2-Phenyl-5-carbomethoxymethylidenethiazolinone-4 (XIIId).—The same reaction was also performed using 1.00 g. (7.3 mmoles) of thiobenzamide and 1.05 g. (7.4 mmoles) of dimethyl acetylenedicarboxylate and resulted in 1.40 g. (77%) of yellowish crystals, m.p. 152–154°, sublimed or recrystallized from benzene–cyclohexane without significant change; infrared band (Nujol): no NH, 5.82, 6.22 (w), and 6.56 μ.

Anal. Calcd. for $C_{12}H_9NO_3S$: C, 58.30; H, 3.67; S, 13.01. Found: C, 58.07; H, 3.77; S, 13.41.

Acknowledgment.—The authors wish to record their grateful appreciation of a grant (GM10715) from the National Institutes of Health which supported a part of this investigation.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, STANFORD UNIVERSITY MEDICAL CENTER, PALO ALTO, CALIF.]

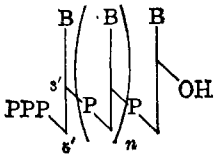
Chemical Synthesis of a Homolog of Deoxyribonucleoside-5′ Triphosphates

BY A. M. DUFFIELD AND A. L. NUSSBAUM

RECEIVED JULY 17, 1963

The synthesis of deoxyadenylyl-(5′ → 3′)-thymidine-5′ triphosphate (d-pppTpA) is reported. Material labeled with ^{32}P at the 5′-terminal ester phosphate does not serve as a substrate for the enzymatic synthesis of deoxyadenylate-thymidylate copolymer (dAT), a DNA-like polymer.

The absolute requirement for deoxyribomononucleoside-5′ triphosphates in the *in vitro* synthesis of DNA-like polymers[1] and related macromolecules[2,3] by enzymes from several sources has been clearly established.[4] There has been some speculation[5] that dinucleotides or higher homologs may be DNA precursors. This idea could be tested in the *in vitro* systems available with such "activated" species as



(where B designates purine or pyrimidine bases native to DNA).[6]

If such a biosynthetic pathway exists, it might be a method for the *in vivo* salvage of naturally occurring oligonucleotide sequences by their incorporation into DNA *via* activation by the pyrophosphate moiety. Furthermore, such structures might be models for the possible natural occurrence of polymeric pppXpY. . .pZ species in biosynthesis. The relative ease with which nucleoside triphosphates can be prepared chemically,[7] and especially the improvements and detailed experimental procedures now available from the work of Moffatt,[8] prompted us to attempt the chemical synthesis of a model triphosphate of the type described and to examine its behavior with the DNA polymerase from *Escherichia coli*.

We chose the structure deoxyadenylyl-(5′ → 3′)-thymidine-5′ triphosphate(d-pppTpA) as a convenient prototype of the general class, since it represents the ideal substrate in an enzymatic synthesis under the priming and template direction of dAT,[2] a copolymer of deoxyadenylate and thymidylate in an alternating sequence. The well-known base-matching requirements of such reactions, the technical advantages of solubility in the reaction media, and availability of precursors make this system especially favorable. The starting material for the triphosphate in question, deoxyadenylyl-5′ → 3′)-thymidylic-5′ acid (IV, Chart I), has already been prepared chemically,[9] and it only remained to be seen whether the pyrophosphorylation methods applicable to mononucleotides would also be useful in this instance.

The conversion of d-pTpA (IV, Chart I) into the desired 5′-terminal triphosphate (VI) would conveniently proceed by way of an activated intermediate, and the phosphomorpholidate (V) was selected. For synthesis of the precursor dinucleotide, a slight modification of the very recently reported[10] procedure of Schaller, *et al.*, was employed, because of its simplicity as compared to the older method[9] and its adaptability to the synthetic sequence where the 5′-phosphate in thymidylic acid is labeled with ^{32}P. The protected thymidylic acid (II) was condensed with $N^6,O^{3'}$-diacetyldeoxyadenylic-5′ acid (III),[11,12] stripped of its protecting groups by saponification, and the desired dinucleotide IV isolated, in moderate yield, by gradient dilution ion-exchange chromatography on DEAE-cellulose. It was characterized by its paper chromatographic migration characteristics, ultraviolet spectrum, hypochromicity,[13] and by the products of the action of snake venom phosphodiesterase[16] (see Experimental).

The next step involved activation of the 5′-terminal phosphate *via* its morpholidate. This is usually accomplished by the slow dropwise addition of dicyclohexylcarbodiimide in *t*-butyl alcohol to a refluxing solution of the nucleotide in aqueous *t*-butyl alcohol containing the reagent. Thus, there is always an excess of nucleotide present, and phosphomorpholidate synthesis takes place at the expense of the competing formation of the strongly basic 4-morpholine-N,N^1-dicyclohexylcarboxamidine,[14] the presence of which strongly inhibits the desired primary event. This procedure was not practical for our purpose because of the small quantities with which we had to work, especially for the preparation of radioactive species. As indicated in the Experimental section, the amidation was carried out in a sealed tube; we were encouraged

(1) I. R. Lehman, M. J. Bessman, E. S. Simms, and A. Kornberg, *J. Biol Chem.*, **233**, 163 (1958).

(2) H. K. Schachman, J. Adler, C. M. Radding, I. R. Lehman, and A. Kornberg, *ibid.*, **235**, 3242 (1960).

(3) C. M. Radding, J. Josse, and A. Kornberg, *ibid.*, **237**, 2869 (1962).

(4) A. Kornberg, "Enzymatic Synthesis of DNA," John Wiley and Sons, Inc., New York, N. Y., 1962.

(5) F. H. C. Crick, in "The Chemical Basis of Heredity," W. D. McElroy and B. Glass, Ed., The Johns Hopkins Press, Baltimore, Md., 1957, p. 534.

(6) For nomenclature and recent chemistry, see H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961.

(7) For a recent review, see J. Baddiley and N. A. Hughes, *Advan. Enzymol.*, **22**, 157 (1960).

(8) J. G. Moffatt, *J. Am. Chem. Soc.*, in press. We wish to thank Dr. Moffatt warmly for making his manuscript available to us prior to publication.

(9) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6217 (1958).

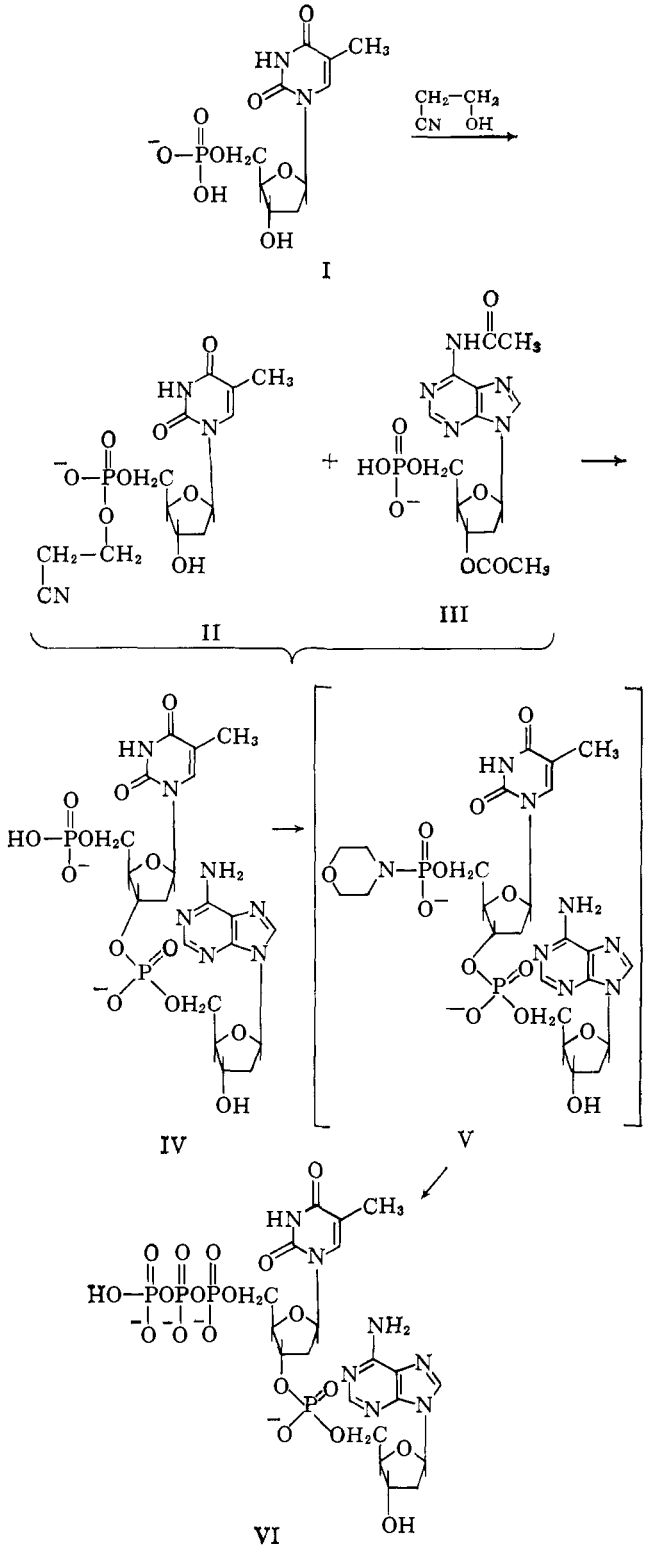
(10) H. Schaller, G. Weimann, and H. G. Khorana, *ibid.*, **85**, 355 (1963).

(11) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2932 (1961).

(12) The condensation described in ref. 10 employs the corresponding N^6-benzoate. However, the problem of a selective hydrolysis of the $O^{3'}$-substituent encountered by these authors does not arise in the present sequence, so the more easily available diacetyl derivative III sufficed for our purposes.

(13) A. M. Michelson, *J. Chem. Soc.*, 1371 (1959).

(14) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).



by the earlier finding[14] that the morpholidate of thymidylic and adenylic acids often formed in excellent yield even without dropwise addition.

The morpholidate V was formed in yields of 65–75% (isolated by electrophoresis). It was not characterized beyond noting its homogeneity and lowered polarity relative to the starting material in electrophoresis and paper chromatography, and its unchanged ultraviolet spectrum. This material was then subjected to the action of tri-*n*-butylammonium pyrophosphate in dimethyl sulfoxide under the carefully anhydrous conditions described by Moffatt.[8] Preparative paper chromatography gave the desired VI in a yield of 37%; (We ascribe this relatively low yield to failure to attain the rigorously anhydrous conditions required.) The material was characterized by its ultraviolet spectrum, polarity, "labile" phosphorus content,[15] susceptibility to venom diesterase,[16] and the nature of the products derived from such a digest.

The entire sequence was repeated, using a 5′-thymidylic acid labeled with ^{32}P.[17] The dinucleotide derivative obtained in this manner had a specific activity of 4.8×10^6 counts per min. per μmole.

The latter was now subjected to the purified DNA polymerase,[18] in the presence of dAT as primer and template, under the standard conditions favorable for the synthesis of this polymer. This assay resulted in the polymerization of the expected amount from d-pp^{32}pA plus pppT, but no detectable amount from d-pp^{32}pTpA(<0.01%). When synthesis was carried out in the presence of d-pp^{32}pTpA,[19] pppT, and d-pppA, a trace of radioactivity was incorporated (less than 0.04% of a comparable assay with d-pp^{32}pA as a source of ^{32}P); however, this amount of incorporation is not considered to be significant since we were unable to exclude the presence of a trace of pp^{32}pT resulting from the action of an enzymatic activity which hydrolyzes d-pp^{32}pTpA to pp^{32}pT and d-pA.

These data do not provide support for the proposal referred to above.[5]

Experimental[20]

Deoxyadenylyl-5′ → 3′)-thymidylic-(5′) Acid (IV).—5′-Thymidylic acid (78 mg. of the hydrated ammonium salt, 0.2 mmole) was converted to the pyridinium salt by passage over a column (3 × 40 cm.) of Dowex 50W-X8 cation exchange resin (pyridinium cycle), the column being washed with 5% aqueous pyridine. The combined eluates were concentrated *in vacuo*. To the residue, 0.25 ml. of hydracrylonitrile was added, and the mixture was dried by several concentrations *in vacuo* in the presence of a few ml. of dry pyridine. The residual oil was dissolved in 2 ml. of anhydrous pyridine, and 420 mg. of dicyclohexylcarbodiimide was added. The reaction vessel was closed tightly and agitated for a few minutes, then allowed to stand in the dark for 62 hr. Water (3 ml.) was added, and the mixture was allowed to stand at room temperature overnight. It was then filtered, the solid material washed well with water, and the latter added to the filtrate. The combined aqueous pyridine solution was concentrated to dryness, redissolved in 25 ml. of water, extracted with petroleum ether, and the aqueous phase was passed once more over a column of Dowex 50W-X8 (hydrogen cycle) cation exchange resin. The eluate was concentrated to a volume of 3.5 ml. and its pH adjusted to 7.5 with a saturated solution of barium hydroxide. Acetone (40 ml.) was added, and the resultant opalescent suspension was stored at −20° overnight. The precipitate was collected by centrifugation, and the solid material was dissolved in a small amount of water. The solution was concentrated again in order to remove any residual acetone; the process was repeated once again. The final residue was dissolved in water and its ultraviolet spectrum measured: a maximum at 267 mμ indicated unchanged thymidine chromophore. This material moved faster than the parent nucleotide in the isobutyric acid–ammonia paper chromatographic system[21] (R_f 1.2), but the original mobility was restored upon treatment with base.[17] The

(15) L. Ernster, R. Zetterstrom, and O. Lindberg, *Acta Chem. Scand.*, **4**, 942 (1950); see also H. B. Steward and K. P. Strickland, *Can. J. Biochem. Physiol.*, **39**, 1141 (1961).

(16) R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.*, **198**, 293 (1952).

(17) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961). In this preparation, the phosphorylation was carried out on thymidine-3′ acetate in order to preclude the presence of any 3′-^{32} phosphate.

(18) C. C. Richardson, C. L. Schildkraut, H. V. Aposhian, and A. Kornberg, *J. Biol. Chem.*, in press.

(19) The presence of this preparation in an assay for dAT synthesis from ^{3}H-pppT and pppA did not inhibit tritium incorporation.

(20) Solvent removal was carried out under oil-pump vacuum at less than 35°. Paper chromatography employed the systems enumerated in the text: individual spots were recovered by excision and threefold extraction with distilled water followed by filtration of the extract through a sintered glass funnel. Ultraviolet measurements were carried out in a Zeiss PMQ II spectrophotometer. Phosphorus was determined by the micromethod of P. S. Chen, T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

(21) B. Magasanik, E. Vischer, R. Doniger, D. Elson, and E. Chargaff, *J. Biol. Chem.*, **186**, 37 (1950).

yield of this intermediate (β-cyanoethyl-5′-thymidylate, II) was 652 optical density units (0.067 mmole, 33.5%). For use in the subsequent condensation, the barium salt was converted into the pyridinium salt as described above for thymidylate.

The protected pyridinium thymidylate (0.066 mmole) was dissolved in 5 ml. of dry pyridine and concentrated to dryness *in vacuo*. Dowex 50 W-X8, pyridinium cycle (prepared by converting the hydrogen form with 5% aqueous pyridine and subsequent suspension of the resin in absolute pyridine), 200 λ, was added to the residue, and the mixture was again dried by coconcentration with a few ml. of dry pyridine. Finally, 0.1 mmole of N^6,$O^{3'}$-diacetyldeoxyadenylic 5′-acid (pyridinium salt) was added and the drying process repeated several times. The residue was dissolved in 0.5 ml. of anhydrous pyridine, and 103 mg. of dicyclohexylcarbodiimide was added. The resulting mixture was tightly stoppered, agitated for several minutes, and allowed to stand in the dark for 72 hr. Water (2 ml.) was added and, after further standing at room temperature for 36 hr., the mixture was concentrated to a volume of approximately 0.5 ml. Concentrated ammonia (1.5 ml.) was added, and the mixture was heated for 1 hr. at 55°; a second portion of ammonia (1.5 ml.) was added and heating continued for another hour. The vessel was cooled and its content diluted with 5 ml. of water. The insoluble components were centrifuged in a clinical centrifuge and the supernatant portion was decanted. The residue was washed several times with water and the mixture was centrifuged each time. The combined aqueous phase was concentrated to a small volume to remove the bulk of pyridine and then brought up to a volume of 30 ml. with distilled water. After adjustment to a pH of 8.8 with ammonia, the solution was applied to the top of a column (2.5 × 15 cm.) of DEAE-cellulose in the bicarbonate cycle. Irrigation with a gradient of 0.3 *M* triethylammonium bicarbonate (pH 7.5, 1.5 l.) into a like volume of distilled water resulted in the elution (at around 0.1 *M* buffer strength) of the desired d-pTpA (IV). Fractions of the symmetrical peak were combined, concentrated to a small volume, and lyophilized. In agreement with its composition, this material had an absorption maximum in the ultraviolet at 260 mμ, 280/260 = 0.43, 250/260 = 0.79 (all measurements at pH 2). A yield of 315 optical density units corresponds to 0.0154 mmole, or 23.4%, based on protected thymidylic acid. This material traveled somewhat slower than pT in the buffered isobutyrate paper-chromatographic system[21] (R_f 0.34) and gave rise to pT and pA (in the ratio of 1.00:1.07) upon treatment with purified venom diesterase.[16] It had an $\epsilon/2P$ = 20,500. Upon complete degradation to mononucleotides with the latter enzyme, an 11% rise in the absorptivity was noted.[13]

Deoxyadenylyl-(5′ → 3′)-thymidine-5′ Triphosphate (VI).—Dinucleotide IV (25 optical density units, 1.2 μmoles) was dissolved in 25 λ of water, transferred to a reaction vessel fashioned from a piece of Pyrex tubing (diameter 3 mm., length 15 cm., sealed at one end) by means of a Pasteur pipet, and the transfer completed by washing with another 25 λ of water. Morpholine (0.8 λ) and 25 λ of a solution of dicyclohexylcarbodiimide (251 mg.) in *t*-butyl alcohol (6.25 ml.) were added, and the open end of the tube was sealed by drawing out in a burner. The tube was immersed in a test tube containing mineral oil, and the latter was placed in a heating bath (mineral oil) which was kept at 132° for 4 hr. The temperature of the inner bath fluctuated between 105 and 110°. The reaction vessel was cooled and opened by means of a triangular file, and the contents were streaked on 7 cm. of Whatman filter paper grade No. 3MM. The paper was saturated with a 0.05 *M* solution of triethylammonium bicarbonate, pH 7.5 (care being taken not to elute or displace the applied product), and subjected to electrophoresis in the apparatus of Markham and Smith[22] at an applied voltage of 1200 v. for a period of 80 min. Under these conditions, the starting dinucleotide had moved a distance of 28 cm. toward the anode; the desired morpholidate V—the major portion—had migrated only 18 cm. in the same direction. The latter, extracted with water and concentrated to a dry residue, had the properties: $\lambda_{max}^{pH\ 2}$ at 260 mμ, 280/260 = 0.49, 250/260 = 0.85; total extracted: 18.9 optical density units (75.6%).

In addition to its homogeneity in paper electrophoresis, the material traveled as a single spot in the paper-chromatographic system 2-propanol–ammonia–water 7:1:2 (after 26 hr. of irrigation, the morpholidate had traveled 29 cm.).

Another preparation on a somewhat larger scale (125 optical density units) resulted in a slightly lower yield (65%). However, the recovery of 15.8 optical density units (12.6%) of unchanged starting material raised the over-all conversion.

The material thus obtained was used directly without further characterization in the conversion to 5′-terminal triphosphate VI. Morpholidate (81.5 optical density units) was dried by repeated coconcentration *in vacuo* with a few ml. of anhydrous pyridine, followed by dry benzene. The residue was treated with 125 λ of a solution consisting of 1 mmole of tri-*n*-butylammonium pyrophosphate in dimethyl sulfoxide (dried thoroughly by storage over a molecular sieve) and the reaction vessel was quickly stoppered and stored in a vacuum desiccator over a drying agent for 84 hr. The reaction was terminated by the addition of 1 drop of water, and most of the solvent was removed by subjecting the vessel to a high vacuum for 5 hr. The residue was then streaked on 28 cm. of Whatman filter paper grade No. 3 MM and developed in the isobutyrate system.[21] Three zones were visible by scanning under ultraviolet light: the most prominent of these, the one migrating most slowly, was extracted with water and concentrated to a small volume. A yield of 30.4 optical density units corresponds to 37.3%. Structure was assigned on the basis of the following properties. The ultraviolet spectrum was very similar to that of IV, $\epsilon^{260}/4P$ = 19,800; R_f thymidylate = 0.58 in the isobutyrate system[21]; 2.09 optical density units of this material (102 mμ-moles) were treated with 1 ml. of *N* hydrochloric acid for 1 min. at 100°.[15] An aliquot (one-fifth) was measured for inorganic phosphate: calcd. 40.8 mμmoles, found 38.8 mμmoles.

The remaining solution was spotted on Whatman filter paper grade No. 1 and developed against standards of adenosine and pTp[23] in the isobutyrate system[21]: approximately equal amounts of two spots traveling as the standards and having the required ultraviolet absorption were observed.

Another aliquot of d-pppTpA (VI) was subjected to the action of venom phosphodiesterase.[16] Paper chromatographic assay indicated the presence of pT, d-pA, and inorganic pyrophosphate in the digest.

Acknowledgment.—This work was carried out with the aid of research grants 5T1 GM 196 and GM 07581 from the National Institutes of Health, U. S. Public Health Service.

(22) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(23) The expected products of concomitant depurination of the dinucleotide. The actual event was observed in a control acid hydrolysis of d-pTpA.

COMMUNICATIONS TO THE EDITOR

Molecular and Electronic Structure of the Bis(maleonitriledithiolato)nickelate(II) Ion[1]

Sir:

The considerable recent interest in square-planar complexes,[2] particularly those containing bidentate sulfur ligands,[3] has prompted us to investigate in detail the molecular structure and the electronic energy levels of a representative example of these compounds. This communication reports the results for the bis(maleonitriledithiolato)nickelate(II) ion, abbreviated $Ni(MNT)_2^{2-}$. The complex $[(CH_3)_4N]_2[Ni(MNT)_2]$ was chosen for the structure determination.

Crystals of $[(CH_3)_4N]_2[Ni(MNT)_2]$ were examined by precession and Weissenberg techniques with Mo Kα radiation and found to be triclinic. A convenient cell is a = 10.16, b = 15.77, c = 8.05 (all ±0.03 Å.), α = 87.4, β = 113.4, γ = 91.4 (all ±0.5). The observed extinctions hkl for $h + k$ odd are consistent

(1) Part of the research was performed under the auspices of the U. S. Atomic Energy Commission. Part of the research was supported by the National Science Foundation.

(2) (a) R. F. Fenske, D. S. Martin, and K. Ruedenberg, *Inorg. Chem.*, **1**, 441 (1962); (b) H. B. Gray and C. J. Ballhausen, *J. Am. Chem. Soc.*, **85**, 260 (1963).

(3) (a) G. N. Schrauzer and V. Mayweg, *ibid.*, **84**, 3221 (1962); (b) H. B. Gray, R. Williams, I. Bernal, and E. Billig, *ibid.*, **84**, 3596 (1962); (c) H. B. Gray and E. Billig, *ibid.*, **85**, 2019 (1963); (d) A. Davison, N. Edelstein, R. H. Holm, and A. H. Maki, *ibid.*, **85**, 2029 (1963).