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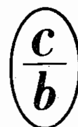

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SUBSTRATE INHIBITORS OF DNA BIOSYNTHESIS

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Data are presented on an analysis of the inhibition of DNA biosynthesis by substrate analogs in solutions containing DNA polymerases of different origin. An attempt has been made to suggest reasons for inhibitor specificity in regard to DNA synthesis catalyzed by different DNA polymerases.

Three approaches are currently employed to study the mode of operation of enzymes and multienzyme complexes catalyzing replication, reverse transcription, genetic recombination, reparation, and other processes. One of these strategies is the study of the properties and composition of multi-protein complexes isolated from cells. A second approach is the reconstruction of multienzyme complexes synthesizing DNA which have been obtained from purified protein components and the study of these enzymes at each stage in the assembly process. The third approach is an inhibitor analysis of the processes involved in DNA biosynthesis. By means of this approach it would be possible to compare complexes which synthesize DNA in cells and in cell-free solutions. A comparative analysis of these processes involving the use of monoclonal antibodies would be of great significance.

The current knowledge of the problem of inhibitor analysis of various processes involved in DNA biosynthesis using substrate inhibitors will be examined in this communication.

It is known that most enzymes which catalyze the polycondensation of dNTP into the DNA chain are template-dependent enzymes. An exception is terminal deoxynucleotidyl transferase, with catalyzes the synthesis of DNA in a cell-free system in the absence of a specific program. Special attention will be devoted to template DNA polymerases.

Information currently available on DNA polymerases is quite fragmented. DNA polymerases from *Escherichia coli* and certain phages parasitizing this bacterium have been studied in considerable detail. Much attention has also been focused on mammalian DNA polymerases, which may be arbitrarily separated into replication (α), reparative (β), and mitochondrial (γ) polymerases. Investigations have also been made into DNA polymerases δ and the mammalian terminal deoxynucleotidyl transferase, although the biological functions of these enzymes are not clear. More detailed information on these enzymes may be found in review articles [1, 2].

A comparative study of mammalian and mammalian virus DNA polymerases would be of particular importance. The differences in the properties of these enzymes provide an opportunity to block selectivity the reproduction of the viral genome in mammalian and human cells. Among the viral DNA-polymerases the DNA polymerases of herpes virus, variola vaccine, and adenoviruses, and among the retroviruses the reverse transcriptase of fowl myeloblastosis virus have been studied in the greatest detail. These polymerases markedly differ from each other, especially in regard to substrate analogs.

Substrate inhibitors of DNA biosynthesis have different modes of action at the molecular level.

1. The competitive inhibitors may compete with substrates for binding with the DNA-synthesizing complex, which, in a cell-free system containing purified DNA polymerases, is usually the template-primer-DNA polymerase complex. In this case the competitive inhibitors are not incorporated into the DNA chain, but merely replace the dNTP in the complex by occupying a position either directly at the site at which dNTP is bound or close to this site.

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2. Terminator substrates also compete with substrates but they are incorporated into the DNA chain and then for various reasons arrest subsequent elongation of the primer chain.

3. Finally, substrate analogs, which also compete with substrates for binding with the DNA complex being synthesized, are incorporated in the DNA chain, but the rate of subsequent elongation of the DNA chain in this case is significantly reduced.

A number of systems are currently being used to study the properties of DNA substrate inhibitors. Firstly there is the so-called activated DNA. This is usually a double-stranded DNA of random composition, and in one of the chains, with the aid of DNase, small nicks or gaps of tens or hundreds of nucleotide residues are formed (the selection of the chain for each nick is random). These damages are "filled in" in the presence of dNTP with the aid of DNA-polymerases, but the results of such experiments provide only general information, and in this case it is difficult to study the mode of action of the substrate inhibitors at the molecular level.

Systems containing individual DNAs (phages, plasmids) are more convenient. Either single spiral circular DNAs (more frequently phage M13 DNA) with a synthetic primer or restrictase fragments in which one chain is partically hydrolyzed from the 3'-end in the presence of *E. coli* exonuclease 111 are used in such systems. Additional systems, which are modifications of the systems described, are also used. With these systems it is possible to study the mode of action of substrate inhibitors at the molecular level and also the effect of the primary structure of the template on the processes involved in the synthesis.

Finally in certain specific cases more complex experimental variants are used. Firstly, the above-mentioned systems with additional auxiliary replication proteins (a protein which stabilizes single-spiral DNA and others) are used. Secondly, cell-free systems containing chromatin or cell nuclei are used. In such systems the DNA polymerases are complexed with many other replication proteins but usually complete replicative complexes are not formed. Thirdly, there are cells which are permeable with respect to dNTP as a result of various reactions and the resultant replicative complexes are apparently similar to complexes of normal cells. Although in this case one should take into account that at least in eukaryotic cells the substrate "flows" differ from those which exist in normal replicating cells and that the relationship between the pathways of biosynthesis of different DNA substrates (de novo and additional) are markedly changed. Finally, there are cell cultures available with the aid of which replication, reparation, reverse transcription, and other processes in the presence of substrate inhibitors may be quite conveniently studied. However, in these systems it is possible to use only 2'-deoxynucleosides, since dNTPs do not penetrate the cell wall. One should also take into account the possibility that analogs of the nucleosides in whole cell systems may be modified by certain metabolic enzymes and may exert their action in the form of other chemical structures.

The principle of selecting substrates with matrix-primer-DNA-polymerase complexes as a physicochemical process has not been studied in detail. One aspect of this selection procedure has been investigated, i.e., the interaction between the substrate base and the template base leading to the formation of hydrogen bonds according to the Watson-Crick law. The triphosphate residue of the substrate which interacts with the enzyme is known to play an important role, but it is not known how significant the remaining elements in the dNTP molecule are, or whether certain other atoms in the bases and sugars are essential for the direct binding with the complex. This information would be much more important in regard to the construction of highly-specific inhibitors of viral DNA-polymerases, which are currently prepared in an empirical manner.

The 6-aryl-aminouracils, 6-arylhydrazinouracils, 6-arylhydrazinocytosines, N^2-n-butylphenylguanine, 2-butylphenylaminoadenine, their 2'-deoxyribosides, and also the 5'-triphosphates of the latter [3] are among the most characteristic and potent representatives of the first type of inhibitor (competitive inhibitors). These agents inhibit the synthesis of DNA catalyzed by mammalian DNA-polymerases α, and by fowl by fowl myeloblastosis reverse transcriptase, and also by a number of bacterial and phage DNA polymerases. These compounds in the form of bases, 2'-deoxynucleosides and 2'-deoxynucleoside-5'-triphosphates bind with the template-primer-DNA-polymerase complex and compete with substrates containing the analogous base. However, these inhibitors are not incorporated into the DNA chain.

Such compounds have a selective action on DNA synthesis catalyzed by certain DNA polymerases, since they form a strong complex with the template (three hydrogen bonds) which is

TABLE 1. Terminator Activity of Substrate Analogs in the Biosynthesis of DNA

dNTP analog	DNA-polymerase					
	I from E. coli	α from calf thymus	β from rat liver	Terminal nucleotidyl transferase from calf thymus	Reverse transcriptase of fowl myeloblastosis virus	Phage T4
ddNTP	+	—	+	+	+	—
dNTP(3'NH_2)	+	+	+	+	+	—
dNTP(3'NHacet)	+	+		+	+	—
dNTP(3'NHbio)	+	—		+	+	
dNTP(3'N_3)	—	—	+—		+	
dNTP(3'F)	+	—	+	+	+	+
dNTP(3'OMe)	—	—			+	
araNTP(3'NH_2)	—	+	+	+	+	+
araNTP(3'N_3)	—	—	—	+	+	
dNTP(3'NH_2) (α-S)	+	—	+		+	
dNTP(3'N_3) (α-S)	—	—	+—		+	
dNTP (3'F) (α-S)	+	—	+		+	

Note. (+), termination; (—), absence of termination; (+ —), termination when there is a 10^2-10^3-fold excess of analog over substrate; ddNTP, 2',3'-dideoxynucleoside-5'-triphosphates; dNTP (3'NH_2), 3'-amino-2',3'-dideoxynucleoside-5'-triphosphates; dNTP (3'NHacet) and dNTP (3'NHbio), 3'-acetyl- and 3'-biotinyl-derivatives of dNTP (3'NH_2) respectively; dNTP (3'F), 3'-fluoro-2',3'-dideoxynucleoside-5'-triphosphates; araNTP (3'NH_2), 3'-amino-3'-amino-3'-deoxynucleoside-5'-triphosphates; araNTP(3'N3), 3'-azido-3'-deoxynucleoside-5'-triphosphates; dNTP (3'OMe), 3'-O-methyl-dNTP; (α-S) denotes that α-phosphate has been replaced by α-thiophosphate.

correctly orientated on account of the strong additional interaction between the aromatic (or other hydrophobic) substituent and the "pocket" of DNA polymerases present at this site. This explanation of the facts is hypothetical. However, other DNA polymerases (β, γ, δ) which do not have a corresponding "pocket" are not sensitive to arylnucleosides and their 5'-triphosphates. However, these inhibitors of DNA biosynthesis are not incorporated into the DNA chain, irrespective of the form in which they are added to the reaction mixture.

Terminator substrates of DNA polymerases have already been studied in considerable detail. There studies commenced in the laboratories of A. Kornberg and F. Sanger [4, 5], who were the first research workers to determine the mode of action of dideoxynucleoside triphosphates (ddNTP) at the molecular level. When ddNTP molecules bound with the template-primer-DNA-polyermase I complex from *E. coli*, the nucleotide residues from ddNTP were incorporated at the 3'-end of the DNA chain in accordance with the complementarity rule, but synthesis subsequently ceased, since a hydroxyl group was not present in the 3'-position of these compounds and therefore a new phosphodiester bond could not be formed. The ddNTP molecules have been extensively used for determination of the primary structure of DNA by the polymerization method [5].

More recent studies have shown that ddNTP molecules are not utilized by all DNA polymerases and are in fact not incorporated into the DNA chain by replicative DNA-polymers [2]. This implied that there could be a possible route for creating new terminator substrates, which are specific for DNA polymerases with different cellular functions.

Extensive studies on such compounds have been carried out in recent years. These studies were initiated when the synthesis of a number of dNTP (3'NH_2) compounds had been achieved, and the latter were studied in experiments with purified DNA polymerases [6, 7], reparation chromatin from liver [8], and sea urchin embryo replicating nuclei [9], and the corresponding nucleosides were studied in experiments with mouse myeloma p_3Ag653 cells in a sea urchin embryo culture [10]. The arsenal of terminator substrates was later further extended, and dNTP(3'F) [11], araNTP(3'NH_2), araNTP (3'N_3) [12], dNTP(3'OMe) and dNTP(3'N_3) were studied. Data on these and certain other compounds have been combined and presented in Table 1 [2, 6, 7, 11, 12].

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TABLE 2. Inhibitors of DNA Synthesis Catalyzed by Various Mammalian and Mammalian Virus DNA-polymerases.

Compounds	DNA-polymerases						
	α	β	γ	δ	Terminal nucleotidyl transferase	Various reverse transcriptases	Herpes viruses
Arylnucleosides	+	−	−	−	−	+	
araNTP	+	−	−		+	+	+
BrdUTP	−	+−	+			−	+
нор-GTP	−	−			−	−	+
araNTP (2′N₃)	+	−			−	−	+
araNTP (2′F)	+−	−	−			+	

Note. araNTP, arabinonucleoside-5'-triphosphates; araNTP $(2'N_3)$, 2'-deoxy-2'-azido-araNTP; araNTP (3'F), 2'-fluoro-2'-deoxy-araNTP; BrdUTP, 5-(β-bromovinyl)-2'-deoxyuridine-5'-triphosphate. (+), inhibition; (−), no inhibition; (+ −), weakly pronounced inhibition. More detailed information may be found in the review [2].

Certain conclusions can be drawn from the data in Table 1.

1. None of the DNA polymerases has the ability to catalyze the formation of phosphoamide bonds. This is apparent from the finding that $dNTP(3'NH_2)$ and $araNTP(3'NH_2)$ act as terminators of DNA synthesis and are not incorporated into the middle of the chain.

2. DNA polymerase α possesses the highest specificity in comparison with other mammalian DNA polymerases examined (β and terminal deoxynucleotidyl transferase). Unfortunately data on the inhibitor analysis of DNA-polymerase γ are not available. Similar data have also been obtained for the DNA-replicase from the Chinese silkworm, *Bombyx mori* [13]. None of the compounds studied from the ddNTP, $dNTP(3'NH_2)$, $dNTP(3'N_3)$, dNTP(3'F) group inhibited the DNA synthesis reaction. However, ddNTP, $dNTP(3'NH_2)$, and dNTP(3'F) markedly inhibited the synthesis of DNA catalyzed by the Chinese silkworm nuclear polyhedrosis virus DNA polymerase [13].

3. On the other hand the reverse transcriptase has the lowest specificity for the terminator substrates. For example all compounds tested in the deoxy- and arabino series terminated DNA synthesis in the presence of reverse transcriptase, but three groups, $dNTP(3'N_3)$, araNTP $(3'N_3)$, and dNTP(3'OMe), were found to be highly specific inhibitors for this enzyme in comparison with the mammalian enzymes, DNA polymerases α and β. Thus, by means of certain alterations in the terminator substrates, it is possible to construct specific inhibitors of DNA synthesis, which is catalyzed by reverse transcriptase. It is not known what laws govern the construction of specific inhibitors of reverse transcriptases in regard to physicochemical calculations. It is also not known whether low substrate specificity with respect to substrate analogs is a feature of different reverse transcriptases and not only the fowl myeloblastosis virus enzyme. There are data in the literature which indicate that the reverse transcriptase of the HTLVIII/LAV virus also has relatively low specificity. For example, 3'-azido-2',3'-dideoxythymidine blocked viral reproduction in a culture of human thymocytes infected with HTLVIII [4].

It is known that reverse transcriptases are less specific towards the template structure (they utilize single-spiral DNA, RNA, and also RNA methylated at the 2'-hydroxyl positions and RNA in which all 2'-hydroxyls have been replaced by fluorine) and the base structure, since the number of mismatches occurring during DNA synthesis catalyzed by reverse transcriptases is 2-3 orders of magnitude higher than in the case of DNA synthesized by DNA polymerases α and β [2].

This property of the reverse transcriptases is partically expressed, when phosphate is replaced by thiophosphate in terminator substrates, for example $dNTP(3'NH_2)$ (α-S) (Table 1). The decrease in specificity with respect to all components in the DNA biosynthesis system is probably a common property of reverse transcriptases.

Another group of terminators of DNA biosynthesis appears, when herpes virus DNA-polymerases are used. The two most active preparations in this group, the triphosphates of 9-(2-hydroxyethoxymethyl)-guanine and 9-(1,3-dioxy-2-propoxymethyl)-guanine (nor-GTP), are se-

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