proved, although not to such an extent as for quinacrine and chloroquine.

Finally, to estimate the effect of the 7-chloro substituent on the electronic properties of the amine salt of chloroquine, we calculated the amine salt of 4-aminoquinoline (Figure 10). Differences between the amine salts of 4-aminoquinoline and chloroquine are slight, indicating that the 7-chloro substituent does not have much influence on the ring structure. In conjunction with the fact that replacement of the 7-chloro substituent diminishes, but does not abolish binding to DNA,[35] this calculation suggests, in accord with Hahn, *et al.*,[8] that perhaps the 7-chloro substituent does enter into specific (though not essential) interaction in the DNA complex.

(35) D. Stollar and L. Levine, *Arch. Biochem. Biophys.*, **101**, 335 (1963).

Acknowledgments.—The authors are grateful to Mr. K. Sundaram for valuable comments on the manuscript and to Dr. L. J. Schaad for his help in computer programming and would like to thank Mr. John M. Clayton for assistance in the calculations.

The authors would also like to acknowledge a Research Contract from the U. S. Army Medical Research and Development Command (DA-49-193-MD-2779) and support from the National Science Foundation (GB-4453). This paper is Contribution No. 183 from the Army Research Program on Malaria. Computer facilities were provided through Grant HE-09495 from the National Institutes of Health.

Nucleic Acids. I. The Synthesis of Nucleotides and Dinucleoside Phosphates Containing *ara*-Cytidine[1a]

WILLIAM J. WECHTER

Research Laboratories of The Upjohn Company, Kalamazoo, Michigan

Received November 14, 1966

With the hope of influencing the mechanism of action, transport, or cross-resistance phenomena of the potent cytotoxic antiviral nucleoside 1-β-D-arabinofuranosylcytosine (*ara*-cytidine, CA), the nucleoside was incorporated into 25 dinucleoside phosphates of the type CApX and XpCA where X represents a second nucleoside. Where possible, all three internucleotide linkages, 2′→5′, 3′→5′, and 5′→5′, were prepared with each structural type, by the condensation of suitably protected nucleosides and nucleotides followed by deblocking. While ion-exchange chromatography was employed for small-scale isolation, continuous-flow film electrophoresis was utilized for larger scale preparations. For biological comparisons, the three mononucleotide derivatives of *ara*-cytidine, as well as two single esters of *ara*-cytidine 5′-phosphate, were prepared. The structures of all phosphorus-containing products were confirmed enzymatically. In the course of the enzymatic characterizations, a new specificity of venom diesterase toward unsymmetrical (with respect to the sugar) 5′→5′-dinucleoside phosphates was uncovered. The structures of all phosphorus-containing intermediates and products were confirmed by their nmr spectra. From these latter studies, we were able to confirm the nature and ratio of heterocyclic bases present, establish the position of nucleotide or internucleotide linkages, differentiate the sugar moieties, and confirm the gross structures of the synthetic products.

For about 14 years[1b,c] chemists, particularly in the United States, have been vigorously engaged in the synthesis of analogs of the naturally occurring nucleosides. The rationale for the most of this work was based on the supposition that one could produce unique selective antimetabolites which would be useful in the treatment of neoplastic and viral diseases. While a number of these compounds are presently being employed clinically, almost all of them are highly toxic to mammalian cells. Further, the infective agent or neoplasm develops resistance to these antimetabolites. In the hope of increasing the cellular selectivity of such antimetabolites, either by alternative mechanisms of action or by alternative transport mechanisms, we have begun a program to incorporate a variety of antimetabolites into oligonucleotides.[1c] By this means we hope to contribute to a partial understanding of the effects of charge, molecular weight, and molecular configuration on cellular penetration incorporation and transport of oligonucleotides into living systems. We thus desire knowledge of the cellular metabolism and possibly biologically unique properties of oligonucleotides. For this purpose, we synthesized a series of dinucleoside phosphates, nucleotides, and simple esters of these nucleotides derived from the cytotoxic,[2a,b] antiviral[2c-e] nucleoside 1-β-D-arabinofuranosylcytosine[2f] (*ara*-cytidine, *ara*-C, CA). Cytotoxicity studies with these compounds will be reported in the accompanying paper.[2g]

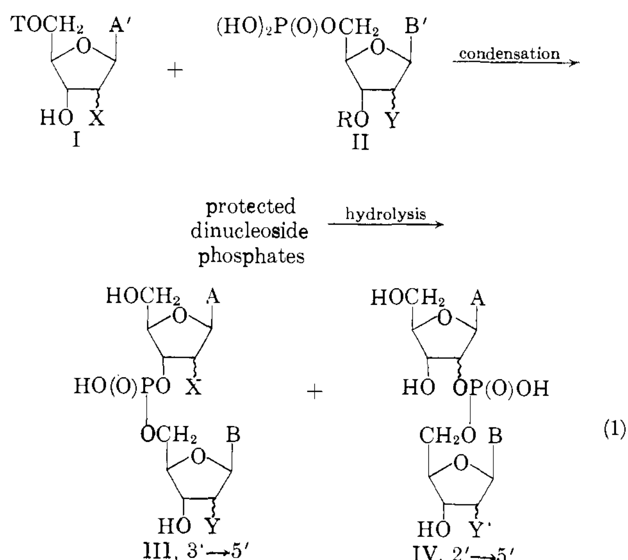
Employing the procedures pioneered principally by Khorana and his co-workers[3] all of the desired compounds were prepared, but on larger scales than those employed in the literature preparations. The products were isolated by ion-exchange chromatography. Large-

(1) (a) Presented at the 152nd National Meeting of the American Chemical Society, Division of Medicinal Chemistry, New York, N. Y., Sept 12, 1966. (b) Early workers in this field included B. R. Baker, G. B. Brown, J. J. Fox, C. A. Dekker, J. A. Montgomery, and their co-workers; leading references may be found in *Advan. Carbohydrate Res.*, **14**, 283 (1959); **17**, 301 (1962). (c) Fradulent 3′→5′- and 5′→5′-linked dinucleoside phosphates have been reported subsequent to the interception of this work: J. A. Montgomery, G. J. Dixon, E. A. Dulinage, H. J. Thomas, R. W. Brockman, and H. E. Skipper, *Nature*, **199**, 769 (1963); D. G. Parsons and C. Heidelberger, *J. Med. Chem.*, **9**, 159 (1966); J. Smrt and F. Šorm, *Collection Czech. Chem. Commun.*, **28**, 61, 887 (1963); R. H. Hall and R. Thedford, *J. Org. Chem.*, **26**, 1506 (1962); A. Block, M. H. Fleysher, R. Thedford, R. J. Maxe, and R. H. Hall, *J. Med. Chem.*, **9**, 886 (1966).

(2) (a) M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.*, **11**, 423 (1962); (b) C. G. Smith, H. H. Buskirk, and W. L. Lummis, *Proc. Am. Assoc. Cancer Res.*, **6**, 60 (1965); (c) G. E. Underwood, *Proc. Soc. Exptl. Biol. Med.*, **111**, 660 (1962); (d) H. E. Renis and H. G. Johnson, *Bacteriol. Proc.*, **45**, 140 (1962); (e) D. A. Buthala, *Proc. Soc. Exptl. Biol. Med.*, **115**, 69 (1964); (f) J. H. Hunter, U. S. Patent 3,115,282 (1963); (g) Part II: G. G. Smith, H. H. Buskirk, and W. L. Lummis, *J. Med. Chem.*, **10**, 771 (1967).

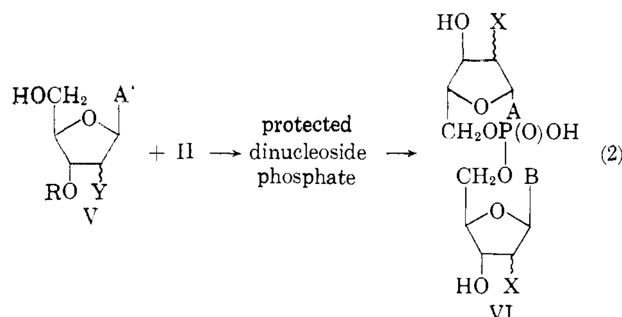
scale column chromatography is, however, time consuming and can lead to rearrangements[3i] and degradations; consequently, a new method was developed. Purification of isomerically pure (*i.e.*, one internucleotide linkage type) products was carried out continuously on the Hannig continuous-flow electrophoresis (cfe) apparatus which employs no stationary phase.[4] All unprotected phosphorus-containing products were characterized by their hydrolytic stability toward the mono- and diesterase of rattlesnake venom, bovine spleen diesterase, ribonuclease I, and bacterial alkaline phosphatase. Nmr spectroscopy was employed to confirm the structures of all of the phosphorus-containing products.

Three routes were employed for dinucleoside phosphate synthesis. The first yielded a mixture of 2'→5' and 3'→5' isomers (both isomers were desired for biochemical and biological evaluation). A nucleoside (I) with the 2' (and 3') hydroxyls unprotected was condensed with a fully protected 5'-nucleotide II. The protecting groups were then removed from the intermediate without prior purification by consecutive base and acid hydrolysis to give the desired products III and IV (see route 1) which were then separated by a suitable procedure (see Table I).

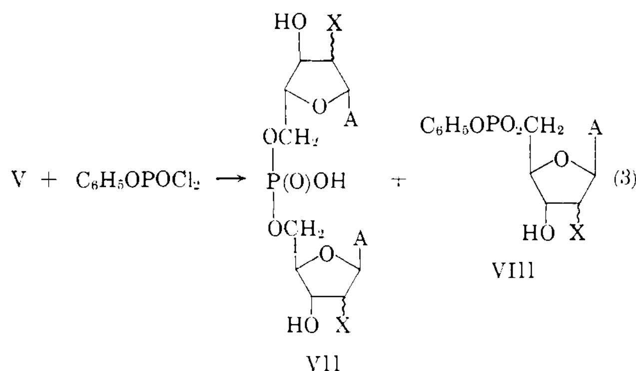


T = trityl or monomethoxytrityl; A', B' = suitable protected purine or pyrimidine bases; X = Y' = α-OH, β-OH, or H; Y = acyl α-OH, acyl β-OH, or H; R = acyl

Route 2 produced only 5'→5'-linked symmetrical or unsymmetrical dinucleoside phosphates. Condensation of 5'-unprotected nucleosides (V) with protected nucleotides as II above followed by base hydrolysis gave an isomerically pure product VI which could then be purified by cfe.



The third sequence afforded only symmetrical 5'→5'-dinucleoside phosphates and phenyl 5'-phosphates. The 5'-unprotected nucleoside (V) was condensed with phenyl phosphodichlorodate according to Michelson[5a] to give products of type VII and VIII after alkaline hydrolysis.



The required protected nucleosides and nucleotide derivatives of *ara*-cytidine were prepared as outlined in Chart I.[6] *ara*-Cytidine (**1**) was monotritylated in good yield giving the ether TrCA (**2**). Benzoylation followed by acid hydrolysis without isolation of the intermediate gave the tribenzoate (**3**), a type V intermediate. Phosphorylation according to Tener[5b] followed by the usual base hydrolysis afforded the crystalline N[4]-benzoyl 5'-phosphate **4**. Acetylation of this product gave the desired protected nucleotide **5**, a type II intermediate. Alternatively, anisoylation of CA followed by base hydrolysis afforded N[4]-anisoyl-*ara*-cytidine (**6**) in relatively poor yield. Tritylation of this substance with *p*-anisyldiphenylmethyl chloride gave the methoxytrityl ether **7** (a type I intermediate) which could not be crystallized, but was homogeneous by tlc. This substance was converted to a type V intermediate by consecutive benzoylation and acid hydrolysis to give the dibenzoyl derivative (**8**), again in poor yield. Consequently, the more desirable type I intermediate was obtained by the benzoylation of TrCA (**2**) followed by hydrolysis giving the crystalline N[4]-benzoyl-5'-O-trityl-*ara*-cytidine (**9**). We then had the necessary protected derivatives of *ara*-cytidine for condensation with known protected derivatives of the natural nucleosides and nucleotides to carry out all dinucleoside phosphate syntheses.

(3) For leading references before 1962 see (a) 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, Chapters 2, 5, and 6; (b) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic Press Inc., New York, N. Y., 1963. More recent work is found in (c) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962); (d) D. H. Rammler and H. G. Khorana, *ibid.*, 3112 (1962); (e) G. Weimann and H. G. Khorana, *ibid.*, 4329 (1962); (f) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); (g) H. Schaller, G. Weimann, B. Lach, and H. G. Khorana, *ibid.*, **85**, 3821 (1963); (h) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963); (i) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3852 (1963); (j) R. Lohrmann and H. G. Khorana, *ibid.*, **86**, 4188 (1964).

(4) Details of this method will be published separately by H. Ko, M. Royer, and W. J. Wechter, *Anal. Biochem.*, in press.

(5) (a) A. M. Michelson, *Biochim. Biophys. Acta*, **55**, 841 (1962); (b) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

(6) The shorthand abbreviations used in this paper are as follows: the 5' linkage is to the left of CA, and the 3' linkage followed by the 2' linkage and the base substituent is to the right of CA. Also, a p to the left of the symbol (CA, etc) indicates a 5' linkage, a superscript p to the right of the symbol indicates a 2' linkage, and a subscript p to the right of the symbol indicates a 3' linkage.

TABLE I

No.	Compd	Abbrev[d]	Route	Isolation[a]	Salt form	$\lambda_{max}^{pH 5}$, mμ	ε × 10⁻³
10	*ara*-Cytidylyl-(2′ → 5′)-*ara*-cytidine	CA^pCA	1	Dowex 1-X2	NH_4^+	272	~17
11	*ara*-Cytidylyl-(3′ → 5′)-*ara*-cytidine	CA_pCA	1		NH_4^+	272	~17
12	*ara*-Cytidylyl-(5′ → 5′)-*ara*-cytidine[j]	pCACA	3	Cfe	Acid	273	~17
13	*ara*-Cytidylyl-(5′ → 5′)-*ara*-cytidine	pCACA	2	DEAE-cellulose	Et_3NH^+	271	~17
14	*ara*-Cytidylyl-(2′ → 5′)-deoxyuridine	CA^pdU	1	Dowex 1-X2	NH_4^+	265	~17.3
15	*ara*-Cytidylyl-(3′ → 5′)-deoxyuridine	CA_pdU	1		NH_4^+	265	~17.3
16	*ara*-Cytidylyl-(5′ → 5′)-deoxyuridine	pCAdU	2	Cfe	Et_3NH^+	267	~17.3
17	Deoxyuridylyl-(3′ → 5′)-*ara*-cytidine[e]	dU_pCA	1	Cfe	Acid	265	~17.5
18	*ara*-Cytidylyl-(2′ → 5′)-adenosine	CA^pA	1	Dowex 1-X2	NH_4^+	262	~21
19	*ara*-Cytidylyl-(3′ → 5′)-adenosine	CA_pA	1		NH_4^+	262	~21
20	*ara*-Cytidylyl-(5′ → 5′)-adenosine	pCAA	2	Cfe	Acid	263	~21
21	Adenylyl-(2′ → 5′)-*ara*-cytidine	A^pCA	1	Dowex 1-X2 and cfe	Acid	263	~21
22	Adenylyl-(3′ → 5′)-*ara*-cytidine	A_pCA	1		Acid	263	~21
23	*ara*-Cytidylyl-(2′ → 5′)-deoxyadenosine	CA^pdA	1	Dowex 1-X2 and cfe	Acid	262	~21
24	*ara*-Cytidylyl-(3′ → 5′)-deoxyadenosine	CA_pdA	1		Acid	262	~21
25	*ara*-Cytidylyl-(2′ → 5′)-uridine	CA^pU	1	Dowex 1-X2	NH_4^+	267	~17
26	*ara*-Cytidylyl-(3′ → 5′)-uridine	CA_pU	1		NH_4^+	267	~17
27	Uridylyl-(2′ → 5′)-*ara*-cytidine	U^pCA	1	Dowex 1-X2	NH_4^+	265	~18
28	Uridylyl-(3′ → 5′)-*ara*-cytidine	U_pCA	1		NH_4^+	265	~18
29	*ara*-Cytidylyl-(2′ → 5′)-thymidine	CA^pT	1	Dowex 1-X2 (formate)	NH_4^+	270	~18.2
30	*ara*-Cytidylyl-(3′ → 5′)-thymidine	CA_pT	1		NH_4^+	270	~18.2
31	Thymidylyl-(3′ → 5′)-*ara*-cytidine	T_pCA	1	DEAE-cellulose	Et_3NH^+	268	~18.0
32	*ara*-Cytidylyl-(5′ → 5′)-deoxycytidine[h]	pCAdC	2	Cfe	Acid	271	17.0
33	*ara*-Cytidylyl-(5′ → 5′)-cytidine	pCAC	2	Cfe	Acid	272	~17.0
34	*ara*-Cytidylyl-(5′ → 5′)-*ara*-uridine	pCAUA	3	DEAE-cellulose	Et_3NH^+	268	~17.3
35	*ara*-Cytidylyl-(5′ → 5′)-tubercidin	pCATu	2	Papergram solv A	NH_4^+	271	
36	*ara*-Cytidine 5′-O-phenylphosphate[i]	PhpCA	3	Cfe	Acid (pH 12)	270	8.85
40	*ara*-Cytidine 5′-phosphate	pCA		See Experimental Section			
41	*ara*-Cytidine 5′-O-methylphosphate	MepCA	1	Silica gel solv A	NH_4^+	271	8.9
44	*ara*-Cytidine 3′-phosphate	CA_p		See Experimental Section			
45	*ara*-Cytidine 2′-phosphate	CA^p		See Experimental Section			

[a] See Experimental Section for details of continuous-flow electrophoresis (cfe) and chromatographic separations. [b] All employed cellulose DF (C) or silica gel G (S) with solvent A unless noted otherwise. [c] Yields are based on the limiting reactant and are individual for route 1 products and no attempt was made to improve yields. [d] High-voltage electrophoresis (hve) was carried out in 0.02–0.05 *M* buffers for 2 hr at 4 → 7000 v on a Shandon double-cooled plate apparatus with tap water cooling. Standards: a, C_p (pH 3.6); b, C_pC (pH 3.6); c, C_pU (6.8); d, A_pC (2.5); e, C_pA (6.8). [e] Prepared by Dr. R. C. Kelly, The Upjohn Company. [f] *n*-Butyl alcohol–acetic acid–H_2O (5:2:3) (solvent G). [g] Ethyl alcohol–1 *M* NH_4OAc (pH 7.5) (5:2) (solvent C). [h] *Anal.* Calcd for $C_{18}H_{25}N_6O_{12}P \cdot H_2O$: C, 38.16; H, 4.80; N, 14.84; P, 5.47. Found: C, 38.74; H, 4.60; N, 15.33; P, 5.26. [i] *Anal.* Calcd for $C_{15}H_{18}N_3O_8P \cdot H_2O$: C, 43.16; H, 4.83; N, 10.07; P, 7.42. Found: C, 42.94; H, 4.87; N, 10.38; P, 7.45. [j] This compound was synthesized in essentially the same manner as was reported by T. Y. Shen, H. M. Lewis, and W. V. Ruyle, Division of Medicinal Chemistry.

In Table I are summarized the products of condensation reactions carried out according to literature procedures[3a–h] *via* reaction routes 1–3. In addition, the starting materials, isolation methods, and thin layer chromatographic, high-voltage electrophoresis, ultraviolet, and shorthand abbreviation data for each of the products are listed in Table I. Twenty-five dinucleoside phosphates are compiled (**10–35**) therein. Compounds which contain *ara*-uridine (UA) arose from deamination of *ara*-cytidine (CA) during the synthetic sequence or chromatography (generally in low yield). The products synthesized by route 1 in which two isomers could be produced (2′→5′ and 3′→5′) gave essentially equal amounts of each isomer where 2′- and 3′-ribosyl hydroxyls were being phosphorylated. On the other hand, isomer distribution where arabinosyl 2′- and 3′-hydroxyls were being phosphorylated favored attack at the 3′-hydroxyl which is less hindered sterically. In these cases, the 3′→5′ isomers were obtained in about 3:1 ratios over the 2′→5′ isomers. This, of course, represents a very small, but significant, energy difference between the phosphorylation of the two hydroxyls.

For the purposes of biochemical and biological comparison, the three nucleotides derived from *ara*-C and the methyl ester of *ara*-cytidine 5′-phosphate (**40**, pCA[6]) were prepared as outlined in Chart II.[6]

ara-Cytidine 5′-phosphate (**40**) was prepared starting with the 5′-O-trityl derivative **2**, which was completely acylated with acetic anhydride and giving in good yield the triacetate (**37**). A number of attempts to selectively hydrolyze the trityl group of **37** were only partly successful. Under our best conditions there was both N and O deacylation. The 2′,3′-O-diacetate (**38**) was isolated in addition to a 30% yield of the desired triacetate (**39**) in our best preparation. Phosphorylation of **39** by the Tener procedure[5b] followed by base hydrolysis afforded the desired nucleotide pCA (**40**) as the crystalline free acid. This synthesis confirmed the structure of the enzymatically prepared nucleotide[7] [structure was confirmed by nmr spectra (Table II)]. Satisfactory uv and ir spectra, p*K* values, and a combustion analysis were obtained. Subsequently, a much more efficient process for the synthesis of **40** was developed. Debenzylation of the crystalline intermediate **4** proceeded to give directly

$\lambda_{max}^{pH\ 2}$, mμ	$\epsilon \times 10^{-3}$	Tlc, R_f[b] or rel R_f	Yield, %[c]	lve rel to std[d]	Starting materials[u] Compd no.	Protected nucleotide	Compd no.	Protected nucleoside
		C 0.22	1.8	1.23 b	5	pCA(AcAcNBz)	9	TrCA(NBz)
		C 0.21	14.5	1.17 b				
		C 1.0 rel to C_pC	10.0	1.22 b		$C_6H_5OPOCl_2$	3	CA(BzBzNBz)
		See no. **12**	35.0	See no. 12	5	pCA(AcAcNBz)	7	MtCA(NBz)
269	~18	C 0.36	1.28	1.07 c		pdU(Ac)[k]	7	MtCA(NAn)
269	~18	C 0.36	3.83	1.0 c				
		S 1.30 rel to C_pU	83.0	0.97 c		pdU(Ac)[k]	3	CA(BzBzNBz)
		C[f] 0.36	2.2	1.1 c		$TrdU_p$[l]	3	CA(BzBzNBz)
		C 0.41	8.7	1.24 d		pA(AcAcNAc)[m]	9	TrCA(NBz)
		C 0.39	22.0	1.08 d				
265	~22	C 0.98 *vs.* C_pA	33.0	0.97 e		pA(AcAcNAc)	3	CA(BzBzNBz)
		C 1.1 *vs.* A_pC		0.72 e	5	pCA(AcAcNBz)	47	TrA(NBz)
255		C 1.1 *vs.* A_pC		1.0				
		C 1.36 *vs.* C_pA	1.7	0.98 e		pdA(AcNAc)[n]	9	TrCA(NBz)
		C 1.25 *vs.* C_pA	7.6	0.85 e				
		C[g] 1.1 *vs.* U_pC	4.5	0.98 c		pU(AcAc)[o]	9	TrCA(NBz)
		C[g] 1.03 *vs.* U_pC	11.0	0.95 c				
		C 1.0 *vs.* C_pU	9.9	1.04 c	5	pCA(AcAcNBz)		TrU[p]
		C 0.9 *vs.* C_pU	10.5	1.00 c				
		S 1.27 rel to U_pC	4	1.04 c		pT(Ac)[k]	9	TrCA(NBz)
		S 1.27 rel to U_pC	14	1.04 c				
		C 0.33	20	1.00 c	5	pCA(AcAcNBz)		TrT[q]
279	26.6		42	1.17 b		pdC(AcNAc)[r]	3	CA(BzBzNBz)
		S 1.0 rel to C_pC	50	1.15 b		pC(AcAcNAc)[s]	3	CA(BzBzNBz)
		C 0.8 rel to pCACA	3				From deamination during prepn of 12	
		C 0.24	<2			pCA(AcAcNBz)		Tu>ip(NBz)[t]
128	12.6	C 1.0 rel to CA	~30			$C_6H_5OPOCl_2$		CA(BzBzNBz)
						See Discussion		
		S 0.54	60	1.41 c	5	pCA(AcAcNBz)		CH_3OH
				0.91 a		See Discussion		
				1.0 a		See Discussion		

148th National Meeting of the American Chemical Society, Chicago, Ill., 1964, p 13p. [k] This material was prepared as described for 3′-O-acetylthymidine 5′-phosphate (pTAc): H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961). [l] This nucleotide was prepared as described for 5′-O-tritylthymidine 5′-phosphate (TrTp): A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959). [m] D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962). [n] R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961). [o] T. Ukita, Y. Takeda, and H. Hayatsu, *Chem. Pharm. Bull.* (Tokyo), **12**, 1503 (1964). [p] R. Lohrman and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 4188 (1964). [q] P. T. Gilham and H. G. Khorana, *ibid.*, **81**, 4647 (1959). [r] P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958). [s] This compound was prepared in the same manner as the corresponding 3′-phosphate [$AcC_p(AcNAc)$].[16] [t] Dr. A. R. Hanze, The Upjohn Company, to be published. [u] See ref 6 for an explanation of the abbreviations used.

and without chromatography the crystalline ammonium salt of the desired acid **40** in 66% yield.

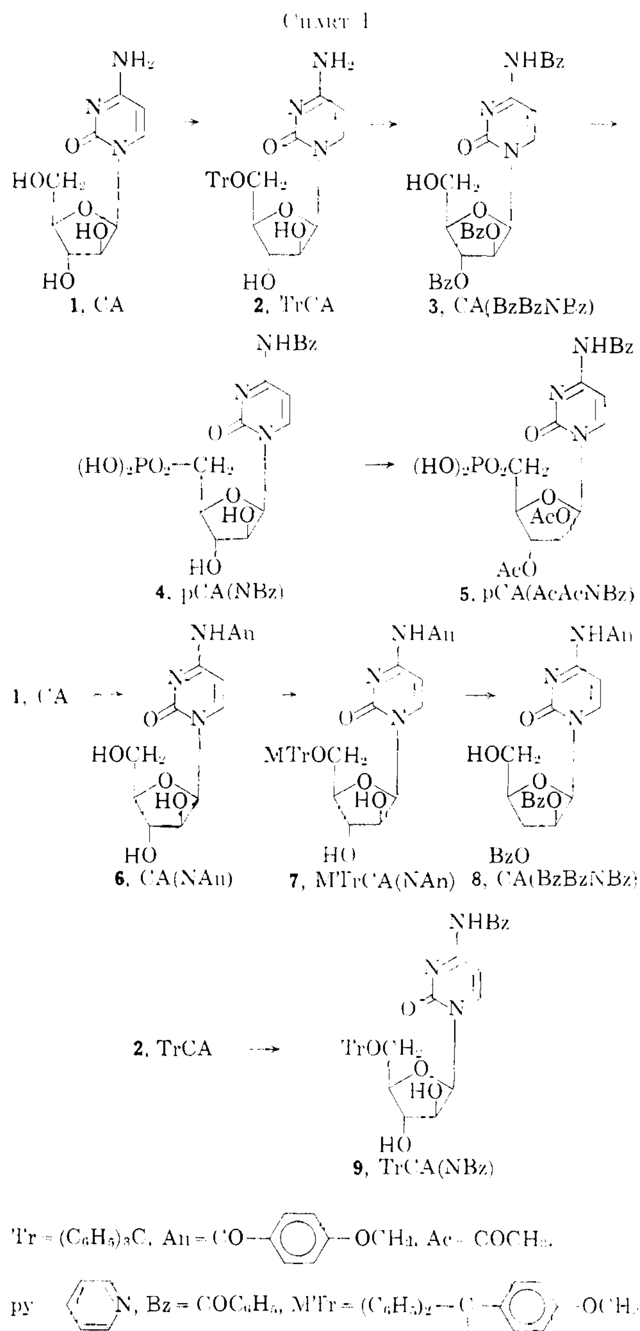
In order to prepare the methyl ester of *ara*-cytidine, **5** was alkylated according to Khorana.[8] Hydrolysis with ammoniacal methanol afforded the ester MepCA (**41**) in 60% yield. For the synthesis of the 3′ and 2′ nucleotides TrCA (**2**) rather than the N-protected TrCA(NBz) (**9**) was chosen as the starting material. Since N-phosphoroamides could be hydrolyzed, the additional steps for the preparation of **9** would have been less efficient over-all. Phosphorylation of **2** gave a mixture of 5-O-trityl phosphates (**42** and **43**) after base hydrolysis. Acid removal of the trityl protecting groups from the two protected nucleotides, **42** and **43**, gave CA_p (**44**) and CA^p (**45**). The free nucleotides were easily separated by ion-exchange chromatography. The identification of isomers was made based on several sets of evidence.

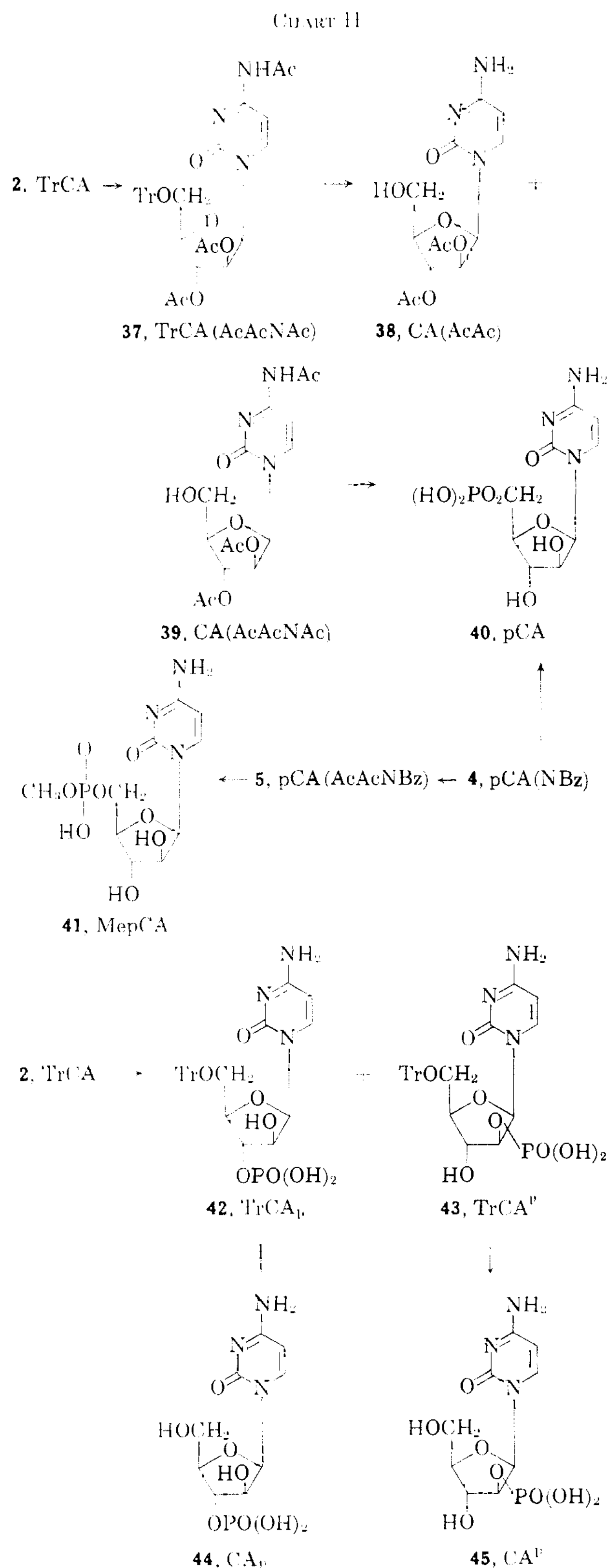
From the known behavior of cytidine 3′- and 2′-phosphates,[9] one would expect the 2′ isomer to be eluted first from the chromatographic column. Our experience with isomer distributions from phosphorylation, by a nucleotide, of the arabinosyl hydroxyls of **9** clearly indicates that the 3′ isomer should predominate in such a reaction by a factor of about 3. Experimentally, we found that the first isomer eluted was, as anticipated, produced in the lower yield and by a factor of one-third. Thus, the former compound was assigned the 2′-phosphate structure (**45**), and the latter compound was obtained in greater yield (the 3′-phosphate structure **44**). These gross structures were supported by the nmr spectra of the two acids which will be detailed later in the discussion. The conclusions derived by nmr concerning the position of phosphate substitution was unfortunately equivocal with respect to the 2′ and 3′ nucleotides. Incorporation of each of these products into dinucleotides gave a product which

(7) (a) The nucleotide was prepared enzymatically by L. I. Pizer and S. S. Cohen, *J. Biol. Chem.*, **235**, 2387 (1960), and later by direct phosphorylation with PPA by P. T. Cardeilhac and S. S. Cohen, *Cancer Res.*, **24**, 1595 (1964), in 6% yield. Later the unprotected nucleoside was phosphorylated in 22% yield by the Tener procedure. The structure of the nucleotide produced in this manner was assigned on the basis of its chromatographic mobility and enzymatic dephosphorylation by the nucleotidase from rye grass. Employing paper chromatography with solvent B, the enzymatically produced nucleotide appears to be the same as our crystalline synthetic product. (b) S. S. Cohen, *Progr. Nucleic Acid Res.*, **5**, 2 (1966).

(8) H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4657 (1959).

(9) E. Chargaff and J. N. Davidson, "The Nucleic Acids," Vol. I, Academic Press Inc., New York, N. Y., 1955, Chapter 6.





allowed enzymatic confirmation of their structures. Spleen phosphodiesterase degraded the dinucleotide derived from the 3′-phosphate (**44**) (spleen enzyme cleaves only 3′→5′ internucleotide linkages) and did not degrade the dinucleotide containing the 2′-phosphate.[10] Therefore, the original assignments based on chromatographic mobility and yield were confirmed enzymatically. It is important to note that all of the products were stable in a sterile aqueous solution at room temperature for several days and that they apparently can be stored indefinitely as frozen aqueous solution. Representative products, listed in Table I (excepting **21**, **22**, **27**, and **28**) are stable in 0.1 *N* potassium hydroxide solution for 16 hr at 37°. Thus, the arabinose 3′- and 2′-phosphate oligonucleotides exhibit the same stability as deoxyribotides toward base. There was no detectable participation of the 2′- or 3′-hydroxyls in the displacement of phosphate with concomitant oxide formation as was demonstrated[11] for cyclohexyl *erythro*-3-hydroxy-2-butyl hydrogen phosphate system (1 *N* NaOH, 100°) which gives pure *meso*-butane-2,3-diol which is clearly derived from an intermediate 2,3-epoxide. That this reaction was not observed with a dinucleoside phosphate of the type CApN (where N is any nucleoside

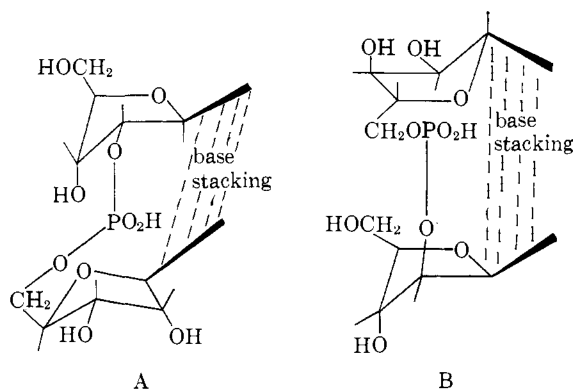
(10) W. J. Wechter, unpublished observations.

(11) D. M. Brown and D. A. Usher, *Proc. Chem. Soc.*, 309 (1963).

and the diester linkage is either 2′→5′ or 3′→5′) is indicative that a 2′,3′-epoxide in the furanose ring is energetically unfavorable.

Enzyme Characterizations (summarized in Table II).—Generally the *ara*-cytidine-containing dinucleoside phosphates and nucleotides react as they might be expected to based on the known enzymology of oligonucleotides.[3b] All of the compounds synthesized herein containing 3′→5′ internucleotide linkages are degraded quantitatively to 5′ nucleotides and nucleosides by venom diesterase and 3′ nucleotides and nucleosides by spleen phosphodiesterase. Each compound has been shown to give equimolar amounts of each component. Hydrolysis rates of all of the compounds were slow when compared with ribodinucleoside phosphates (*i.e.*, require more enzyme and longer reaction time for quantitative cleavage). As expected RNAase degraded only U_pCA (**28**) since the enzyme requires a 3′-ribopyrimidine nucleoside. Contrary to the experience of Thedford, *et al.*,[12] who found that the RNAase degradation of $U(5,Me)_pU(5,Me)$ was completely inhibited by the presence of an equimolar amount of the 2′→5′ isomer $U(5,Me)^pU(5,Me)$, U_pCA (**27**) did not inhibit the hydrolysis of U_pCA (**28**).[13]

The 2′→5′-linked dinucleoside phosphates hydrolyzed very slowly with venom diesterase. The most outstanding examples of this phenomenon were CA_pCA (**10**) and CA_pdU (**14**) for which no hydrolysis could be detected after the 2 hr. These compounds require more than 24 hr for complete hydrolysis. This unusual enzymatic stability is probably due to the fact that such 2′→5′ internucleotide bonds involve the 2′-hydroxy of an arabinoside and, consequently, results in a folded conformation of the dinucleoside phosphate. This conformation in turn adversely effects the adsorption of the substrate to the enzyme active site [*cf.* ribo-2′→5′ link (A) with *ara*-2′→5′ link (B)]. The



A B

trans configuration of the arabinose hydroxyls should not affect this hydrolysis since the enzyme ostensibly attaches to the 3′-hydroxyl of the sugar which becomes the nucleotide. Ribose dinucleoside 2′→5′ phosphates are hydrolyzed neither by spleen diesterase nor RNAase. Similarly, none of our 2′→5′ dinucleosides were hydrolyzed by these enzymes.

The simple nucleotide esters **36** and **41** of pCA (5′) were subject to hydrolyses by venom diesterase and were quite slow, but did, with time, give *ara*-cytidine quantitatively.

Unexpectedly, the 5′→5′ dinucleoside phosphates, which are substrates for the venom enzyme only, exhibited an unanticipated specificity. One would expect an unsymmetric (with respect to base) 5′→5′ dinucleoside phosphate to give upon enzymatic cleavage essentially equimolar amounts of the two nucleosides and two nucleotides. All of the arabinose-containing 5′→5′ dinucleoside phosphates, pCACA (**12**), pCAdU (**16**), pCAA (**20**), pCAdC (**32**) pCAC (**33**), pCATu (**35**), gave on enzyme hydrolysis either *ara*-cytidine or *ara*-uridine as the exclusive nucleoside product. The 5′ nucleotide was exclusively that derived from the remaining nucleoside. We can conclude from the several examples that (1) selectivity is not dependent on the heterocyclic base; therefore, the sugar configuration is responsible for the direction of cleavage; (2) for practical purposes, ribosides and deoxyribosides are interchangeable and do not affect specificity in competition with arabinosides; and (3) arabinosides can be substrates. Considering the possible explanations of this specificity, one can speculate, in the light of the total enzyme cleavage picture, that the arabinofuranosyl sugar moiety is far less favored as a site of enzyme attachment, so much so, that it is not competitive with ribo- and deoxyribosides.

The three nucleotides of *ara*-cytidine were characterized enzymatically first by the ability of alkaline phosphatase to dephosphorylate the 2′-, 3′-, and 5′-phosphates (compounds **45**, **44**, and **40**, respectively) to *ara*-cytidine. Secondly, the monoesterase of crude snake venom, when incubated separately with the three nucleotides, degraded only the 5′-phosphate (**40**).

Nmr Characterizations (assignments summarized in Table II).—Where sufficient compound was available, the nmr spectra of all products and most of the intermediates and starting materials were examined. From these studies we were able to establish the following: (1) which nucleoside bases were present and their ratio to one another, (2) independent confirmation of the class of internucleotide linkage, (3) differentiation of the sugar moieties present, and (4) unequivocal differentiation of the three nucleotides of *ara*-cytidine.

By noting the chemical shift and integrating the peaks resulting from the H-6, H-5, H-8, H-2, and H-1′ protons in a spectrum, one can identify, employing models, the five common as well as other heterocyclic bases present in dinucleoside phosphates or dinucleotides and ascertain from the integration of these peaks the ratios of bases to one another. With higher oligonucleotides some of these details may be obscured by overlap of many contributors to the spectrum. The internucleotide linkages of dimers can be classified as 5′→5′ if there is no characteristic 5′-hydroxymethylene resonance in the 230–235-cps area. While at 60 Mc/sec the 2′→5′ and 3′→5′ linkages may not be assigned, there are sufficient differences in the 240-280-cps portion of the spectrum as well as shifts in the base protons to allow the differentiation of the isomers. The sugars employed in this study can be identified. Compounds containing deoxyriboses have a 2′-CH_2 multiplet at about 140 cps and their downfield H-1′ protons appear as triplet absorptions (J = 6–7 cps). Ribose and arabinose derivatives may be distinguished by the coupling of the H-1′ protons in

(12) R. Thedford, M. H. Fleysher, and R. H. Hall, *J. Med. Chem.*, **8**, 486 (1965).
(13) W. J. Wechter and L. D. Zeleznick, to be published.

TABLE II: CHARACTERIZATION

No.	Abbrev[c]	Solvent/Reference	Nmr[a] (J, cps) H-6(8)	H-5(2)	H-1'	H-2'	H-3'
1	CA	DMF-d_7/TMS D_2O/SDSS	467.5 (7) 466 (7.5)	354.5 (7) 360 (7.5)	374 (3–3.5) 370 (5.0)	 265 (q) ($J_{2,3}$ 3.5)	251 m
2	TrCA	DMF-d_7/TMS	458 (7.5)	349 (7.5)	379 (4.0)		248 m
3	CA(BzBzNBz)	DMF-d_7/TMS	524 (7.5)	Under arom	402 (4.5)	367 (q) ($J_{2,3}$ ~2)	352 (q) ($J_{3,4}$ ~2)
4	pCA(NBz)	D_2O/NaOD eq py–H_2O std set at 300	499 (8)	443 (8)	373.5 (5)	275 m	255 m
6	CA(NAn)	DMSO-d_6/TMS	515.5 (7.5)	453 (7.5)	369.5 (3.5)		252 m, 241 m
8	CA(BzBzNAn)	d_7DMF-d_7/TMS	520 (7.5)	Under arom	400 (4.5)	365 (q) ($J_{2,3}$ ~2)	350 (q) ($J_{3,4}$ ~2)
10	CA^{p}CA	D_2O/SDSS (ext)[e]	466 (7.5) 468.5 (7.5)	Under 370 peak		272 m	262 m
11	CA$_p$CA	D_2O/SDSS (ext)[e]	473 (8) 475 (8)	372 (8) 367 (8)	375 (3.0)		Mult at 273, 264, 250
12, 13	pCACA	D_2O/SDSS (ext)[e]	474 (7.5)	Under 368	371 (5.0)	267	248 m
14	CA^{p}dU	D_2O/SDSS (ext)[e]	469 (8)	356 (8)	369 (3.5) 377 (4)	 140 m	260 m–245 m
15	CA$_p$dU	D_2O/SDSS (ext)[e]	475 (8)	367 (8) 357 (8)	372 (3.5) 380 (4, 7)	 145	252 m
16	pCAdU						
17	dU$_p$CA	Insufficient material for nmr					
18	CA^{p}A	D_2O/5-CH_2 set as 231 cps	462 (7.5) 500±	365 (7.5) 489±	365		257
19	CA$_p$A	D_2O/5'-CH_2 set as 231 cps	464 (8) 499±	360 (8) 478±	360 m	268 m	252, m 268 m
20	pCAA	D_2O/SDSS ext	454 (8) 495	349 (8) 482	~355 (~4.5)		260 m
21	A^{p}CA	D_2O/SDSS ext	496 443 (7.5)	479 350 (7.5)	367 (4) 373 (3)	260 m	250 m
22	A$_p$CA	D_2O/SDSS ext	499 466 (7.5)	487 352 (7.5)	362 (~3) 366 (~4)		250 m
23	CA^{p}dA	D_2O/H_2O std	464 (8) 497	376 (8) 494±	<376 390 (4)	 160 m	249 m, 257 m
24	CA^{p}dA	D_2O/H_2O std	466 (8) 498	Under 384 483	355 (2.5) 382 (4, 6.5)	264 m 160 m	242 m
25	CA^{p}U	D_2O/SDSS ext	481 (7.5)	363 (7.5) 357 (7.5)	380 (3.5) 358 (4)		254 m
26	CA$_p$U	D_2O/SDSS ext	473 (8) 471.5 (8)	359 (8) 352 (8)	379 (3) 350 (4)	268 m	255 m
27	U^{p}CA	D_2O/SDSS ext	468 (8) 462 (7)	343 (8) 366 (7)	359 (5) 267 (7)		265 m, 243 m
28	U$_p$CA	D_2O/SDSS ext	474 (8) 473 (7)	351 (8) 351 (7)	354 (4.5) 373 (5)		267, 270 and 249 m
29	CA^{p}T	D_2O/SDSS ext	469 (8) 456	360 (8)	375 (4) 375 (4, 7)	260 m 136 m	241 m
30	CA$_p$T	D_2O/SDSS ext	471 (8) 458	363 (8)	363.5 (4) 374 (1, 6)	271 m 139 m	246 m
31	T$_p$CA	D_2O/SDSS	462 473 (8)	 364 (8)	Under 370 370 (4)	152 m 270 m	252 m
32	pCAdC	D_2O-HCl/SDSS	478 (8) 481 (7.5)	374.5	Under 374 374 (4, 6)	270 m 147 m	252 m
33	pCAC	D_2O/SDSS	479 (8) 474 (8)	Complex in 300	373 (5) Under 370	*Ca.* 270 m	254 m
34	pCAUA	Insufficient material for nmr					
35	pCATu	Insufficient material for nmr					
36	PhpCA	DMSO-d_6/TMS	477 (7)	359 (7)	362 (4–5)		270 m, 239 m
39	CA(AcAcNAc)	DMF-d_7/TMS	500 (7.5)	446 (7.5)	379 (4)		262 m
40	pCA	D_2O eq NH_4OH/SDSS	477 (8)	366 (8)	373 (5)		267 m, 251 m
41	MepCA	D_2O eq NH_4OH/SDSS ext	476 (8)	368 (8)	374 (4.5)	268 m	250 m
44	CA$_p$	D_2O/SDSS	487 (8)	377 (8)	373 (3.5)		268 m, 258 m
45	CA^p	D_2O/SDSS	483 (8)	379 (8)	376 (4)		265 m, 255 m

[a] Nmr spectra were determined on 5–15% solutions in the indicated solvents with a Varian A-60 or A-60-A spectrometer employing Me_4Si (TMS) in nonaqueous and sodium 2,2-dimethyl-2-silapentane 5-sulfonate (SDSS) in D_2O as internal references. Frequencies are reported in cycles per second (cps) relative to TMS or SDSS as 0 cps. [b] Enzyme assays are run as described in the Experimental Section. All cleavages were run for 2 hr except where noted and all of these hydrolyses were quantitative except where noted. [c] External reference refers to the spectrometer having been set to 0 with the appropriate standard just before the spectrum is run and assumes

H-4′	H-5′	Other	OD units and substrate	Enzyme[b,c]: Venom diesterase products	Spleen diesterase products	RNAase products	Bact alk phos products
		230					
244 m		233 m					
	203 m	ArH 445 m					
275 m	247 m	o-H 485, m-H 456.5, p-H 474					
		ArH and pyH 460 m, 523, 517					
		222					
273 m	245 (br)	CH_3O 233					
240 m	232 m		5.2	CA, pCA (24 hr)	No reaction		
	235 m		7.0	CA, pCA	CA, CA_p (24 hr)		
			9.4	CA, pCA	No reaction	No reaction	
	233 m		2.6	CA, pdU (incomp 24 hr)	No reaction	No reaction	
	236 m		2.5	CA, pdU	CA_p, dU	No reaction	
			7.5	pdU, CA	No reaction		
				pCA, dU	dU_p, CA		
	231 m		4.8	pA, CA	No reaction		
	231 m		5.6	CA, pA	CA_p, I		
	237 m		5.0	CA, pA	No reaction		
	234 m		5.3	A, pA	No reaction		
	232 sh?						
	237 m		6.4	A, pCA	I_p, A_p, CA		
	232 m		4.0	CA, pdA	No reaction		
	227 m		6.5	CA, pdA	CA_p, dI		
	234 m		9.0	CA, pU	No reaction	No reaction	
	231 m		7.2	CA, pU	U, CA_p	No reaction	
	236 m		4.7	pCA, U	No reaction	No reaction	
	232 m		5.6	pCA, U	CA, U_p	CA, U_p	
	232 m	CH_3 112	6.0	pT, CA	No reaction		
	232 m	CH_3 111	4.7	pT, CA	CA_p T		
	230 m	CH_3 118	15	T, pCA	T_p CA		
	248 (broad singlet)		3.0	CA, pdC	No reaction		
	248 (broad singlet)		5.0	CA, pCA	No reaction		
			1.8	UA, CA, pUA, pCA	No reaction		
			5.0	pTu, CA	No reaction		
		ArH 432.5 (broad)	0.5 mg	pCA	No reaction		
	230 m	CH_3CONH 117 CH_3CO_2 130, 135					
			15	CA[d]			CA
		CH_3O 218 (J_{HP} 13)	9.6	pCA (18 hr)	No reaction		
		239 m	0.5 mg	No reaction[d]			CA
		235 m	0.5 mg	No reaction[d]			CA

both stability of the spectrometer for the time required for a single spectrum and the identity of influence of the individual samples on the resonance frequency of the calibration line. Where water is used as the standard (two cases) all values are probably ±10 cps. [d] In this enzymatic hydrolysis crude rattle snake venom (20 mg/ml) was substituted for the purified diesterase in order to determine reactivity toward the 5′-mononucleotidase of this substance. [e] See ref 6 for an explanation of the abbreviations used.

some cases as $J_{1,2}$ for ribosides is generally greater than for arabinosides.

The structures of the *ara*-cytidine nucleotide derivatives were confirmed independently of their assignment based on synthesis and enzymology. The 5′-phosphate (**40**) clearly shows functionalization of the 5′-OH resulting in a downfield shift of the 5-CH_2, normally at 233 cps, to about 250 cps. Many of the details of the spectra of 3′- and 2′-phosphates differ greatly so that they are easily distinguished from one another and the 2′ isomer which was assigned its structure owing to a small but definite upfield shift of the H-5, H-6, and H-5′ protons owing ostensibly to phosphate anisotropy. Certainly at 100-Mc/sec, spin and heteronuclear (P) decoupling, and prior exchange of the OH protons, the downfield shift and splitting due to P could be determined for the 2′-H and 3′-H independently. [The P-H coupling in MepCA (**41**) is 13 cps.]

These dinucleosides phosphates and their intermediates were studied biologically in terms of their cytotoxicity toward KB cells, L5178Y leukemia cells, and antiviral properties *in vitro* and *in vivo*. This work will be reported in part in the accompanying papers.[2g,14]

Experimental Section

General.—Paper chromatography was performed by the descending technique employing Whatman chromatographic paper no. 40, 44, 3HR, and 3MM. Thin layer chromatography was carried out on plates coated (100–250-μ thick) with either Camag silica gel G or Camag cellulose DF (A. H. Thomas) by the ascending technique. The solvent systems used routinely were isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2 v/v) (solvent A); isobutyric acid–1 *M* NH_4OH (0.1 *N*)–Na_2EDTA (100:60:1.6) (solvent B); ethyl alcohol–1 *M* NH_4OAc (pH 7.5) (5:2) (solvent C); *n*-propyl alcohol–concentrated NH_4OH–H_2O (55:10:35) (solvent E); *n*-butyl alcohol–acetic acid–H_2O (5:2:3) (solvent G); isopropyl alcohol–concentrated NH_4OH–0.1 *M* boric acid (7:1:2) (solvent I). Reagent grade pyridine was stored over CaH_2 before use; purified pyridine refers to reagent pyridine that has been purified by the method of Khorana.[15] Melting points are uncorrected (Thomas–Hoover Uni Melt apparatus). Petroleum ether employed was the commercial fraction having a boiling range of 40–60°. For chromatography on cellulose, S and S No. 389 pulp was used. Evaporations were carried out at 30°, except where noted otherwise, on a Buchi Rotavap evaporator (Brinkman Instrument Co.).

5′-O-Trityl-*ara*-cytidine (2).—A solution of 5.0 g of *ara*-cytidine hydrochloride and 20 g of impure trityl bromide in 150 ml of pyridine was stirred at room temperature overnight. The resulting suspension was filtered free of solid and poured into 1.5 l. of H_2O. The resulting solid was isolated, washed with H_2O, and dried (*in vacuo*, 60°). The dry material was triturated three times with 100 ml of boiling heptane, then recrystallized from acetone with a Darco treatment to give 6.54 g (75%) of the desired product, mp 227.5–228.0° dec, λ_{max}^{EtOH} 273 mμ (ε 9100). A sample was recrystallized from acetone for analysis, mp 228.5–229.5°.

Anal. Calcd for $C_{28}H_{27}N_3O_5$: C, 69.26; H, 5.61; N, 8.86. Found: C, 69.09; H, 5.67; N, 8.93.

N^4-Benzoyl-2′,3′-O-diacetyl-*ara*-cytidine (3).—5′-O-Trityl-*ara*-cytidine (**2**) (6.2 g, 12.8 mmoles) in 40 ml of dry pyridine was treated with benzoyl chloride (6 ml, 50 mmoles) and allowed to stand at room temperature overnight. The reaction mixture was poured into 500 ml of a stirred mixture of ice and water and stirring was continued for 3 hr. The aqueous portion was decanted from the residual gum and the residue was washed twice with water. The residue was taken up in methylene chloride (150 ml) and this solution was washed consecutively with H_2O (two times) and saturated NaCl and dried (Na_2SO_4). The solution was taken to dryness under reduced pressure and the residue was dissolved in 50 ml of $CHCl_3$ and treated with 6.7 ml of 30% HBr in acetic acid. After 3 min at room temperature the solution was reduced to about 10 ml under reduced pressure. This solution was adsorbed onto a column of silicic acid[16] made up with $CHCl_3$ (100 g of silica gel, 18 × 70 cm) after dilution with an equal volume of $CHCl_3$. The column was then eluted with 550 ml of ethanol-stabilized $CHCl_3$ (flow *ca.* 3.5 ml/min) and the effluent was discarded. The column was then eluted with 1.2 l. of ordinary $CHCl_3$ to which 3% (v/v) of methanol had been added. This effluent was collected in 20-ml fractions. The fractions were examined for the presence of triphenylcarbinol or trityl ether by placing a spot of each fraction on filter paper and examining all of the spots for uv followed by a 50% H_2SO_4 spray. In this manner the approximate position of the desired material (trityl negative but uv positive) was determined. The marginal fractions were evaluated for purity by tlc (silica G; methanol–benzene, 1:9). On this basis fractions 25–43 were combined and washed with 200 ml of water containing 0.5 ml of pyridine. The $CHCl_3$ solution was then dried (Na_2SO_4), and taken to dryness under reduced pressure. The residue was crystallized from acetone–petroleum ether. Three crops were collected which were homogeneous by tlc giving 3.13 g (44%), mp 177.5–178° dec. A sample was recrystallized from ethyl acetate for analysis; mp 177.5–178° dec; ν_{max}^{Nujol} 2540, 3380, 1720, 1695, 1660, 1615, and 1600 cm^{-1}; uv, λ_{max}^{EtOH} 234 mμ (ε 35,900), 261 (25,400), and 304 (10,350).

Anal. Calcd for $C_{30}H_{25}N_3O_8$: C, 64.9; H, 4.5; N, 7.57. Found: C, 64.95; H, 4.67; N, 7.29.

Pyridinium N^4-Benzoyl-*ara*-cytidine 5′-Phosphate Hydrate (4).—Approximately 50 mmoles of pyridinium β-cyanoethyl phosphate was rendered anhydrous by several evaporations with anhydrous pyridine. The residue was then dissolved in 10 ml of dry pyridine, **3** (2.77 g, 5 mmoles) was added, and the solution again was taken to dryness. The mixture was then dissolved in 25 ml of pyridine, dicyclohexylcarbodiimide (DCC) (3.09 g, 150 mmoles) was added, and the mixture was shaken at room temperature for 5.5 days. About 15 ml of water was then added and the mixture was extracted twice with petroleum ether and filtered free of the urea. The solution was then diluted to 40 ml with pyridine, chilled to ice temperature, and made 1 *N* in NaOH by the addition of 40 ml of ice-cold 2 *N* NaOH. The reaction was terminated with excess pyridinium Dowex 50 after 20 min. The resin was separated and washed with water, and the aqueous portion was evaporated under reduced pressure to about 25 ml after the addition to 200 mg of $(NH_4)HCO_3$. The precipitate was removed by filtration. The solvents were removed under reduced pressure and the residue was taken up in a solvent system composed of 1 *M* NH_4OAc (pH 6) and isopropyl alcohol (2:5), then absorbed onto a cellulose column (5.8 × 65 cm, cv 1850 ml) made up with this same system. The column was then eluted and the first 600 ml of eluate was discarded after which 20-ml fractions (325 total) were collected and examined at intervals by uv and tlc (solvent A on silica gel G). Fractions 55–110 proved to contain almost pure pCA(NBz), 54,000 OD units at 304 mμ (*ca.* 4.5 mmoles, 90% of theory). The combined fractions were taken to a small volume in the presence of 10 ml of pyridine, the residue was diluted to 50 ml with water, the product was adsorbed onto pyridinium Dowex 50W-X8 (45 × 3.8 cm), and the column was eluted with 3 l. of deionized water. The total effluent was concentrated under reduced pressure and rediluted with 1% aqueous pyridine four times, followed by concentration. Finally, the residue was taken up in diluted aqueous pyridine and lyophilized twice from this solvent. The residue, a white solid, was submitted for analysis: $\lambda_{max}^{pH\ 5.9}$ 257.5 mμ (ε 26,300), 303 (14,050).

Anal. Calcd for $C_{16}H_{18}N_3O_8 \cdot 2.5H_2O \cdot$ pyridine: C, 46.58; H, 4.29; N, 10.35. Found: C, 46.46; H, 4.58; N, 10.35.

N^4-Benzoyl-2′,3′,O-diacetyl-*ara*-cytidine 5′-Phosphate (5).—The above product pCA(NBz) (**4**) (1.79 g, *ca.* 3.4 mmoles) was suspended in 30 ml of pyridine–acetic anhydride (1:1) and stirred overnight at room temperature. The solution (homogeneous) was then diluted with 15 ml of water and stirred for 3 hr at room temperature. The solvent was then removed at 30° (high vacuum) and the residue was triturated with ether. The gum was freed from ether *in vacuo* and then taken up in 10 ml of dry pyridine and stored at 4°, concentration *ca.* 0.31 mmole/ml.

(14) Part III: H. E. Renis, C. A. Hollowell, and G. E. Underwood, *J. Med. Chem.*, **10**, 777 (1967).

(15) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964).

(16) Brinkman silicic acid for chromatography, Brinkman Instruments Inc., Westbury, N. Y.

Tlc on silica gel G (solvent A) showed one major material and was employed without further purification.

N^4-Anisoyl-*ara*-cytidine (6).—*ara*-Cytidine (1) hydrochloride (5.0 g) and anisoyl chloride (25 ml) were dissolved in pyridine (100 ml) and the solution was stirred at room temperature for 6 hr. Acid (400 ml, 1.5 *N* HCl) was then added and the solution was allowed to stand overnight at room temperature. The solid was filtered, washed, ground thoroughly with H_2O, and air-dried. The residue was suspended in H_2O (275 ml) and ethanol (250 ml) and warmed to 70° on a steam bath. The cool suspension was then chilled to 4°, the pH was adjusted to 8 with 1 *N* NaOH, and the solid was filtered immediately, washed with water, air dried, then washed with three portions of ether (100 ml). The solid was then stirred rapidly with 100 ml of ether, filtered, and air-dried to give 16.6 g of crude product containing some anisic acid by tlc (5% MeOH–C_6H_6, silica gel G). The crude product was taken up in pyridine (195 ml) and water (65 ml) and chilled to ice temperature. The solution was then treated with vigorous stirring with 350 ml of 1.5 *N* NaOH for 0.5 hr. The reaction was terminated by the addition of *ca.* 350 ml of Dowex 50-X8 (50–100 mesh) pyridinium resin followed by stirring for 20 min (pH 7.0). The solution was filtered free of insoluble material. The residue was washed with water. The combined filtrates were taken to dryness *in vacuo* at <50°, and the residue was stirred with three 200-ml portions of ether and filtered. The solid was then suspended in 300 ml of boiling water and filtered (three times). The combined filtrates were evaporated to a small volume under reduced pressure giving 2.0 g of product, mp 197–200° dec. A sample was recrystallized four times from H_2O and once from methanol for analysis; mp 200.5–201.5° dec; $\lambda_{max}^{H_2O}$ 216 mμ (ε 20,450), sh 256 (12,800), sh 286 (20,850), 304 (25,200).

Anal. Calcd for $C_{17}H_{19}N_3O_7$: C, 54.11; H, 5.08; N, 11.14. Found: C, 54.38; H, 4.82; N, 11.31.

5′-O-Monomethoxytrityl-N^4-anisoyl-*ara*-cytidine (7).—N^4-Anisoyl-*ara*-cytidine (4.8 g), dissolved in pyridine (50 ml), was treated with *p*-anisyldiphenylmethyl chloride (9.5 g). After 9 hr methanol (10 ml) was added and the pyridine solution was poured into 600 ml of H_2O and stirred. When the gum had coagulated, the solution was decanted. The gum was washed with H_2O several times by decantation. The gum was then taken up in methylene chloride, washed with H_2O (two times) and saturated NaCl solution, dried (Na_2SO_4), and taken to dryness at 30° *in vacuo*. The residue dissolved in benzene was adsorbed onto a column of silica gel (5.8 × 48 cm) made up with benzene and eluted with twenty 100-ml fractions of 2% methanol–benzene, then forty 100-ml fractions of 5% methanol. Fractions 49–60 were triturated with ether, giving a crystalline solid which was collected and washed with ether (combined in acetone). The product, 4.21 g (36–39% for the three steps from CA), exhibited a single uv-absorbing spot by tlc (10% MeOH–C_6H_6 on silica gel G), but carbinol impurities could be detected with 50% H_2SO_4 spray. Attempts to recrystallize this material were unsuccessful. A sample was precipitated from THF with ether at −20° and this material (*i.e.*, not analytical sample) was submitted for analysis: λ_{max} 285 mμ (ε 23,800), sh 303 (21,350); ν_{max} 3340, 1690, 1645, and 1600 cm^{-1}.

Anal. Calcd for $C_{37}H_{35}N_3O_7$: N, 6.47. Found: N, 6.27.

N^4-Anisoyl-2′,3′-O-dibenzoyl-*ara*-cytidine (8).—A 4.0-g sample (6.2 mmoles) of MTCA(NAn) in dry pyridine (20 ml) was treated with 3 ml (25 mmoles) of benzoyl chloride. The sealed reaction mixture was allowed to stand 18 hr at room temperature. The mixture was then poured into ice–water and stirred for 3 hr at ambient temperature, giving a gummy solid. The crude product was extracted into two 50-ml portions of CH_2Cl_2, after which the combined extracts were washed consecutively with water (five times), saturated NaCl solution, dried (Na_2SO_4), and taken to dryness *in vacuo*. The residual pyridine was codistilled with toluene at reduced pressure and the residue was taken up in dioxane (50 ml) and treated with 80% acetic acid (50 ml). After 24 hr the reaction was incomplete as evaluated by tlc (10% MeOH–C_6H_6 on silica gel G); consequently, the solution was made 0.03 *N* in HCl. After 5 hr hydrolysis was complete. The solvents were then removed at 40° *in vacuo* then reevaporated with 100 ml of 1:1 $CHCl_3$–ethanol. The residue was taken up in $CHCl_3$ and absorbed onto a column of silicic acid (2.8 × 40 cm) made up with $CHCl_3$. The column was eluted with four 250-ml fractions of chloroform (0.75% EtOH), followed by six 250-ml fractions of $CHCl_3$ with 3% methanol added. Fractions 5–8 were combined and absorbed onto a fresh silica gel column (2.8 × 50 cm) made up with alcohol-free $CHCl_3$ in the same $CHCl_3$; it was then eluted with four column volumes of ordinary $CHCl_3$, followed by 2 l. of 3% methanol–$CHCl_3$ collected in 20-ml fractions at a column rate of 5.0 ml/min. Fractions 46–54 contained the desired material (220 mg), mp *ca.* 167–174° (from ethyl acetate–Skellysolve B). A sample was recrystallized for analysis; mp 172.0–173.0°; ν_{max} 3540, 3380, 1720, 1690, 1655, and 1605 cm^{-1}; λ_{max}^{EtOH} 232 mμ (ε 35,700), 288 (24,650), and sh 303 (22,350).

Anal. Calcd for $C_{31}H_{27}N_3O_9$: C, 63.58; H, 4.65; N, 7.18. Found: C, 63.62; H, 5.12; N, 7.23.

N^4-Benzoyl-5′-O-trityl-*ara*-cytidine (9).—5′-O-Trityl-*ara*-cytidine (5.0 g, 10.3 mmoles) in 35 ml of pyridine was treated with benzoyl chloride (5.0 ml) and stirred overnight. The suspension was poured into 400 ml of H_2O, and the mixture was stirred overnight at room temperature. The gummy product was filtered, washed thoroughly with H_2O, and then taken up in CH_2Cl_2 (300 ml). This solution was washed with H_2O (100 ml), saturated NaCl solution (100 ml), and then taken to dryness under reduced pressure. The residue was taken up in 400 ml of absolute methanol and 200 ml of dry THF, and the solution was chilled to ice temperature. The cold solution was treated with 10 ml of 25% $NaOCH_3$ in methanol and the course of the reaction was followed by tlc (silica gel G, 10% MeOH–C_6H_6). After 30 min at 0°, 110 ml of Dowex 50W-X8 (pyridinium) resin (20–50 mesh) was added, whereupon the pH of the solution dropped to about 7.0. The suspension was filtered free of resin and the resin was washed with two 50-ml portions of methanol. The combined filtrates were taken to dryness at 30° (water pump) and then dissolved in a minimum volume of benzene. The benzene solution was adsorbed onto a column of silica gel (58 × 4.8 cm) made up with benzene. The column was eluted with twenty 100-ml portions of 2% methanol in benzene followed by forty 100-ml portions of 5% methanol–benzene. Fractions 34–41 were ascertained by tlc (as above) to be the product, combined in acetone, and crystallized as microclusters from acetone–Skellysolve B, giving 2.29 g (38%), mp 208.0–209.5°. A sample was recrystallized once for analysis; mp 210.5–211.5° (from acetone); ν_{max} 3440 sh, 3340 sh, 3210, 1705, 1640, 1610, 1600, and 1560 cm^{-1}; λ_{max}^{EtOH} 260 mμ (ε 23,750), 307 (11,750).

Anal. Calcd for $C_{35}H_{31}N_3O_6$: C, 71.9; H, 5.32; N, 7.19. Found: C, 71.41; H, 5.59; N, 7.46.

General Procedure for Condensation Reactions and Subsequent Hydrolyses.—The protected nucleotide (3 mmoles) was mixed with the protected nucleoside (6–10 mmoles) together with 500 mg of dry pyridinium Dowex 50W-X8 in 25 ml of purified pyridine. The suspension was taken to dryness at 30° under reduced pressure (water aspirator). The coevaporation with purified dry pyridine was repeated five times. The residue was then suspended in 25 ml of purified pyridine, 10 g of dicyclohexylcarbodiimide (DCC) was added, and the mixture was shaken in the dark for 4 days. Water (10 ml) was then added and the suspension was stirred at room temperature overnight. The insoluble urea and resin were filtered and washed with two 20-ml portions of 50% aqueous pyridine. The combined filtrates were washed with four 50-ml portions of petroleum ether and then the aqueous portion was taken to dryness *in vacuo* at 40°. The residue was resuspended in aqueous pyridine and again taken to dryness as above and the last traces of H_2O were removed under high vacuum. The residue was then taken up in 50 ml of methanol saturated with anhydrous NH_3 and stirred at room temperature for 24 hr. This solution was then taken to dryness under reduced pressure. If there were any methoxytrityl or trityl protecting groups present (route 1) the residue was taken up in 50 ml of 80% acetic acid and stirred at room temperature for 6 or 24 hr, respectively, for the protecting groups. The solution was once again taken to dryness under reduced pressure at 30°. The product from the base or acid hydrolysis was then taken up in 1 l. of H_2O, filtered free of insoluble material, and extracted exhaustively with ether. The resulting solution was then freeze-dried and the residue was subjected to one or more of the separation procedures described below.

Separation Methods. A. Ion-Exchange Chromatography over Dowex 1-X2 (Formate).—Chromatography was carried out by the procedure of Taylor and Hall[17] on AG 1-X2 (formate) resin. The total product from the condensation procedure carried out on the scale described above was adsorbed onto the "1-mmole" column of Taylor and Hall (200–400 mesh, 1.8 × 27

(17) P. R. Taylor and R. H. Hall, *J. Org. Chem.*, **29**, 1078 (1964).

cm) in H_2O. The column was then eluted by gradient with from 0.04 to 0.08 *M* ammonium formate (pH 5.0) employing 2 l. of each salt solution. The column was monitored at an appropriate wavelength (near the λ_{max} of the product) by means of a Vanguard 1056 OD uv scanner with 0.1-mm path length cells so the optical densities of up to about 30 could be conveniently read. The fractions were collected in 20-ml portions in a fraction collector (photoelectric or precise volume collector). Based on the elution diagram generated by the uv scanner with the peaks over 30 OD's filled in by manual dilution and reading appropriate fractions were combined and freeze-dried several times from deionized water in order to rid the product of salts. The resulting freeze-dried solid was then dissolved in D_2O and its pmr spectrum was run (see Table II). The sample was then freeze-dried once again, then taken up in sterile H_2O so that the optical density of the solutions were in the range of 100–500 and the uv spectra, high voltage electrophoresis (hve), tlc, and enzymatic splitting were run. In those cases, where the products were not free of nucleoside or nucleotide at this point, they were further purified by cfe (below).

B. DEAE-Cellulose Chromatography.—Chromatography on DEAE-cellulose (S and S) was carried out as described by Smith and Khorana.[18] For a condensation in which the total product was of the order of 44,000 OD units, the product, after suitable hydrolyses, was adsorbed onto a column of purified DEAE-cellulose (60 × 5.8 cm) which had been prepared as described in the above reference. The column was eluted first with 600 ml of 0.02 *M* triethylamine bicarbonate (pH 7.5), then over a gradient of from 0.02 to 0.12 *M* salt employing 5 l. of each buffer. The column was monitored as described in section A above and the products were treated after combining the fraction as described above. The products of the section A chromatography were ammonium salts of the dinucleoside phosphates and in this case the triethylamine salts.

C. Continuous Flow Electrophoresis (Cfe).—For cfe separations, the Hannig Model FF electrophoretic separator (Brinkman Instruments Co.) was employed. The experimental details for each of these runs will be reported separately.[4] Compounds **12**, **16**, **17**, **20**, **21**, **32**, **33**, and **36** were purified in this manner.

N^4,2′,3′-O-Triacetyl-5′-O-trityl-*ara*-cytidine (37).—A 750-mg sample of the trityl ether (**2**) was suspended in 9 ml of pyridine and treated with 3 ml of acetic anhydride at room temperature with stirring until solution was complete. Stirring was continued for 2 hr whereupon the solution set up to a crystalline mass. The suspension was then poured into 90 ml of water and the white crystalline product was isolated, washed thoroughly with water, and dried (vacuum, 60°) giving 950 mg, mp 248–249.5°. Recrystallization from ethanol afforded colorless rosettes (800 mg): mp 251–252°; ν_{max} 3210, 1760, 1720, 1670, and 1620 cm^{-1}; λ_{max}^{EtOH} 250 mμ (ϵ 15,500), 300 (7650).

Anal. Calcd for $C_{34}H_{33}N_3O_7$: C, 66.76; H, 5.44; N, 6.87. Found: C, 67.04; H, 5.47; N, 7.00.

2′,3′-Diacetyl- and N^4,2′,3′-Triacetyl-*ara*-cytidine (38 and 39).—A 1.3-g sample of the trityl triacetate (**37**) was suspended in 10 ml of 80% acetic acid and heated to reflux for 10 min. The suspension was then refrigerated, filtered free of triphenylcarbinol, and taken to dryness *in vacuo* at 30–40°. The product was absorbed onto silica gel (2.8 × 33 cm) (column volume 200 ml) and eluted with thirty 20-ml fractions of methanol–benzene (25:75). Fractions 5–11 were combined (455 mg) and recrystallized from acetone–Skellysolve B giving 240 mg (30%), mp 171.0–172.5°. A sample was recrystallized once for analysis; mp 174.5–175.5°, ν_{max} 3400, 3280, 3240, 1750, 1710, and 1655 cm^{-1}; λ_{max}^{EtOH} 213 mμ (ϵ 18,150), 249 (15,750), and 299 (7500), which is consistent for the N^4,O$^{2'}$,O$^{3'}$-triacetate.

Anal. Calcd for $C_{15}H_{19}N_3O_8$: C, 48.78; H, 5.19; N, 11.38. Found: C, 48.79; H, 4.81; N, 11.66.

Fractions 26–29 contained a very small amount of the O$^{2'}$,O$^{3'}$-diacetate identified by chromatographic mobility on silica gel G tlc (MeOH–C_6H_6, 25:75), λ_{max} 271 mμ (free 4-NH_2 group).

***ara*-Cytidine 5′-Phosphate (40).**—The above triacetate containing a few per cent of the N-deacylated material (2.5 g) was dissolved in 40 ml of pyridine 0.325 *M* in cyanoethyl phosphate. An additional 20 ml of pyridine was added containing 5.6 g (26 mmoles) of DCC. The mixture was shaken in the dark for 2 days, after which time 10 ml of H_2O was added. The solution was warmed and then shaken for 1 hr, after which time an additional 35 ml of H_2O was added, and the solution was filtered free of dicyclohexylurea. The filtrate was taken to dryness, diluted with 50 ml of H_2O, and again taken to dryness to remove residual pyridine. The residue was then partitioned between H_2O and ether (150 ml, 1:1) and the aqueous portion freed of ether *in vacuo* after a second extraction. The aqueous solution (90 ml) was then treated with 2.16 g (90 mmoles) (*i.e.*, 0.1 *M*) of LiOH and the solution was heated to 100° for 1 hr, after which time the suspension was chilled and filtered free of lithium phosphate. The solid was washed with 0.01 *N* LiOH, after which time the pH of the filtrate was adjusted to 7 by the addition of Dowex 50 (H$^+$) resin. The resin-free solution was distilled to 25 ml under reduced pressure (40°) and the solution was passed over fresh Dowex 50 (75 ml). The resin was eluted with water until the pH of the eluate was in the range 4–5. The pH of the solution was adjusted to 7.5 by the addition of concentrated NH_4OH. The product solution (*ca.* 200 ml) was absorbed onto a column of Dowex Ag-1 (formate) of dimensions 1.88 × 44.5 cm and the column was eluted with 125 ml of H_2O. The column was then eluted with 0.15 *M* formic acid, and the elution was followed at 260 mμ on a Vanguard, 1056 OD uv scanner. The eluate was collected in 20-ml fractions at a flow rate of 2.0 ml/min. Fractions 13–33 (major scale) were combined, after a 200-ml forerun, and lyophilized to give a white crystalline solid, yield 250 mg (11%). This material was identical by paper chromatography with a sample prepared enzymatically,[7] papergram R_f (solvent B) 0.38, R_f (solvent G) 0.36. A sample was recrystallized twice for analysis from water at 4° to give fine needles: $\lambda_{max}^{0.1\ N\ HCl}$ 212 mμ (ϵ 10,100) and 280 (13,400); p$K_a{}^1$ = 4.05, p$K_a{}^2$ = 6.15.

Anal. Calcd for $C_9H_{14}N_3O_8P$: C, 33.44; H, 4.36; N, 13.00; P, 9.58. Found: C, 33.37; H, 4.88; N, 12.61; P, 9.75.

Ammonium *ara*-Cytidine 5′-Phosphate Hemihydrate (40, NH_4^+ Salt).—A 4.0-g sample (7.3 mmoles) of pCA(NBz) (**4**) was taken up in a mixture of methanol (100 ml) and anhydrous NH_3-saturated methanol (170 ml) and stirred at room temperature overnight. About one-half of the solvent was distilled under reduced pressure. The resulting suspension was chilled to 4° and the crystalline ammonium salt was filtered and washed with a small amount of cold methanol. Tlc (cellulose DF, solvent A) exhibited a single spot whose mobility was identical with that of an authentic sample of pCA (**41**). Dried under reduced pressure (0°), the product weighed 1.66 g (66%), $\lambda_{max}^{pH\ 6}$ 272 mμ (ϵ 8980), $\lambda_{max}^{pH\ 2}$ 279 mμ (ϵ 12,550)).

Anal. Calcd for $C_9H_{14}N_3O_8 \cdot NH_3 \cdot 0.5H_2O$: N, 16.05; H_2O, 2.58. Found: N, 16.22; H_2O (Karl Fisher), 3.2.

Methyl-*ara*-cytidine 5′-Phosphate (41).—A 1-mmole sample of pypCA(AcAcNBz) (**5**) was coevaporated (*in vacuo*) with pyridine three times, then dissolved in 20 ml of dry pyridine and treated with tri-*n*-butylamine (1.2 ml), methanol (40 ml), and DCC (7.0 g). The mixture was shaken for 18 hr at room temperature and then taken to dryness under reduced pressure. The liquid residue was triturated twice with ether and the residue was diluted to 35 ml with H_2O and the insoluble urea was filtered. This solution exhibited λ_{max} 271 mμ (21,600 OD units, 84% recovery). The solution was made basic (NH_4OH) and taken to a small volume at 40° (aspirator vacuum). The aqueous residue was taken up in 200 ml of methanol and 25 ml of concentrated NH_4OH and allowed to stand at ambient temperature overnight. Tlc (silica gel G, solvent A) indicated a single major material plus two other small amounts of uv-absorbed material, one of them obviously benzamide. The solution was taken to almost dryness, then adsorbed onto a column of 50 g of silica gel made up with solvent A. The column was eluted with 5.0-ml fractions of solvent A. Fractions 17–21 were combined, freeze-dried, and proved homogeneous by tlc. The freeze-dried material was dissolved in 8.0 ml of sterile water and filtered; $\lambda_{max}^{pH\ 6.5}$ 271 mμ (3040 OD units) indicative of a 60% yield; λ_{min} 251 mμ. The structure of this compound was confirmed both enzymatically and by pmr spectra (Table II).

***ara*-Cytidine 3′- and 2′-Monophosphates (44 and 45).**—5′-O-Trityl-*ara*-cytidine (970 mg, 2 mmoles) was added to 4.0 ml of 1 *M* cyanoethyl phosphate (4 mmoles) in pyridine and the solution was taken to dryness under reduced pressure. The residue was evaporated with three fresh portions of dry pyridine and finally taken up in 10 ml of purified pyridine and treated with 1.2 g of DCC at room temperature with shaking for 5 days in the dark. Water (10 ml) was added and the suspension was stirred at ambient temperature for 3 hr. The urea was filtered and the filtrate was washed twice with petroleum ether. The nitrogen-flushed ether-free solution was then treated with 13 ml of 2 *N*

(18) M. Smith and H. G. Khorana, *Methods Enzymol.*, **6**, 667 (1963).

NaOH at ambient temperature for 20 hr. The reaction was terminated by the addition of Dowex 50-X8 (pyridinium, 30 ml). The solution was filtered free of resin and the resin was washed with water. The combined filtrates were taken to dryness at 40° (*in vacuo*).

The above gummy product was dissolved in 100 ml of 80% acetic acid and allowed to stand at room temperature for 3 days. The solvent was removed under reduced pressure at 40°, the residue was taken up in a small amount of H_2O, filtered free of triphenylcarbinol, and adsorbed onto a column of AG 1-X8 (formate) ion-exchange resin of dimensions 2.8 × 24 cm, and eluted with 7 l. of 0.01 *M* formic acid, followed by 5 l. of 0.02 *M* formic acid, collecting 20-ml fractions. Fractions 195–210 were combined and freeze-dried and the product was crystallized from H_2O to give 10 mg of *ara*-cytidine 2′-phosphate as colorless rosettes: $\lambda_{max}^{pH\ 4.5}$ 212 mμ (ε 9600), 274 (9950); $\lambda_{max}^{pH\ 2}$ sh 213 mμ (ε 11,550), 279 (13,650).

Anal. Calcd for $C_9H_{14}O_8N_3P$: C, 33.44; H, 4.36; Found: C, 33.91; H, 4.71.

Fractions 426–270 were combined, freeze-dried, and isolated as a crystalline solid (112 mg) of *ara*-cytidine 3′-phosphate: $\lambda_{max}^{pH\ 4.5}$ 274 mμ (ε 10,700), sh 213 (10,750); $\lambda_{max}^{pH\ 1}$ sh 213 mμ (ε 10,750), 279 (13,900).

Anal. Calcd for $C_9H_{14}N_3O_8P$: C, 33.44; H, 4.36; N, 13.00; P, 9.58. Found: C, 33.12; H, 4.58; N, 12.99; P, 9.65.

5′-O-Trityl-N⁶-benzoyladenosine (46).—N^6-Benzoyladenosine (2.09 g, 5.4 mmole)[19] was dissolved in 20 ml of anhydrous pyridine and treated with trityl chloride (3.0 g, 10.8 mmoles) at room temperature for 2 days. The reaction mixture was then poured into 500 ml of ice–water and allowed to stir at room temperature. The product was filtered, washed thoroughly with water and ether, then crystallized from methanol containing a trace of pyridine, mp 198–201.5°. A sample was recrystallized in the same manner for analysis; mp 203.0–204.5°; λ_{max}^{EtOH} 230 mμ sl sh (ε 20,250), 260 sl sh (12,800), and 279 (19,900).

Anal. Calcd for $C_{36}H_{31}N_5O_6$: C, 70.46; H, 5.09; N, 11.41. Found: C, 70.10; H, 5.12; N, 11.59.

Enzyme Hydrolyses. A. Snake Venom Diesterase.—Purified phosphodiesterase was obtained from Worthington Biochemicals Corp., Freehold, N. J., and a standard solution was prepared in sterile H_2O so that the concentration of protein was from 5 to 10 mg/ml adjusted so that the potency (as described by Worthington) remained constant. The incubation mixture contained the substrate [1–15 OD units (in water), see Table II], 10 μl of 1 *M* pH 9.0 Tris buffer, 40 μl of enzyme solution (*ca.* 0.2 mg of protein), and sufficient H_2O to make the total volume 100 μl. The incubation was carried out at 37° for 2 hr except where noted and all of the incubations were carried to complete hydrolysis. After heating at 100° for 2 min to precipitate protein, the entire reaction mixture was streaked on 3MM paper and developed with solvent A *vs.* suitable controls. The hydrolysis products were then extracted from the paper and examined by uv. In every case, the ratio (millimicromoles) of nucleoside and nucleotide products was consistant within experimental error with that anticipated (*i.e.*, 1:1 for dinucleoside phosphates). The recovered material, where desirable, was then chromatographed by tlc (cellulose) or by paper high-voltage electrophoresis in order to unquestionably identify every product.

B. Spleen Phosphodiesterase.—The diesterase was obtained as above from Worthington Biochemicals and was taken up in H_2O so that the activity (Worthington) was 20–30 units/ml. The incubation mixture as described by Razzell,[20] contained 20 μl of 1 *N* ammonium acetate (pH 5.7), 5 μl of 1% Tween 80 in H_2O, 1–15 OD units of dinucleoside phosphate (in H_2O), 40 μl of enzyme, and sufficient H_2O to make 105 μl. The incubation, termination, and identification of products was carried out as described for venom in A.

C. RNAase.—Crystalline bovine pancreatic RNAase (Worthington, highly purified) was used to make up a stock solution of the enzyme whose concentration was 5 ml/ml. The incubation mixture contained 2.5–10 OD units of substrate in H_2O, 10 μl of RNAase stock solution, and sufficient H_2O to make 100 μl of solution. Incubations were carried out for 2–6 hr at 37° after which time, to total reaction mixtures, the solutions were streaked onto 3MM paper and developed with solvent A. The identification and uv quantitation of the products was carried out as described for venom in section A.

D. Bacterial Alkaline Phosphatase.—Commercial enzyme from Worthington Biochemicals (concentration 2 mg/ml) was diluted tenfold with 0.05 *M* Tris (pH 9). The incubation was carried out according to Khorana and Vizsolzi[21] and the mixture contained 0.1–1 μmole of substrate nucleotide, 10 μl of Tris (pH 9.0), 10 μl of enzyme solution, and sufficient H_2O to make 100 μl. The mixture was incubated for 2 hr at 37° after which time the nucleoside products were isolated and identified as for the venom enzyme (A) above.

(19) R. Lohrmann and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 4188 (1964).

(20) W. E. Razzell, *Methods Enzymol.*, **6**, 245 (1963).

(21) H. G. Khorana and J. P. Vizsolzi, *J. Am. Chem. Soc.*, **83**, 675 (1961).