protein induced	Product	Reference
1. dTMPase (dUMPase	dThd + Pi dUrd + Pi at high K_m)	Kahan abstract (4) Price and Fogt (5)
2. dCTP deaminase	dUTP + NH_3	Tomita and Takahashi (6) Price (7)
3. DNA polymerase	DNA	Price and Cook (2)
4. dUMP kinase (ATP)	dUDP + ADP	Kahan abstract (4)
5. inhibitor of host's dUTPase	less dUMP made	Price and Frato (8,9)
6. dTTPase (?)	dTDP	Takahashi (pers. commun.)
7. RNA polymerase	mRNA	Price and Frabotta (10) Clark et al. (11) Price et al. (12)
8. inhibitor of host's N-glycosidase	less uracil made	Tomita and Takahashi (13) Friedberg et al. (14)

We have been investigating these PBS2-induced functions to learn how this unique virus can accumulate dUTP for uracil-DNA synthesis in a cell which normally makes dTTP for thymine-DNA synthesis. Our work for the past year is summarized below:

PBS2 inhibitor of host's N-glycosidase

Last summer Takahashi (13) and Friedberg (14) reported the discovery of an enzymatic activity in B. subtilis extracts which specifically degraded the uracil-containing DNA of PBS2 phage. Takahashi (13) reported that the enzyme was a Ca^{2+}-activated "endonuclease" releasing deoxyuridine, and that this nuclease was totally inhibited by a PBS2 protein (#8 above) at 20 minutes after infection. On the other hand, Friedberg (14) reported that the enzyme was an EDTA-stable N-glycosidase releasing uracil, and it was totally inhibited by 4 minutes after infection.

We have set up the Takahashi and Friedberg assays to confirm their results. We indeed see degradation of PBS2 DNA by B. subtilis extracts under both conditions. Our results (see Fig. 1) demonstrate that PBS2 infection results in the inhibition of the activities in parallel from 3 to 7 minutes. By mixing infected extracts with an aliquot of uninfected extract, we could show that the synthesis of this inhibitor continues from 10 to 30 minutes after infection. Furthermore, this inhibitor seems to be the earliest known PBS2-induced protein, appearing earlier than the PBS2 dTMPase, DNA polymerase, dUMP kinase, and dUTPase inhibitor (see Fig. 1) as well as the PBS2 dCTP deaminase (6,7) and RNA polymerase (12).

The parallel time course of inhibition after infection, as well as the coincident elution on our Sephadex G100 column, suggests that Takahashi (13) and Friedberg (14) are probably both looking at the same basic enzyme and one PBS2-induced inhibitor. However, it will be important to separate the inhibitor(s) from the enzyme(s) and to characterize them to prove this point. There are weaknesses in both papers (13,14), which we hope to help resolve. Friedberg (personal communication) is also proceeding with the purification and differentiation of these proteins.

It is likely that this PBS2-induced inhibitor of the host's N-glycosidase is important to allow the PBS2 uracil-DNA to replicate and not be degraded by the B. subtilis enzyme. We wondered whether this enzyme would attack injected PBS2 DNA early in infection. But our experiments with [^{3}H]uracil-labelled PBS2 phage suggest that [^{3}H]uracil is not released, even at multiplicities of infection of 0.5 to 10 in cells growing in 100 µg uracil/ml.

PBS2 dUMP kinase

PBS2 induces an activity catalyzing the reaction (enzyme #4 above): dUMP + ATP → dUDP + ADP (4). We assay this enzyme by conversion of [^{3}H]dUMP

to higher phosphates which are eluted from AG1 columns at higher formate concentrations. This is one of the high activity PBS2 enzymes (4 μmoles/hr/mg protein in extracts). For some unknown but useful reason, its level uniquely doubles if infection is performed in rifampicin at 100 μg/ml (to which PBS2 infection is resistant; see refs. 10-12).

We have partially purified the dUMP kinase by streptomycin sulfate treatment (it stays in the supernatant), DEAE-cellulose column (it elutes at 0.25 M NaCl), Sephadex G100 column (Stokes radius of about 26 A, corresponding to about 40,000 daltons), and isoelectric focusing (pI = 4.5). The PBS2 dUMP kinase can thus be separated from the B. subtilis dUTPase and its PBS2 inhibitor (protein # 5 above; see refs. 8,9). We want to study its specificity, K_m, and properties, as we did for the PBS2 dTMPase-dUMPase (enzyme #1; see ref. 5). This PBS2 dUMP kinase may play a role in producing dUDP and dUTP for PBS2 uracil-DNA synthesis.

PBS2 DNA polymerase purification

We have made considerable progress in obtaining a good, reproducible method to purify our PBS2 phage-induced DNA polymerase (enzyme # 3; see ref. 2). We make extracts of infected cells using lysozyme and brief sonication; then we precipitate the PBS2 DNA polymerase with the DNA by streptomycin sulfate treatment. The resuspended pellet is digested with DNase, RNase and venom phosphodiesterase to degrade the nucleic acids, leaving the DNA polymerase soluble. The enzyme is precipitated by ammonium sulfate, and the redissolved material is passed through a Sephadex G200 column to remove the nucleases. The pooled fractions are passed through a DEAE-cellulose column in 0.4 M NaCl; this removes the remaining traces of nucleic acids and allows the enzyme to bind to a phosphocellulose column (for gradient elution in 0.5 M NaCl). Then a DNA-cellulose column (eluting with a linear gradient in 1M NaCl) gives an overall purification of about 300- to 400-fold.

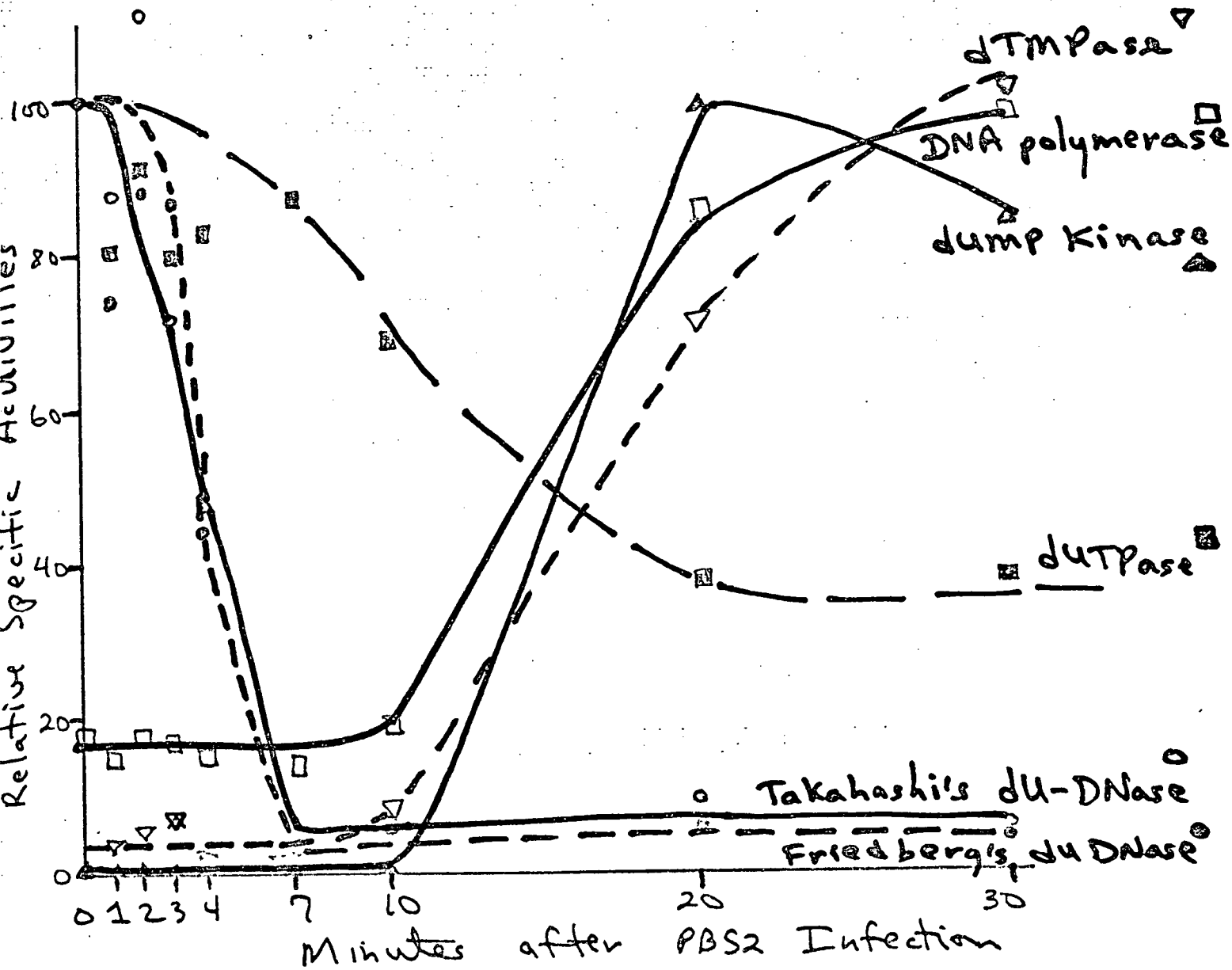
We are studying the properties of the PBS2 DNA polymerase. It appears to aggregate in low salt buffers. It is most active on single-stranded (or DNase-degraded native) DNA, especially at high (70 mM) $MgCl_2$ concentrations. Nuclease activity seems to copurify with the polymerase.

The evidence indicates that the PBS2 DNA polymerase is distinct from the 3 B. subtilis DNA polymerases. We use a polA strain to eliminate the host's DNA polymerase I. Polymerases II and III appear to elute earlier off the phosphocellulose column than the PBS2 enzyme. Also, the PBS2 polymerase is essentially inactive in the host polymerase assays (using activated DNA and 6 mM $MgCl_2$), and here only the host's DNA polymerase III can be inhibited (15,16) by 6-(p-hydroxyphenylazo)uracil [HPUra] or N-ethylmaleimide.

Fig. 1

Enzyme Levels after PBS2 phage infection of Bacillus subtilis 5B19.

Lysozyme/sonication extracts were made in Takahashi's buffer [50 mm Tris Cl, pH 7.5]. (Similar results were obtained with Friedberg's buffer = 10mm Tris Cl, pH 8.0].



We want to characterize the purified PBS2 DNA polymerase, since it is likely that this enzyme is involved in PBS2 uracil-DNA synthesis in vivo. Does the phage polymerase prefer uracil-DNA to thymine-DNA? Is the K_m for dUTP much lower than that for dTTP? Is there an associated 3'-exonuclease activity (17)?

PBS2 infection of B. subtilis pol III mutants

Further evidence that PBS2 DNA polymerase is unique is its normal induction in host mutants defective in DNA polymerase III. We compared Cozzarelli's F2 (polA59) and F25 (polA59 polC25 HPUra-resistant) strains (18). We found that gently-lysed F25 cells contained normal F2-levels of polymerases II and III (Cozzarelli had never been able to observe polymerase III activity in F25 extracts -- so our method may be useful for isolating mutant cell polymerase III). But sonic extracts of F25 had very little (less that 10% of F2 sonic extracts) N-ethylmaleimide-sensitive DNA polymerase III. Yet the 50-fold increase in PBS2 DNA polymerase was identical in sonic extracts after F2 and F25 infection. And the heat-inactivation curves at 55° of PBS2 DNA polymerase in F2- and F25-infected extracts was identical (half-life of 40 minutes). This inactivation was much slower than uninfected extracts (half-life less than 1 minute). Thus, PBS2 DNA polymerase seems distinct from the host's DNA polymerase III (and I).

We previously demonstrated (19,20) that PBS2 DNA polymerase in vitro and PBS2 DNA synthesis in vivo were resistant to HPUra, a potent inhibitor of DNA polymerase III in uninfected cells. Similar results were observed by Marcus (21) using the HPUra-resistant ϕe phage [whose DNA contains 5-hydroxymethyluracil and not thymine]. Marcus (21) also used a temperature-sensitive mutant (polA mut-1, strain 2355) which had a ts DNA polymerase III (pol III^{ts}) and stopped DNA synthesis at 48°. Surprisingly, ϕe phage could make DNA in pol III^{ts} at 37°, but not at 48° [although a ts^+ revertant (R2355) could do so]. This suggested that the host's DNA polymerase III was needed for ϕe DNA replication, but that the ϕe phage seemed to modify it to be HPUra-resistant.

We did similar experiments with PBS2 phage infection of Marcus' pol III^{ts} strain. In our broth, uninfected pol III^{ts} cells make DNA unless the temperature is raised to 49°. Infected cells made DNA only at 37°, similar to Marcus' results. But infection of pol III^{ts} is poor: there are many survivors, especially those in long chains; lysis is poor; and there is a poor burst of new phage. [Even wild-type cells infected above 47° give little or no new phage.] And Marcus' "revertant" gave similar results. Thus the involvement of B. subtilis DNA polymerase III in PBS2 infection is not clear.

So we tried another ts polymerase III mutant (BD317, polA59 mut-1) from Cozzarelli (18). We had to go to 50° to get a 90% reduction in uninfected BD317 DNA synthesis. In this case, this was also a poor infection, but some PBS2 DNA was made at 50° [as separated from uninfected host DNA on a CsCl gradient (1)]. Very surprizingly (see Fig. 2), HPUra inhibited both host and phage DNA synthesis at 50° (although PBS2 DNA was made in HPUra in BD317 at 37°). So these results are counter to Marcus' φe data (21). This pol III^{ts} strain allows some PBS2 DNA synthesis at 50°, but it is modified to being HPUra-sensitive! We are pursuing these experiments in the above and other pol III^{ts} versus parental strains.

E. coli DNA polymerase and N-glycosidase on uracil-DNA

Wovcha and Warner (22) reported that partially-purified E. coli DNA polymerase I attacked uracil-containing DNA (synthesized in vitro by repair of exonuclease-treated thymine DNA) releasing oligonucleotides, apparently by use of its 5'-to'3' exonuclease activity. On thymine-DNA, it gave only mononucleotides. We wanted to confirm these results on our double-stranded uracil-DNA of PBS2 phage.

We purchased from Boehringer-Mannheim these E. coli DNA polymerase I preparations: fraction IV, fraction VII, and its fragment which lacks the 5'-exonuclease activity. Using high and equivalent units of polymerase activity, we observed that both thymine-DNA and uracil-DNA were attacked in the same manner: a) the IV polymerase was contaminated by an endonuclease giving large oligonucleotides; b) the VII polymerase gave mainly mononucleotides; and c) the fragment did not degrade either DNA. Thus, we could not confirm Wovcha's results.

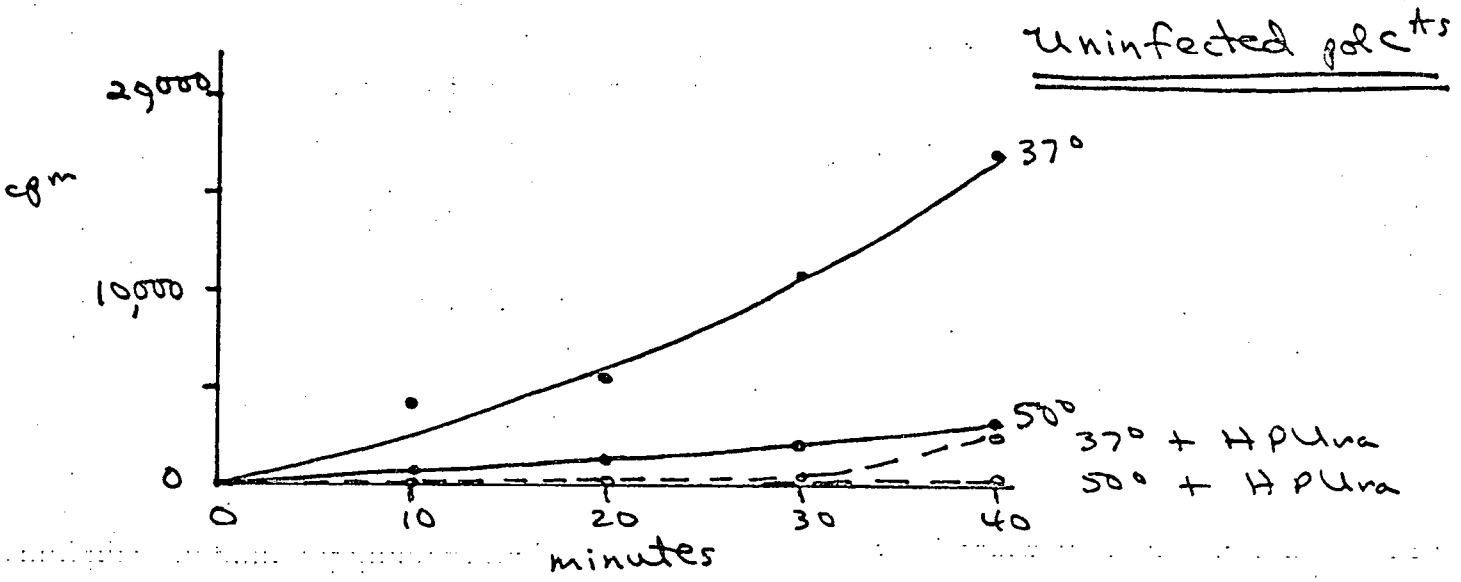
Recently we learned from Duncan and Warner (personal communication) of their similar results. They showed that Wovcha's DNA polymerase fractions were contaminated by the N-glycosidase which specifically attacks uracil-DNA (see above) and by exonuclease III, which together give uracil-containing oligonucleotides. Thus, the E. coli DNA polymerase I does not really act endonucleolytically on uracil-DNA.

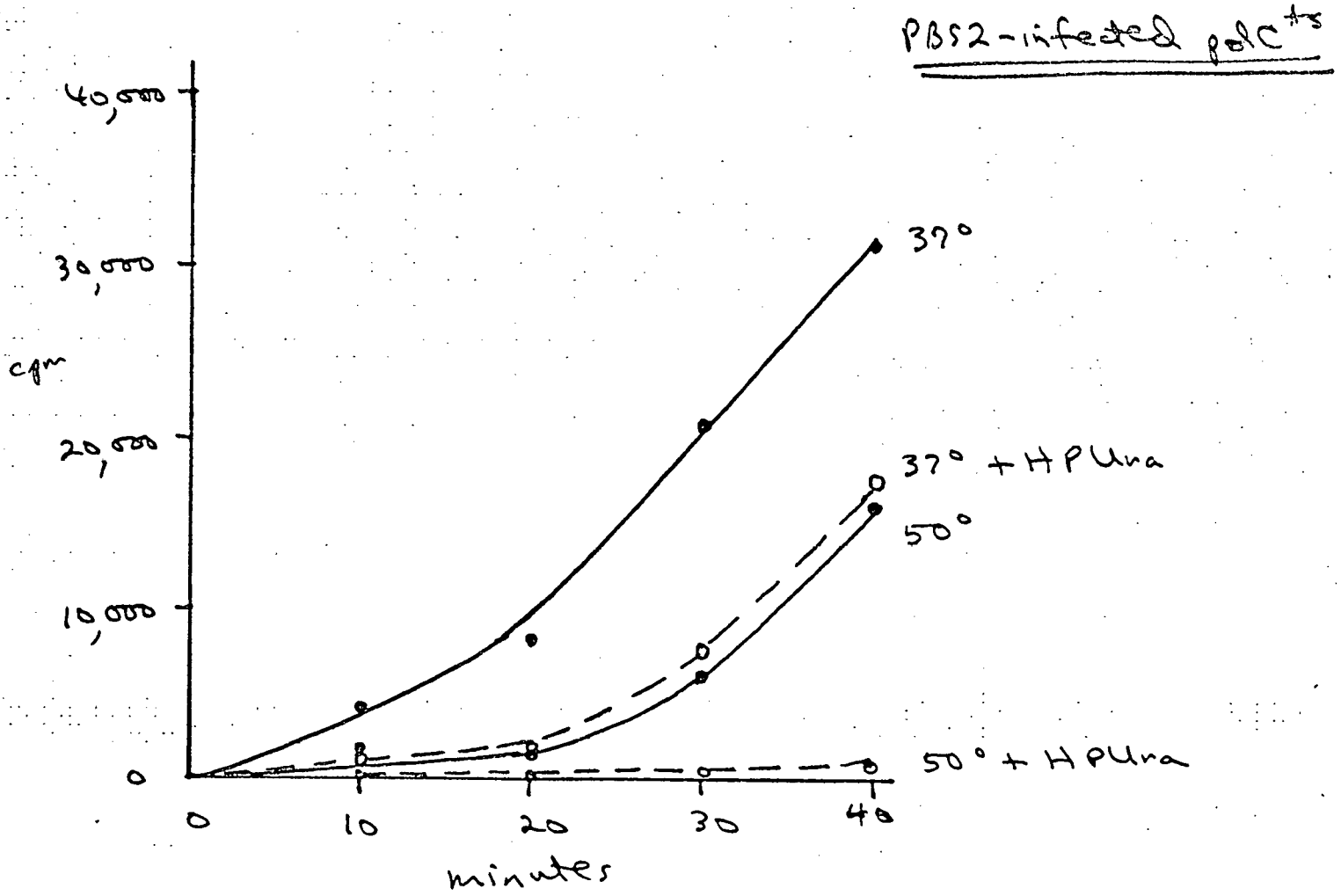
Deoxyribonucleotide pools after PBS2 infection

Our model for PBS2-induced proteins altering the B. subtilis deoxyribonucleotide metabolism (see scheme above) predicts that: (a) the dTTP in uninfected cells will disappear after PBS2 infection; and (b) dUTP, which has never been detected in uninfected cells (23), should accumulate after PBS2 infection for uracil-DNA synthesis. We have modified the 2-dimensional thin-layer cellulose chromatography system of Neuhard (23) and others, so that we can now separate dCTP, dTTP, and dUTP from one another and from all other common pyrimidine ribo- and deoxyribonucleoside mono-, di-, and triphosphates.

Incorporation of [6-^{3}H]uracil into DNA in uninfected or PBS2-infected *B. subtilis* BD317 *polA*$^-$ *polC*^{ts} at 37° or 50° w/wo 430 μm HPUra.

Fig. 2





We are perfecting the system for labelling all pyrimidine nucleotides in vivo with [6-^{3}H]uracil. [We found that labelled deoxyuridine will not selectively label deoxyribonucleotides, since it gets degraded to uracil -- even in excess uridine and in Warner's B. subtilis mutant with low levels of uridine/deoxyuridine/thymidine phosphorylase.] We then quickly extract the cells with trichloroacetic acid, which itself is removed by ether extraction. The solution is evaporated and the redissolved material is applied with cold standards to cellulose plates. After methanol treatment to remove traces of salts, the chromatogram is developed in: (a) butanol/methanol/HCL/water at 4° for 24 hours; then in (b) 0.5 M and 0.7 M ammonium sulfate. We may have to use [^{32}P]labelling to get significant radioactivity to detect dUTP (and then show also that purine nucleotides do not interfere). We are pursuing this method to prove that PBS2 infection really alters the nucleotide pools as we predict.

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*Six reprints of this paper, now in final form, are enclosed.