

# Inhibition by aphidicolin and dideoxythymidine triphosphate of a multienzyme complex of DNA synthesis from human cells

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A multienzyme complex consisting of DNA polymerase and several DNA precursor-synthesizing enzymes was solubilized by gentle lysis of cultured human cells. This complex channelled the distal precursor [ $^3\text{H}$ ]dTMP into DNA. The patterns of inhibition of the complex by aphidicolin and dideoxythymidine triphosphate (ddTTP) suggested that the complex contained the replicative DNA polymerase, polymerase  $\alpha$ . Inhibition by ddTTP was competitive with dTTP. This was exploited to estimate the effective concentration of [ $^3\text{H}$ ]dTTP at the site of DNA synthesis during channelling of [ $^3\text{H}$ ]dTMP into DNA. The estimated concentration (about 50  $\mu\text{M}$ ) was so high as to suggest that the solubilized complex was able to functionally compartmentalize DNA precursors.

*DNA polymerase*

*Functional compartmentation*

*DNA precursor-synthesizing enzyme*

*HPB-All cell*

*Thymine nucleotide*

*Gentle lysis*

## 1. INTRODUCTION

The enzymes of DNA precursor biosynthesis form a multienzyme complex with DNA polymerase in both prokaryotic [1,2] and eukaryotic [3–5] cells. The channelling of distal precursors through the complex results in a functional compartmentation of deoxynucleoside triphosphates, the immediate precursors for DNA synthesis. Consequently, a higher concentration of these species is maintained at the replication fork than could be produced by unlinked enzymes. We have developed a procedure for the gentle lysis of HPB-ALL cells (a lymphoblastoid cell line from a patient with thymic acute lymphoblastic leukaemia) using phospholipase C and micrococcal nuclease to digest membranes and nucleic acids, respectively. The resulting soluble preparation

channelled the distal DNA precursors thymidine, dTMP and dUMP into DNA [4]. The measured overall concentration of dTTP produced during these reactions was insufficient to account for the observed levels of incorporation, suggesting that the preparation retained the ability to functionally compartmentalize DNA precursors. We have also demonstrated the presence of a multienzyme complex consisting of DNA polymerase and several DNA precursor-synthesizing enzymes by gel-filtration of the lysate [5].

Here, we report the sensitivity of the preparation to the inhibitor aphidicolin, which suggests that the major DNA polymerase involved is the replicative enzyme, DNA polymerase  $\alpha$ . We have also used the competitive inhibitor dideoxythymidine triphosphate (ddTTP) to estimate the effective concentration of [ $^3\text{H}$ ]dTTP at the site of DNA polymerase action during incorporation of the distal precursor [ $^3\text{H}$ ]dTMP into DNA. The results show that the effective concentration of [ $^3\text{H}$ ]dTTP at the site of DNA synthesis was almost 30-fold greater than the measured overall concentration in the reaction mixture.

**Abbreviations:** ddTTP, dideoxythymidine triphosphate; HPB-ALL cells, a lymphoblastoid cell line from a patient with thymic acute lymphoblastic leukaemia; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

## 2. MATERIALS AND METHODS

Aphidicolin was the generous gift of Dr A. Todd (Imperial Chemical Industries, Alderley Park, Cheshire). ddTTP was obtained from Boehringer (Mannheim). Procedures for cell growth and lysis, the preparation of salt extracts of cells and the separation of labelled thymine nucleotides by PEI-cellulose chromatography have been described in [4,6].

Incorporation of labelled thymine nucleotides into DNA was assayed in 75  $\mu$ l reaction mixtures containing 25  $\mu$ l enzyme fraction (equivalent to  $2-3 \times 10^6$  cells), 50 mM Hepes (pH 7.4), 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, 250  $\mu$ M each of dATP, dGTP and dCTP, 50  $\mu$ M of either [ $^3$ H]dTTP or [ $^3$ H]dTMP (250 cpm  $\cdot$  min $^{-1}$   $\cdot$  pmol $^{-1}$ ), 200  $\mu$ g  $\cdot$  ml $^{-1}$  activated DNA, 100  $\mu$ g  $\cdot$  ml $^{-1}$  bovine serum albumin and 1 mM EGTA. Incubations were at 37°C. Tubes were processed for determinations of radioactivity in DNA and for the separation of labelled thymine nucleotides by PEI-cellulose chromatography as in [4].

## 3. RESULTS

Fig.1 shows the results of an experiment in which increasing amounts of aphidicolin were added to reactions in which incorporation of either [ $^3$ H]dTTP or [ $^3$ H]dTMP into DNA was catalyzed by the HPB-ALL cell lysate. Both reactions were inhibited to a similar extent. DNA polymerases  $\beta$  and  $\gamma$  (the presumed repair and mitochondrial polymerases, respectively) have been reported to be totally resistant to aphidicolin, whereas polymerase  $\alpha$  was sensitive [7-9]. We concluded that the major DNA polymerase present in the lysate was DNA polymerase  $\alpha$ , the presumed replicative enzyme [10], and that this enzyme was involved in the multienzyme complex which was responsible for the channelling of [ $^3$ H]dTTP into DNA. In our hands the DNA polymerase  $\alpha$  of the lysate appeared slightly more resistant to aphidicolin than reported by others [7-9]. This appeared to be due to the high levels of dCTP (250  $\mu$ M) used in our assay to optimise the channelling of distal substrates. dCTP is competitive with aphidicolin [11]. When the assay was repeated using 25  $\mu$ M dCTP, 50% inactivation of

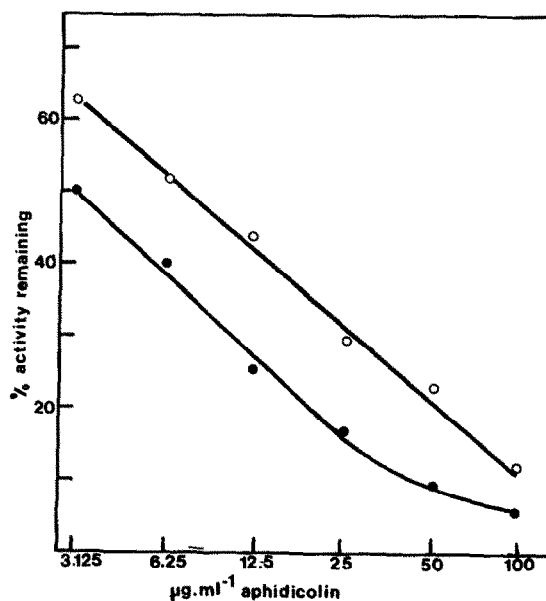


Fig.1. Effect of aphidicolin on the incorporation of [ $^3$ H]dTTP or [ $^3$ H]dTMP into DNA. Aliquots (25  $\mu$ l) of a lysate of HPB-ALL cells were incubated in final volumes of 75  $\mu$ l of DNA incorporation mixture. The labelled precursor was either 50  $\mu$ M [ $^3$ H]dTTP (●) or 50  $\mu$ M [ $^3$ H]dTMP (○). Increasing levels of aphidicolin were added as indicated. Incubations were for 1 h.

polymerase activity was observed at 2  $\mu$ g  $\cdot$  ml $^{-1}$  aphidicolin, in agreement with the results of others (not shown).

The sensitivity of ddTTP of two different preparations from HPB-ALL cells was then studied (fig.2). DNA polymerase activity in gently lysed cells was resistant up to dTTP:dTTP ratios of 0.1 but was inhibited at higher levels of ddTTP. This pattern of sensitivity was very similar to that found for purified DNA polymerase  $\alpha$ , polymerase  $\beta$  and  $\gamma$  being much more sensitive [12,13]. This result also suggested that the major polymerase present in the lysate was polymerase  $\alpha$ . In contrast, a preparation extracted from the same cell culture using 1 M NaCl followed by dialysis was considerably more sensitive to ddTTP (fig.2). This suggested that salt extracts contained higher proportions of non- $\alpha$  polymerases than lysates, in agreement with our finding of significant levels of non- $\alpha$  polymerases following gel-filtration of a salt extract [4].

The sensitivity of the [ $^3$ H]dTTP and [ $^3$ H]dTMP

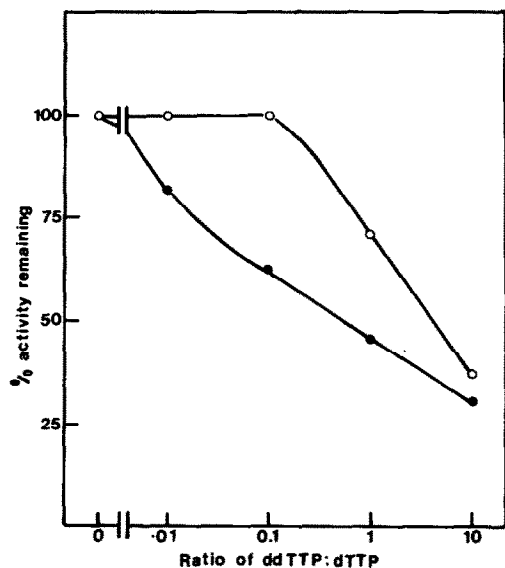


Fig. 2. Effect of ddTTP on the incorporation of [ $^3\text{H}$ ]dTTP into DNA. Aliquots (25  $\mu\text{l}$ ) of gently lysed (○) or salt-extracted (●) HPB-ALL cells were incubated in 75  $\mu\text{l}$  of DNA incorporation mixtures containing 50  $\mu\text{M}$  [ $^3\text{H}$ ]dTTP. Increasing amounts of ddTTP were added as indicated. Incubations were for 1 h.

incorporation reactions to inhibition by ddTTP were compared (fig. 3). Whereas the [ $^3\text{H}$ ]dTTP incorporation reaction was 78% and 34% resistant, respectively, at ddTTP levels of 50 and 500  $\mu\text{M}$

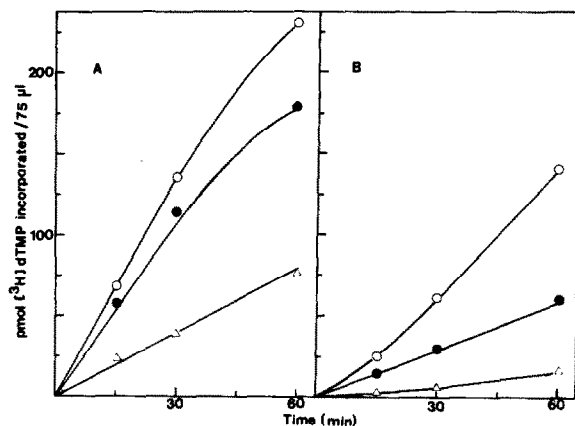


Fig. 3. Effect of ddTTP on the incorporation of [ $^3\text{H}$ ]dTTP or [ $^3\text{H}$ ]dTMP into DNA. Aliquots (25  $\mu\text{l}$ ) of a lysate of HPB-ALL cells were incubated in standard incorporation reactions containing 50  $\mu\text{M}$  [ $^3\text{H}$ ]dTTP (A) or 50  $\mu\text{M}$  [ $^3\text{H}$ ]dTMP (B): (○) control; (●) + 50  $\mu\text{M}$  ddTTP; (Δ) + 500  $\mu\text{M}$  ddTTP.

(fig. 3A), [ $^3\text{H}$ ]dTMP incorporation was more sensitive, being 35% and 14% resistant at 50 and 500  $\mu\text{M}$  ddTTP, respectively (fig. 3B). Since inhibition by ddTTP is competitive with dTTP (see fig. 5), we reasoned that the greater sensitivity of [ $^3\text{H}$ ]dTMP incorporation was due to the lower concentration of [ $^3\text{H}$ ]dTTP present at the site of polymerase action when [ $^3\text{H}$ ]dTMP was the substrate (fig. 3B) than when [ $^3\text{H}$ ]dTTP was supplied at 50  $\mu\text{M}$  (fig. 3A). However, when the [ $^3\text{H}$ ]dTTP level was permitted to reach a plateau prior to addition of ddTTP, the incorporation of added [ $^3\text{H}$ ]dTMP into DNA was less sensitive to the inhibitor: 38% of the incorporation was maintained when 500  $\mu\text{M}$  ddTTP was added 60 min after commencement of the incorporation reaction (fig. 4). In the control reaction, the apparent overall concentration of [ $^3\text{H}$ ]dTTP plateaued at 0.7  $\mu\text{M}$  after 10 min. Upon addition of 500  $\mu\text{M}$  ddTTP, this level rose to a new plateau at 1.7  $\mu\text{M}$ , presumably due to the inhibition of utilisation of [ $^3\text{H}$ ]dTTP (fig. 4). Addition of ddTTP to [ $^3\text{H}$ ]dTTP incorporation reactions after 1 h of in-

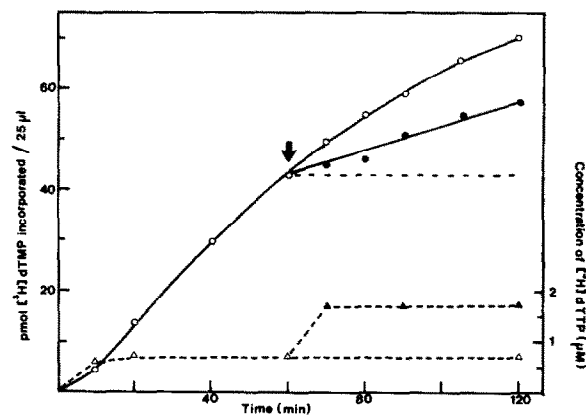


Fig. 4. Effect of addition of ddTTP to a [ $^3\text{H}$ ]dTMP incorporation reaction after 1 h incubation. A 750  $\mu\text{l}$  reaction containing cell lysate and 50  $\mu\text{M}$  [ $^3\text{H}$ ]dTMP was incubated at 37°C. Aliquots (25  $\mu\text{l}$ ) were withdrawn as indicated and were processed for the determination of radiolabel in DNA and in dTTP. At 1 h, the mixture was divided into two. One portion was made 500  $\mu\text{M}$  in ddTTP, while the other served as control. Incubation and sampling were carried out for a further 1 h. Circles, radiolabel in DNA, triangles, apparent overall concentration of [ $^3\text{H}$ ]dTTP: open symbols, control; closed symbols, + 500  $\mu\text{M}$  ddTTP.

cubation did not alter the degrees of inhibition seen in fig.3A (not shown).

We then attempted to use the data of fig.4 to estimate the effective concentration of [ $^3$ H]dTTP at the active site of DNA polymerase during incorporation of [ $^3$ H]dTTP into DNA. Fig.5A shows the lysate-catalyzed incorporation of increasing levels of [ $^3$ H]dTTP into DNA in the absence or presence of 500  $\mu$ M ddTTP. In fig.5B the percentage of [ $^3$ H]dTTP incorporation resistant to inhibition is plotted as a function of the concentration of added [ $^3$ H]dTTP. This showed clearly that inhibition by ddTTP was overcome in a competitive fashion by dTTP. Comparing fig.4 and 5B, it is

evident that the measured concentration of [ $^3$ H]dTTP (1.7  $\mu$ M) during channelling of [ $^3$ H]dTTP in the presence of 500  $\mu$ M ddTTP would have resulted in a more profound inhibition of incorporation than was actually observed, with only about 15% of the incorporation being resistant. The observed degree of resistant in fig.4 (38%), when compared with the graph in fig.5B, suggests that the effective concentration of [ $^3$ H]dTTP at the active site was about 50  $\mu$ M. Thus the [ $^3$ H]dTTP produced from [ $^3$ H]dTMP was highly localized at the DNA polymerase active site, at a concentration about 30-fold greater than the apparent overall concentration measured in the reaction mixture.

#### 4. DISCUSSION

Here, we have studied the effect of the DNA polymerase inhibitors aphidicolin and ddTTP on the incorporation of [ $^3$ H]dTTP or [ $^3$ H]dTMP into DNA. Incorporation was catalyzed by a gently lysed preparation from HPB-ALL cells containing a multienzyme complex consisting of DNA polymerase and several DNA precursor-synthesizing enzymes [4,5]. The pattern of inhibition of incorporation by aphidicolin and by ddTTP suggested that a single DNA polymerase, polymerase  $\alpha$ , was responsible for the major proportion of the incorporation detected in the lysate. Since DNA polymerase  $\alpha$  is thought to be involved in replication [10] we propose that the multienzyme complex also participates in this process. In contrast, salt extracts from HPB-ALL cells appeared to contain higher levels of polymerases  $\beta$  and  $\gamma$ , presumably due to selective inactivation of polymerase  $\alpha$  or to increased efficiency of extraction of the  $\beta$  and  $\gamma$  enzymes from subcellular structures.

When ddTTP was added at the commencement of the incubation, inhibition of [ $^3$ H]dTMP incorporation was much greater than inhibition of [ $^3$ H]dTTP incorporation. However, if incorporation of [ $^3$ H]dTMP was allowed to proceed for 1 h prior to addition of ddTTP, the observed inhibition was considerably reduced. This appeared to be due to the build-up of a small localized pool of [ $^3$ H]dTTP close to the active site of DNA polymerase  $\alpha$ . Inhibition of incorporation by ddTTP was competitive with dTTP. Thus, by titrating the inhibition by a fixed concentration of

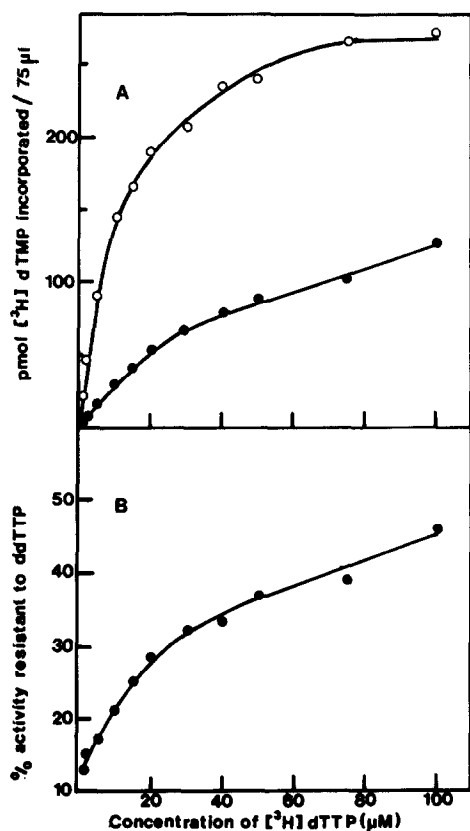


Fig.5. Competitive inhibition by ddTTP of incorporation of [ $^3$ H]dTTP into DNA. (A) Standard reaction mixtures were incubated for 1 h, except that the concentration of [ $^3$ H]dTTP was varied as indicated: (○) control; (●) + 500  $\mu$ M ddTTP. (B) Data of (A) were replotted, showing the variation of resistance to inhibition by 500  $\mu$ M ddTTP as a function of [ $^3$ H]dTTP concentration.

ddTTP against increasing levels of [ $^3\text{H}$ ]dTTP we estimated that the effective concentration of [ $^3\text{H}$ ]dTTP at the DNA polymerase active site was about 50  $\mu\text{M}$ , although the measured overall concentration in the reaction mixture as a whole was only 1.7  $\mu\text{M}$ . This showed that the multienzyme complex was able to functionally compartmentalize DNA precursors in vitro.

Functional compartmentation of metabolites by the operation of multienzyme complexes is a well-documented phenomenon [13]. The use of competitive inhibitors to estimate the effective concentrations of substrates in such systems could be generally applicable.

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