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EFFECT OF CYTOSINE ARABINOSIDE TRIPHOSPHATE ON DEOXYRIBONUCLEIC ACID SYNTHESIS IN PERMEALYSED CELLS FROM EHRLICH ASCITES TUMOUR

STUDIES OF PHOSPHORYLATED DRUG METABOLITES ON QUASI-NORMAL DEOXYRIBONUCLEIC ACID REPLICATION

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Abstract—Cytosine arabinoside triphosphate (ara-CTP) is a strong inhibitor of DNA synthesis in permealysed Ehrlich ascites cells, and this effect is rapidly reversed by dCTP but not by d-cytidine. Cytosine arabinoside is a weak inhibitor of DNA synthesis in permealysed Ehrlich ascites cells, and this effect is reversed by both dCTP and d-cytidine. A slightly improved assay system is described, and the permealysed ascites cells are shown to slowly lose activity during assays although DNA polymerase activity is not lost. An explanation of the effect of ara-CTP on DNA synthesis in permealysed cells and other systems is presented.

IN THIS paper the effect of cytosine arabinoside triphosphate (ara-CTP) on permealysed ascites cells[1] is compared to the known effects of ara-CTP on other systems *in vitro*. This was to allow further information to be gained on the mechanism of cytosine arabinoside (ara-C) inhibition of DNA replication and also to allow the evaluation of the permealysed cell system as a technique for the study of the action of phosphorylated drug metabolites.

Phosphorylated drug metabolites do not generally pass through the outer cell membranes readily, and the small amounts that do pass may be negligible compared to the rate of passage of the drug after dephosphorylation by the membrane phosphatases. However, the study of phosphorylated drugs on isolated polymerases has a disadvantage in that the normal action of a drug may depend on the target site having a high degree of organization. Thus there is a requirement for a system with a degree of organization between that of the intact cell and isolated enzymes. One such system has been developed[1] with mammalian cells, and this paper describes its application to some features of ara-C action.

In normal cells, most of the ara-C incorporated into DNA is found in internucleotide links,[2] whereas in model systems *in vitro* using ara-CTP it has mainly been observed to be incorporated into the DNA 3′-terminal position.[3–5] It should be noted that even in these studies small amounts of internucleotide ara-C were observed in the model systems, although this was always much less than the terminal ara-C.

Another point of difference was that in intact cells, ara-C inhibition of DNA synthesis is rapid and readily reversible,[6] whereas in at least one system *in vitro*, ara-CTP inhibition took many min to become completely effective,[4,7] and the inhibition could not be reversed by dCTP alone.[5]

This paper will attempt to resolve some of these differences.

MATERIALS AND METHODS

Permealysed Ehrlich ascites cells. These were prepared from cells grown in Swiss albino mice which were transferred or harvested at intervals of 4–6 days. The method of preparation and properties of the permealysed cells have been described in detail[1] and entail the permealysation of cells by mild homogenisation in high viscosity buffers that contain chelating agents. The permealysed cells are readily separated from normal cells by their buoyancy in a ficoll step-gradient.

The nuclei of the permealysed cells appeared to be metabolically similar to those in intact cells with respect to DNA synthesis.[1] The cell's outer plasma membrane was disrupted and the cytoplasmic structure was expanded, vesicularized and, presumably, highly depleted in soluble proteins. These cells appear to utilize deoxyribonucleotides directly. They have a very limited ability to use thymidine, which appears to be due to the presence of residual kinases and not to the presence of low numbers of intact cells.[1]

Assay procedure. All components were obtained from commercial sources. However, the dATP, dCTP and dGTP were repurified by chromatography in isobutyric acid–1 M ammonium hydroxide–0·1 M EDTA (100:60:1·6, v/v) before use. α-[^{32}P]dTTP was prepared by a method previously described.[8] [^{3}H]dTTP was purchased from Amersham and diluted to the specific activities stated in the text.

For assay, the permealysed cells were suspended in 15% ficoll (w/w), 100 μM EDTA, 100 μM EGTA,* 15 mM 2-mercaptoethanol, 100 μM spermidine, 10 mM Hepes† and 70 mM Tris. Final pH was adjusted to 7·4 with HCl. In addition to this buffer, the complete assays contained 3 mM ATP, 2 mM phosphoenolpyruvate, 4 mM $MgCl_2$, 6 mM α-glycerophosphate and 50 μM each of dATP, dGTP and dCTP. Radioactive dTTP was present at 10 μM. This assay system has a lower Mg^{2+} concentration than the system previously reported[1] and contains α-glycerophosphate. These modifications gave improved over-all rates when compared to the system previously reported.

Unless otherwise stated, the standard assay volume was 0·05 ml. All assays were at 37°. Permealysed cells in standard assays or in standard samples from time-course incubations were lysed with $\frac{1}{5}$ vol of 1·8 M KOH, 100 mM EDTA and the lysate was dried onto 25-mm discs of Whatman No. 50 paper when using ^{32}P, or nitrocellulose membrane when using ^{3}H. The discs were then washed thirteen times with 0·12 M H_2SO_4, 0·05 M H_3PO_4, 0·25 M Na_2SO_4. In any experiment, all papers were washed together in the acid-saline and each wash was for a period of 1 hr. The papers were finally washed in 1% trichloroacetic acid, neutralized in ammonia vapour, dried and counted.

* EGTA, ethanedioxy bis(ethylamine)tetra-acetate.

† Hepes, 2-(*N*-2-hydroxyethyl piperazin-*N'*-yl)ethane sulphonic acid.

TABLE 1. DNA SYNTHESIS IN PERMEALYSED CELLS—INTERACTIONS BETWEEN ARABINOSIDE AND DEOXYRIBOSE DERIVATIVES OF CYTOSINE*

Part A	Incorp. (%)	Part B	Incorp. (%)	Part C	Incorp. (%)
A_1-control	100 (99–100)	B_1-minus dCTP	51 (51–50)	C_1-minus dCTP plus d-cyt. (500 μM)	60 (55–64)
A_2-plus ara-cyt. (50 μM)	98 (97–99)	B_2-minus dCTP plus ara-cyt. (50 μM)	37 (37–37)	C_2-minus dCTP plus ara-cyt. (50 μm) plus d-cyt (500 nm)	53 (55–51)
A_3-plus ara-CTP (50 μM)	47 (48–45)	B_3-minus dCTP plus ara-CTP (50 μM)	7 (6–8)	C_3-minus dCTP plus ara-CTP (50 μm) plus d-cyt. (500 μM)	9 (9–10)

* All assays are the standard complete system with modifications as stated. Assay procedure and complete system are as described in Materials and Methods. All assays containing permealysed cells equivalent to 37·5 μg DNA were incubated for 20 min. [^{3}H]TTP sp. act. was 2·0 Ci/m-mole and was counted with an efficiency of 11·1 per cent. All results are expressed as a percentage of the control which had an average incorporation of 0·713 pmole TMP/mg of DNA over this time period. The results are the means of replicates whose ranges are shown in parentheses. Abbreviations: ara-CTP, cytosine arabinsoside triphosphate; d-cyt., deoxycytidine; and ara-cyt., cytosine arabinoside.

Cytosine arabinoside triphosphate (ara-CTP). This was prepared by enzymatic phosphorylation of cytosine arabinoside-5'- monophosphate with *Escherichia coli* kinases. The ara-CMP was received as a gift from Dr. W. J. Wechter.

RESULTS

Inhibition by ara-C nucleoside and ara-C triphosphate. The results in Table 1 suggest that the permealysed cells appear to have a limited endogenous source of dCTP, as ara-CTP has an effect in the absence of exogenous dCTP. This is discussed below. The interactions of the added cytosine deoxynucleosides and nucleotides with their corresponding arabinosides demonstrate that the nucleoside triphosphates are utilized readily and have large effects where as the nucleosides have very weak effects that are readily reversed by competing nucleotide triphosphates.

There was a large inhibitory effect of ara-CTP when no dCTP was added to the assay mix (Table 1, B_3). This inhibitory effect was markedly reduced by added dCTP (Table 1, A_3) but not by a large excess of d-cytidine (Table 1, C_3).

The presumptive, weak endogenous supply of dCTP may have a number of sources, e.g. it may be due to small amounts of degrading DNA having its products phosphorylated. However, the supply appears to be variable between preparations and has been estimated to be equivalent to some value below 2 μM dCTP.

Reversal of ara-CTP inhibition. The experiment shown in Fig. 1 demonstrates that after 3 min of inhibition with ara-CTP, dCTP-induced reversal of inhibition is just as effective as in a control experiment in which there was no preincubation with ara-CTP (i.e. D_1 approx = D_2 in Fig. 1). This pattern of inhibition superficially suggests that ara-CTP is acting in a simply competitive way, and not acting as a chain-terminator. However, other circumstantial evidence suggests that this is probably better described as pseudo-competitive inhibition (see Discussion).

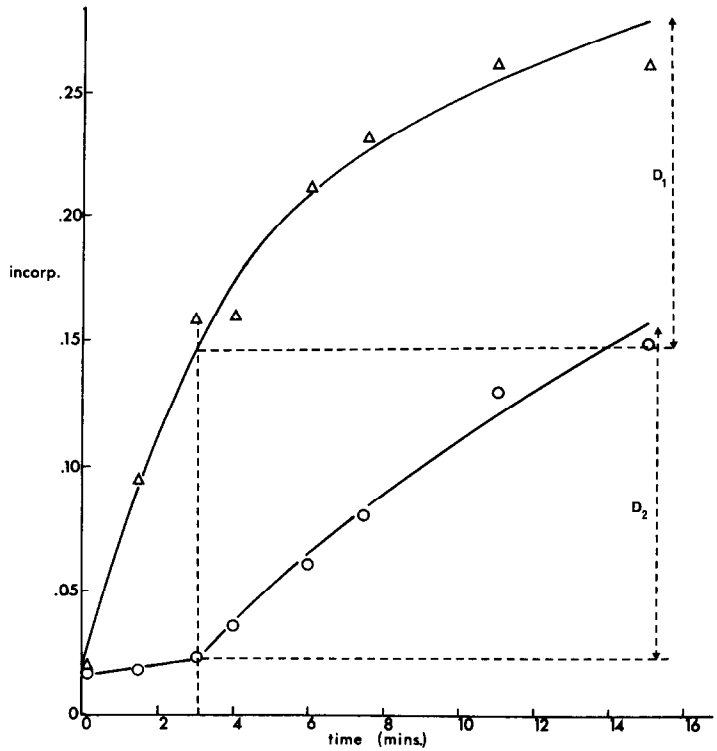

FIG. 1. Reversal of ara-CTP inhibition when the dCTP/ara-CTP ratio is changed. Incorporations are pmoles TMP/mg of cells DNA. Assay procedure and complete assay system are as described in Materials and Methods except that dCTP levels were varied as below. Assay volumes were 0·5 ml initially and 0·05-ml samples were taken. Each 0·05-ml sample contained permealysed cells equivalent to 9·25 μg DNA. α-[^{32}P]dTTP sp. act. was 4·4 × 10^8 cpm/μmole at the counting efficiency of this experiment. Symbols: △, initial vol. of 0·5 ml contains 2 μM dCTP. At 3 min, dCTP and ara-CTP were added to give final concentrations of 100 and 73 μM respectively; and ○, Initial vol. of 0·5 ml contains 2μM dCTP and 73 μM ara-CTP. At 3 min, dCTP was added to give a final concentration of 100 μM.

These results are also different to some systems *in vitro*,[4,5,7] with respect to the time taken for ara-CTP to have its maximal effect. In the experiments reported here, ara-CTP inhibition was almost complete at times less than 2 min whereas calcium-activated isolated nuclear systems have been reported as showing a progressive inhibition that may take more than 15 min.[4,5,7]

This difference probably reflects the different kinetics of the two systems—systems like those reported here—one having fast DNA chain growth at few primer sites and the other type of system[4,5,7] having slow chain elongation but occurring at many sites simultaneously (see Discussion).

Effect of exogenous DNA on the system. Figures 2 and 3 show the effect of ara-CTP on the time-course of incorporation of permealysed cells without and with exogenous priming of DNA synthesis. There is a large over-all difference in incorporation rates and the exogenously primed system is linear with time (Fig. 3) while the unprimed system is not (Fig. 2). In addition, ara-CTP inhibits the unprimed system much more than the primed system (Figs. 2 and 3). This could be due to the extra

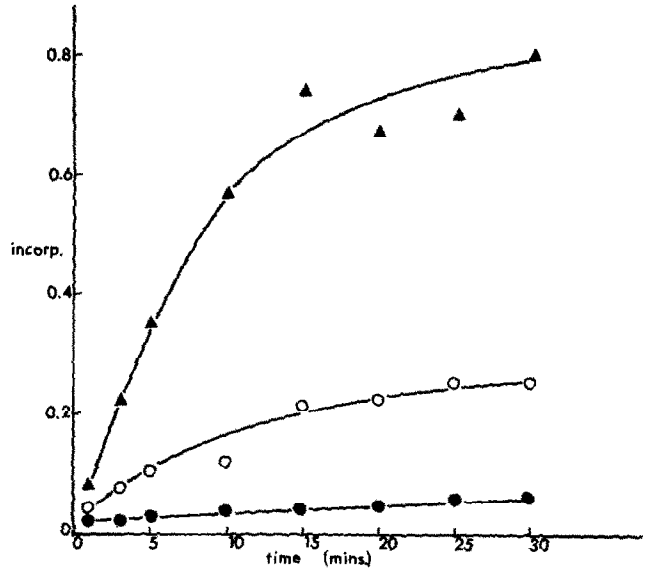

FIG. 2. Effect of ara-CTP on permealysed cells without exogenous primer. Incorporations are pmoles TMP/mg of cell DNA. Assay procedure and complete system are as described in Materials and Methods Assay volumes were 0·1 ml and contained permealysed cells equivalent to 26 μg DNA. α-[^{32}P]dTTP sp. act. was $3{\cdot}8 \times 10^8$ cpm/mole at the counting efficiency of this experiment. Symbols: ▲, complete system; ○, complete system minus dCTP; and ●, complete system minus dCTP, plus 50 μM ara-CTP.

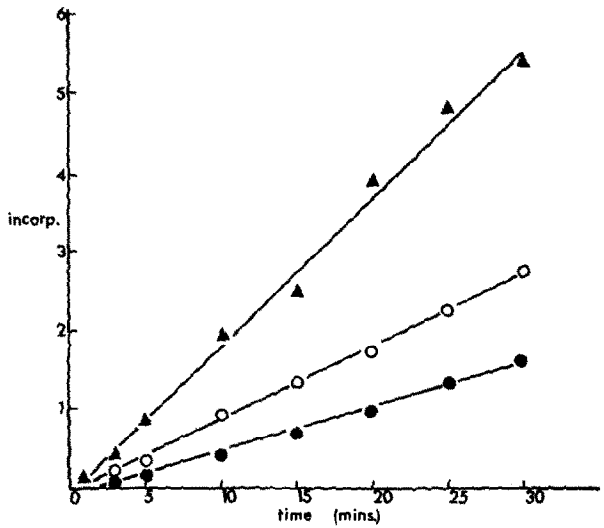

FIG. 3. Effect of ara-CTP on permealysed cells with exogenous primer. Incorporations are pmoles TMP/mg of cell DNA. Assay procedure and complete system are as described in Materials and Methods. All assays contain 7 μg native calf thymus DNA that has been activated by brief exposure to bovine DNAase I. Assay volumes were 0·1 ml and contained permealysed cells equivalent to 19 μg DNA. α-[^{32}P]dTTP sp. act. was $3{\cdot}3 \times 10^8$ cpm/μmole at the counting efficiency of this experiment. Symbols: ▲, complete system; ○, complete system minus dCTP; and ●, complete system minus dCTP, plus 50 μM ara-CTP.

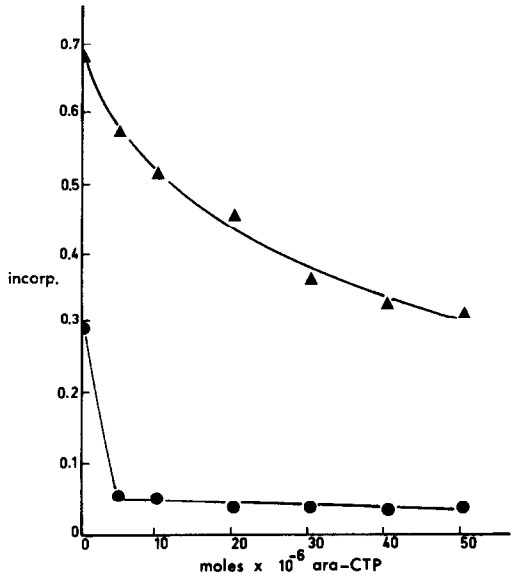

FIG. 4. Residual, or ara-CTP-resistant, DNA synthesis. Incorporations are pmoles TMP/mg of cell DNA. Assay procedure and complete system are as described in Materials and Methods. Assay times were 20 min. Assay volumes were 0·05 ml and contained permealysed cells equivalent to 4·5 μg DNA. [^{3}H]dTTP sp. act. was 2·0 Ci/m-mole and counting efficiency was 11·1 per cent. Symbols: ▲, complete system (contains dCTP, 100 μM), ara-CTP as stated; and ●, complete system minus dCTP, ara-CTP as stated.

primer providing dCTP from the products of its degradation and/or due to the primer providing more priming sites than can be terminated by the presumptive chain-terminator, ara-CTP.

Residual rate of DNA synthesis after inhibition. Figure 4 shows a comparison of the effect of increasing concentrations of ara-CTP on permealysed cells with and without additional dCTP in the assay mixture. In the absence of dCTP, 94 per cent inhibition is the maximal inhibition observed. After 94 per cent inhibition has been achieved, further additions of ara-CTP have little effect. Thus the 6 per cent residual DNA synthesis appears to be a process that is distinctly different to the bulk of the normal DNA synthesis.

Non-linearity of the standard system. The rate of DNA synthesis in the standard, unprimed preparation falls off with time, but this decay in rate does not appear to be entirely, or even mainly, caused by the exhaustion of assay components (e.g. triphosphates) because the decay in rate is only weakly dependent on the cell density in the assay (Fig. 5). Moreover, this insufficiency, or progressive damage, does not affect the activity of the exogenously primed system (compare Figs. 2 and 3). Thus, the decay in incorporation is apparently not due to loss of DNA polymerase activity nor to simple exhaustion of the medium. It appears that some more subtle fault develops in the latter stages of the assay.

The improved assay system described in this paper has given higher initial rates of activity, and this is probably what has exposed the problems that develop in the latter phases of the assay. Because of this, these differences were not clearly observed in the previous work[1] which claimed a more linear time-course than is reported here.

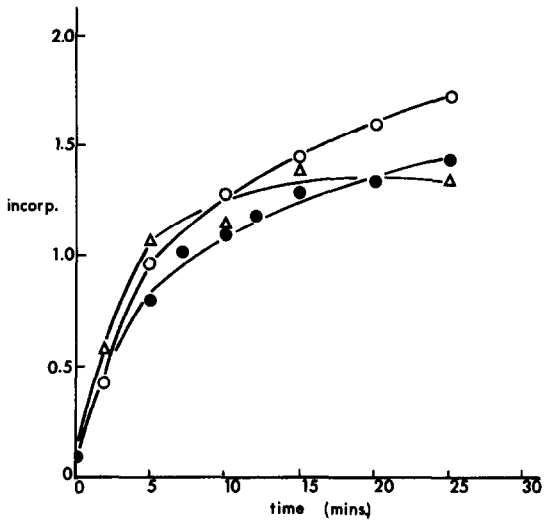

FIG. 5. Effect of cell density in assay on incorporation curves. Incorporations are pmoles TMP/mg of cell DNA. Assay procedure and complete system are as described in Materials and Methods. Assay volumes were 0·05 ml. α-[^{32}P]dTTP sp. act. was 5·8 × 10^8 cpm/μmole at the counting efficiency of this experiment. Symbols: ○, high density assay contained permealysed cells equivalent to 6·3 μg DNA/assay; ●, intermediate density assay contained permealysed cells equivalent to 1·26 μg DNA/assay (i.e. $\frac{1}{5}$ of high density); and △, low density assay contained permealysed cells equivalent to 0·315 μg DNA/assay (i.e. $\frac{1}{20}$ of high density).

DISCUSSION

The results obtained with ara-CTP in permealysed cells provide a useful connecting link between results obtained in living cells and results obtained with much simpler systems *in vitro* such as isolated nuclei and semi-purified DNA polymerases.

Permealysed cells are inhibited rapidly and reversibly, like normal cells, but are utilizing phosphorylated substrates like systems *in vitro*. The DNA synthesis of permealysed cells also has the characteristics of cellular DNA-replication in that the product has, apparently, a long average chain length and synthesis proceeds via short fragments.[1]

Effective reversal of ara-CTP inhibition in permealysed cells requires dCTP and not d-cytidine, which would be expected for intact cells.

When examined closely, the ara-CTP inhibition of permealysed cells is compatible with previous results obtained with both systems *in vivo* and *in vitro*. An explanation is as follows.

Virtually all of the effects of ara-C and its derivatives on DNA synthesis *in vivo* and *in vitro* can be now satisfactorily explained if we accept three reasonably secure facts: (a) DNA-polymerizing systems *in vivo* and *in vitro* incorporate ara-CMP into DNA from ara-CTP,[2–5] (b) during DNA synthesis *in vitro*, the 5′ position of ara-CTP has a similar reaction rate with primer sites as dCTP does,[2,4,9] and (c) *in vitro*, the 3′-position of ara-CMP has a very low but not negligible reactivity when acting as primer terminus on a growing DNA chain; this can be deduced from previous results[3,5] which show low, but not negligible, incorporation of ara-CMP into internucleotide links.

If one accepts the above three statements for both *in vivo* as well as *in vitro*, then the effect of ara-C derivatives on the process of DNA chain elongation can be described in terms of two theoretically extreme cases: case I, systems with high potential rates relative to the number of primer termini being extended (e.g. high ratios of polymerase to primer); and case II, systems with large numbers of available primer termini relative to the total rate of DNA synthesis (e.g. low ratios of polymerase to primer).

The first case produces few but very long chains; the second case produces many very short chains— in one case,[5] two or less nucleotides long in 30 min. Now a simple consideration of the statistics of chain growth shows that the first case should be rapidly but reversibly inhibited by ara-C derivatives. The inhibition should be rapid because at any one time all the available ends are in the process of extension; thus the proportion of primer termini that has received one or more ara-CMP residues in a given time is high, relative to the second case. Once this has occurred, DNA synthesis is limited by the rate of reaction of the ara-CMP 3′ sites, and this rate may largely explain the residual DNA synthesis or ara-CTP-resistant DNA synthesis in permealysed cells.

In the second case, the proportion of primer termini covered with ara-CMP per unit of time is small, and it thus takes a relatively longer time for the rate of DNA synthesis to become dependent on the rate of extension of chains that have received one or more ara-CMP residues.

The factors affecting the rate of reversal of ara-CMP inhibition should be almost identical to the factors affecting the rate at which inhibition develops. However, during inhibition-reversal, the relevant reaction is the attachment of normal nucleotides to 3′-sites of terminal ara-CMP. Thus, normal DNA synthesis *in vivo* is thought to approximate case I and DNA synthesis *in vitro*, in the past, to usually approximate case II. This is because case II is the operative situation when an assay system has maximal primer added in order to maximize observable enzymic activity.

It can also be seen that in case I, the ara-CMP which was incorporated would have a high proportion of ara-CMP incorporated into internucleotide links relative to case II. This is because the residual rate of DNA synthesis in case I would be almost entirely determined by the slow, but significant, rate of reaction of the terminus-ara-CMP-3′-site, whereas the true residual rate in case II would not be achieved without long incubation times, depending on the assay conditions. In the time prior to achieval of the true residual rate, the incorporation of ara-CMP would be predominantly due to addition of ara-CMP to the excess of primer termini. This would result in a high proportion of terminal ara-CMP to internucleotide ara-CMP.

Case II has been demonstrated to exist[5] and case I appears to explain the results obtained in normal and permealysed cells.

Thus, at present, there appears to be no inexplicable observations that relate to the effect of ara-C or its derivatives on simple DNA synthesis *in vitro* or *in vivo*.

Thus in summary, the effect of ara-CTP on DNA synthesis *in vivo* and *in vitro* is determined by the ratio of potential catalytic activity to available priming sites. The two extreme theoretical limits of this ratio give rise to two extreme cases (case I and case II above). One of these extreme cases approximately describes the events occurring during DNA replication *in vivo* and the other approximately describes the situation during DNA synthesis in many systems *in vitro*.

The residual DNA synthesis of ara-CTP-inhibited permealysed cells allows a determination of the maximum rate of reaction at the arabinosyl 3′-site relative to the arabinosyl 5′-site. If all the residual DNA synthesis is due to the extension of a fixed number of chains, then the rate of DNA synthesis will be limited by the slow rate of reaction at the arabinosyl 3′-site. This figure is only an upper limit because it assumes no chain initiation. If there is a high rate of chain initiation, it will be an over-estimate of the reaction rate of the 3′-site relative to reaction rates at the 5′-site.

Table 1 and Fig. 4 indicate that this rate is approximately equal to or less than 7–6 per cent of the rate of reaction at the 5′-site under the conditions of these experiments.

It is important to distinguish between a satisfactory explanation and a proven explanation in these systems. Nuclear systems and permealysed cell systems are highly complex, and thus there is always a number of potential explanations for any group of observations. However, studies of simple, purified polymerases are also insufficient, as it is now obvious that many more enzymes are involved in DNA polymerisation than the polymerases alone.

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