DNA-DEPENDENT RNA POLYMERASE MEDIATED FORMATION OF METHYLPHOSPHONYL INTERNUCLEOTIDE LINKAGE

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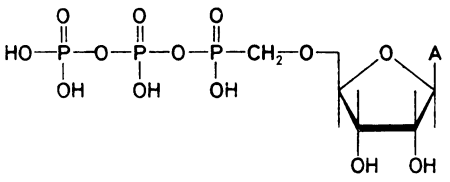
Analogues of ATP modified in the triphosphate moiety by introducing phosphonylmethyl ether residue(s) were used as substrates for bacterial DNA-dependent RNA polymerase. Although the analogues function as poor substrates they enter the initiation as well as the elongation site of the enzyme. The ATP analogue containing the phosphonylmethyl ether group in the α position in the presence of UTP and poly [d(A – T)] gives rise to alternating di- and trinucleotides with one anomalous internucleotide linkage per molecule.

Transcription of genetic sequences in bacteria is mediated by DNA-dependent RNA polymerase[1,2]. In this enzymatic reaction ribonucleoside triphosphates and some of their analogues function as substrates for the synthesis of polyribonucleotide chains. Modified substrates were shown to be valuable tools for the study of the mechanism of enzyme action as well as its catalytic site[3,4].

The modification concerns mostly the nucleoside moiety of natural substrates so that the corresponding oligomer and polyribonucleotide contain the same ribose phosphate backbone as the product formed from natural substrates. This does not apply to analogues containing C_5'—S—P bonds[5] and adenosine 5'-O-(1-thiotriphosphate) (ref.[6]) which represent a special group of modified substrates. It was therefore of considerable interest to investigate a new type of ATP analogues (*I*–*III*) with phosphonylmethyl groups localized in different positions of the acidic part of the molecule. The most interesting among them seems to be analogue *I* where modification occurs close to the adenosine moiety, i.e., at that part of the molecule which is involved in the formation of sugar phosphate backbone. The main difference in comparison with ATP is characterized by the occurence of ether instead of ester bond to the primary 5'-hydroxyl group of nucleoside moiety. Analogues of dinucleotides ApU and UpA modified in the same way were synthesized recently and their

* Abbreviations used: ATP, UTP and their tetraphosphates, di- and trinucleotides are pppA, pppAp, pppApU, pppApUpA, resp. pppU, pppUp, pppUpA, pppUpApU. The position of the radioactive phosphorus is marked by an asterisk. The phosphonylmethyl group in analogues is designated as p(c) for instance ppp(c)A, pppUp(c)A etc.

elongation to corresponding analogues of trinucleotides ApUpA and UpApU using both RNA polymerase from *E. coli*[7] and RNA polymerase *II* from wheat germ[8] were studied. The present paper deals with the possibility to employ analogues *I*–*III* as substrates for RNA polymerase from *E. coli* and with the characterization of abortive oligonucleotides originating from analogue *I*.



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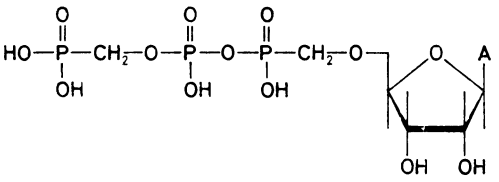

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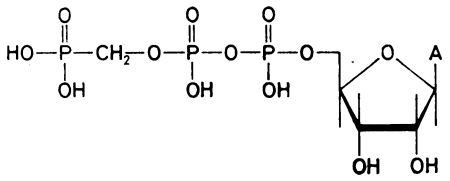

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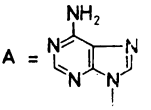


EXPERIMENTAL

Reagents. The synthesis of 5′-O-diphosphorylphosphonylmethyladenosine (*I*) was published earlier[9], analogue *II* was synthesized in a similar way and for the synthesis of analogue *III* see ref.[10]. All of them were homogeneous according to HPLC analysis. ATP and UTP (HPLC grade) were obtained from Serva (F.R.G.), [α^{32}P]-UTP from Amersham, [^{14}C]-ATP was from the Institute for Research, Production and Application of Radioisotopes (Czechoslovakia), poly-[d(A — T)] from Boehringer (Austria), DEAE-cellulose DE 52 from Whatman (England,) X-ray films from Fotochema (Czechoslovakia). All buffer components were reagent grade; redistilled water was used.

Enzymes. DNA-dependent RNA polymerase was prepared from *E. coli* K 12 by the method of Burgess and Jendrisak[11] and purified up to the DNA-cellulose chromatography step. Since this report deals with incorporation of different ATP analogues into the polyribonucleotide chains it was necessary to eliminate the possibility of ATPase contamination of RNA polymerase[12–14]. Therefore both holoenzyme and core enzyme were purified using phosphocellulose column according to Gonzales et al.[15] and were found to be ATPase free; this contaminating activity was present in the 50 mmol l^{-1} KCl wash as was shown by ATPase assay[16]. The purity of the holoenzyme was 98% as judged by scanning of SDS-polyacrylamide gel. Specific act;vity of the holoenzyme used was 20 500 units/mg and 8 700 units/mg using T7 DNA as template according to the standard assay[15]. Bovine pancreatic RNase A (five times crystallized) was purchased from Calbiochem (U.S.A.), RNase T_2 *Aspergillus oryzae* was from Sankyo (Japan), bacterial alkaline phosphatase was from Pharmacia (Sweden).

ATPase assay. The reaction mixture (15 μl) contained 40 mmol l^{-1} Tris-HCl (pH 7·9), 10 mmol l^{-1} $MgCl_2$, 80 mmol l^{-1} KCl, 0·1 mmol l^{-1} dithiothreitol, 50 μmol l^{-1} [^{14}C]-ATP ($1{\cdot}2 \cdot 10^3$ c.p.m. μmol^{-1}) and 1—5 μg of enzyme protein. The reaction mixtures were incubated for 10 min at 37°C and the reactions were terminated by adding EDTA to a final concentration of 80 mmol l^{-1}. The reaction mixtures were analyzed by paper electrophoresis (Whatman 3 MM) in 0·05 mol l^{-1} sodium citrate, pH 3·8 at 1 000 V for 70 min. Electrophoreograms were cut and counted in toluene scintillation cocktail.

Transcription assay. Standard reaction mixture (15 μl) contained 40 mmol l^{-1} Tris-HCl (pH 7·9), 80 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 0·1 mmol l^{-1} dithiothreitol, 50 μmol l^{-1} ATP or ATP analogues, 5 μmol l^{-1} UTP and [α^{32}P]-UTP ($7{\cdot}4 \cdot 10^4$ Bq), 1—2 μg poly [d(A — T)], 5 μg of enzyme protein. After preincubation of the enzyme with template at 37°C for 10 min the reactions were initiated by the addition of substrates. The time of incubation is given in the legends to the figures. The reactions were stopped by adding them to the lyophilisate of 15 μl of the so called "reaction-stop mixture" containing 9 mol l^{-1} urea and 0·05 mol l^{-1} EDTA. Bromphenol blue (0·1%) and xylene cyanol (0·1%) were added to the samples which were then analyzed by gel electrophoresis.

Conditions of gel electrophoresis and its evaluation. The reaction mixtures were analyzed in 24% polyacrylamide gels (0·04 × 13 × 40 cm) at room temperature according to Maxam and Gilbert[17]. The gels contained methylene bis(acrylamide) and acrylamide at a ratio of 1 : 29, 7 mmol . l^{-1} urea, 89 mmol l^{-1} Tris-borate pH 8·3 and 2·5 mmol l^{-1} EDTA. Before the application of samples the gels were subjected to a prerun at 1 000 V at least for 30 min. The electrophoresis was allowed to proceed at 1 000 V until the bromphenol blue marker moved approximately 17 cm from the start. After completion of the electrophoresis run the gels were evaluated by autoradiography. Selected bands were cut out for quantitative evaluation and counted according to Cerenkov.

Preparation of abortive products. Abortive products for enzyme treatment were prepared by the RNA polymerase reaction as given above with some modifications. In the case of natural substrate the reaction mixture contained 50 μmol l^{-1} ATP, 5 μmol l^{-1} UTP and [α^{32}P]-UTP ($1{\cdot}85 \cdot 10^5$ Bq); time of incubation was 5 min. The reaction mixture with ATP analogue *I* contained 2 mmol l^{-1} diphosphorylphosphonylmethyladenosine, 5 μmol l^{-1} UTP and [α^{32}P]-UTP ($3{\cdot}7 \cdot 10^5$ Bq) and was incubated for 30 min. The reaction mixtures were analyzed on 24% polyacrylamide gels as described above. After autoradiography the bands corresponding to the abortive products were cut out, crushed and eluted overnight at 4°C with a solution containing 0·5 mol l^{-1} ammonium acetate, pH 5·0, 0·1% sodium dodecylsulphate, 0·1 mmol l^{-1} EDTA and 50 μg/ml yeast tRNA. Polyacrylamide was removed by centrifugation and supernatants (1—2 ml) were loaded onto a small DEAE cellulose column (1 ml). After extensive washing with water to remove urea the abortive products were eluted with 1 mol l^{-1} triethylammonium bicarbonate. The eluate was collected into Eppendorf tubes containing Dowex 50-X8 200—400 mesh in pyridine cycle. After removal of the resin by centrifugation the radioactive fractions were neutralized with NH_4OH and evaporated several times with water in a dessicator in vacuo. The residues were dissolved in a small amount of water and aliqouts were subjected to enzymes treatment.

Enzyme treatment. Aliquots of abortive products (10^4 c.p.m.) were digested for 30 min at 37°C *a*) with RNase A in 0·1 mol l^{-1} Tris-HCl, pH 7·5, *b*) with RNase T_2 in 0·1 mol l^{-1} Tris-HCl, pH 7·0 and *c*) with bacterial alkaline phosphatase in 0·1 mol l^{-1} Tris-HCl, pH 8·0. Controls were incubated under the same conditions in the absence of enzymes. The reaction mixtures were analyzed either on 24% polyacrylamide–urea gels or by paper electrophoresis in citrate

buffer as described above. Electrophoreograms were autoradiographed overnight at −70°C using intensifying screen Perlux (VEB Kali Chemie, G.D.R.).

Kinetic experiments. The affinity of analogue *I* to enter the initiation and/or the elongation binding site of RNA polymerase was compared using the apparent Michaelis constants for this analogue in the course of ppp(c)ApU and pppUp(c)A formation. The reactions were performed for 10 min at standard reaction conditions using 100, 200, 500 and 750 μmol l^{-1} of analogue *I* and constant concentration of 5 μmol l^{-1} labelled UTP as well. The reaction mixtures were analyzed by polyacrylamide electrophoresis and after autoradiography the ratio of both dinucleotides was estimated using Cerenkov counting of corresponding spots and scanning of films (LKB Ultroscan XL). The kinetic data were computed using least square procedure (Enzfitter software) on a personal computer.

RESULTS AND DISCUSSION

First it was attempted to establish whether analogues *I*–*III* are accepted as substrates by RNA polymerase. The easy way to show their incorporation would be to label them in the phosphonylmethyl moiety. However, on account of the difficulties connected with the introduction of the label into the analogues we have chosen a different strategy. As a template for RNA polymerase we used the alternating copolymer poly[d(A–T)] mostly used in studies dealing with analogues of natural substrates. In presence of ATP and [α^{32}P]-labelled UTP a polymer pppAp*UpAp*UpA... having the length of several hundred nucleotides is formed[18,19]. Concomitantly the release of short oligonucleotides from the unstable transcription complex (abortive synthesis) takes place[20,21]. Distribution of radioactivity after RNA polymerase mediated incorporation of the [α^{32}P]-labelled uridylyl residue between unreacted substrate, abortive oligonucleotides and high molecular polymer is summarized in Table I.

We have found that only analogue *III* is incorporated into the high molecular polymer (Fig. 1, lane d), although to a lesser degree than its natural counterpart ATP (Table I). In view of the fact that the product formed contains a normal ribose phosphate backbone it is evident that the phosphonylmethyl group in position β causes either less favourable conditions for the analogue *III* to enter the elongation site or decreases the rate of the diphosphonylmethyl residue release. Analogues *I* and *II* are on the other hand very poor substrates for RNA polymerase (Fig. 1, lanes b and c, respectively). In comparison with the control only abortive oligonucleotides were found.

Next we have characterized the abortive products 3–5 (Fig. 1, lane b) from the reaction mixture using analogue *I* as a substrate. We have compared them with the abortive products 1 and 2 originating from the natural substrates (Fig. 1, lane a). The abortive oligonucleotides mentioned were purified from polyacrylamide gels and digested with pancreatic RNase A, RNase T_2 and bacterial alkaline phosphatase[22–24].

Enzymatic treatment of the abortive oligonucleotides 1 and 2 has shown that these oligonucleotides are pppAp*U and ppp*UpA, respectively. After digestion of oligonucleotide 1 with alkaline phosphatase a product with mobility corresponding to the standard ApU (using paper electrophoresis analysis) is formed. The oligonucleotide was further found to be resistant to pancreatic RNase A. On the other

TABLE I

Distribution of radioactivity after RNA polymerase reaction with ATP or its analogues and labelled UTP as substrates.

Substrates	Time of incubation min	Nonincorporated UTP, %	Products, %	
			Abortive oligonucleotides	High molecular products[a]
ATP, UTP*	5	17·0	7·7	73·8
I, UTP	30	87·3	9·0	2·9
II, UTP*	30	90·3	7·1	2·6
III, UTP*	15	41·0	12·7	45·1

[a] Products with mobility in gel electrophoresis slower than xylene cyanol blue.

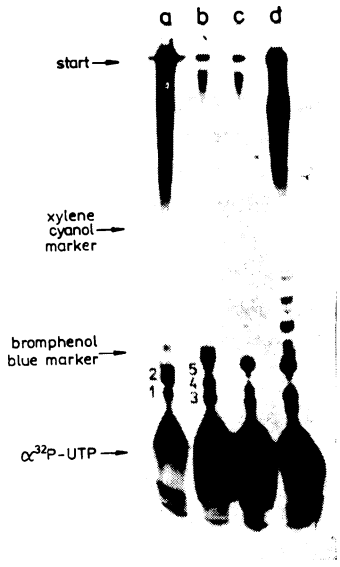


FIG. 1

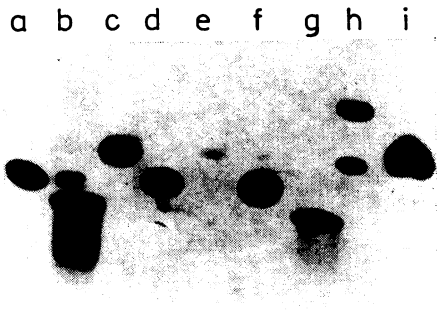
Products of RNA polymerase reaction with ATP and its analogues and labelled UTP as substrates. Composition of reaction mixture and conditions of electrophoresis see Experimental. Radioactive products formed from the following substrates: lane a ATP, UTP; lane b analogue *I*, UTP; lane c analogue *II*, UTP; lane d analogue *III* and UTP. Time of incubation lanes a, b, c, and d was 5, 30, 30 and 15 min, respectively. Spots 1—5 abortive products subjected to analysis

hand treatment with RNase T_2 resulted in the formation of a product with the electrophoretic mobility higher than the original substance indicating the release of uridine from 3'-end of the dinucleotide. The abortive oligonucleotide 2 was analyzed in a similar way and the results show that digestion by alkaline phosphatase resulted in splitting off radioactive orthophosphate, and that digestion with both RNase A and RNase T_2 resulted in the formation of an identical product ppp*Up. The quantitative analysis shows that dinucleotide ppp*UpA was synthesized to a higher extent than pppAp*U (2·4 pmol and 1·7 pmol, respectively). This indicates that the higher affinity of ATP for the initiation binding site is not the only reason for the predominance of the adenosine moiety at the 5'-prime end of high molecular RNA.

The abortive oligonucleotides 3–5 (Fig. 1, lane b) were treated in a similar way. In view of the similar mobility of abortive oligonucleotides originating from ATP and analogue *I* (Fig. 1, lanes a and b) oligonucleotides 3 and 4 were expected to be anomalous dinucleotides containing the phosphonylmethyl group located either at the acidic part of the molecule or in the internucleotide bond. The first possibility was confirmed by treatment of oligonucleotide 3 with bacterial alkaline phosphatase. In this case a product has been obtained which had a higher electrophoretic mobility both in gel and on paper to compare with ApU and a lower mobility than the original substance (Fig. 2, lanes a and c, respectively). The electrophoretic mobility of this product was the same as the mobility of the standard prepared from p(c)AMP and [^{32}P]-labelled UTP (unpublished results). Thus we concluded that alkaline phosphatase splits off from the abortive oligonucleotide 3 only diphosphate and p(c)Ap*U is formed. Following digestion with RNase A and RNase T_2 no difference in the splitting pattern between pppAp*U and the anomalous dinucleotide was

FIG. 2

Splitting pattern of abortive products 3 to 5 digested by alkaline phosphatase or RNase T_2. For the composition of the reaction mixture and conditions for electrophoresis see Experimental. Lane a product 3 control, lane b product 3 treated with RNase T_2, lane c product 3 treated with alkaline phosphatase, lane d product 4 treated with RNase T_2, lane e product 4 treated with alkaline phosphatase, lane f product 4 control, lane g product 5 treated with RNase T_2, lane h product 5 treated with alkaline phosphatase, lane i product 5 control



observed. The anomalous dinucleotide is also resistant to RNase A (Fig. 3, lanes a and b) whereas by digestion with RNAse T_2 uridine splits off and ppp(c)Ap* is formed. These results are in accordance with the anomalous dinucleotide structure ppp(c)Ap*U where the phosphonylmethyl residue is located at the 5'-end of the molecule.

Abortive oligonucleotide 4 (Fig. 1, lane b) is resistant to digestion with RNase T_2 (Fig. 2, lanes d and f) as well as with RNase A (Fig. 3, lanes c and d); the treatment with alkaline phosphatase resulted in the complete release of the triphosphate group i.e., the radioactive label disappeared (Fig. 2, lane e). We therefore concluded that abortive oligonucleotide 4 is ppp*Up(c)A, an anomalous dinucleotide containing the phosphonylmethyl group in the internucleotide bond.

Abortive oligonucleotide 5 migrates on the gel slower than dinucleotides and faster than the bromphenol blue marker which under given conditions moves with tetra- and pentanucleotide[25]. After treatment with alkaline phosphatase the mobility of the split product is close to the mobility of the original substance 5. As the splitting of the 5'-terminal triphosphate would bring about a considerable decrease in mobility the obtained product apparently contains a phosphonylmethyl group at the 5'-end and alkaline phosphatase evidently splits off only the terminal diphosphate[7] (Fig. 2, lanes h and i). Oligonucleotide 5 is resistant to RNase A (Fig. 3, lanes f and g) in accordance with the presumed occurrence of an anomalous backbone. On RNase T_2 treatment only one radioactive product is formed which is identical with RNase T_2 product of oligonucleotide 3 (Fig. 2, lane d). This indicates a trinucleotide character of oligonucleotide 5 since longer oligonucleotides digested by RNase T_2 would yield an additional radioactive product. The resulting structure is thus ppp(c)Ap*. .Up(c)A. We were not able to detect the phosphonylmethyl group in the abortive oligonucleotide found close to the anomalous trinucleotide ppp(c)Ap*Up(c)A. All results mentioned above are summarized in Table II.

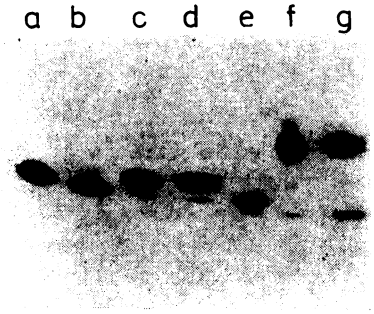


FIG. 3

Splitting pattern of abortive products 3 to 5 digested by pancreatic RNase A. For the composition of the reaction mixture and conditions for electrophoresis see Experimental. Lanes a, c and f nontreated abortive products 3, 4 and 5, respectively; lanes b, d and g abortive products 3, 4 and 5 split by RNase A

Thus we conclude that 5'-O-diphosphorylphosphonylmethyladenosine productively enters the initiation as well as the elongation site of bacterial DNA-dependent RNA polymerase with different affinity as shown by respective K_m^{app} and V_{max}^{app} constants (Table III). It is not surprising that the K_m^{app} constant for entering the initiation site for this analogue is lower than the corresponding value for binding to the elongation site since it has been established that structural requirements for the acidic moiety of the initiation substrate are rather flexible[26]. On the other hand, the interaction of elongation substrate with the polymerase is mediated mainly by its ribose phosphate part[27,28] whose structure and conformation is disturbed by the introduced modification. Especially the crucial interaction of substrates mediated by divalent cation activator Mg^{2+} seems to be affected[29,30]. The stability of ternary complexes containing modified oligonucleotide is sufficient in the case of dinucleotide ppp(c)Ap*U which is elongated according to the probability ppp(c)Ap*Up(c)A/(ppp(c)Ap*Up(c)A + ppp(c)Ap*U) = 0·2. The inability of *E. coli* RNA polymerase to form longer oligo-

TABLE II

Scheme of enzymatic digestion of abortive products

Abortive product	Spot[a]	Digestion products		
		alkaline phosphatase	RNase A	RNase T_2
pppAp*U	1	Ap*U + 3 P	no splitting	pppAp* + U
ppp*UpA	2	UpA + 3 P*	ppp*Up + A	ppp*Up + A
ppp(c)Ap*U	3	p(c)Ap*U + 2 P	no splitting	ppp(c)Ap* + U
ppp*Up(c)A	4	Up(c)A + 3 P*	no splitting	no splitting
ppp(c)Ap*Up(c)A	5	p(c)Ap*Up(c)A + P	no splitting	ppp(c)Ap* + Up(c)A

[a] See Fig. 1.

TABLE III

Apparent kinetic constants of analogue *I* in the course of ppp(c)Ap*U and ppp*Up(c)A formation

Dinucleotide	K_{mA}^{app} μmol l^{-1}	V_{max}^{app} pmol min^{-1}
ppp(c)ApU	130	1·32
pppUp(c)A	298	0·89

nucleotides may be caused by various factors. One of them is the inability of the enzyme to translocate along the template due to the steric hindrance in hybrid duplex DNA–RNA. This may be significant since there is no detectable amount of tetranucleotide ppp(c)Ap*Up(c)Ap*U although the next substrate to elongate ppp(c)Ap*Up(c)A is UTP. A similar phenomenon is observed using phosphonylmethyl analogues of dinucleotides[16] where anomalous trinucleotides U(c)pApU and Up(c)ApU formed from dinucleotide precursors are not elongated to corresponding tetranucleotides by 3'-dATP (cordycepin 5'-triphosphate) (ref.[31]). On the other hand, dinucleotide ppp(c)ApU is elongated to ppp(c)ApUp(c)A since in this case the phosphonylmethyl group is not localized inside the chain. The *E. coli* RNA polymerase can thus utilize ppp(c)A only for the formation of one modified internucleotide bond.

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REFERENCES

1. Chamberlin M. J.: Enzymes *15*, 61 (1982).
2. von Hippel P. H., Bear D. C., Morgan W. D., McSwiggen J. A.: Ann. Rev. Biochem. *53*, 389 (1984).
3. Krakow J. S., Rhodes G., Jovin T. M. in: *RNA Polymerase* (R. Losick and M. J. Chamberlin Eds), p. 127. Cold Spring Harbor Laboratory, New York 1976.
4. Wu C. W., Tweedy N.: Mol. Cell Biochem. *47*, 129 (1982).
5. Stütz A., Scheit K. H.: Eur. J. Biochem. *50*, 343 (1975).
6. Yee D., Armstrong V. W., Eckstein F.: Biochemistry *18*, 4116 (1979).
7. Cvekl A., Horská K., Šebesta K., Rosenberg I., Holý A.: Int. J. Biol. Macromol. *11*, 33 (1989).
8. Cvekl A., Horská K., Šebesta K., Rosenberg I., Holý A.: Collect. Czech. Chem. Commun. *54*, 811 (1989).
9. Holý A., Rosenberg I.: Collect. Czech. Chem. Commun. *47*, 3447 (1982).
10. Simon L., Myers T., Mednieks M.: Biochim. Biophys. Acta *103*, 189 (1965).
11. Burgess R. R., Jendrisak J. J.: Biochemistry *14*, 4634 (1975).
12. Paetkau V., Coy G.: Can. J. Biochem. *50*, 142 (1972).
13. Ishihama A., Ikeuchi T., Yura T.: J. Biochem. (Tokyo) *79*, 917 (1976).
14. Burgess R. R.: Ref.[3], p. 69.
15. Gonzales N., Wiggs J., Chamberlin M. J.: Arch. Biochem. Biophys. *182*, 404 (1977).
16. Cvekl A.: *Thesis*. Czechoslovak Academy of Sciences, Prague 1987.
17. Maxam A. M., Gilbert W.: Methods Enzymol. *65*, 495 (1980).
18. Maitra V., Hurwitz J.: Proc. Natl. Acad. Sci. U.S.A. *54*, 815 (1965).
19. Schulz W., Zillig W.: Nucleic Acid Res. *9*, 6889 (1981).
20. Johnston D. E., McClure W. R.: Ref.[3], p. 413.
21. McClure W. R., Cech C. L., Johnston D. E.: J. Biol. Chem. *253*, 8941 (1978).
22. Donis-Keller H., Maxam A. M., Gilbert W.: Nucleic Acid Res. *4*, 2527 (1977).
23. Rosenberg I., Holý A.: Collect. Czech. Chem. Commun. *50*, 1507 (1985).
24. Rosenberg I., Holý A.: Nucleic Acids Res., Symp. Ser. *14*, 275 (1983).

25. Carpousis A. J., Gralla J. D.: Biochemistry *19*, 3245 (1980).
26. Smagowicz W. J., Scheit K. H.: Nucleic Acid Res. *9*, 2397 (1981).
27. Sylvester J. E., Dennis D.: Biochem. Biophys. Res. Commun. *75*, 667 (1977).
28. Dennis D., Jurgenssen S., Sylvestér J.: Biochim. Biophys. Res. Commun. *94*, 205 (1980).
29. Szafranski P., Smagowicz W. J., Wierzchowski K. L.: Acta Biochim. Pol. *32*, 327 (1985).
30. Cvekl A., Horská K., Šebesta K., Rosenberg I., Holý A.: Biocatalysis *1*, 275 (1988).
31. Cvekl A., Horská K., Šebesta K.: Unpublished results.

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