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Syntheses of Deoxynucleoside 3′-Triphosphates*

John Josse and J. G. Moffatt

ABSTRACT: Efficient chemical syntheses of the 3′-triphosphates derived from the four commonly occurring deoxyribonucleosides are described. The syntheses involve conversion of the chemically synthesized or enzymatically isolated deoxynucleoside 3′-phosphates into the 3′-phosphoromorpholidates, which are then condensed with tributylammonium pyrophosphate in anhydrous dimethyl sulfoxide.

Isolated yields of 52–72% of the pure triphosphates were obtained by ion-exchange chromatography, and the structures were confirmed both chemically and enzymatically.

The findings of Canellakis *et al.* (1965) that extracts of *Bacillus subtilis* catalyze phosphorylation of thymidine 3′-diphosphate (3′-TDP)[1] to the corresponding triphosphate and of Josse (unpublished) that kinases specific for deoxynucleoside 3′-monophosphates can be purified from extracts of *Escherichia coli* have raised the question of what role, if any, such deoxynucleoside 3′-triphosphates might play in the cell. (A recent report by Coutsogeorgopoulos and co-workers (1965) has documented formation of 3′-TTP by extracts of regenerating rat liver.) In an effort to investigate this question, which could conceivably have important

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[1] Abbreviations used are: 3′-dAMP, 3′-dGMP, 3′-dCMP, 5-Me-3′-dCMP, and 3′-TMP: the deoxynucleoside 3′-monophosphates, respectively, of adenine, guanine, cytosine, 5-methylcytosine, and thymine; 3′-d( )M: deoxynucleoside 3′-phosphoromorpholidates of the various bases; 3′-d( )DP and 3′-d( )TP: deoxynucleoside 3′-di- and triphosphates, respectively, of the various bases; α^{32}P-3′-TDP and α^{32}P-3′-TTP: thymidine 3′-di- and triphosphate, respectively, labeled with ^{32}P in the α-phosphate (that esterified to sugar).

implications for the synthesis of DNA, a general method for syntheses of deoxynucleoside 3′-triphosphates was sought. Earlier work by Moffatt (1964) on the synthesis of both ribo- and deoxyribonucleoside 5′-triphosphates had shown the utility of initial conversion of a nucleoside 5′-phosphate to the corresponding nucleoside 5′-phosphoromorpholidate. Such phosphoromorpholidates in the presence of tributylammonium pyrophosphate in rigorously anhydrous dimethyl sulfoxide were converted in good yield to the nucleoside 5′-triphosphates, which could be purified directly by column chromatography. It seemed reasonable that an analogous synthetic route beginning with deoxynucleoside 3′-phosphates might lead readily to formation of the corresponding deoxynucleoside 3′-triphosphates, and it is the purpose of this report to confirm that expectation and give the details of these syntheses. In addition we report the detailed synthesis of thymidine 3′-triphosphate labeled with ^{32}P in the α position ($\alpha^{32}P$-3′-TTP).[1] Canellakis *et al.* (1965) and Ward (1963; personally communicated by Dr. G. M. Tener) have also synthesized 3′-TTP.

Experimental Section and Results

General Methods. Inorganic phosphorus (P_i) was measured by the method of King (1932) or, when greater sensitivity was desired, by the procedure of Chen *et al.* (1956); if labile phosphate esters were present, the Lowry and Lopez technique (1946) was employed. Acid-labile P was determined after heating at 100° for 8 min in the presence of 1 N $HClO_4$, and bound (total) P was measured after digestion with 70% $HClO_4$ (King method) or after ashing with 10% $Mg(NO_3)_2$ in 95% ethanol (Chen method). Purine-bound deoxyribose was estimated by the diphenylamine method of Dische (1955) with the modification that 1 hr at 100° was required for total color development in the case of 3′-deoxynucleotides. Ultraviolet spectra (200–300 mμ) were recorded on a Cary Model 15 spectrophotometer. Elemental analyses were obtained in the laboratory of Dr. A. Bernhardt, Mülheim (Ruhr), Germany, on samples dried overnight at room temperature on an oil diffusion pump.

Paper chromatography was conducted by the descending technique at room temperature using the following solvent systems: solvent I, isobutyric acid–1 M NH_4OH–0.1 M Na_4EDTA (100:60:1.6 by volume); solvent II, 1-propanol–concentrated NH_4OH–water (6:3:1 by volume); solvent III, 2-propanol–concentrated NH_4OH–water (7:1:2 by volume). The R_F values of all pertinent compounds in solvents I and II are given in Table I. Paper electrophoresis was performed by the general technique of Markham and Smith (1952) in 0.05 M ammonium bicarbonate, pH 7.5, or 0.05 M ammonium formate, pH 3.5, using a 1.5-kv potential difference. Schleicher and Schuell No. 589 Orange Ribbon paper was used throughout. Compounds were located on paper by observation under an ultraviolet lamp and/or with the phosphate-detecting spray of Hanes and Isherwood (1949), using ultraviolet development of color (Bandurski and Axelrod, 1951). When ^{32}P compounds were present, autoradiograms were made by exposure to Kodak medical X-ray film. Radioactivity was measured in a thin window, gas flow counter.

TABLE I: R_F Values of Reported Compounds.[a]

Compound	Solvent I	Solvent II[b]
3′-dAMP	0.70	0.23
3′-dAM	0.76	0.61
3′-dADP	0.60	0.15
3′-dATP	0.47	0.11
3′-dGMP	0.40	0.11
3′-dGM	0.59	0.40
3′-dGDP	0.25	0.06
3′-dGTP	0.18	0.05
3′-dCMP	0.58	0.21
3′-dCM	0.75	0.57
3′-dCDP	0.47	0.14
3′-dCTP	0.36	0.10
3′-TMP	0.50	0.26
3′-TM	0.66	0.60
3′-TDP	0.37	0.18
3′-TTP	0.31	0.13

[a] Paper chromatography was conducted by the descending technique at room temperature on Schleicher and Schuell No. 589 Orange Ribbon paper. [b] The absolute R_F values in solvent II were somewhat variable; however, the migration velocities of all of the compounds were always related proportionately.

Enzymes. Micrococcal DNAase was prepared according to Cunningham *et al.* (1956); the final fraction had a specific activity of 9000 units/mg of protein (see footnote 2 of Josse *et al.*, 1961, for assay). Calf spleen phosphodiesterase was purified through step 6 of Hilmoe's procedure (1960) and had a specific activity of 25 units/mg of protein. Snake venom phosphodiesterase, isolated according to Sinsheimer and Koerner (1952), had a specific activity of 1000 units/mg of protein (where 1 unit is that amount of enzyme which in 1 hr at 37° will release 1 μmole of mononucleotide from a limit pancreatic DNAase digest of native calf thymus DNA). The aforementioned enzymes, with the exception of calf spleen phosphodiesterase, were devoid of phosphomonoesterase activity; the contaminating phosphomonoesterase present in the calf spleen fraction was effectively inhibited by 0.025 M NaF, which did not affect the diesterase activity. Human semen phosphomonoesterase devoid of nucleoside di- and triphosphatase activity was prepared as described by Wittenberg and Kornberg (1953) and had a specific activity of 1100 units/mg of protein (see footnote 3 of Josse *et al.*, 1961, for assay). *Crotalus adamanteus* venom (Ross Allen Reptile Institute, Silver Springs, Fla.) was rich in diesterase as well as 5′-nucleotidase activity at pH

TABLE II: Properties of Deoxynucleoside 3′-Phosphates.

Compound	λ_{max} (mμ) pH 2	ϵ_{max} × 10^{-3}	Molar Ratios			Sensitivity to Enzymes[a]		
			Base	Deoxy-ribose	Total P	Semen P'tase	*E. coli* P'tase	Snake Venom
						(% degradation)		
3′-dAMP	257	14.8	1.00	1.03	0.98	>96	>99	<1
3′-dGMP	255	12.3	1.00	0.97	1.02	>97	>98	<2
3′-dCMP	278	13.0	1.00		1.00	>96	>99	<2
3′-TMP	267	9.6	1.00		0.97	>98	>96	<2
^{32}P-3′-TMP[b]	267	9.6	1.00		1.01	>97	>96	<2
					(0.98)	(>99)	(>99)	(<1)

[a] Incubations (0.05 ml) of nucleotides with enzymes were as follows: (1) Human semen phosphomonoesterase (semen P'tase): The mixture contained sodium acetate, pH 5 (10 μmoles), nucleotide (0.2 μmole), and enzyme (10 units); (2) *E. coli* alkaline phosphatase (*E. coli* P'tase): The mixture contained Tris buffer, pH 8 (10 μmoles), $MgCl_2$ (1 μmole), nucleotide (0.2 μmole), and enyzme (10 μg); (3) *Crotalus adamanteus* venom (snake venom): The mixture contained glycine, pH 8.5 (10 μmoles), $MgCl_2$ (1 μmole), nucleotide (0.2 μmole), and venom (0.1 mg). After 30 min at 37° the mixtures were chilled to 0°, 0.01 ml was chromatographed on paper in solvent I, and to the remainder were added: 0.1 ml of 2 N HCl, 0.25 ml of a Norit suspension (20% packed volume), and 0.61 ml of water. After 5 min at 0° the suspension was centrifuged, and total P in the supernatant was determined by the method of Chen *et al.* (1956). The numbers shown indicate the per cent of starting nucleotide which was degraded to nucleoside and P_i as detected by release of P into the Norit supernatant and by appearance of nucleoside and P_i (as well as disappearance of nucleotide) on the paper chromatogram. [b] Values in parentheses are based upon radioactivity measurements.

8.5 in 0.02 M $MgCl_2$ but showed virtually no activity with 2′- or 3′-nucleotides of either the ribo or deoxyribo series. *E. coli* alkaline phosphatase was purchased from the Worthington Biochemical Corp., Freehold, N. J.; this preparation was an equally potent nucleoside di- and triphosphatase as well as a phosphomonoesterase (Heppel *et al.*, 1962).

Deoxynucleoside 3′-Phosphates. These were obtained either by chemical syntheses following the method of Tener (1961) for the pyrimidine derivatives and of Schaller *et al.* (1963) for the purines, or they were isolated from enzymatic digests of DNA. Such digests were prepared by dissolving 1 g of calf thymus DNA (prepared by the method of Kay *et al.*, 1952) in 250 ml of 0.01 M Tris buffer, pH 8.6, and heating the solution at 100° for 30 min followed by rapid cooling. After addition of $CaCl_2$ (0.5 mmole) and micrococcal DNAase (100,000 units) the mixture was kept at 37° for 60 min with intermittent additions of 1 N NaOH to maintain the pH between 8.4 and 9.0. Following neutralization with 2 N HCl (0.5 to 1.0 ml), NaF (6.2 mmoles, 260 mg) and calf spleen phosphodiesterase (40 units) were added, and incubation at 37° was continued. (Turbidity due to precipitated CaF_2 did not affect the digestion.) Completeness of digestion was judged by conversion of ultraviolet-absorbing material to four mononucleotide spots on paper electrophoresis (0.05 M ammonium formate, pH 3.5); 2 hr was usually sufficient, after which time 0.5 ml of 5 N NaOH was added to bring the pH to 9, and the solution was heated (100°, 10 min). After passage through a coarse sintered-glass filter, the filtrate (containing 1.5 mmoles of nucleotide phosphorus) was applied to a 3.8 cm^2 × 12.0 cm column of Dowex 1-acetate (8% cross-linked, 200–400 mesh) and washed on with 200 ml of water. Chromatography was carried out at 2° by stepwise elution with solutions of ammonium acetate at pH 4.3 according to Sinsheimer and Koerner (1951). The following deoxynucleoside 3′-phosphates in the amounts shown in parentheses were eluted with the indicated resin bed volumes of ammonium acetate buffers: 5-Me-3′-dCMP (0.005 mmole), 30–35 volumes of 0.05 M buffer; 3′-dCMP (0.276 mmole), 40–80 volumes of 0.05 M buffer; 3′-TMP (0.408 mmole), 15–45 volumes of 0.15 M buffer; 3′-dAMP (0.391 mmole), 20–80 volumes of 0.25 M buffer; 3′-dGMP (0.288 mmole), 5–30 volumes of 1.0 M buffer. The respective nucleotide pools were chilled to 0°, glacial acetic acid was added to bring the pH to 3.5, and equal amounts of Darco G-60[2] and Celite 535[2] were added (2 g each/mmole of nucleotide). After stirring for 10 min in the cold, each suspension was filtered through a coarse sintered-glass filter, and the filtrate, which invariably contained less than 2% of the original ultraviolet-absorbing material, was discarded. Following exhaustive washing of the charcoal with cold water until the odor of acetic acid disap-

[2] Darco G-60, a product of the Atlas Powder Co., Wilmington, Del., was treated first with boiling concentrated HCl, then washed exhaustively with water with removal of fines, partially inactivated with 2-octanol according to Threlfall (1957), and finally washed with water and dried at 110°. Celite 535 (Johns Manville Co.) was washed several times with water with concomitant removal of fines.

TABLE III: Product Distributions of 3′-Deoxynucleoside Polyphosphates.

Deoxynucleoside	% Yields Based upon Starting Material				
	Phosphoro-morpholidate	Mono-phosphate	Di-phosphate	Tri-phosphate	Isolated Na Salt of Triphosphate
Deoxyadenosine	3	5	16	76	72
Deoxyguanosine	17	6	7	59	56
Deoxycytidine	19	8	11	57	52
Deoxythymidine[a]	8 (12)	6 (5)	6 (3)	75 (74)	71 (67)

[a] The values in parentheses are those obtained in the preparation of ^{32}P-labeled deoxythymidine derivatives; they are based upon radioactivity as well as absorbancy measurements.

peared, 50% ethanol containing 3 ml of concentrated NH_4OH/l. was percolated through the charcoal at room temperature over a period of 15–30 min. The filtrate (1 ml of ammoniacal ethanol/μmole of nucleotide adsorbed to charcoal) now contained over 90% and usually close to 100% of the previously adsorbed nucleotide in a salt-free form; evaporation to dryness followed by two evaporations with 25-ml portions of water removed all traces of ammonia and ethanol.

In all cases the isolated deoxynucleoside 3′-phosphates were homogeneous on paper chromatography with R_F values given in Table I. They had spectra (200–300 mμ) indistinguishable from the corresponding deoxynucleoside 5′-phosphates except for 3′-dCMP, which had an absorption maxima at 278 mμ (pH 2) rather than 280 mμ. The molar ratios of purine deoxyribose and bound P to base (using the indicated molar absorptivities) are recorded in Table II. No free P_i by the method of Lowry and Lopez (1946) was detected in any of the preparations. The enzymatic studies also cited in Table II show that greater than 96% of the P could in each case be released from the nucleotide (rendered nonadsorbable to charcoal with resultant disappearance of nucleotide and formation of nucleoside and P_i as detected by paper chromatography) by treatment with either human semen phosphomonoesterase or *E. coli* alkaline phosphatase. However, an amount of crude snake venom sufficient to release greater than 98% of the P from a deoxynucleoside 5′-phosphate had no significant effect on any of the deoxynucleoside 3′-phosphates (Table II).

Deoxynucleoside 3′-Phosphoromorpholidates. Syntheses followed the procedure of Moffatt and Khorana (1961) except that in most cases the system was scaled down by a factor of 5. In these instances 0.2 mmole of deoxynucleoside 3′-phosphate (unless originally free acid) was first converted to the morpholinium salt by passage over a 0.25 $cm^2 \times 10$ cm column of Dowex 50-morpholinium resin (2% cross-linked, 200–400 mesh). The effluent and washings were concentrated to 5 ml and added to 5 ml of *t*-butyl alcohol containing 0.6 mmole of morpholine (0.05 ml) and a Hengar granule. A solution of dicyclohexylcarbodiimide (0.8 mmole, 165 mg) in 5 ml of *t*-butyl alcohol was added dropwise to the refluxing mixture over 2 hr and washed in with 0.5 ml of *t*-butyl alcohol. Refluxing was continued until paper electrophoresis (0.05 M ammonium bicarbonate, pH 7.5) indicated complete conversion of deoxynucleoside 3′-phosphate to the more slowly moving phosphoromorpholidate. If after 3 hr starting material still remained, a mixture of 0.2 mmole of dicyclohexylcarbodiimide (40 mg) and 0.2 mmole of morpholine (0.02 ml) in 1 ml of *t*-butyl alcohol was added, and refluxing was continued another hour. By this time conversion to phosphoromorpholidate was invariably 98% or greater, although on paper electrophoresis there was often a small amount (less than 2%) of ultraviolet-absorbing material traveling just ahead of the phosphoromorpholidate spot but well behind the original, more rapidly moving, deoxynucleoside 3′-phosphate. In the case of the thymine derivatives this third spot had a mobility identical with that of authentic thymidine 3′,5′-cyclic phosphate. The mixture was then cooled to room temperature, concentrated *in vacuo* to remove most of the *t*-butyl alcohol, and shaken with 15 ml of ether. The suspension was filtered through glass wool, and the filtrate was extracted twice with 15-ml portions of ether. The remainder of the procedure was exactly as described by Moffatt and Khorana (1961), and the final white powders (4-morpholine *N,N′*-dicyclohexylcarboxamidinium salts of the respective deoxynucleoside 3′-phosphoromorpholidates) were analyzed as follows.

(a) Deoxyadenosine 3′-phosphoromorpholidate was obtained in 98% yield as a tetrahydrate. *Anal.* Calcd for $C_{31}H_{52}N_9O_7P \cdot 4H_2O$: C, 48.68; H, 7.90; N, 16.47; P/adenine, 1.00. Found: C, 48.61; H, 8.09; N, 16.08; P/adenine, 1.04.

(b) Deoxyguanosine 3′-phosphoromorpholidate was obtained in 98% yield as a hexahydrate. *Anal.* Calcd for $C_{31}H_{52}N_9O_8P \cdot 6H_2O$: C, 45.54; H, 7.90; N, 15.41; P/guanine, 1.00. Found: C, 45.01; H, 7.35; N, 15.97; P/guanine, 0.98.

(c) Deoxycytidine 3′-phosphoromorpholidate was obtained as the dihydrate in 77% yield. *Anal.* Calcd for $C_{30}H_{52}N_7O_8P \cdot 2H_2O$: C, 51.10; H, 8.00; N, 13.91; P/

TABLE IV: Properties of Deoxynucleoside 3′-Di- and Triphosphates.

Compound	Molar Ratios				Sensitivity to Enzymes[a]					
	Base	Deoxy-ribose	Acid-Labile P	Total P	Venom P'diest.	Spleen P'diest.	Microc. DNAase	Semen P'tase	*E. coli* P'tase	Snake Venom
					(% degradation)					
3′-dADP	1.00	1.01		1.91	<1	11	6	<2	>98	<2
3′-dATP	1.00	0.99		2.97	<1	7	5	<1	>96	<2
3′-dGDP	1.00	1.05		2.06	<2	9	4	<3	>96	<1
3′-dGTP	1.00	0.98		2.95	<1	10	5	<1	>99	<2
3′-dCDP	1.00		1.01	1.98	<1	10	4	<2	>97	<2
3′-dCTP	1.00		2.04	2.98	<2	7	3	<3	>98	<2
3′-TDP	1.00		0.98	2.01	<2	9	5	<2	>98	<2
3′-TTP	1.00		2.07	3.08	<1	8	6	<2	>98	<2
α^{32}P-3′-TDP[b]	1.00		1.03	1.94	<1	8	4	<2	>99	<2
α^{32}P-3′-TTP[b]	1.00		2.05	2.94	<1	9	5	<3	>97	<1

[a] Incubations (0.05 ml) of nucleotides with enzymes were as follows: (1) Snake venom phosphodiesterase (venom P'diest.): the mixture contained glycine, pH 8.5 (10 μmoles), $MgCl_2$ (1 μmole), nucleotide (0.2 μmole), and enzyme (15 units); (2) calf spleen phosphodiesterase (spleen P'diest.): the mixture contained sodium succinate, pH 6.5 (10 μmoles), NaF (1 μmole), nucleotide (0.2 μmole), and enzyme (1 unit); (3) micrococcal DNAase (microc. DNAase): the mixture contained Tris buffer, pH 8.6 (10 μmoles), $CaCl_2$ (1 μmole), nucleotide (0.2 μmole), and enzyme (2000 units). Incubations with semen P'tase, *E. coli* P'tase, and snake venom were as described in Table II. In all cases after 30 min at 37° the mixtures were chilled and treated exactly as outlined in Table II. The numbers shown indicate the per cent of starting nucleotide which was degraded by the respective enzymes as measured by release of P into the Norit supernatant and by appearance of compounds other than starting material on the paper chromatogram. [b] Radioactivity measurements indicated α^{32}P/thymine ratios of 1.03 and 0.99 for α^{32}P-3′-TDP and α^{32}P-3′-TTP, respectively.

cytosine, 1.00. Found: C, 51.00; H, 8.06; N, 14.00; P/cytosine, 1.05.

(d) Thymidine 3′-phosphoromorpholidate was obtained as the tetrahydrate in 84% yield. *Anal.* Calcd for $C_{31}H_{53}N_6O_9P \cdot 4H_2O$: C, 49.20; H, 8.12; N, 11.10; P/thymine, 1.00. Found: C, 48.56; H, 8.06; N, 11.24; P/thymine, 1.05.

Ultraviolet spectra (200–300 mμ) were similar to those of the respective deoxynucleoside 3′-phosphates (Table II) except for increased end absorption below 240 mμ due to the carboxamidine salt. Except for the above-noted contamination, probably with the respective deoxynucleoside 3′,5′-cyclic phosphates, the products appeared homogeneous both by paper electrophoresis and chromatography with the R_F values recorded in Table I.

Deoxynucleoside 3′-Di- and Triphosphates. Synthesis and isolation procedures previously outlined for nucleoside 5′-triphosphates (Moffatt, 1964) were closely followed and scaled down when necessary. The limited solubility of 3′-dCM and 3′-dGM in anhydrous pyridine during the drying procedure was overcome by preliminary solution of these in 3 ml of dimethylformamide (stored over Molecular Sieve type 4A, Linde Co., Los Angeles) followed by addition of 5 ml of pyridine (dried with calcium hydride). The condensation reactions were followed by paper chromatography in solvents I and II and were largely complete within 2 days. Chromatography on columns of DEAE-cellulose (Brown Co., Berlin, N. H.) in the bicarbonate form, using linear gradients of triethylammonium bicarbonate as outlined earlier, yielded discrete peaks of ultraviolet-absorbing material with recoveries of various products shown in Table III. The diphosphates were invariably contaminated with large amounts of PP_i, and in the cases of 3′-dATP and 3′-dGTP there was also slight PP_i contamination. In these instances the nucleotides were further purified by adsorption on charcoal (Darco G-60) following the technique described above (see under Deoxynucleoside 3′-Phosphates).

The tetrasodium salts of 3′-dATP, 3′-dGTP, 3′-dCTP, and 3′-TTP were obtained as the octahydrate, hexahydrate, trihydrate, and trihydrate, respectively. The products appeared homogeneous by paper chromatography in solvents I and II with R_F values recorded in Table I. All had ultraviolet spectra (200–300 mμ) identical with those of the respective deoxynucleoside 3′-phosphates (Table II). Table IV lists the stoichiometry of base, sugar, and P, as well as the results of enzymatic studies. The deoxynucleoside 3′-di- and triphosphates were virtually unaffected by incubation with snake venom phosphodiesterase, less than 2% degradation occurring under conditions that completely degraded the corresponding 5′-derivatives (Razzell, 1963). Similar results were obtained using crude snake venom. Large excesses of calf spleen phosphodiesterase and micrococcal DNAase led to only

slight degradation of the 3′-tri- and diphosphates to P_i and di- and monophosphates, respectively (Table IV). It is likely, however, that these marginal hydrolyses are the results of contaminating nonspecific phosphatases since hydrolysis of the corresponding 5′-di- and triphosphates occurred to the same extent. All of the 3′-di- and triphosphates were totally hydrolyzed to the nucleoside and P_i by *E. coli* alkaline phosphatase but were almost totally resistant to the action of human semen phosphomonoesterase. In addition to these purely enzymatic tests it was possible to degrade chemically the pyrimidine deoxynucleoside 3′-di- and triphosphates. Treatment with 1 N $HClO_4$ at 100° for 8 min released the expected amounts of acid-labile phosphate leaving the appropriate deoxynucleoside 3′-monophosphates which could be isolated by paper chromatography. The latter compounds, when tested by the procedures in Table II, were then found to be susceptible to human semen phosphomonoesterase and *E. coli* alkaline phosphatase (greater than 96% degradation to nucleoside and P_i in each case), but refractory (less than 2% hydrolysis) to an amount of crude snake venom which completely degraded the 5′-nucleotides obtained by a similar acid treatment of the corresponding 5′-di- or triphosphates.

α-^{32}P-Labeled Thymidine 3′-Di- and Triphosphates. 1. ^{32}P-LABELED THYMIDINE 3′-PHOSPHATE. The general procedure of Tener (1961) was modified as follows: the synthesis of ^{32}P-2-cyanoethyl phosphate, described by Pfitzner and Moffatt (1964), was carried out on a 0.15-mmole scale using 15 mcuries of ^{32}P-orthophosphoric acid (Oak Ridge National Lab.). The yield of crystalline barium salt was 45 mg (93%), and the product was greater than 98% pure as judged by autoradiography of a paper chromatogram run in solvent III. After conversion of the barium salt to the free acid with Dowex 50-H^+ (0.25 cm^2 × 10 cm column) ^{32}P-2-cyanoethyl phosphate (0.14 mmole) was taken to dryness and rendered anhydrous by three evaporations with 5-ml portions of pyridine. 5′-O-Tritylthymidine (0.42 mmole, 236 mg) prepared according to Gilham and Khorana (1958) was added, followed by a final evaporation with 5 ml of pyridine. The residue was dissolved in 5 ml of pyridine, dicyclohexylcarbodiimide (0.42 mmole, 87 mg) was added, and the sealed mixture was incubated in the dark at room temperature for 2 days. (Autoradiography of a paper chromatogram run in solvent III showed that less than 5% of the ^{32}P-2-cyanoethyl phosphate remained.) Water (1 ml) was added, and after 1 hr at room temperature the mixture was taken to dryness and then evaporated with 10 ml of water and two 10-ml portions of ethanol. The residue was suspended in 10 ml of 25% acetic acid, heated at 100° for 30 min, and filtered through glass wool. The clear filtrate plus water washings of the glass wool were taken to dryness and evaporated once with 5 ml of water and twice with 5-ml portions of ethanol. This residue was dissolved in 0.5 N LiOH (5 ml), heated at 100° for 45 min, treated with Dowex 50-H^+ resin until the pH reached 9, and filtered through glass wool. Barium acetate (0.42 mmole) was added to the filtrate, and the mixture was quickly centrifuged to remove traces of barium phosphate. The barium salt of ^{32}P-3′-TMP was then precipitated by addition of three volumes of ethanol and collected by centrifugation; following washings with 10-ml portions of 50% ethanol, ethanol, and ether, the precipitate was dried *in vacuo*. The product obtained in 80% yield (62 mg) as the tetrahydrate was homogeneous and identical with unlabeled 3′-TMP by paper electrophoresis (0.05 M ammonium bicarbonate, pH 7.5) and by paper chromatography in solvents I and II. The specific activity was 10^8 cpm/μmole; other analytical properties are summarized in Table II.

2. ^{32}P-LABELED THYMIDINE 3′-PHOSPHOROMORPHOLIDATE. The technique followed that described above for 0.2 mmole of unlabeled nucleotide except that it was proportionately scaled down by a factor of 2 for treatment of 0.1 mmole of ^{32}P-3′-TMP, and all steps after the third ether extraction were omitted. The yield based upon radioactivity was 90%, and the P/thymine ratio was 1.03. Except for slight contamination (less than 5%), presumably with the ^{32}P-thymidine 3′,5′-cyclic phosphate, autoradiograms indicated that the product was homogeneous and identical with unlabeled 3′-TM by paper electrophoresis (0.05 M ammonium bicarbonate, pH 7.5) and by paper chromatography in solvents I and II.

3. α-^{32}P-LABELED THYMIDINE 3′-DI- AND TRIPHOSPHATES. The aqueous phase from step 2 above was taken to dryness and evaporated once with 10 ml of water. The residue was treated with anhydrous pyridine and benzene, as with unlabeled 3′-TM, and the phosphorylation reaction, scaled down for 0.1 mmole of starting phosphoromorpholidate, was complete in 2 days. Chromatography on DEAE-cellulose (bicarbonate form) yielded four discrete radioactive and ultraviolet-absorbing peaks with recoveries recorded in Table III. The α-^{32}P-3′-TDP and α-^{32}P-3′-TTP (specific activity: 10^8 cpm/μmole) products were homogeneous and identical with unlabeled markers in their paper chromatographic behavior in solvents I and II and spectral properties (Table II). Analytical properties and results of enzymatic studies are listed in Table IV. In addition the 3′ nature of the sugar phosphate linkage was confirmed by enzymatic testing of the mononucleotide obtained after treatment with 1 N $HClO_4$ for 8 min at 100°. These procedures were exactly like those described above for unlabeled 3′-TDP and 3′-TTP and, in addition, radioactivity determinations indicated less than 1% degradation of the resultant ^{32}P-3′-TMP by crude snake venom.

Discussion

The successful syntheses of the four deoxynucleoside 3′-triphosphates should permit a careful search for the possible roles of these derivatives in pathways of nucleotide metabolism and possibly in nucleic acid synthesis. Although the recoveries were in general slightly less than those in the corresponding syntheses of the 5′ derivatives (Moffatt, 1964), and there was the

above-noted contamination of certain of the compounds with minor amounts of PP_i following chromatography on DEAE-cellulose, these difficulties have not represented a serious handicap, and the general utility of the phosphoromorpholidate route of nucleoside polyphosphate synthesis has been further confirmed. The yields of deoxynucleoside 3′-diphosphates in these syntheses were small and incidental; predominant yields of diphosphates should be specifically obtained if tributylammonium orthophosphate (rather than pyrophosphate) is employed in the phosphorylation step.

In view of the occurrence of enzymes capable of synthesizing deoxynucleoside 3′-triphosphates, it is interesting to speculate upon the possible existence of a DNA polymerase utilizing these substrates. Such an enzyme, together with the normal Kornberg polymerase, would rationalize the *in vivo* synthesis of both polydeoxynucleotide chains of the DNA helix which are of opposite polarity (Schildkraut *et al.*, 1964). The search for such an enzyme is in progress. No such polarity problem is evident in *in vivo* RNA synthesis, which appears to entail transcription of only one of the complementary DNA chains (Spiegelman and Hayashi, 1963; Marmur *et al.*, 1963; Hall *et al.*, 1963; Bautz, 1963), and a single enzyme utilizing ribonucleoside 5′-triphosphates should suffice.

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