5′-O-DIPHOSPHORYLPHOSPHONYLMETHYLRIBONUCLEOSIDES — A NEW GROUP OF DNA-DEPENDENT RNA POLYMERASE INHIBITORS

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Received August 10th, 1982

The effect of 5′-O-diphosphorylphosphonylmethylribonucleosides *I* on the transcription reaction catalyzed by DNA-dependent RNA polymerase from *Escherichia coli* was studied. These analogues of ribonucleoside 5′-triphosphates are not enzyme substrates but inhibit the transcription reaction, competing specifically with the natural substrates. According to the inhibition constants, the pyrimidine derivatives *Ia,b* are more potent inhibitors than the purine derivatives *Ic,d*.

Mechanism and specificity of the transcription reaction, catalyzed by DNA-dependent RNA polymerase, were studied using a number of ribonucleoside 5′-triphosphate analogues which are natural substrates for this enzyme. In addition to analogues, modified in the base[1,2] or sugar component[2–4], also analogues modified in the triphosphate grouping were studied[5–7]. However, the interpretation of the results obtained with the latter analogues is difficult: no correlation has been found between their inhibition potency and size or charge of the modified triphosphate grouping or modifying substituent[8]. In the present work we investigated the inhibition of the transcription reaction using compounds *I* as a new type of analogues of ribonucleoside 5′-triphosphates[9,10]. In these compounds the modification concerns the phosphoric acid moiety, bonded in α-position of the triphosphate part of the molecule[7,11]. The phosphoric acid residue in compounds *I* is replaced by methanephosphonic acid moiety, linked by an ether instead of ester bond to the primary 5′-hydroxyl group of the nucleoside moiety.

EXPERIMENTAL

DNA-dependent RNA polymerase was prepared from *E. coli* K12 by the modified method of Burgess[12], including the chromatography on heparin-Sepharose according to Sternbach and coworkers[13]. The solution of enzyme in 50% glycerol containing 10 mg protein per ml was stored at −20°C. DNA from *Bacillus subtilis* SB 19 was prepared by the method of Marmur[14]. Poly(dA-dT) (Na salt) was purchased from Miles Laboratories (U.S.A.). Unlabelled nucleoside 5′-triphosphates were purchased from the California Corporation for Biochemical Research (U.S.A.). [^{14}C]-UTP and [^{14}C]-ATP were obtained from the Institute for Research, Production

and Application of Radioisotopes, Prague. Synthesis of phosphonylmethyl analogues of ribonucleoside 5′-triphosphates was described elsewhere[9,10]. Their purity was checked by HPLC analysis, UV-spectrophotometry and phosphorus determination. Omnifluor was purchased from New England Nuclear (U.S.A.) and Soluene 300 was from Packard (The Netherlands). Whatman GF/C glass fibre filters (diameter 24 mm) were used.

Enzyme assay: The reaction mixture (final volume 0·125 ml) contained: 50 mmol l^{-1} Tris-HCl (pH 7·5), 0·8 mmol l^{-1} Mg^{2+}, 1 mmol l^{-1} Mn^{2+}, 4 μmol l^{-1} dithiothreitol, 80 μmol l^{-1} UTP, CTP and GTP, respectively, 16 μmol l^{-1} ATP and 0·8 μmol l^{-1} [^{14}C]-ATP (25 nCi, 925 Bq) (in experiments with labelled UTP, the concentration of ATP was 80 μmol l^{-1}, of UTP 16 μmol l^{-1} and of [^{14}C]-UTP 0·8 μmol l^{-1}, 25 nCi, 925 Bq); the reaction mixture contained further 12 μg of DNA and 10 μg of enzyme protein. After incubation at 37°C for 10 min, 0·01 ml of bovine serum albumin (7·5 mg/ml) was added as a carrier, followed by 1·0 ml of 5% (w/v) trichloroacetic acid. After standing for 30 min at 0°C the acid-precipitable material was collected on Whatman GF/C glass fibre filters and washed successively with 5 ml of 5% and 5 ml of 0·5% trichloroacetic acid. After drying (10 min, 80°C) the filter was suspended in 0·2 ml of Soluene 300 for 30 min. The radioactivity was measured 60 min after addition of 5 ml of a scintillation liquid (4 g of Omnifluor per 1 000 ml of toluene) in an Isocarb liquid scintillation counter.

Metal-ion catalyzed hydrolysis: Reaction mixture consisted of 0·8 mmol l^{-1} ATP or ATP_c (*Ic*) in 1 mmol l^{-1} $MgCl_2$, $MnSO_4$, $ZnSO_4$ or 10 mmol l^{-1} HCl or NaOH. After 15 h at 37°C, EDTA solution was added to final concentration 10 mmol l^{-1} (except for HCl or NaOH mixtures) and 5 μl of the resulting mixture was applied on Separon SI C18 column (3·3 × 150 mm, 5 μ). HPLC analysis was performed with 0·1M triethylammonium hydrogen carbonate (pH 7·5) containing 5% methanol. Elution rate, 0·4 ml/min, UV detector LCD 254 with EZ 11 recorder (Laboratory Equipment, Prague).

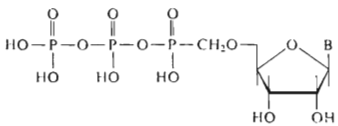
Stability of ATP_c (Ic) *in the* DNA-*dependent* RNA *polymerase reaction*: The reaction mixture (final volume 0·125 ml) consisted of 50 mmol l^{-1} Tris-HCl (pH 7·5), 0·8 mmol l^{-1} Mg^{2+}, 1 mmol l^{-1} Mn^{2+}, 4 μmol l^{-1} dithiothreitol, 80 μmol l^{-1} UTP, 10 μg of poly(dA-dT) and 10 μg of enzyme protein. After incubation at 37°C for 60 min EDTA solution (36 mM, 10 μl) was added and 10 μl sample was applied on the HPLC column and the analysis performed as above (sensitivity: 0·16 absorbancy units per full scale).

RESULTS AND DISCUSSION

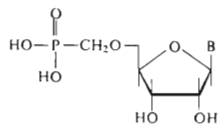
We studied first whether the 5′-O-diphosphorylphosphonylmethylribonucleosides derived from uracil, cytosine, adenine and guanine (*Ia*–*Id*) can act as substrates of DNA-dependent RNA polymerase in the transcription reaction. Replacement of one of the four natural ribonucleoside 5′-triphosphates by the same or a double concentration of the corresponding analogue *I* practically stopped the RNA synthesis.

Diphosphorylphosphonylribonucleoside analogues of triphosphates (*I*) also affect the transcription reaction which takes place in the presence of all the four natural substrates. This inhibitory effect is specific with respect to the base and can be partially suppressed by an increased concentration of the ribonucleoside 5′-triphosphate the base of which is identical with the analogue used for the inhibition. The extent of inhibition by compound *Ic* and its reversal by ATP are depicted in Fig. 1; the same results were obtained also with the other three analogues *Ia*–*Ic*. The inhibition is

competitive with respect to the corresponding ribonucleoside 5'-triphosphate (Fig. 2). As shown by the K_i values (Table I), the inhibitory effect of 5'-O-diphosphoryl-phosphonylmethylribonucleosides (*I*) is about the same as that of thuringien-



I



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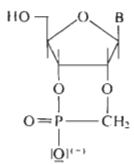
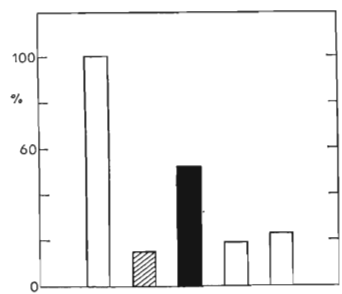


III

In formulae *I*—*III*, *a*: B = uracil-1-yl (UTP_c); *b*: B = cytosin-1-yl (CTP_c); *c*: B = adenin-9-yl (ATP_c); *d*: B = guanin-9-yl (GTP_c).



ATP_c	—	60	60	60	60
ATP	10	10	30	10	10
CTP	10	10	10	30	10
GTP	10	10	10	10	30

FIG. 1

Inhibition of DNA-dependent RNA polymerase reaction by ATP_c and its reversion by ATP. For concentration of salts, template, radioactive triphosphate and enzyme see Experimental. Amount of the inhibitor (ATP_c) and remaining nonlabelled triphosphates (ATP, CTP and GTP, nmol) in the single experiments is given under the bars

sin[6,15,16]. The pyrimidine analogues are more effective inhibitors than the purine derivatives; a similar observation has been made recently with 2,4-dinitrophenylaminoethyl derivatives of ribonucleoside 5'-triphosphates[8].

On the basis of these results we can deduce that 5'-O-diphosphorylphosphonylmethyl ribonucleosides *I* enter without difficulty the binding site of DNA-dependen-RNA polymerase which is specific for the corresponding natural substrate containing

TABLE I
Inhibition constants K_i

Inhibitor	K_i, μmol l^{-1}	Inhibitor	K_i, μmol l^{-1}
ATP_c	30	CTP_c	10
GTP_c	40	UTP_c	15
		Thuringiensin	30

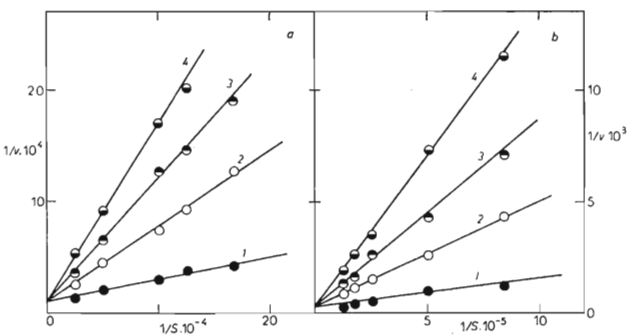


FIG. 2

Double reciprocal plot of RNA polymerase at different ATP_c/ATP (*a*) and UTP_c/UTP (*b*) ratios. For composition of the reaction mixture and conditions of incubation see Experimental. Final concentration in the assay: (*a*) CTP, GTP 80 μmol l^{-1}, [^{14}C]-UTP 16 μmol l^{-1}, ATP 6—40 μmol l^{-1}; values for 0, 56, 113 and 226 μmol l^{-1} concentration of ATP_c (curves *1*, *2*, *3*, *4*, respectively); (*b*) CTP, GTP 80 μmol l^{-1}, [^{14}C]-ATP 16 μmol l^{-1}, UTP 1·2—8 μmol l^{-1}; values for 0, 30·5, 61 and 112 μmol l^{-1} concentration of UTP_c (curves *1*, *2*, *3*, *4*, respectively)

an identical base. This conclusion is supported by the K_i constants of these compounds which are very close to the K_m values of the natural substrates (K_m for ATP, GTP, CTP and UTP are $20 . 10^{-6}$, $50 . 10^{-6}$, $3{\cdot}3 . 10^{-6}$ and $5 . 10^{-6}$ mol l^{-1}, respectively). The interaction with the analogue takes place in spite of the presence of the inserted CH_2 group which increases the distance between the $C_{(5')}$ and the phosphorus atom in α-position. The ether bridge, linking this methylene group with the 5'-hydroxyl of the nucleoside, allows perfect conformational adaptability of the analogue molecule; thus, in the complex with the enzyme, the conformation of the analogue may not differ significantly from the conformation of the natural substrate molecule. An analogous situation occurs *e.g.* in the case of 5'-ribonucleotide analogues *II* of the same type which are not substrates but effective inhibitors of 5'-nucleotidases[17]. Using poly(dA-dT) as a template we have confirmed that in the presence of UTP as the only natural substrate, the adenine derivative *Ic* itself was not affected by the enzyme. No cleavage of the bonds between the α, β or γ phosphorus atoms in this analogue was observed. Although the analogues *I* enter the binding site of the enzyme, no enzyme-catalyzed reaction takes place, *i.e.* no internucleotide bond between the phosphonic acid moiety in the analogue *I* and the 3'-hydroxy group in the oligonucleotide is formed. The reason is undoubtedly in the different character of the immediate vicinity of the phosphorus atom in the methanephosphonic acid moiety (increase of its hydrophobicity) which influences interactions in the catalytic center. At the same time, substitution at the phosphorus atom changes also its ability of transition into the corresponding activated state of the enzyme-catalyzed reaction. A similar marked effect of the CH_2 group on reactivity at the phosphorus atom is illustrated also by the extraordinarily facile chemical hydrolysis of six-membered cyclic esters of compounds *III* in comparison with such reaction of six-membered cyclic esters of phosphoric acid[18]. Also the extent of acid hydrolysis of ATP and compound *Ic* is quite different: whereas under the experimental conditions ATP affords only 19% of ADP and 1% of AMP, the phosphonate analogue *Ic* is hydrolyzed to much higher degree (14% of ADP-analogue, and 61% of AMP_c (*IIc*)). In an alkaline solution both ATP and its analogue *Ic* are stable. None of them undergoes any changes in the presence of either magnesium, manganese or zinc ions in neutral solutions.

A structural change in the α-position of the phosphorus acid moiety (which results in enzymatic as well as chemical stability of the α-phosphorus-nucleoside bond) may not necessarily influence the reactivity of the phosphoryl moieties in the positions β and γ: the purine derivatives *Ic*, *d* can, even though with a reduced effectivity[19], act as phosphate donors in phosphorylation of uridine, catalyzed by uridine kinase from mouse leukemic cells. (On the other hand, the pyrimidine analogues *Ia*, and *Ib*, are, similarly to natural UTP and CTP, allosteric inhibitors of this reaction[20].) Obviously, in this case, an activation of the β- or rather γ-phosphorus atom and a subsequent transfer of the γ-phosphoric acid moiety take place. Thus, according to our results, the course of the catalyzed reaction with DNA-dependent RNA

polymerase apparently requires formation of a transition state which includes an activation of the α-phosphorus atom of the natural substrate.

The authors wish to express their thanks to Mrs D. Veverková for skillful technical assistance and to Mr J. Hanzlik for careful radioactivity measurements of the samples.

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Translated by M. Tichý.