nature of the bases is critically important in producing precipitates. Although there may be other explanations of the phenomena which also fit all the data, the present postulates seem to us to be the most reasonable in light of the presently available evidence.

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Conversion of Mono- and Oligodeoxyribonucleotides to 5′-Triphosphates[1]

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Reaction of the phosphorimidazolidate formed from a nucleotide and 1,1′-carbonyldiimidazole with inorganic pyrophosphate provides the nucleoside triphosphate in good yield. The method is convenient, generally applicable, and particularly suitable for microscale syntheses from mono- or oligonucleotides.

The immediate precursors in the replication of DNA are the deoxyribonucleoside 5′-triphosphates.[2] An interesting question is the possible ability of oligonucleotides bearing 5′-triphosphate end groups to function as substrates in enzymatic DNA and polynucleotide syntheses. Despite a negative result recently reported,[3] the question warrants further investigation in view of preliminary indications in this laboratory that certain dinucleotide derivatives are incorporated, without degradation, into DNA synthesized by DNA polymerase from *E. coli*.[4] Adequate inquiry into this and related questions requires the availability of suitable substrates. A method for the synthesis of 5′-triphosphates of mono- and oligonucleotides, adaptable to a microscale and suitable for radioactively labeled materials, is thus highly desirable.

Chemical methods, as opposed to enzyme-catalyzed synthesis,[5] would appear to be more uniformly applicable for this purpose. The method which has been most widely employed,[6] however, is not readily adaptable to small-scale preparations. An approach involving condensation of an activated nucleotide derivative, *e.g.*, a nucleoside 5′-phosphoromorpholidate,[7] with inorganic pyrophosphate[8] offers greater versatility and a minimum of side reactions, although preparation of the intermediate in optimal yield on a small scale is difficult owing to the requirement of slow addition of a reagent to a reaction mixture. Cramer and co-workers[9] have demonstrated that phosphorimidazolidates are useful intermediates for synthesis of pyrophosphate bonds, and that these amides can be readily prepared under very mild conditions from a phosphomonoester and 1,1′-carbonyldiimidazole.

The series of reactions illustrated in Figure 1 have been successfully applied[10] to microscale preparations of 5′-triphosphates of various deoxyribonucleotides. Formation of the imidazolidate (III) from a nucleotide (I) and excess 1,1′-carbonyldiimidazole (II) is complete in about 1 hr. at room temperature. Unreacted II is decomposed with methanol, before inorganic pyrophosphate (IV) is added, to eliminate formation and subsequent necessity for separation of inorganic polyphosphates. Owing to the precipitation of imidazolium pyrophosphate, an excess of IV is used. The phosphorylation is essentially complete within 24 hr.,

Table I. Distribution of Products in Triphosphate Synthesis

Parent nucleotide (I)[a]	Monophosphate (I)	Imidazolidate (III)	Diphosphate (VI)	Triphosphate (V)	Other[b]	Isolated salt of triphosphate
pT	8	3	8	66	2	50
d-pC	7[c]	0	7	56	4	45
d-pA	1	6	4	73[d]	5	36[e]
d-pG	1	3	8[d]	70[f]	3	59[f]
pTpT	11	..	8[f]	76	4	60
d-pTpC	3	..	8[f]	51[g]	3	20[e]
pTpTpT	3[f]	..	6[f]	81	2	70
d-pApT	17	2	19	50[f]	4	..

[a] Abbreviations conform to *J. Biol. Chem.*, **238**, 6 (1963). [b] Includes all ultraviolet-absorbing material eluted after triphosphate peak. [c] P^1,P^2-dinucleoside pyrophosphate (VII) also detected by paper chromatography. [d] Incompletely resolved from an impurity having parent nucleotide spectrum. [e] From homogeneous portion of peak only. [f] A second substance with parent nucleotide spectrum detectable by paper chromatography. [g] Incompletely resolved from material with altered spectrum.

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.
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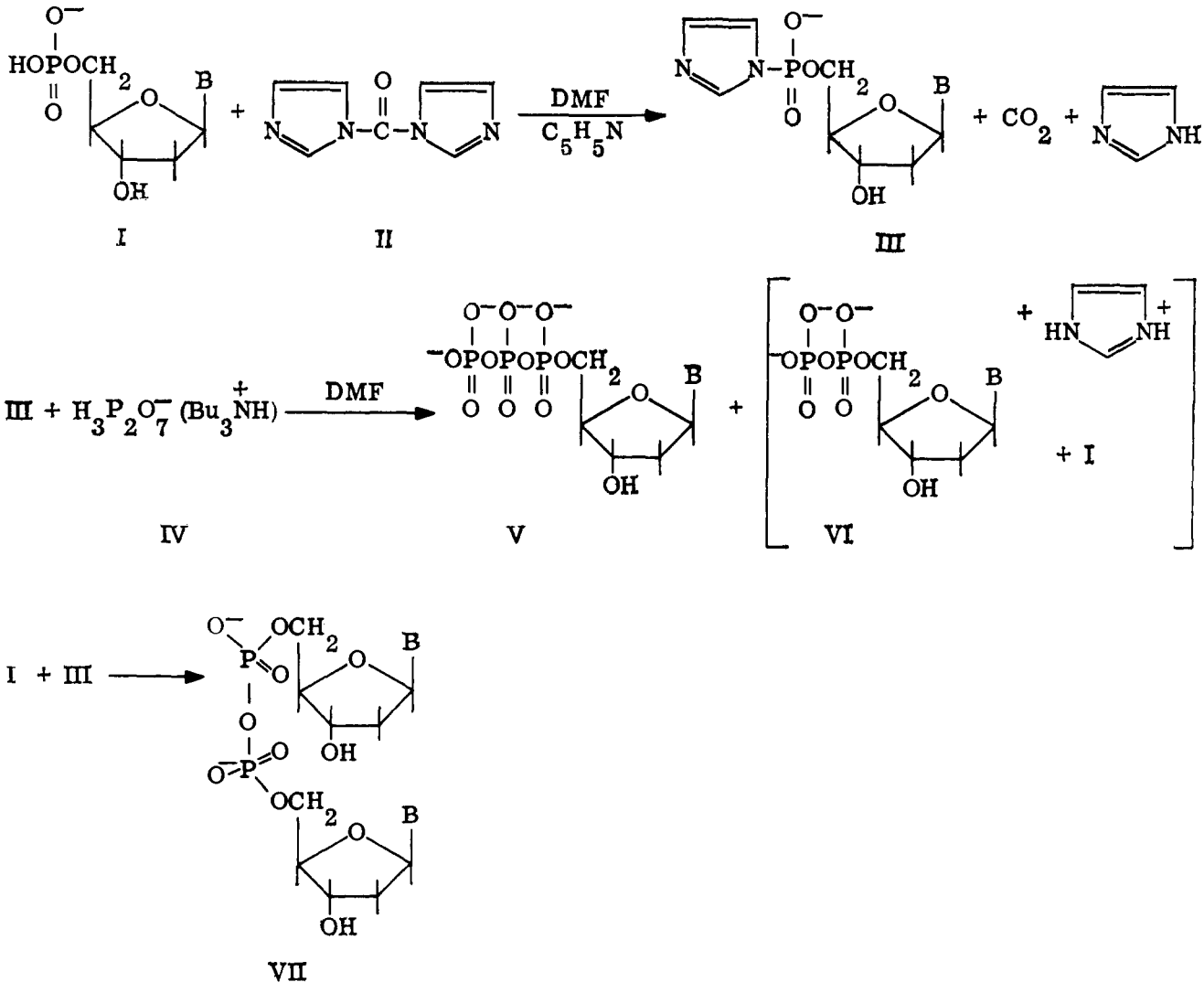

and the product is purified by anion-exchange chromatography on DEAE-cellulose followed by conversion to the sodium salt.

Results obtained with various mono- and oligonucleotides are given in Table I. The desired triphosphates (V) are the principal products formed. During the phosphorylation, limited hydrolysis of a portion of III gives some monophosphate I, and further reaction of these two materials gives rise to the symmetrical pyrophosphate VII.[7,11] This by-product, under the slightly alkaline conditions used for chromatography, has the same anionic charge as the monophosphate I if from a mononucleotide, the same charge as the diphosphate VI if from a dinucleotide, and the same charge as the triphosphate V if from a trinucleotide. Consequently, elimination from the product V of any VII which may be formed requires further purification only in the case of the trinucleotide, *e.g.*, by ion-exchange chromatography at a lower pH where V has one less charge than VII. Other by-products, such as the diphosphate VI and the analogous tetraphosphate, were well resolved from the triphosphate. In certain cases the product peak from ion-exchange chromatography was shown by paper chromatography to be inhomogeneous or incompletely resolved from a contaminant, and only the homogeneous portion was converted to the sodium salt. These impurities, and the one persisting in deoxyguanosine 5′-triphosphate, have not as yet been characterized nor have attempts been made to separate them by other techniques, although these would provide better yields of products in such cases.

It is evident that this method for synthesis of triphosphates is applicable to oligonucleotides as well as mononucleotides and that gross decomposition or rearrangement does not occur. The over-all yields are not as good as those reported for the morpholidate method[8]; this may reflect the lower reactivity of the imidazolidate or may be a consequence of moisture in the system, since rigorously anhydrous conditions were not stressed. The imidazolidate procedure does offer the advantages of simplicity and adaptability to microscale syntheses.

Experimental

Analytical Methods. Descending paper chromatography was carried out on Whatman No. 31 paper employing the following solvent systems: solvent A, 2-propanol–concentrated ammonium hydroxide–water (65:10:25, v./v.); solvent B, 2-propanol–0.25 *M* aqueous ammonium bicarbonate (65:35 v./v.); solvent C, isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* aqueous disodium ethylenediaminetetraacetate (100:60:1.6, v./v.). Mean R_f values appear in Table II

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Table II. Paper Chromatography R_f Values of Reaction Products and Intermediates[a]

Parent nucleotide (I)	Monophosphate (I)			Imidazolidate (III)			Triphosphate (V)			Diphosphate (VI)			Pyrosphosphate (VII)		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
pT	0.40			0.79			0.24			0.31			0.55		0.31
d-pC	0.35		0.56	0.73			0.18		0.24	0.29		0.35	0.51		0.75
d-pA		0.42	0.77		0.78			0.22	0.63		0.34	0.72		0.51	0.87
d-pG		0.23	0.56		0.61			0.13	0.38		0.18	0.47			
pTpT		0.39	0.57					0.23	0.38						
d-pTpC		0.32	0.69					0.22	0.48						
pTpTpT	0.23		0.46				0.18		0.30						
d-pApT			0.63						0.47			0.54			

[a] Solvents A, B, and C as defined in Experimental.

Nucleotide spots were located by observation under an ultraviolet lamp. Phosphorus-containing material on chromatograms was located with the spray reagent of Hanes and Isherwood,[12] using ultraviolet irradiation[13] for final color development of the spots. Samples were analyzed for total phosphorus[14] after ashing by the method of Ames and Dubin[15] using a modification of the procedure outlined by Bartlett.[16]

Tributylammonium Pyrophosphate. To an aqueous solution of pyridinium pyrophosphate, obtained by passing a solution of tetrasodium pyrophosphate decahydrate (446 mg., 1 mmole) through a column of Dowex 50W-X4 (pyridinium) resin (17 ml.), was added tributylamine (0.24 ml., 1 mmole). The solution was concentrated under vacuum and the sirupy residue was dried by repeated addition and evaporation of anhydrous pyridine followed by addition and evaporation of two 10-ml. portions of N,N-dimethylformamide (DMF).

Deoxyribonucleoside 5'-Triphosphates. General Procedure.[17] To a solution or suspension of the mono- or oligonucleotide (0.1 mmole) as the anhydrous tributylammonium salt,[18] in 1 ml. of DMF, was added 1,1'-carbonyldiimidazole (80 mg., 0.5 mmole) in 1 ml. of DMF. The mixture, in a tightly stoppered container, was shaken for 30 min., held in a desiccator at room temperature for 4 hr. or overnight, and then treated with methanol (33 μl., 0.8 mmole). After 30 min. at room temperature, tributylammonium pyrophosphate (0.5 mmole) in 5 ml. of DMF was added with vigorous mixing, and the stoppered mixture was held at room temperature in a desiccator for 1 day. The precipitate (imidazolium pyrophosphate) was removed and washed with DMF (four 1-ml. portions) by centrifugation.[19] The supernatant was treated with an equal volume of methanol, and the solution was evaporated under vacuum to dryness. The residue was chromatographed on a DEAE-cellulose column (2 × 20 cm.) with a linear gradient of triethylammonium bicarbonate (usually 0–0.4 *M* in 3 l.), pH 7.5. Appropriate fractions were evaporated under vacuum, and the triethylammonium bicarbonate was removed by addition and evaporation of ethanol. The residue of triethylammonium nucleoside triphosphate was dissolved in methanol to give a concentration of *ca.* 0.05 *M*, and five volumes of an acetone solution of sodium perchlorate (15 equiv.) was added. The precipitated sodium salt was collected by centrifugation, washed with acetone (four 1-ml. portions), and dried over phosphorus pentoxide under vacuum; yields for this step varied from 71 to 99%. Yields and distribution of by-products for various syntheses are recorded in Table I. Analytical and spectral data for the various products are given below.

(*a*) *Thymidine 5'-triphosphate* gave at pH 2 λ_{max} 267 mμ (ε (P) 3180).

Anal. Calcd. for $C_{10}H_{13}N_2O_{14}P_3Na_4 \cdot H_2O$ (588): P, 15.8. Found: P, 16.0.

(*b*) *2'-Deoxycytidine 5'-triphosphate* gave at pH 2 λ_{max} 280 mμ (ε (P) 4240); at pH 7 λ_{max} 270 mμ (ε (P) 2960).

Anal. Calcd. for $C_9H_{12}N_3O_{13}P_3Na_4 \cdot 2H_2O$ (591): P, 15.7. Found: P, 15.7.

(*c*) *2'-Deoxyadenosine 5'-Triphosphate.* The product was not completely resolved by column chromatography from an unidentified impurity which had R_f 0.78 in solvent C (slightly greater than for the mononucleotide) and was not resolved from the triphosphate in solvent B. The sodium salt was isolated from the homogeneous portion of the peak; at pH 7 λ_{max} 259 mμ (ε (P) 4880).

Anal. Calcd. for $C_{10}H_{12}N_5O_{12}P_3Na_4 \cdot 2H_2O$ (615): P, 15.1. Found: P, 15.1.

(*d*) *2'-Deoxyguanosine 5'-Triphosphate.* The product from column chromatography contained a second component having the parent nucleotide spectrum and R_f 0.62 in solvent C, but with the same R_f as the triphosphate in solvent B. This contaminant was still present in the isolated sodium salt; at pH 7 λ_{max} 252 mμ (ε (P) 4830).

Anal. Calcd. for $C_{10}H_{12}N_5O_{13}P_3Na_4 \cdot 4H_2O$ (667): P, 13.9. Found: P, 13.6.

(*e*) *Thymidylyl-(5'→3')-thymidine 5'-triphosphate*[20] gave at pH 2 λ_{max} 267 mμ (ε (P) 4670).

(12) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).
(13) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(14) D. F. Petersen, L. B. Cole, E. H. Lilly, and V. E. Mitchell, Los Alamos Scientific Laboratory Report LAMS-2780, 1962, p. 300.
(15) B. N. Ames and D. T. Dubin, *J. Biol. Chem.*, **235**, 769 (1960).
(16) G. R. Bartlett, *ibid.*, **234**, 466 (1959).
(17) Quantities are given for 0.1-mmole scale reaction; satisfactory syntheses have been performed at the 2-μmole level by using proportionate amounts.
(18) Prepared from the pyridinium salt by addition of 1 mole of tributylamine per g.-atom of phosphorus, followed by repeated evaporation of anhydrous pyridine and DMF. Prior to addition of carbonyldiimidazole, nucleotides d-pA and d-pC were largely insoluble in DMF.
(19) Recovery at this point (spectrophotometric evaluation) was quantitative except in the preparations from d-pC (80%), pTpT (90%), and d-pTpC (87%).
(20) The starting material, pTpT, was prepared by condensation of $O^{3'}$-acetylthymidine 5'-phosphate and 2-cyanoethyl 5'-thymidylate with DCC.[10] The specifically double-labeled analog was prepared by D. L. Williams *via* the sequence H^3-T + pT-2-C^{14} ⟶ (H^3-T)p(T-2-C^{14}) → p(H^3-T)p(T-2-C^{14}).

Anal. Calcd. for $C_{20}H_{25}N_4O_{21}P_4Na_5 \cdot 5H_2O$ (986): P, 12.3. Found: P, 12.3.

The doubly labeled compound, *thymidylyl-2-C*^{14}*-(5′→3′)-H*^{3}*-thymidine 5′-triphosphate*, was similarly prepared but on a 10-μmole scale.

(f) 2′-Deoxycytidylyl-(5′→3′)-thymidine 5′-triphosphate[21] gave at pH 2 λ_{max} 273 mμ (ε (P) 4785).

Anal. Calcd. for $C_{19}H_{24}N_5O_{20}P_4Na_5 \cdot 7H_2O$ (1007): P, 12.3. Found: P, 12.3.

(g) Thymidylyl-(5′→3′)-thymidylyl-(5′→3′)-thymidine 5′-Triphosphate.[22] Paper chromatography of the sodium salt [λ_{max} 267 mμ (ε (P) 5560)] obtained from an apparently symmetrical peak on column chromatography gave a second spot, R_f 0.50, in solvent C. Gel filtration chromatography on Sephadex G-75[23] gave a single symmetrical peak which emerged somewhat in advance of tetrathymidylic acid (pTpTpTpT) used as a reference. Rechromatography on DEAE-cellulose, chloride form, with a linear gradient of lithium chloride buffered at pH 5.6 with 0.01 *M* lithium acetate, effected no noticeable change of the paper chromatography results. Elution and rechromatography of the triphosphate spot in solvent C gave the R_f 0.50 spot, in addition to the product spot, indicating, at least in part, an artifact of the chromatographic procedure. The hygroscopic lithium salt was isolated by evaporation of the column effluent and repeated extraction with methanol and acetone to remove lithium chloride and lithium acetate; at pH 2 λ_{max} 267 mμ (ε (P) 5660).

Anal. Calcd. for $C_{30}H_{37}N_6O_{28}P_5Li_6 \cdot 16H_2O$ (1414): P, 11.0. Found: P, 11.0.

(h) Thymidylyl-(5′→3′)-2′-deoxyadenosine 5′-triphosphate[24] gave at pH 8 λ_{max} 259 mμ (ε (P) 5500).

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(21) The starting material, d-pTpC, was prepared by D. L. Williams by condensation of N^4, O$^{3'}$-diacetyl-2′-deoxycytidine 5′-phosphate and 2-cyanoethyl 5′-thymidylate with DCC.[10]

(22) The starting material, pTpTpT, was obtained by polymerization of pT with DCC by the method of H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

(23) F. N Hayes, E Hansbury, and V. E. Mitchell, *J Chromatog.*, **16**, 410 (1964).

(24) The starting material, d-pApT, was isolated following condensation of N^6-benzoyl-2′-deoxyadenosine 5′-phosphate and excess O$^{3'}$-acetylthymidine 5′-phosphate with DCC.[10]

On the Steady-State Method of Enzyme Kinetics

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A unified analytical treatment has been developed for the continuous description of the transient-state and steady-state phases of reaction according to the enzymic mechanism of Michaelis and Menten. This allows a separation of the two sources of errors attending the usual steady-state solution. The relative error δ_c arises from the omission of the complementary function from the unified solution, and the relative error δ_p accompanies an approximation to the particular integral. Equations and inequalities relating δ_c and δ_p to experimental observables have been derived. The consideration of errors indicates that the validity of the steady-state assumption does not depend upon the concentrations of reaction intermediates remaining stationary, but merely upon the elimination of the time as an explicit variable governing these concentrations.

Introduction

Since exact solutions to the differential equations arising from the formulation of enzyme kinetics are not obtainable, approximate solutions are required as the basis of experimental analysis. Among these, the steady-state solutions introduced by Briggs and Haldane[1] have come to constitute the principal means for defining the kinetic properties of enzymic systems. More recently, a complete generalization of the steady-state method has been achieved in the form of the schematic rule by King and Altman[2] describing the relative concentrations of enzymic species and the structural rules by Wong and Hanes[3] describing the structure of rate equations. The use of structural rules makes possible the direct interpretation of steady-state kinetics in terms of correlations between features of reaction mechanism and features of rate behavior. In contrast to these developments in the application of the steady-state solutions, there is only limited understanding of the underlying implications of the steady-state assumption itself. There is no continuous transition between the transient-state (or, presteady-state) and steady-state solutions now in general use, and, even during the steady-state phase, the validity of the solutions remains undefined by any rational analysis of errors. It is the purpose of the present study to consider the preliminary delineation of these problems.

Conventional Derivations of Approximate Solutions

The simplest representative of an enzymic mechanism is given by the mechanism of Michaelis and Menten[4] for irreversible one-substrate reactions

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