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$$v = k_{im}\,[\text{O-acyl peptide}]\,[\text{imidazole}]_{(\text{free base})},$$

is approximately $10^{-3}\ M^{-1}\ \text{min}^{-1}$. At pH 8, in 1 M imidazole, catalysis by imidazole predominates over hydroxide catalysis by a factor of 10. Thus, although imidazole catalysis is clearly demonstrable, the absolute velocity of hydrolysis would be minute, even in 10 M imidazole (a concentration at which bimolecular reactions are comparable in velocity to the corresponding intramolecular first-order reaction rates[19]). The rate of hydrolysis of native furylacryloyl-Novo is approximately 1 min^{-1}. On this basis, straightforward intramolecular general-base catalysis by imidazole in the hydrolysis of an O-acyl serine ester would be eliminated as a potential mechanism of acyl-enzyme hydrolysis.

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*SYNTHETIC DEOXYRIBOPOLYNUCLEOTIDES AS TEMPLATES FOR RIBONUCLEIC ACID POLYMERASE: THE FORMATION AND CHARACTERIZATION OF A RIBOPOLYNUCLEOTIDE WITH A REPEATING TRINUCLEOTIDE SEQUENCE**

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Thymidine oligonucleotides have been shown previously to serve as templates for the synthesis of ribopolyadenylate in the presence of DNA-dependent RNA polymerase.[1, 2] In continuing this work, we have been interested in the use of this enzyme for the preparation of ribopolynucleotides containing repeating di- and trinucleotide sequences complementary to those contained in the chemically synthesized short chain deoxyribopolynucleotides.[3] In the present communication we report on the utilization of the nonanucleotide, d-$(TTC)_3$,[4, 5] containing the

repeating trinucleotide sequence, thymidylyl-thymidylyl-deoxycytidylate, as a template by the RNA polymerase. The product formed in the presence of ATP and GTP has been characterized as a ribopolynucleotide containing the repeating trinucleotide sequence, adenylyl-adenylyl-guanylate. A useful feature of the reaction is that the ribopolynucleotide product is much longer (containing probably more than 150 nucleotide residues in a chain) than the short deoxyribopolynucleotide used as the template.

Materials and Methods.—Deoxyribopolynucleotides, d-$(TTC)_2$, d-$(TTC)_3$, d-$(TTC)_3$-TT, and d-$(TTC)_4$ were synthesized as described elsewhere.[3, 4] ATP-8-C^{14} and GTP-8-C^{14} were purchased from Schwarz BioResearch, Inc. α-P^{32}-ATP and α-P^{32}-GTP were purchased from International Chemical and Nuclear Corp. and were further purified by paper chromatography whenever necessary. Unlabeled ribonucleoside 5'-triphosphates were also commercial products.

Enzyme assay: RNA polymerase was prepared from *E. coli* B according to Chamberlin and Berg.[6] The preparation represented a purification of 100-fold over the crude extract, and the specific activity corresponded to an incorporation of 2,000 mμmoles of C^{14}-ATP/hr/mg of fraction 4 using calf thymus DNA as the template under the assay conditions of Chamberlin and Berg.[6] Fraction 4 was precipitated by adding an equal volume of saturated ammonium sulfate, and the precipitate was dissolved in 0.1 *M* Tris-HCl, pH 8.0, containing 0.001 *M* $MgCl_2$ and 0.005 *M* glutathione and stored in liquid nitrogen.[7]

For assay using deoxyoligonucleotides, the typical reaction mixture contained per ml: 40 μmoles of Tris-HCl buffer, pH 7.9; $MgCl_2$, 4 μmoles; $MnCl_2$, 1 μmole; β-mercaptoethanol, 12 μmoles; ATP, 0.36 μmole; GTP, 0.18 μmole; d-$(TTC)_3$ or homologous template, 0.12–0.48 μmole as based on mononucleotide concentration; and RNA polymerase, 0.41 mg. The incubation was carried out at 14° unless otherwise stated. This low temperature was chosen in keeping with the findings on temperature dependence of the efficiency of thymidine oligonucleotides of varying chain length as templates for RNA polymerase.[8]

For routine measurement of the incorporation of the labeled triphosphate into acid-insoluble product, either C^{14}-ATP or C^{14}-GTP was used at a specific activity of 5 μc/μmole. Aliquots (0.01–0.02 ml) of the reaction mixture (usually 0.0625–0.125 ml total volume) were applied to Whatman 3 MM filter paper disk (approximately 1 sq cm in area). The filter paper disks were immersed in cold 5% trichloroacetic acid, washed twice with fresh portions of the acid solution at intervals of about 15 min, then with ethyl alcohol–ether (1:1) mixture in the cold, and finally with ether. After drying in the air, the disks were counted for radioactivity in a Packard TriCarb scintillation counter as reported previously.[9]

Isolation of C^{14}-labeled poly AAG: The reaction mixture (total volume, 1 ml) contained, in addition to the assay components described above, C^{14}-ATP and C^{14}-GTP of equal specific activity (5 μc/μmole) and 0.48 μmole of d-$(TTC)_3$. After incubation at 14° for 120 min, when the reaction mixture was turbid, it was treated with 0.20 ml of 0.1 *M* EDTA, pH 7.0, for 2 min at 37°. A clear solution now resulted and after cooling this in an ice bath, 0.015 ml of acetic acid was added. After 5 min at 0°, the precipitate was collected by centrifugation. The precipitate was washed three times with 0.5 ml of 0.05 *M* acetic acid, then dissolved in 0.04 ml of 0.5 *M* ammonium bicarbonate, and diluted to 0.8 ml with distilled water.

Degradation of poly AAG with T_1-RNase: C^{14}-poly AAG (1.6 mμmole, 7,300 cpm) was incubated with 0.04–2 μg of T_1-RNase[10] in 0.025 ml of 0.04 *M* ammonium bicarbonate for 15 min at 37°. The reaction mixtures were applied on strips of Whatman paper no. 1 and chromatographed either in *n*-propanol–concentrated ammonium hydroxide–water (55:10:35, v/v/v), (Solvent I), or in *iso*butyric acid–concentrated ammonium hydroxide–water (661:329:10, v/v/v) (pH 3.7) (Solvent II). Markers of $(Ap)_5$, $(Ap)_4$, $(Ap)_3$, $(Ap)_2$, Ap, and Gp were run alongside. For determination of the radioactivity, the paper chromatograms were cut into 1-cm-wide strips and the strips counted in the liquid scintillation counter.

Determination of chain length of poly AAG: The filter paper disks containing poly AAG (21,600 cpm) prepared by using C^{14}-ATP and C^{14}-GTP of equal specific activity (5 μc/μmole), were well soaked in 0.3 *N* sodium hydroxide (0.5 ml for a total area of 1 sq cm) for 20 hr at 37°. Cold Ap, Gp, A, and G (100 mμmoles of each) were added as carriers, and the total supernatant was collected by centrifugation. The filter paper was washed twice with 0.5 ml of distilled water. The

combined alkaline solution was neutralized by the addition of pyridinium Dowex-50 ion exchange resin. The resin was removed by centrifugation and washed twice with 0.5 ml of 0.1 *M* ammonium hydroxide. The combined supernatant and wash was evaporated in a rotary evaporator at low temperature, the dry residue dissolved in 0.05 ml of water, and 0.025 ml applied to a DEAE-cellulose paper strip. The strip was chromatographed in 0.3 *M* ammonium formate, pH 5.0, for $5^1/_2$ hr. The areas corresponding to the carrier nucleosides and nucleotides and the remainder of the chromatogram were scanned for radioactivity by counting in the liquid scintillation counter.

Nearest-neighbor frequency analysis of poly AAG: Poly AAG was prepared by using either α-P^{32}-GTP or α-P^{32}-ATP as one of the two triphosphates. The isolation of the polymer and the alkaline hydrolysis was as described above. Carrier Ap and Gp (100 mμmoles) were added during alkaline hydrolysis. The hydrolysate was worked up as described above and chromatographed on Whatman paper no. 1 in Solvent II for 16 hr. The nucleotides, Ap and Gp, were well separated as shown by inspection of the chromatogram under an ultraviolet lamp. The areas corresponding to the nucleotides were cut out and their radioactivity measured in the liquid scintillation counter.

Sucrose density centrifugation: Linear gradients of 5–20% sucrose were used containing 0.01 *M* Tris-HCl buffer, pH 7.5, and 0.0005 *M* EDTA. The total volume of the gradient solution was 5.0 ml. The centrifugation was done in Spinco model L centrifuge using SW-39 rotor at 32,000 rpm for 14.5 hr. Fractions consisting of 2 drops each were applied directly on filter paper disks after piercing the bottom of the centrifuge tube with a needle. The filter paper disks were counted for radioactivity both simply after drying, and after washing with 5% trichloroacetic acid, ether-ethyl alcohol mixture, and ether as described above.

Results.—Stoichiometry and characteristics of poly AAG synthesis: Figure 1 shows the kinetics of incorporation of C^{14}-ATP and of C^{14}-GTP using d-$(TTC)_3$ as template in RNA polymerase catalyzed reaction. Under the conditions of this experiment, as theoretically required for poly AAG synthesis, ATP incorporation was close to twice that of GTP incorporation throughout the incubation period.

While the above conditions gave faithful duplication of the triplet sequence in the template, variations in the temperature used or in the relative concentrations of the triphosphates caused marked distortions in the incorporation of the triphosphates. Thus, as shown in Figure 2, at 37°, the ATP incorporation was more than four times the incorporation of GTP. On the other hand, at 25°, GTP incorporation exceeded that of ATP incorporation. Furthermore, at higher temperatures, the total incorporation of the triphosphates was much reduced (cf. Figs. 1 and 2).

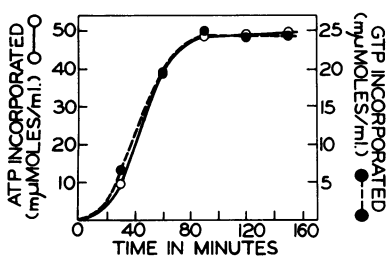

FIG. 1.—Incorporation of triphosphates at 14° in presence of d-$(TTC)_3$ template. The components of the system were exactly as described under *Materials and Methods*. Tube 1 contained C^{14}-ATP, 0.36 μmole/ml, sp. act. 5 μc/μmole, and cold GTP, 0.18 μmole/ml. Tube 2 contained C^{14}-GTP, sp. act. 5 μc/μmole, but ATP was cold. Both tubes contained d-$(TTC)_3$, 0.12 μmole/ml. Incubation was at 14°. Aliquots (0.02 ml) were removed at intervals and assayed by the filter paper disk asay.

Further characteristics of poly AAG synthesis were studied using conditions of Figure 1. Table 1 shows the requirement of both of the triphosphates for d-$(TTC)_3$-mediated synthesis of poly AAG.

However, it is clear that omission of either of the triphosphates did not completely abolish the incorporation of the second triphosphate and that therefore the template significantly stimulated the formation of polyadenylic or of polyguanylic acid. In order to minimize these side reactions, the ratio of ATP to GTP provided in the reaction mixtures for poly AAG synthesis was kept at 2:1 (as in Fig. 1).

The amount of poly AAG formed did not exceed the amount of the template d-$(TTC)_3$ added (Fig. 1). This conclusion was further supported by the results of Figure 3, in which the amount of C^{14}-ATP incorporation was again proportional to the amount of the template added (amounts of templates shown in individual curves). Addition of more triphosphates or variations in magnesium or manganese concentrations did not alter this result. In a further experiment (Fig. 4) more RNA polymerase or more template, d-$(TTC)_3$, was added after the synthesis had leveled off. The addition of the template gave a further burst of synthesis, corresponding to the amount of the template, whereas addition of the enzyme made little difference.

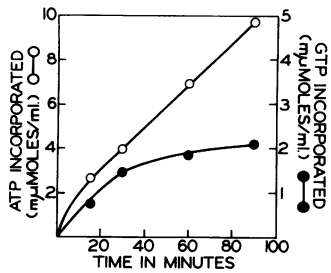

FIG. 2.—Incorporation of triphosphates at 37° in presence of d-$(TTC)_3$ template. The system was identical to that of Fig. 1, the concentration of d-$(TTC)_3$ being 0.48 μmole/ml. Incubation was at 37°.

Chain length of poly AAG by alkaline hydrolysis: C^{14}-labeled poly AAG was prepared, hydrolyzed with alkali, and the products were separated as described under *Methods*. The total radioactivity present in Ap + Gp was 7,838 cpm, while the total radioactivity in A + G was 44 cpm. This result therefore showed a chain length of 181. Because of the low counts in the nucleoside, the chain length may be regarded as between 150 and 200.

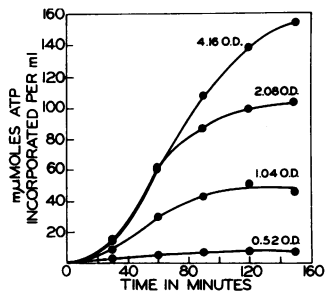

FIG. 3.—Dependence of poly AAG synthesis on concentration of d-$(TTC)_3$. The system was as in Fig. 1. The concentrations of d-$(TTC)_3$ used/ml are shown on the individual curves.

Sedimentation properties of poly AAG: Figure 5 shows the sedimentation behavior of samples of poly AAG obtained after 50 min and 100 min incubation. As can be seen, the polymer sedimented faster than C^{14}-valyl-transfer RNA. Using the generally accepted value (4S) for the transfer RNA, the sedimentation constant values correspond to 6.7 and 8.7, respectively, for the 50-min and the 100-min products. While the patterns of Figure 5 were obtained by removing the acid-soluble radioactivity from each fraction with trichloroacetic acid, direct measurement of total radioactivity in each fraction showed some (10–20% of acid-insoluble poly AAG) moving between C^{14}-poly AAG and C^{14}-ATP. Exam-

TABLE 1

REQUIREMENTS FOR POLY AAG SYNTHESIS

		Incorporation of Nucleoside Triphosphates	
		C^{14}-ATP, mμmole/ml	
	Components	60 min	120 min
Expt. 1	Complete	89	157
	-GTP	12	24
	-GTP, - $(TTC)_3$	1.5	5.0
	-$(TTC)_3$	0.8	3.2
		C^{14}-GTP, mμmoles/ml	
Expt. 1	Complete	57	98
	-ATP	4.0	6.9
	-ATP, - $(TTC)_3$	0.6	1.7
	-$(TTC)_3$	0.2	2.1

The components of the complete system were as described under *Materials and Methods*. The concentration of d-$(TTC)_3$ in both experiments was 0.48 μmole/ml.

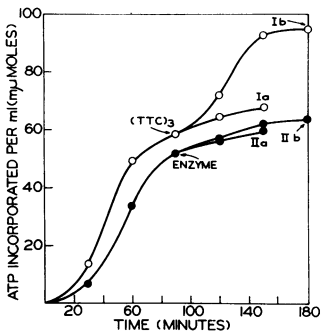

FIG. 4.—Characteristics of poly AAG synthesis. The reaction mixture of curves *Ia* and *Ib* contained at 0 time 0.41 mg of RNA polymerase and 0.12 μmole of d-$(TTC)_3$ per ml. After 90 min incubation at 14°, more d-$(TTC)_3$, 0.12 μmole/ml, was added in the reaction mixture of curve *Ib*. The reaction mixture of curves *IIa* and *IIb* contained 0.21 mg of RNA polymerase and 0.12 μmole of d-$(TTC)_3$ per ml at 0 time. After 90 min incubation, another 0.21 mg of RNA polymerase was added in the reaction mixture of curve *IIb*.

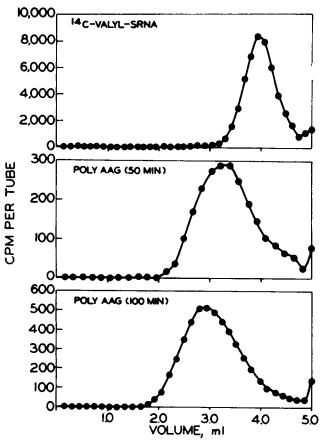

FIG. 5.—Sucrose gradient centrifugation of poly AAG. 5 μc/μmole of C^{14}-ATP was used as the labeled nucleoside triphosphate. Acid-insoluble counts were 300,000 cpm per ml at 50 min incubation, and 663,000 cpm per ml at 100 min incubation. 0.05 ml of each reaction mixture after 50 and 100 min incubation was diluted to 0.5 ml with 0.05 *M* sodium citrate, pH 5.5, and shaken with 0.5 ml of 90% phenol. The phenol layer was re-extracted once with 0.5 ml of 0.05 *M* sodium citrate, pH 5.5. The combined aqueous fraction was dialyzed against 0.005 *M* Tris-HCl buffer pH, 7.5, and 0.0005 *M* EDTA for 20 hr. The recovery of acid-insoluble radioactivity from the starting material was about 90%. 0.3 ml of each dialysate was used per tube for density gradient centrifugation.

ination of a number of reaction mixtures directly by the DEAE-cellulose paper assay[2] also supported this result, in that some radioactivity was present in the area between the origin (poly AAG) and C^{14}-ATP. This radioactivity presumably indicates the presence of acid-soluble short chain oligonucleotides which have not, however, been characterized further.

Nearest-neighbor frequency analysis: Table 2 shows the results of nearest-neighbor analysis performed on poly AAG obtained by using α-P^{32}-ATP and by using α-P^{32}-GTP. The values showed detectable deviations from the theoretical values expected for pure poly AAG. Thus, either slight amounts of homopolymers of A and of G were formed or runs of A and of G contaminated the strictly repeating

TABLE 2

NEAREST-NEIGHBOR FREQUENCY ANALYSIS

Substrates	Time of incubation	Radioactivity in Isolated Nucleotide 2′-(3′)AMP	2′-(3′)GMP	Transfer, % Found	Theoretica
α-^{32}P-ATP + GTP	40	486	350	42	50
α-^{32}P-ATP + GTP	80	1258	1113	48	50
α-^{32}P-ATP + GTP	120	1973	1793	48	50
α-^{32}P-GTP + ATP	40	127	4	97	100
α-^{32}P-GTP + ATP	80	607	16	97	100
α-^{32}P-GTP + ATP	120	924	26	97	100

Incorporation of α-^{32}P-ATP into acid-insoluble fraction was 188,000 cpm at 40 min, 600,000 cpm at 80 min, and 818,090 cpm per ml at 120 min incubation. Incorporation of α-^{32}P-GTP was 32,000 cpm at 40 min, 137,000 cpm at 80 min, and 224,000 cpm per ml at 120 min incubation. 0.015 ml of the reaction mixture was applied to a filter paper disk, washed, and subjected to alkaline hydrolysis as described in *Materials and Methods*.

AAG sequences. It is clear, however, that about 95 per cent of the total polynucleotide formed consisted of the repeating trinucleotide sequence. The same result was further confirmed by the experiments below on digestion by T_1-ribonuclease.

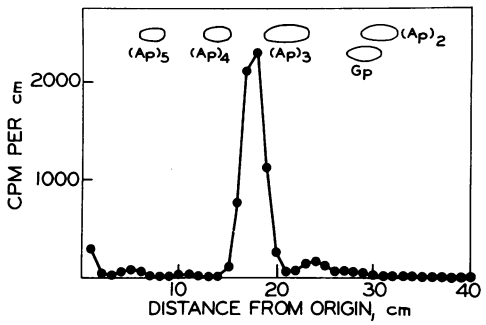

FIG. 6.—T_1-RNase digestion of poly AAG. Conditions as in text; 0.2 μg of T_1-RNase was used in this experiment. Chromatography was for 36 hr in Solvent I.

Degradation of poly AAG by T_1-RNase: Figure 6 shows the distribution of radioactivity in the products obtained on digestion of the C^{14}-labeled poly AAG by T_1-RNase. Over 90 per cent of the radioactivity was present in one product which is concluded to be ApApGp. (The latter on alkaline hydrolysis gave C^{14}-Ap and C^{14}-Gp in the ratio of 1.8:1.) The minor radioactive band moving just ahead could be ApGp. The oligonucleotide moving slower than ApApGp is probably ApApApApGp, and the result shows that occasionally the C residue in d-$(TTC)_3$ is omitted from being "read" by RNA polymerase. The minor radioactivity at the origin could represent poly A and/or poly G. While the above separations were formed using Solvent I, chromatography in Solvent II provided further confirmation of the above results.

Priming by other deoxyoligonucleotides: d-$(TTC)_2$ failed to stimulate incorporation of any nucleoside triphosphate when tested under the conditions of d-$(TTC)_3$ experiments. The characteristics of poly AAG synthesis using d-$(TTC)_4$ were like those described above for d-$(TTC)_3$ reactions and, in particular, there was marked deviation from faithful duplication of the triplet sequence in 37° reactions as noted above for d-$(TTC)_3$. The use of d-$(TTC)_4$ therefore did not offer any advantage over the use of d-$(TTC)_3$. d-$(TTC)_3$-TT stimulated quite well the incorporation of the two triphosphates, but an analysis of the product by T_1-RNase degradation followed by chromatography showed, in addition to ApApGp, the presence of ApApApApGp (50% of ApApGp) and of ApApApGp (25% of ApApGp). The mechanism of the reaction with d-$(TTC)_3$-TT as template requires further study.

Discussion.—The total evidence shows that poly AAG prepared using d-$(TTC)_3$ as template is much longer in size than the template and that it contains more than 90 per cent of the total internucleotidic linkages in the strictly repeating trinucleotide sequence, ApApGp. The evidence for the latter conclusion came from the nearest-neighbor analysis and from the analysis of T_1-RNase digestion products, and results of both techniques were in good accord. Regarding the size, the results of chain length determination by alkaline hydrolysis shows that the high-molecular-weight product does not consist of aggregates of short chain oligonucleotides. The estimate of chain length obtained this way appears to be consistent with the sedimentation behavior of the product in the sucrose density gradient.

Previously,[2] short chain thymidine oligonucleotides have been shown to serve as templates for the synthesis of long ribopolyadenylate, and short chains of oligonucleotides containing alternating thymidylate and deoxyadenylate residues

similarly bring about the synthesis of apparently high-molecular-weight poly AU.[8] The present work demonstrates the utility of RNA polymerase for the purpose of preparing long ribopolynucleotides of repeating trinucleotide sequences by using chemically synthesized deoxyribo-oligonucleotides of complementary sequences.[3]

The amount of poly AAG was proportional to the amount of d-$(TTC)_3$ template provided in the reaction mixture. The result suggests that a hybrid between the short template chains and the long poly AAG product is formed and that the template becomes effectively unavailable to the enzyme as a result of hybridization. The results of Figure 4 further demonstrate that the enzyme remains fully active at the point when the leveling off of the reaction occurs, since addition of more d-$(TTC)_3$ at this stage gives a new burst of poly AAG synthesis. This finding is in contrast to the situation obtained with RNA polymerase and high-molecular-weight DNA. Thus Bremer and Konrad[11] demonstrated a rather irreversible formation of a complex between the enzyme, the DNA template, and the product.

RNA polymerase catalyzes the synthesis of ribopolyadenylate from single-stranded DNA in the presence of ATP alone.[6, 7] The conclusion that short runs of thymidylate residues are responsible for this synthesis is supported by the work with the short chain chemically synthesized thymidine oligonucleotides.[2] The stimulation of polyadenylate synthesis in the presence of ATP alone using d-$(TTC)_3$ as the template was observed in the present work. The results show that, in this template, the nonpairing base C present between the pairs of thymidylate residues can loop out so as to permit the synthesis of continuous ribopolyadenylate. The same situation may readily apply to single-stranded DNA, and for the extensive homopolymer formation reported in the presence of single triphosphates,[7, 12, 13] it does not now appear to be essential to have continuous runs of one base in the DNA template. A similar explanation has been advanced recently for the priming capacity of various DNA's in the synthesis of alternating d-AT polymer by DNA polymerase.[14]

Poly AAG and related polynucleotides of repeating nucleotide sequences are clearly of interest as messengers in the *in vitro* amino acid incorporation system. The binding of poly AAG to ribosomes has already been demonstrated in this laboratory by the sucrose density gradient technique. Further work on polypeptide synthesis using ribopolynucleotides of known sequences is in progress.

Summary.—The nonanucleotide, d-$(TTC)_3$, containing the repeating sequence thymidylyl-thymidylyl-deoxycytidylate, serves as a template for the RNA polymerase and brings about the synthesis of the complementary ribopolynucleotide containing the repeating sequence, adenylyl-adenylyl-guanylate. The size of this product has been estimated to be in the range of 150–200 nucleotide units.

We are grateful to Drs. P. Berg, M. Chamberlin, and O. W. Jones for advice and assistance in the preparation of the RNA polymerase.

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[5] Most of the abbreviations are as in current use in *J. Biol. Chem.*: d-$(TTC)_3$ is abbreviation for the nonanucleotide d-TpTpCpTpTpCpTpTpC, bearing no phosphomonoester end group, and similarly, d-$(TTC)_2$ and d-$(TTC)_4$ are homologues containing, respectively, 2 and 4 triplets; d-$(TTC)_3$-TT is undecanucleotide, bearing at the right-hand terminus the fragment -TpT; poly AAG, ribopolynucleotide containing the repeating sequence, adenylyl-adenylyl-guanylate.

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*THE ROLE OF DPNASE IN THE MECHANISM OF ACTION OF AN ANTITUMOR ALKYLATING AGENT ON EHRLICH ASCITES CELLS**

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Inhibition of glycolysis of tumor cells is one of the most conspicuous metabolic effects of carcinostatic alkylating agents.[1-3] This inhibition coincides with a profound diminution of the steady-state concentration of DPN of tumor cells, as observed by Roitt,[3] and Holzer *et al.*,[4] and amply confirmed by numerous investigators.[5-11] The mechanism responsible for the decrease in DPN content of tumor cells by carcinostatic alkylating agents has been unknown. Holzer *et al.*[12, 13] suggested that carcinostatic alkylating agents inhibit at some point the biosynthesis of DPN. On the other hand, Green and Bodansky[14] as well as Hilz *et al.*[15] believe that an activation of DPNase is the primary reason for a decrease of DPN content of tumor cells. A hint regarding the mechanism of carcinostatic alkylating agents on glycolysis has already been provided by the work of Roitt,[3] who found that nicotinic acid amide antagonizes the inhibitory effect of these drugs. Interpretation of this observation remains ambiguous, however, since nicotinic acid amide is a well-known biosynthetic precursor of DPN as well as an inhibitor of DPNase. We have reinvestigated this question with the aid of structural analogues of nicotinamide. In order to eliminate the uncertainty concerning the possible dual action of nicotinamide on DPN metabolism, DPNase inhibitors had to be found which could not serve as precursors of DPN. Two among the nicotinamide homologues tested