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Modified polynucleotides. I. Investigation of the enzymatic polymerization of 5-alkyl-dUTP-s

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ABSTRACT

The chemical synthesis of 5-alkyl-dUTP-s and their participation as substrates in poly[d(A-T)] primed polymerization reactions with dATP by E. coli DNA polymerase I enzyme has been described. In comparison with dTTP, at saturating substrate concentrations, the rate of hypochromic effect was found to be 17.3 % higher for dUTP and was lower by 27.4 % for 5-ethyl-dUTP, 29.5 % for 5-n-propyl-dUTP, 31.4 % for 5-n-butyl-dUTP and by 85.0 % for 5-n-pentyl-dUTP. No hypochromic effect could be observed, however, with 5-iso-propyl-, 5-tert.butyl- and 5-n-hexyl--dUTP-s. Polydeoxynucleotides have also been isolated from the reaction mixture and some of their structural properties determined.

INTRODUCTION

DNA-s and synthetic polynucleotides containing analogous bases[1] show altered physical properties e.g. in stability and buoyant density. In order to study correlation between the structure of nucleic acid components and the physical and biological properties of polymeric products, we attempted to prepare polydeoxyribonucleotides containing a series of 5-substituted uracils due to the numerous natural 5-substitutions of pyrimidine bases found in the DNA-s of phages e.g. uracil[2,3], 5-hydroxymethyluracil[4,5], 5-(4',5'-dihydroxypentyl)-uracil[6] and 5-(4-aminobutylaminomethyl)--uracil[7] as substitutions of thymine. 5-Ethyl-2'-deoxyuridine has also been incorporated into the DNA of phages[8], bacteria[9] and also mammalian cells[10].

In order to synthesize the polydeoxynucleotides in enzymatic reactions we had to clarify whether our modified triphosphates are substrates of the DNA polymerase enzyme. Therefore, we first studied the relationship between substrate structure and enzymatic activity. For this purpose we synthesized a series of 5-alkyl-2'--deoxyuridines (β-anomers).[11,12] In our present communication we

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report the synthesis of the corresponding 5'-mono- and 5'-triphosphates and our results on poly [d(A-T)] primed E. coli DNA polymerase reactions with 5-substituted-dUTP-s and dATP.

MATERIALS AND METHODS

The triphosphates dATP, dTTP and dUTP and the enzyme E.coli DNA polymerase I (corresponding to fraction VII, in Jovin's procedure; 5300 units/mg protein) were purchased from Boehringer-Mannheim. The preparation of poly [d(A-T)] template-primer is described in Results.

Phosphate analysis of nucleotides was carried out as described by Allen[13] with the modification that digestion was performed by the Schöniger method.

Column chromatographic separations were carried out with the help of a Spectromom 204 spectrophotometer (MOM, Hungary) furnished with flow-through cells (Starna Ltd.) and a potenciometric recorder (Type OH 814/1, Radelkis, Hungary).

NMR spectra of natural abundance ^{13}C were recorded at 25.16 MHz using a Varian XL-100-15 instrument equipped with Varian S124-XL-FT accessory and 16 k 620L computer.

DNA polymerase reactions and hyperchromicity measurements were carried out in a Unicam SP 8000 thermostated spectrophotometer, spectra were measured in a Spectromom 204 spectrophotometer (MOM, Hungary).

Determination of the base composition of acid hydrolysed polydeoxynucleotides was performed in a Hupe-Busch 1010 B high pressure liquid chromatograph equipped with Autolab System IV computing integrator. We used a 250x4x6 mm column filled with Nucleosil 10 SA (10 μm) cation exchanger (Macherey-Nagel Co.) at 90 atm pressure and 70 ^{o}C with ammonium formiate buffer (pH 4.75); flow rate was 1.5 ml/min.

RESULTS

Preparation of 5-alkyl-2'-deoxyuridine-5'-monophosphates

The preparation of these compounds was carried out with a slight modification of the selective phosphorylation method of Yoshikawa et al.[14].

A solution of 5-alkyl-2'-deoxyuridine (8.5 mmole) and triethyl phosphate (20 ml) was cooled to 0-(-4) ^{o}C. To this solution, 2 ml

(21.8 mmole) of distilled $POCl_3$ was added dropwise under constant stirring for 20 min. After the elapse of 5-24 hours, 100 ml of water was added and the mixture was allowed to stand overnight.

The reaction mixture was then extracted with 5x8 ml of ether or with chloroform (in case R = propyl or a longer carbon chain), the extract was evaporated to 60 ml and after the addition of $Ba(ClO_4)_2$ (10 g) the solution was adjusted with an ammonium solution to pH = 4 and after filtration to pH = 8. The filtrate was then evaporated to turbidity. After the addition of double volume of ethanol the precipitate formed was centrifuged and washed with alcohol and acetone. The crude barium salt was purified by repeated precipitation.

Free acids were obtained from Ba-salt of at least 70 % nucleotide content with a cation exchange resin (DOWEX 50 Wx4 H^+). The aqueous solution of the nucleotide was evaporated in vacuo at 40 °C, washed with ethyl acetate and evaporated with anhydrous ethanol. After repeated evaporation with anhydrous ether from the half- solid material, a white hygroscopic powder was recovered in 50- 70 % yield and stored over P_2O_5 in a desiccator.

According to UV absorption data (for molar absorbances see Table 1), the material was recovered in ≥95 % purity. Phosphorus

Table 1

UV-spectral data of 5-R-2'-deoxyuridine-5'-phosphates*

R	λmax (nm)	ε max $\times 10^{-3}$	λmin (nm)	ε min $\times 10^{-3}$
-H**	262	10.32	230.5	2.23
-methyl***	267	9.68	234.5	2.25
-ethyl	267	9.68	234.5	2.30
n-propyl	268	9.73	235.5	2.36
i-propyl	267	9.95	235	3.15
n-butyl	268	9.6	235.5	2.51
tert.-butyl	265	9.52	233.5	2.69
n-pentyl	268	9.48	236	2.53
n-hexyl	268.5	9.4	236	2.73

* Spectra were taken in aqueous solutions

** Literature[15]: 262; 10.2; 231; 2.2

*** Literature[15]: 267; 9.65; 235; 2.2

content related to $C_{9+n}H_{11+2(n+1)}O_8N_2P.2H_2O$, showed a deviation of ± 5 % from the calculated value.

The structure of the products was confirmed by ^{13}C NMR spectroscopy. It is known that in acyclic 5'-nucleotides the vicinial $^3J_{P-O-CC}$ spin-spin coupling is consistently larger than the geminal $^2J_{P-O-C}$ (approx. 8 and 5 Hz, respectively)[16]. The observed $^{31}P-^{13}C$ couplings appeared on the resonances of C-5' and C-4' nuclei. Since C-4' resonances exhibit large coupling the site of esterification may be located conclusively at C-5'. The assignment of ^{13}C resonances to the indivicual carbons in the molecules was based on literature data[16-18] and confirmed the structure expected.

Preparation of 5-alkyl-2'-deoxyuridine-5'-triphosphates

These syntheses were based on the morpholidate-method as described by Moffatt[19]. A solution of tri-n-butylammonium pyrophosphate (4 mmole) and anhydrous DMSO (16 ml) was added to the morpholidate (1 mmole) dehydrated with pyridine. After 2-4 days, 100 ml of water was added to the reaction mixture which was then applied to an ion-exchange resin column (DOWEX 2x8 $(HCO_3)^-$; 2,4x60 cm), fitted with a cooling jacket. The column was washed with water till UV absorption of the effluent ceased. The components were eluted with the exponential gradient of the ammonium hydrogen carbonate solution. The mixing vessel contained 1500 ml of water and the reservoir held 3-5000 ml of aqueous solution of NH_4HCO_3 (0.6-2 M). Triphosphate fractions (peak 4) were evaporated at 30-35 °C until NH_4HCO_3 was fully decomposed. A solution of 5 ml acetone and 1 g of $NaClO_4$, was added to the aqueous solution (0.5 ml) of the syrupy substance. The white powder obtained was centrifuged, washed with acetone and dried in vacuo. The product was stored over P_2O_5 at 4 °C.

In all cases the ratio of the readily hydrolyzing phosphorus (boiled with 1 N HCl at 100 °C for 15 min) to the overall phosphorus content calculated by the chemical formula $C_{9+n}H_{9+2(n+1)}O_{14}N_2P_3Na_4.2H_2O)$ was: 2:2.94 ± 2 %.

Enzymatic polymerization of 5-alkyl-dUTP-s: kinetic aexperiments

The relationship between nucleotide structure and velocity of the enzymatic reactions could be studied most easily in simple systems, e.g. in the template directed synthesis of poly[d(A-T)] from dATP

and dTTP in the presence of E.coli DNA polymerase I as described by Schachman et al.[20]. Under such conditions the enzyme was capable of synthesizing alternating copolymers with analogous triphosphates, i.e. poly[d(A-U)], poly[d(A-br^{5}U)][21], poly[d(A-hm^{5}U)][4] and poly[d(A-HgU)] with various mercaptans as ligands[22,23]. Since our chief aim has been to determine whether our analogous triphosphates are substrates of the enzyme, i.e. whether hypochromicity develops at 260 nm during incubation and if so at what rate the reaction takes place, the above system of Schachman et al. primed by poly[d(A-T)] was used.

Reaction mixtures contained 60 mM KH_2PO_4 (pH 7.4), 6 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.25 mM dATP, 0.25 mM 5-substituted-dUTP, 5 μM(P) poly[d(A-T)] and 0.8 μg of E.coli DNA polymerase I, in a final volume of 0.33 ml. Reactions were started by the addition of enzyme in enzyme-diluting solution to the preincubated reaction mixtures in 1 mm cuvettes at 37 $^\circ$C. All measurements were carried out in triplicate.

Figure 1 shows two characteristic primed reactions: the development of hypo- and hyperchromicity as a function of incubation time under conditions described above. The initial rate of polymerization is calculated from the initial slope of the curve. The apparent linearity of the curves are valid up to 30-50 minutes. In the synthesis of poly[d(A-T)] from dATP and dTTP this rate was 0.410 % hypochromicity/min, which is equivalent to 1.47 nM total nucleotide incorporation/min. This velocity was taken as 100 %.

Table 2 contains the data of enzymatic reactions with two compositions of analogous pyrimidine triphosphate substrates: relative polymerization rate and total hypochromicity both given in percentage. (Since saturating substrate concentrations of triphosphates and template-primer /free 3'-OH/[24] were used the rate values are considered to be maximal rates.)

It is noteworthy that the relative rate increased by 17 % when dTTP was replaced by dUTP in the primed reaction. If the mixture contained dUTP-s substituted in position 5 by straight alkyl chains longer than methyl there was also some hypochromic effect, however, the relative rate decreased in all cases. In the presence of 5-ethyl-dUTP this decrease was nearly 30 % and about the same relative rates developed with 5-n-propyl-

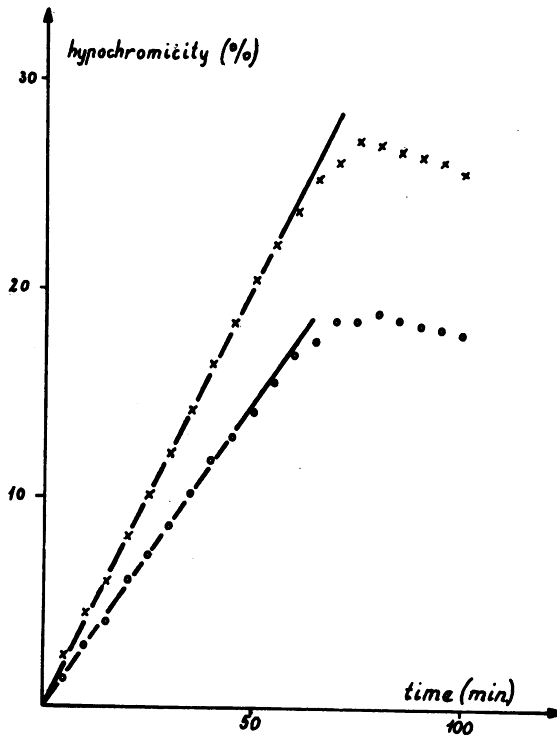

Figure 1. Development of hypochromicity in the presence of dATP and dTTP /x/, dATP and 5-ethyl-dUTP /o/. Reaction conditions described in Results.

and 5-n-butyl-dUTP in the mixture, respectively. Total hypochromicities were on an average by 30 % lower than that of the dTTP--dATP reaction. Drastic decrease (85 %) in the relative velocity of polymerization reaction could be observed with 5-n-pentyl-dUTP and dATP as substrates. With 5-n-hexyl-dUTP as well as with 5-i-propyl- and 5-tert.-butyl-dUTP, respectively, there was no hypochromic effect during a period of 240 minutes.

In all above reactions with hypochromicity polymers in covalent linkage to primers, presumably the alternating type of copolymers were synthesized, according to the template-primer: poly[d(A-U)], poly[d(A-et^5U)], poly[d(A-pr^5U)], poly[d(A-bu^5U)] and poly[d(A-pe^5U)]. (There was no hypochromicity in the presence of only one triphosphate substrate.)

If the reaction mixture was composed of 1:1 dTTP:5-substituted--dUTP in a final concentration of 0.25 mM, a decrease in A_{260}

Table 2
Data of DNA polymerase I reactions

5-substituted-dUTP-s in final conc. of 0.25mM	Relative velocity (%)	Total hypochromicity (%)
100 % -H-	117.3±4.3	28.4±0.4
50 % -H- 50 % -methyl-	108.8	27.1
100 % -methyl	100.0	26.6±0.8
100 % -ethyl-	72.6±1.6	17.7±1.4
50 % -ethyl- 50 % -methyl-	88.1±0.8	22.6±0.6
100 % -n-propyl-	70.5±2.4	22.0±3.3
50 % -n-propyl- 50 % -methyl-	85.5±3.2	23.8±0.6
100 % -n-butyl-	68.6±1.3	18.9±1.6
50 % -n-butyl- 50 % -methyl-	88.3±2.6	22.0
100 % -n-pentyl-	15.0±4.8	5.3±2.0*
50 % -n-pentyl- 50 % -methyl-	60.7±1.5	18.4±0.1
100 % -n-hexyl-	0	0
50 % -n-hexyl- 50 % -methyl-	63.1±0.2	13.6±1.4
100 % -i-propyl-	0	0
50 % -i-propyl- 50 % -methyl-	90.9±0.5	14.3±0.7
100 % -tert.-butyl-	0	0
50 % -tert.-butyl- 50 % -methyl-	87.2±1.6	14.4±0.1

* measured at 140 minutes

developed with all analogues. In the dUTP-dTTP mixture the relative rate was a mean value of the rates obtained in the two separate reactions. Probably, a randomly mixed copolymer was synthesized by the enzyme. The same type of polymerization may be assumed in 5-ethyl-, 5-n-propyl- and 5-n-butyl-dUTP - dTTP mixtures where the average relative rate was 87 %. Total hypochromicity values here were also mean values of the separate reactions. Surprisingly, the same relative rates have developed in the mixtures of 5-i-propyl-dUTP - dTTP (90.9 %) and 5-tert.-butyl--dUTP - dTTP (87.2 %). These results do not necessarily mean incorporation of the analogues into the polydeoxynucleotides they may be merely due to the inhibition of dTTP polymerization. The

same is probably valid in the mixture of 5-n-hexyl-dUTP - dTTP where the rate was similar to that of the 5-n-pentyl-dUTP - dTTP mixture.

Preparation of polydeoxynucleotides by de novo reactions

In all above reactions hypochromic effects could be observed also in the absence of the poly[d(A-T)] template-primer but only after 100-200 min lag periods. These de novo reactions were used for the preparation of polydeoxynucleotides, in quantities which allowed the determination some of their structural properties.

For the preparation of poly[d(A-T)] and polymers containing various 5-substituted uracils instead of thymine, we used the same mixtures as applied in the kinetic experiments without the addition of a template-primer and with 1/3 amount of enzyme. At maximum hypochromicity value this de novo volume (0.33 ml) was scaled up to 9.9 ml. The progress of polymerization was monitored also in a 1 mm light path cuvette. The other part of the mixture was kept also at 37 °C. Exchange and mixing of these solutions ensured approximately the same rates. At the end of polymerization, reactions were stopped by NaCl-EDTA solutions as described by Burd and Wells[25]. Heat inactivated protein was removed by chloroform extraction. The separated solution was concentrated by ethanol precipitation. Fractionation of the reaction products was performed on a Sephadex G-200 column (1.5x18 cm) with 100 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA (pH 7) solution, flow rate was 0.2 ml/min. The first peak contained the excluded polydeoxynucleotides. This was the only peak collected, which was then concentrated and dialyzed against 1 mM NaCl, 0.1 mM EDTA (500 ml) and redistilled water (500 ml) at 4 °C in a Bio-Fiber 50 Minibeaker (Bio-Rad Laboratories GmbH, München) with a flow rate of 5 ml/min. The solution was then filtered through a 0.45 μ membrane filter (Sartorius GmbH, Göttingen) and freeze-dried. These de novo reactions were carried out with dTTP, 5-ethyl-, 5-n-propyl-, 5-n-butyl- and 5-n-pentyl-dUTP-s as pyrimidine substrates. 2-500 μg of higher molecular weight polymers were obtained.

About 50 μg of the freeze-dried polydeoxynucleotides were hydrolysed in a glass tube with 25 μl of 90 % HCOOH at 175 °C for 30 minutes. HCOOH and then 3x50 μl water was removed by

lyophilization and the residue was dissolved in 0.1 N HCl. Base composition was determined in a high pressure liquid chromatograph as described in Mat. and Met. by using standard solutions of adenine and 5-alkyluracils and their 1:1 mixtures, respectively. In all cases examined a 1:1 ratio of adenine: 5-alkyluracil could be observed within ± 2 % experimental error. This proves that the polymers contain always the two bases present in the initial reaction mixture as triphosphates.

There is hardly any difference in the ultra-violet spectral properties of polydeoxynucleotides (for data see Table 3). They all have their λ_{max} values at 262 nm. The A_{280}/A_{260} ratios are between 0.55 and 0.57 showing the degree of purity and nativity of the products. With the exception of poly[d(A-pe^{5}U)] the polymers display about the same degree of thermal hyperchromicity (45-47%). After rapid cooling of the solution of heat-denaturated polymers to 25 °C, all substances regained the initial A_{260} values showing fully reversible renaturation of helices. These heating-cooling cycles were repeated two-three times in the same solution with hardly any lowering in the initial hyperchromicity values.

The exclusion of the reaction products on Sephadex G-200 proves the relatively high molecular weights of polydeoxynucleotides. They always contain two bases in equivalent molar ratios and the similarity of some spectral properties of poly[d(A,r^{5}U)] to poly[d(A-T)] points to a poly[d(A-r^{5}U)] structure, where r equals straight chain alkyl groups up to pentyl. Naturally this

Table 3

Some spectral data of poly[d(A,r^{5}U)] polymers*

r	λmax (nm)	λmin (nm)	A_{250}/A_{260}	A_{270}/A_{260}	A_{280}/A_{260}	A_{290}/A_{260}	A_{260} at 75°C / A_{260} at 25°C
methyl-	262	235	0.762	0.905	0.570	0.175	1.469
ethyl-	262	235	0.767	0.893	0.549	0.165	1.471
n-propyl-	262	235	0.774	0.899	0.573	0.218	1.451
n-butyl-	262	235	0.775	0.898	0.559	0.188	1.467
n-pentyl-	262	235	0.794	0.896	0.570	0.222	1.385

* Spectra were taken in 0.1 M NaCl, 0.02 M NaH_2PO_4 (pH 7.0) at 25 °C

assumption requires further investigation, the results of which will be published subsequently.

A biological interpretation of the results presented here is that antiviral nucleoside analogues, e.g. 5-ethyl-, 5-n-propyl-2'-deoxy-uridines[26], if present in cells as 5'-triphosphates may also exert activity in the polymerization reaction.

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1. Abbreviations: we used two letter symbols for 5-substitutions of uracil in polynucleotides e.g. poly [d(A-pr^{5}U)], and poly [d(A-pe^{5}U)] for propyl and pentyl substitutions, respectively.
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