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Studies on Polynucleotides. XI.[1] Chemical Polymerization of Mononucleotides. The Synthesis and Characterization of Deoxyadenosine Polynucleotides[2]

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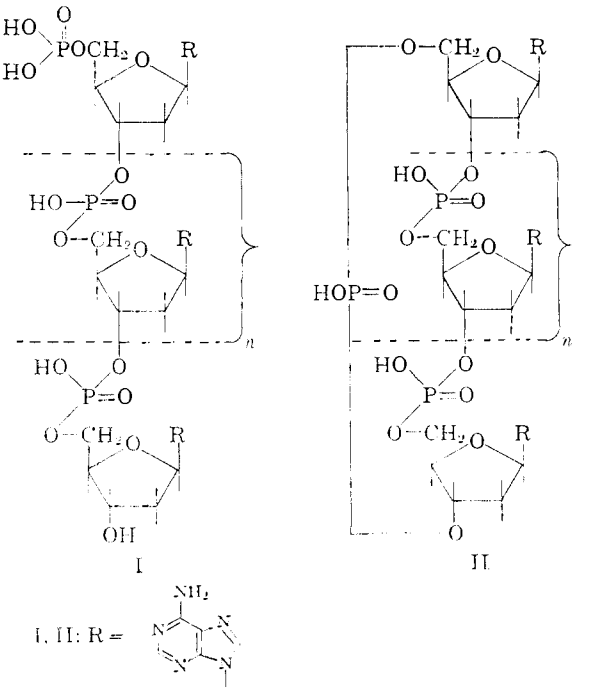
Benzoylation of deoxyadenosine-5′ phosphate with an excess of benzoyl chloride followed by controlled alkaline hydrolysis gives *N*-benzoyldeoxyadenosine-5′ phosphate in essentially quantitative yield. The reaction of a mixture of *N*,3′-*O*-diacetyl-(or dibenzoyl-)-deoxyadenosine-5′ phosphate and *N*-benzoyldeoxyadenosine-5′ phosphate in anhydrous pyridine with dicyclohexylcarbodiimide at room temperature for several days gives polymeric products which, after the removal of the protecting groups, are separated by chromatography on a diethylaminoethyl (DEAE)-cellulose (bicarbonate form) column. Subsequent purification of the individual peaks either by paper chromatography, or, preferably, by rechromatography on DEAE-cellulose (chloride form) columns at *p*H 5.5 gives pure homologous polynucleotides containing repeating C_3'–C_5' internucleotide bonds (I) as the major products. Members containing up to eight units in a chain have been fully characterized. A concomitant reaction during polymerization is the intramolecular cyclization involving the 5′-phosphomonoester and the 3′-hydroxyl end groups. In contrast, however, with the previously described polymerizations of pyrimidine deoxyribomononucleotides, none of the cyclic dinucleotide (II, $n = 0$) is observed and, instead, the cyclic trinucleotide (II, $n = 1$) is a major product. A series of compounds which contaminate the homologous polynucleotides bearing 5′-phosphomonoester end groups is the pyrophosphates formed, presumably, by linking mono- and/or oligonucleotides through their phosphomonoester groups.

Synthetic work in the polynucleotide field which has been in progress in this Laboratory during the past several years[4,5] aims at the development of methods for the stepwise synthesis of polynucleotides with predetermined sequences of nucleotide units and for the polymerization of mononucleotides to form relatively simple polymers containing one kind of mononucleotide residue. In the latter part of the synthetic program, studies were first carried out with the isomeric thymidine mononucleotides[6–8] (thymidine-5′ and -3′ phosphates) and satisfactory methods for the synthesis, separation and characterization of homologous polynucleotides containing up to twelve units in a chain were developed. Difficulties were encountered when a direct extension of the synthetic method to other deoxyribomononucleotides was attempted. The most serious problem was that of the reactivity of the amino groups present in the pyrimidine and purine rings, and to realize the exclusive formation of the naturally occurring C_5'–C_3' internucleotide bonds it was necessary to prepare mononucleotides containing suitably protected amino groups. More recently, *N*-anisyl and related derivatives of deoxycytidine-5′ phosphate were prepared and these served as suitable starting materials for the synthesis of deoxycytidine polynucleotides.[9] It was desirable to extend these studies to the synthesis of polynucleotides derived from purine deoxyribomononucleotides and the present communication reports on a simple method for the preparation of *N*-benzoyldeoxyadenosine-5′ phosphate and the synthesis from it of deoxyadenosine polynucleotides[10] (general structure I). Homologous members containing up to eight members in a chain have been obtained pure and characterized. An interesting difference between the previous polymerization of pyrimidine deoxyribonucleotides and the present polymerization result is the absence of the cyclic dinucleotide (II, $n = 0$) and the virtual replacement of this by the cyclic trinucleotide (II, $n = 1$). A number of observations has been made from time to time in this Laboratory on the ease of acylation and deacylation of the amino groups in the cytosine, adenine and guanine moie-



(1) Paper X, W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **236**, 1144 (1961).

(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and the National Research Council of Canada, Ottawa.

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(4) H. G. Khorana, in "The Nucleic Acids," Vol. III, E. Chargaff and J. N. Davidson, eds., Academic Press, Inc., New York, N. Y., 1960, p. 105.

(5) H. G. Khorana, "Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., in press.

(6) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6224 (1958).

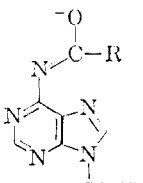
(7) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

(8) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).

(9) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).

(10) Brief mention of this work has been made previously [ref. 4, 5 and H. G. Khorana, *Federation Proc.*, **19**, 931 (1960)].

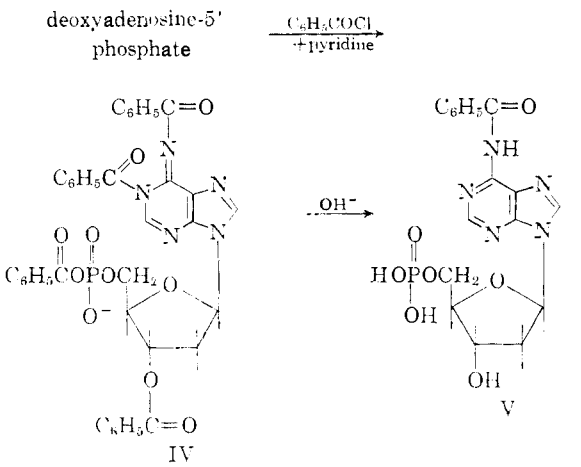
ties of the corresponding nucleosides (*cf.* ref. 9). In general, these weakly basic amino groups cannot be selectively acylated in the presence of free hydroxyl functions in the sugar part of the nucleosides.[11] Additional complications in the use of limited amounts of acylating agents arise in the case of nucleotides due to anhydride exchange reactions.[12] Consequently, the general approach taken in the preparation of *N*-acylmononucleotides has been to acylate fully the starting material and then remove all of the acyl groups except that on the amino group. For success in this approach, knowledge is required of the approximate rates of deacylation of the *N*- and *O*-acyl groups in the different mononucleotides.[13] An earlier study of the alkaline hydrolysis (see Experimental) of *N*,3′-*O*-diacetyldeoxyadenosine-5′ phosphate at *p*H 11.3 showed that the rate of *N*- and *O*-deacetylation was competitive. Subsequently it was found that there were marked differences in the rates of *O*- and *N*-deacylation in rather strongly alkaline (*e.g.*, 1 *N* sodium hydroxide) solutions. The cause of the "stability" of the *N*-acyl groups at high *p*H appears to be the ionization of the amide group as shown in the partial formula III. This ionization according to spectrophotometric determination occurs at about *p*H 11.



III

Using the above observations and working with benzoyl and anisyl derivatives, a simple and highly satisfactory method for the preparation of *N*-benzoyl and *N*-anisyl deoxycytidine-5′ phosphate from the parent nucleotide was recently developed. Analogously, in the present work, deoxyadenosine-5′ phosphate was benzoylated with an excess of benzoyl chloride and the product, presumably IV,[14,15] treated with sodium hydroxide under carefully controlled conditions. *N*-Benzoyldeoxyadenosine-5′ phosphate (V) was isolated in a high yield.

The half-life of the *N*-benzoyl group of V in 1 *N* sodium hydroxide at room temperature was found



to be about 52 hr., the group thus being much more stable than the corresponding group in *N*-benzoyldeoxycytidine-5′ phosphate.[9] As found earlier with the latter compound, ammonia was much more effective for removal of the benzoyl group, the half-life in 9 *N* ammonia at room temperature being 3.8 hr.[16]

With a view to minimizing the intramolecular cyclization reaction leading to the formation of compounds of the type II, polymerizations of mononucleotides were described recently in which some 25% of the starting material contained a suitably protected 3′-hydroxyl group.[8,9] In the present work, initially, a mixture of *N*,3′-*O*-diacetyldeoxyadenosine-5′ phosphate and *N*-benzoyldeoxyadenosine-5′ phosphate (V) was used for polymerization. Subsequently, a suitable mixture of *N*,3′-*O*-dibenzoyldeoxyadenosine-5′ and *N*-benzoyldeoxyadenosine-5′ phosphates, prepared directly from the fully benzoylated product IV, was used. Reaction with dicyclohexylcarbodiimide under the standard conditions[8,9] gave, after work-up and removal of the protecting groups, a mixture which was separated on a diethylaminoethyl (DEAE)-cellulose column in the bicarbonate form. A typical elution pattern is shown in Fig. 1 and the distribution of the nucleotidic material in the major peaks which were further investigated is shown in Table I.

TABLE I

CHROMATOGRAPHY OF DEOXYADENOSINE POLYNUCLEOTIDES

Distribution of nucleotide material in peaks of Fig. 1[a]			
Peak no.[b]	% of total nucl. mater. in peaks of Fig. 1	Peak no.[b]	% of total nucl. mater. in peaks of Fig. 1
2	3.27	9	12.05
3	26.30	10	9.16
4	18.31	11	4.95
5	8.29	12	3.09
7	14.20	13	1.50

[a] Total recovery of nucleotidic material was 0.72 mmole (10,300 optical density units at 260 mμ) representing an over-all yield from deoxyadenosine-5′ phosphate of 72% or greater since this figure does not make allowance for the hypochromicity which occurs during polymerization. [b] For the composition of the peaks see Table II.

Linear Polynucleotides.—The homologous members di- to octa-nucleotides (I, n = 0–6) were present, respectively, in peaks 4, 7, 9, 10, 11, 12 and

(11) The amino group on the cytosine ring is an exception. Thus, for example, the amino group in 5-*O*-triphenylmethyldeoxycytidine may be selectively benzoylated by using a limited amount of benzoic anhydride. B. Lerch and H. G. Khorana, unpublished work (*cf.* ref. 5 and 9). Similarly, Michelson [*J. Chem. Soc.*, 3655 (1959)] has reported on the selective acetylation of the amino group in cytidine-2′,3′-cyclic phosphate.

(12) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959).

(13) The *N*- acyl groups in different nucleosides vary in stability among themselves. In alkali, the order of stability is guanosine > adenosine > cytidine (unpublished work of P. T. Gilham, ref. 9 and present work).

(14) Benzoylation with an excess of benzoyl chloride readily introduces two benzoyl groups on the adenine ring of the corresponding nucleosides; *cf.* M. Smith, D. H. Rammler, I. Goldberg and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, in press (1961).

(15) The position of the second benzoyl group on the adenine ring is shown arbitrarily to be N^1. Some support for this has been provided by the analogous study of cytidine derivatives; D. M. Brown, A. R. Todd and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956).

(16) This determination was carried out by Dr. A. F. Turner.

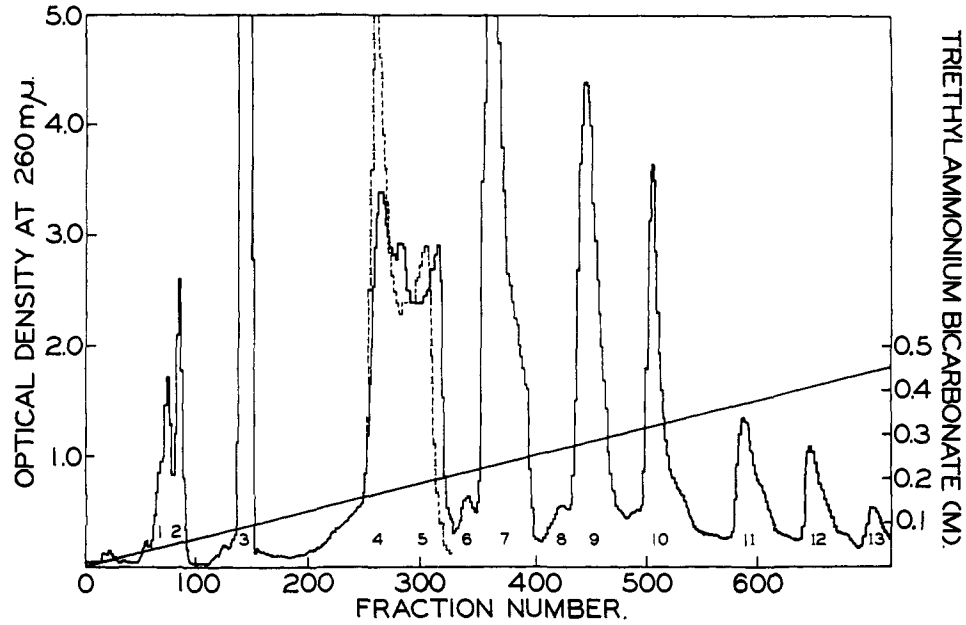


Fig. 1.—Chromatography of deoxyadenosine polynucleotides (total polymeric mixture) on a DEAE-cellulose (bicarbonate) column. Rechromatography of fractions (250–325) separated only two peaks as shown by the broken line. For further details of the procedure see text. The sloping line shows linear triethylammonium bicarbonate gradient.

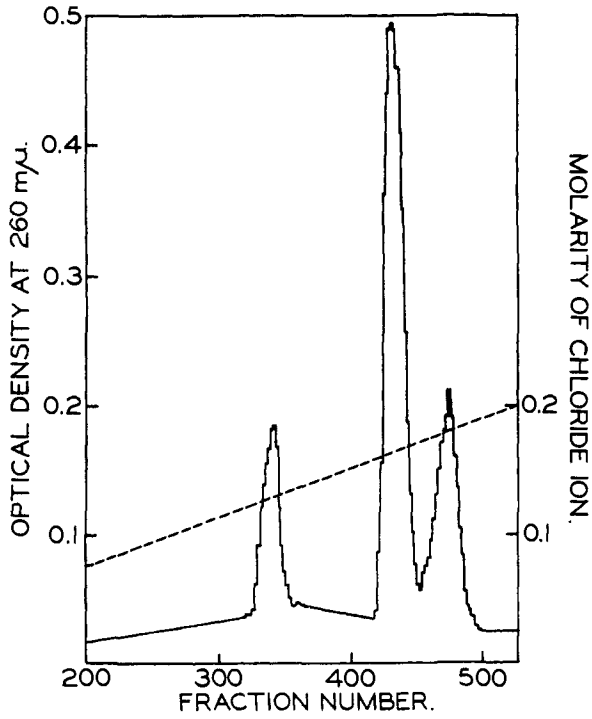


Fig. 2.—Rechromatography of the pentanucleotide peak (peak 10 of Fig. 1) on a DEAE-cellulose (chloride) column at *p*H 5.5. Elution carried out using a linear gradient (broken line) of lithium chloride adjusted to *p*H 5.5 with lithium acetate buffer. For details see Table II.

13. While further purification of the lower homologs could be accomplished by preparative paper chromatography, a general and satisfactory technique for separating the products involved rechromatography on DEAE-cellulose columns in the chloride form by carrying out the elution at *p*H 5.5. This *p*H was chosen because the impurities (see below) that were present in the linear polynucleotides were those that carried the same negative charge at *p*H 7.5, the *p*H of the initial chromatography. At the lower *p*H, the secondary phosphoryl dissociation of the phosphomonoester end groups would be largely suppressed, without significant protonation of the amino groups in the adenine moieties of the polynucleotides. The procedure was uniformly successful in effecting complete purification of the linear polynucleotides up to the octanucleotide, the largest member studied. The conditions used for rechromatography of the different peaks and the percentages of the pure products obtained are given in Table II. A typical elution result is that which was obtained with the pentanucleotide and is shown in Fig. 2. The three distinct peaks obtained here were obtained on rechromatography of all of the other homologs as well. The major peak invariably was the middle one and it corresponded to the polynucleotide bearing a 5′-phosphomonoester end group (I).

The characterization of the homologous linear polynucleotides was accomplished in a variety of ways. They were all homogeneous on paper chromatograms in a number of solvents (R_f's. in Table III) and on paper electrophoresis at both *p*H 5 and 7.5. As discussed previously,[8,9] a stringent test of purity was provided by enzymic removal of the terminal phosphomonoester end groups followed by paper chromatography of the products. The spots corresponding to the starting materials disappeared completely and were replaced by single faster traveling spots corresponding to the series (d)A(pA)$_n$pA.[17] Furthermore, on incubation of the latter series of compounds with

(17) Abbreviations used in this paper are as currently used by *J. Biol. Chem.*, and as further developed in ref. 8.

TABLE II

RECHROMATOGRAPHY OF INDIVIDUAL PEAKS OF FIG. 1 ON DEAE-CELLULOSE (CHLORIDE) COLUMNS AT *p*H 5.5 (FURTHER DETAILS AS IN TEXT)

Peak no. from Fig. 1	Condit. of rechromatography and concn. of salt—Mixing vessel (LiAc)	Reservoir		Concn. of chloride ion at mid-point of peaks		Products[17]	% of oligonucleotides in peaks after rechromat.
3	2 l. of 0.05 *M*	2 l. of	0.05 *M* LiAc + 0.1 *M* LiCl	1	0.01	(d)pA	82.6
				2	.08	(d)ApApA	11.0
				3	.11	Pyrophosphate	6.4
4	2 l. of 0.05 *M*	2 l. of	0.05 *M* LiAc + 0.1 *M* LiCl	1	.013	(d)ApA	15.0
				2	.071	(d)pApA	35.6
				3	.096	Cyclic trinucleotide	49.5
5	4 l. of 0.01 *M*	4 l. of	0.01 *M* LiAc + 0.2 *M* LiCl	1	.095	Cyclic trinucleotide	100
7	4 l. of 0.01 *M*	4 l. of	0.01 *M* LiAc + 0.2 *M* LiCl	1	.048	(d)ApApA	35
				2	.095	(d)pApApA	57
				3	.12	Cyclic tetra + pyrophosphates	7.9
9	4 l. of 0.01 *M*	4 l. of	0.01 *M* LiAc + 0.2 *M* LiCl	1	.092	(d)A(pA)$_2$pA	20.2
				2	.137	(d)pA(pA)$_2$pA	64.5
				3	.156	Pyrophosphates	15.3
10	4 l. of 0.01 *M*	4 l. of	0.01 *M* LiAc + 0.2 *M* LiCl	1	.13	(d)A(pA)$_3$pA	19.0
				2	.164	(d)pA(pA)$_3$pA	55.7
				3	.182	Pyrophosphates	25.3
11	4 l. of 0.01 *M*	4 l. of	0.01 *M* LiAc + 0.3 *M* LiCl	1	.17	(d)A(pA)$_4$pA	21.2
				2	.21	(d)pA(pA)$_4$pA	60.0
				3	.238	Pyrophosphates	18.7
12	6 l. of 0.01 *M*	6 l. of	0.01 *M* LiAc + 0.3 *M* LiCl	1	.204	(d)A(pA)$_5$pA	19.2
				2	.23	(d)pA(pA)$_5$pA	59.9
				3	.247	Pyrophosphates	20.9
13[a]	6 l. of 0.01 *M*	6 l. of	0.01 *M* LiAc + 0.3 *M* LiCl	1	.15	(d)A(pA)$_6$pA	11.2
				2	.178	(d)pA(pA)$_6$pA	63.3
				3	.19	Pyrophosphates	25.9

[a] Column used in this experiment was 23 cm. long × 1 cm. dia.

spleen phosphodiesterase[1,18] (*cf.* ref. 9) complete degradation to the mononucleotide, identified as deoxyadenosine-3′ phosphate, and deoxyadenosine occurred. These results proved that the synthetic polynucleotides contained exclusively the C_3'–C_5' internucleotide linkages and that no amino groups were involved. Degradation of the above series of compounds lacking terminal phosphomonoester groups by venom phosphodiesterase[19] also proceeded to completion and gave deoxyadenosine-5′ phosphate and deoxyadenosine in the ratios consistent with the chain length. The results of this analysis are given in Table IV.

The first peaks from the rechromatography on DEAE-cellulose (chloride) columns (Table II) were also identified by the methods discussed above as the homologous series (d)A(pA)$_n$pA. Thus the first peak on rechromatography of the dinucleotide peak was (d)ApA and that from the trinucleotide (d)ApApA. These peaks must have arisen by the non-enzymic loss of the terminal phosphomonoester groups subsequent to the initial chromatography on the bicarbonate column. The extent of the terminal dephosphorylation varied and in subsequent work[20] was almost eliminated by maintaining low temperature and avoiding prolonged evaporation during the removal of the triethylammonium bicarbonate from the eluted peaks.[21]

The third series of peaks from the DEAE-cellulose (chloride) columns, which would be expected to contain one negative charge in excess of the corresponding middle peaks at *p*H 5.5, contained either the cyclic oligonucleotides (II) or the pyrophosphates formed by joining mononucleotides and/or different oligonucleotides. These compounds are discussed below.

Cyclic Oligonucleotides.—Analogously to the formation of thymidine and deoxycytidine-3′,5′-cyclic phosphate in the previous polymerizations, deoxyadenosine-3′,5′ cyclic phosphate was identified as essentially the sole component of peak 2 (Fig. 1). Its paper chromatographic and electrophoretic properties were consistent with its structure. A direct comparison was made of the electrophoretic mobilities at *p*H 5 and 7.5 with those of adenosine-3′,5′ cyclic phosphate.[22] Furthermore, hydrolysis with barium hydroxide, which, presumably, gave a mixture of deoxyadenosine-3′ and

(18) L. A. Heppel and R. J. Hilmoe, in "Methods in Enzymology," Vol. II, S. P. Colowick and N. P. Kaplan, eds., Academic Press, Inc., New York, N. Y., 1955, p. 565.

(19) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

(20) In the previous work on deoxycytidine and thymidine polynucleotides, similar dephosphorylation occurred to a negligible extent.

(21) In general, phosphomonoester groups are extremely stable in the *p*H range above 7–8. It is possible that during repeated evaporations from aqueous solutions, in order to ensure complete removal of triethylammonium bicarbonate, the *p*H fell to the range 4–5. Triethylamine itself is lost readily from solutions of those nucleotides which contain amino groups on the heterocyclic rings. Prolonged exposure of the polynucleotides at *p*H 4–5, where spontaneous hydrolysis of phosphomonoester groups is at a maximum, probably was the cause of terminal dephosphorylation.

(22) M. Smith, G. I. Drummond and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 698 (1961).

TABLE III

R_f'S OF POLYNUCLEOTIDES ON PAPER CHROMATOGRAMS

Solvent systems and technique as in text

Compound	Solvent B R_f	Solvent B R_f relative to pA	Solvent C R_f	Solvent C R_f relative to pA	Solvent D R_f	Solvent D R_f relative to pA	Solvent G R_f	Solvent G R_f relative to pA
(d)pA	0.61	1.0	0.145	1.0	0.22	1.0	0.42	1.0
(d)pApA	.583	0.956	.073	0.500	..	0.13	..	0.76
(d)pApApA	.558	.915	.029	.2	..	0.037	..	.585
(d)pA(pA)$_2$pA	.544	.892	..	.1	..	...	..	.43
(d)pA(pA)$_3$pA	.520	.850	..	..	..	...	..	.31
(d)pA(pA)$_4$pA	.475	.780	..	..	..	...	..	.207
(d)pA(pA)$_5$pA	.415	.680	...	..	..	...	..	.136
(d)pA(pA)$_6$pA	.378	.620	...	..	..	...	..	.10
(d)ApA	.795	1.29	0.394	2.72	..	1.51	..	1.4
(d)ApApA	.693	1.125	...	..	..	0.65	..	1.125
(d)A(pA)$_2$pA	.628	1.02	...	..	..	.21	..	0.85
(d)A(pA)$_3$pA	.565	0.92	...	..	..	.046	..	.65
(d)A(pA)$_4$pA	.545	.885	...	..	..	.01	..	.475
(d)A(pA)$_5$pA	.488	.79	...	..	..	...	..	.275
(d)A(pA)$_6$pA	...	..	...	..	..	...	..	.092
Deoxyadenosine-3′,5′ cyclic phosphate	.702	1.14	.38	2.6	..	1.85	0.6	1.43
Cyclic trinucleotide	.685	1.11	.202	1.39	..	0.96	..	1.24

TABLE IV

RESULTS OF DEGRADATION BY SNAKE VENOM PHOSPHODIESTERASE OF DEOXYADENOSINE POLYNUCLEOTIDES (I) AFTER ENZYMIC REMOVAL OF TERMINAL PHOSPHOMONOESTER GROUPS

Compound	Product of hydrolysis (d)pA Optical density of spot/ml. at 260 mμ	Product of hydrolysis (d)A Optical density of spot/ml. at 260 mμ	Ratio (d)pA/(d)A Found	Ratio (d)pA/(d)A Calcd.
(d)pApA	0.36	0.37	1	1
(d)pApApA	1.25	.56	2.2	2
(d)pA(pA)$_2$pA	2.055	.68	3.02	3
(d)pA(pA)$_3$pA	1.355	.35	3.9	4
(d)pA(pA)$_4$pA	3.56	.687	5.18	5
(d)pA(pA)$_5$pA	3.655	.600	6.09	6
(d)pA(pA)$_6$pA	2.53	.33	7.7	7

-5′ phosphates, followed by enzymic removal of the phosphomonoester groups gave deoxyadenosine.

No evidence was obtained for the presence of cyclic deoxyadenosine dinucleotide (II, n = 0), the corresponding compound being the most abundant (14–18%) single product in the polymerizations of pyrimidine deoxyribomononucleotides. The place of the cyclic dinucleotide was evidently taken by the next homolog, the cyclic trinucleotide (II, n = 1) which was present in a total amount of about 15%, being the main constituent of peak 5 and emerging after the linear dinucleotide. (This order of emergence from the bicarbonate column corresponds to those of analogous compounds in the pyrimidine series.[8,9]) Further purification was accomplished by chromatography on a DEAE-cellulose (chloride) column at pH 5.5 as described above for linear polynucleotides. The cyclic trinucleotide corresponded to the third peak obtained on rechromatography of the linear dinucleotide peak.

The structural assignment for the cyclic trinucleotide followed from its position of elution in the initial bicarbonate column, its resistance to phosphomonoesterase, and its electrophoretic properties relative to the linear trinucleotide at pH 5 and 7.5. Further, as expected from previous studies of the venom phosphodiesterase,[9,19,23] the compound was degraded extremely slowly by this enzyme to give deoxyadenosine-5′ phosphate; no degradation occurred under the conditions that sufficed for complete degradation of the linear trinucleotide (I, n = 1). Finally, the technique which was developed previously for determination of the absolute size of the cyclic oligonucleotides was also adapted to the present case. In this technique (Chart I) the aim was to isolate after acidic hydrolysis of one of the glycosyl bonds, the largest linear product of degradation, namely, the product containing all the phosphorus of the original molecule but one less nucleoside unit. For this purpose, the cyclic trinucleotide (II, n = 1) was treated with 0.1 N hydrochloric acid at 50° for 20 min. to form VI and the reaction mixture then was treated with concentrated ammonia to give, after the elimination reactions,[24] the substance (d)pApAp (VII). Digestion of the latter with phosphomonoesterase gave (d)ApA (VIII) which was identified by paper chromatographic comparison with a sample prepared by phosphomonoesterase treatment of (d)pApA. Although other products were also formed during this series of degradations (see Experimental), the fact that the dinucleoside phosphate (d)ApA, was recognized as a product showed that the original molecule was larger than the cyclic dinucleotide.

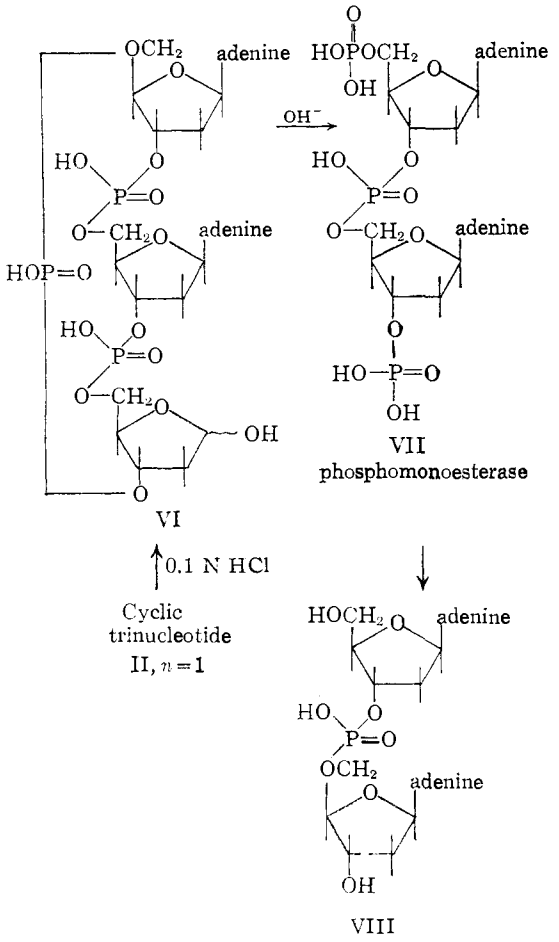
Cyclic tetranucleotide (II, n = 2) would be expected to be in peak 7 of Fig. 1 and after rechromatography on the chloride column, it would emerge after the linear trinucleotide. Pyrophosphates formed by linking two linear dinucleotides or a tri- and a mononucleotide would also emerge at the same position. The evidence used for distinguishing between the two classes of compounds was the rate of degradation by the venom phos-

(23) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2114 (1959).

(24) β-Eliminations releasing phosphate anions would be expected to be very rapid in this series. In an unpublished experiment, deoxyribose-3 phosphate was found to be considerably degraded to inorganic phosphate on paper chromatography in an ammoniacal solvent system (solvent C in Experimental).

CHART I

DEGRADATION OF CYCLIC TRINUCLEOTIDE (II, n = 1)



phodiesterase. Most of the nucleotidic material obtained in the third peak on rechromatography of peak 7 was rapidly degraded as expected for linear structures bearing 3′-hydroxyl end groups and only a small part seemed to be resistant. The latter part probably was the cyclic tetranucleotide.

Other Minor Products.—Beyond the trinucleotide level, the amounts of peaks that emerged after the linear polynucleotide peaks during rechromatography on DEAE-cellulose (chloride) columns (third series of peaks) increased with the increase in the size of the polynucleotide chain. These peaks, in view of their rapid degradation by venom phosphodiesterase probably consisted of pyrophosphates linking up different oligonucleotides. Similar side products have been found in previous polymerization reactions, although their amounts vary with the mononucleotide.[7-9]

Peak 1 contained two substances, in addition to some deoxyadenosine-3′,5′ cyclic phosphate, which had R_f's of 0.066 and 0.85 on paper chromatograms developed in the isopropyl alcohol–ammonia–water (7:1:2) solvent system. The fast traveling substance, which contained phosphorus, was probably an N-phosphorylurea, similar minor products having been encountered previously.[7-9] The slow traveling substance (slower than deoxyadenosine-5′ phosphate) had all the properties of pyridinium-nucleoside phosphates that were also encountered earlier.[7-9] Thus its ultraviolet absorption was additive of those of *N*-methylpyridinium cation and deoxyadenosine. Treatment of the compound with sodium borohydride to reduce the pyridinium chromophore gave an absorption spectrum similar to that of deoxyadenosine. On paper electrophoresis at *p*H 7, the compound moved toward the anode while at *p*H 3.5, it moved toward the cathode.

Concluding Remarks.—The present work has reported on the extension of the methods developed previously to the synthesis of purine (adenine) deoxyribonucleotide polymers. The general features of the present results are similar to those obtained with the pyrimidine deoxyribonucleotides.

A notable difference between the present and the earlier results was the absence of the cyclic dinucleotide (II, n = 0) and the presence, instead, of a large amount of the cyclic trinucleotide (II, n = 1). The formation of the cyclic dinucleotide must somehow be inhibited by the conformation of the reactive intermediates in the cyclization reaction, although, as judged by molecular models, the formation of the cyclic dinucleotide does not appear to be prohibited.

Although the extent of polymerization has varied somewhat with the mononucleotide studied, it has so far not been possible to form higher than dodecanucleotide in any appreciable amount by the chemical methods available at present. Further studies are necessary to see whether chemical methods can be made to give much larger polymers.

Experimental

General Methods.—Paper chromatography was carried out by the descending technique using Whatman No. 1 or 40 (double acid-washed) paper. The solvent systems used were: solvent A, isopropyl alcohol–concd. ammonia–0.1 *M* boric acid (7:1:2, v./v.); solvent B, isobutyric acid–1 *M* ammonium hydroxide–disodium ethylenediamine tetraacetate (0.1 *M*) (100:60:1.6, v./v.); solvent C, isopropyl alcohol–concd. ammonia–water (7:1:2, v./v.); solvent D, ethyl alcohol–1 *M* ammonium acetate (*p*H 7.5) (5:2, v./v.); solvent E, isopropyl alcohol–0.5 *M* ammonium acetate (*p*H 6) (5:2, v./v.); solvent F, *n*-butyl alcohol–acetic acid–water (5:2:3, v./v.); solvent G, *n*-propyl alcohol–concd. ammonia–water (55:10:35, v./v.).

The R_f's of different compounds are listed in Table III. Solvent G proved to be particularly useful for separating the higher oligonucleotides in periods of 2–3 days.

Paper electrophoresis was carried out on Whatman 3MM strips in an apparatus similar to that designed by Markham and Smith.[25] The buffers used were 0.05 *M* triethylammonium bicarbonate (*p*H 7.5), 0.05 *M* ammonium acetate (*p*H 5) and 0.05 *M* ammonium formate (*p*H 3.5).

Pyridine was dried over calcium hydride for a period of several days. All evaporations were carried out under reduced pressure and at outside bath temperature of below 40°. Phosphorus analysis was carried out by King's method.[26]

Deoxyadenosine-5′ phosphate used was a commercial preparation. It was checked carefully for its purity by paper chromatography in solvents A and B by applying approximately 1 μmole of material on a 1″ band. The former solvent detected ribonucleotide contaminants which were present in some samples. By these checks and determination of ultraviolet absorption characteristics its freedom from other deoxyribonucleotides or ribonucleotides

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

(26) E. J. King, *ibid.*, **26**, 292 (1932).

or any other ultraviolet light absorbing materials was made completely certain.

Purification of commercial samples containing ribonucleotide analogs and other impurities (*e.g.*, adenine) was accomplished as follows. Ammonium deoxyadenosine-5′ phosphate (100 mg.) was dissolved in 0.005 *M* sodium tetraborate (20 ml.) and the solution applied to a column (6 cm. × 1 cm.) of Dowex-1 (chloride) ion exchanger (200–400 mesh). The column was eluted with a linear gradient of sodium tetraborate and ammonium chloride. The mixing vessel contained 2 l. of 0.005 *M* sodium tetraborate and the reservoir 2 l. of 0.005 *M* sodium tetraborate in 0.1 *M* ammonium chloride. Fractions of 18 ml. per 10 min. were collected and the eluted solution was estimated spectrophotometrically at 260 mμ. Three peaks were obtained: the first contained adenine, the second contained deoxyadenosine-5′ phosphate and the third contained adenosine-5′ phosphate.

Fractions containing the second peak were combined; charcoal (Norit A) was added with stirring until the optical density at 260 mμ (1-cm. cell) of the solution fell to ≤0.05 and the charcoal was then collected by filtration. After washing the charcoal with water, the nucleotide was eluted with a solution of 50% aqueous ethanol containing 5% concd. ammonium hydroxide until the optical density at 260 mμ (1-cm. cell) of the eluate became negligible.

The ammoniacal solution was removed *in vacuo* (<30°) to give 80 mg. of chromatographically homogeneous deoxyadenosine-5′ phosphate.

Enzyme Experiments: (a) Removal of Terminal Phosphomonoester Groups.—The prostatic phosphomonoesterase prepared by the method of Boman[27a] was used in early work: later, the alkaline phosphatase from *Escherichia coli* as prepared by Garen and Levinthal[27b] was used. The manner of the use of these enzymes has been described in an earlier publication.[8]

(b) Degradations Using Venom and Spleen Phosphodiesterases.—Venom phosphodiesterase was purified as described previously.[19] Spleen phosphodiesterase was a sample given by Dr. Razzell[1] and had been prepared essentially by the method of Hilmoe.[28] The procedures for the use of these enzyme preparations have been described previously.[8,9] For the identification of the mononucleotide formed after degradation of the polynucleotides lacking 5′-phosphate end groups with spleen diesterase, the material corresponding to the nucleotide was eluted and rechromatographed in saturated ammonium sulfate–isopropyl alcohol–water (80:2:18, v./v.) with ammonium hydroxide added to adjust the *p*H to 6. This solvent has been utilized by Cunningham[29] to separate deoxyadenosine-3′ and -5′ phosphates. A single ultraviolet light absorbing spot was obtained alongside authentic deoxyadenosine-3′ phosphate.

***N*-3′-*O*-Diacetyldeoxyadenosine-5′ Phosphate.**—An aqueous solution of pyridinium deoxyadenosine-5′ phosphate (0.3 mmole), prepared by exchanging the diammonium salt by passage through a bed of Amberlite IR-120 (pyridinium resin), was lyophilized. The resulting finely divided powder was suspended in anhydrous pyridine (5 ml.) and acetic anhydride (3.5 ml.) was added. The sealed reaction mixture was shaken in the dark overnight when a clear solution resulted. After a further 24 hours in the dark at room temperature, water (10 ml.) was added and the mixture kept for a further 1 hour. The solution was evaporated *in vacuo* at <20° and the residue redissolved in 20 ml. of 10% aqueous pyridine. The solution was reevaporated and the process of dissolution and evaporation repeated several times to remove most of the pyridinium acetate. The residual gum was next dissolved in some water containing 1 ml. of pyridine and the solution lyophilized. The white powder thus obtained was dissolved in dry pyridine and stored at 4°. Paper chromatography in solvent E showed two ultraviolet light absorbing spots. The faster traveling was non-nucleotidic (λ_{max} 295 mμ) and is that which is encountered in all acetylations, arising evidently from the reaction of acetic anhydride with pyridine. The single nucleotidic spot was the desired *N*,*O*-diacetyldeoxyadenosine-5′ phosphate λ_{max} 272 mμ, *p*H 7.[30,31]

(27) (a) H. G. Boman, *Arkiv. Kemi*, **12**, 453 (1958); (b) A. Garen and C. Levinthal, *Biochem. Biophys. Acta*, **38**, 470 (1960).

(28) R. J. Hilmoe, *J. Biol. Chem.*, **235**, 2117 (1960).

(29) L. Cunningham, *J. Am. Chem. Soc.*, **80**, 2546 (1958).

(30) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).

Benzoylation of Deoxyadenosine-5′ Phosphate.—Finely divided pyridinium deoxyadenosine-5′ phosphate (1 mmole) prepared by lyophilization of a dilute aqueous solution was suspended in 20 ml. of dry pyridine and freshly distilled benzoyl chloride (2.5 ml., 20 mmoles) was added. The clear light yellow solution which soon resulted was kept in the dark under exclusion of moisture. After 1 hour at room temperature it was poured into a mixture of chloroform (50 ml.) and water (50 ml.) in the cold. After about 15 minutes the chloroform layer was separated and the aqueous layer extracted again with chloroform (2 × 25 ml.). The combined chloroform extracts, which contained virtually all of the nucleotidic material, were evaporated *in vacuo* to a gum. Further processing and isolation of *N*-benzoyldeoxyadenosine-5′ phosphate was carried out in one of the several ways described below.

***N*-Benzoyldeoxyadenosine-5′ Phosphate.**—(a) The gum as obtained above was dissolved in pyridine (28 ml.) and to the solution was added 28 ml. of 1 *N* sodium hydroxide. After swirling 10 minutes at room temperature the solution was treated with a large excess of Amberlite IR-120 (pyridinium) resin to remove all the sodium ions. The resin was removed by filtration and washed thoroughly with water. The combined filtrate and washings were concentrated under reduced pressure to about 20 ml. and benzoic acid which separated was removed by ether extraction. The aqueous solution was lyophilized and the resulting powder dissolved in 5 ml. of pyridine. A 0.5-ml. aliquot of this solution (0.1 mmole of nucleotide) was diluted with 10 ml. of a mixture of isopropyl alcohol and water (3:1, v./v.) and the solution applied to the top of a cellulose powder column (28 cm. × 4.1 cm.) packed in the same isopropyl alcohol–water mixture. Elution was carried out with the solvent mixture at a flow rate of 1 ml. per 4 min., 5-ml. fractions being collected. Fractions 60–80 contained benzoic acid, while fractions 80–100 contained *N*,3′-*O*-dibenzoyldeoxyadenosine-5′ phosphate. *N*-Benzoyldeoxyadenosine-5′ phosphate appeared in fractions 140–220, which were combined and evaporated *in vacuo* after the addition of 5 ml. of pyridine. The residue was dissolved in dilute aqueous pyridine and the solution lyophilized. The resulting white powder was dissolved in the minimum volume of anhydrous methyl alcohol and to the solution was added a 1 *M* methyl alcoholic solution of calcium chloride. The precipitate of calcium *N*-benzoyldeoxyadenosine-5′ phosphate was collected by centrifugation and washed repeatedly with a mixture of methyl alcohol and acetone (1:3) to remove calcium chloride, then ether and finally dried at 70° *in vacuo*. The yield of the hydrated salt was 36 mg. (70%). The ultraviolet absorption characteristics were: *p*H 2: λ_{max} 288 mμ, ϵ_{max} 27,000; λ_{min} 240 mμ, ϵ_{min} 9,280; *p*H 8: λ_{max} 280 mμ, ϵ_{max} 18,300; λ_{min} 243 mμ, ϵ_{min} 9,300; *p*H 11.3: λ_{max} 299 mμ, ϵ_{max} 10,970; λ_{min} 255 mμ, ϵ_{min} 6,500.

Anal. Calcd. for $C_{17}H_{16}N_5O_7P\cdot Ca\cdot 3H_2O$: C, 38.72; H, 4.18; N, 13.28; P, 5.88. Found: C, 38.81; H, 4.32; N, 12.61; P, 6.11.

(b) For preparative purposes, the procedure used was as follows. The gum as obtained above from a 1-mmole run was dissolved in a cold mixture of pyridine (20 ml.) and water (10 ml.). To the solution was added 30 ml. of cold 2 *N* sodium hydroxide and the clear solution, which soon resulted, was kept for 9 min. in an ice-bath. An excess of Amberlite IR-120 (pyridinium) resin was added rapidly to neutralize and to remove the sodium ions. To ensure complete removal of the metal ions, the solution, after removal of the initial lot of added ion exchange resin, was passed through a column (15 cm. × 2 cm.) of the ion exchanger in the pyridinium form. The resin was washed thoroughly with water and the combined aqueous pyridine solution of the product, when examined by paper chromatography, showed only *N*-benzoyldeoxyadenosine-5′ phos-

(31) A 25 molar excess of acetic anhydride is routinely used for acetylation of nucleotides in order to avoid the exchange reactions leading to pyrophosphate formation [(H. G. Khorana and J. P. Vizsolyi, *ibid.*, **81**, 4660 (1959)]. However, in the acetylation of the amino group in adenine nucleotides a much larger excess is desirable since the rate of this acetylation is much slower than that of the acetylation of the 3′-hydroxyl group in the molecule. Thus using an approximately tenfold excess of acetic anhydride, Hayes, Michelson and Todd [*J. Chem. Soc.*, 808 (1955)] obtained from deoxyadenosine after overnight reaction, a good yield of 3′,5′-*O*-diacetyldeoxyadenosine.

phate and benzoic acid. The solution was evaporated *in vacuo* to about 20 ml. when benzoic acid largely crystallized. The mixture, which was kept cold in an ice-bath, was repeatedly extracted with ether to remove benzoic acid. The *p*H of the final solution was around 4 and paper chromatography[32] in solvent E showed complete absence of benzoic acid. Some pyridine was added and the solution of the nucleotide was lyophilized to give a dry powder (405 mg.) which was stored in the cold as its solution in pyridine.

In an alternative procedure, the separation of benzoic acid from *N*-benzoyldeoxyadenosine-5′ phosphate was achieved on a DEAE-cellulose column in the acetate form as described previously.[9] The yield of the lyophilized pyridinium salt was 45 mg. (85%).

Preparation of a Mixture of N-Benzoyl- and N,3′-O-Dibenzoyldeoxyadenosine-5′ Phosphate.—The procedure b described above for *N*-benzoyldeoxyadenosine-5′ phosphate was modified so as to give directly a mixture of *N*-benzoyldeoxyadenosine-5′ phosphate and *N*,3′-*O*-dibenzoyldeoxyadenosine-5′ phosphate in the approximate ratio of 7:3. The sirupy benzoylated product from 1 mmole of deoxyadenosine-5′ phosphate (see above) was dissolved in pyridine (100 ml.) and water (100 ml.) and to the solution was added 200 ml. of 0.5 *N* sodium hydroxide in an ice-bath. An excess of Amberlite IR-120 (pyridinium) resin was added rapidly after 23 min. to remove the alkali. Sodium ions were removed completely by passage of the total solution through a column of the same resin and the subsequent workup involving removal of benzoic acid by evaporation and extraction with ether was as described above. The yield of the lyophilized powder was 470 mg.

Polymerization of a Mixture of N,3′-O-Diacetyl- or Dibenzoyl-deoxyadenosine-5′ Phosphate and N-Benzoyldeoxyadenosine-5′ Phosphate.—The starting material used in the first experiment on polymerization was a mixture of *N*,3′-*O*-diacetyldeoxyadenosine-5′ phosphate (0.3 mmole) and *N*-benzoyldeoxyadenosine-5′ phosphate (0.7 mmole). Later a mixture of *N*,3′-*O*-dibenzoyldeoxyadenosine-5′ phosphate and *N*-benzoyldeoxyadenosine-5′ phosphate in approximately the same proportions prepared as described above was used as the starting material.

A pyridine solution of the mixture (total 1 mmole of nucleotide) was evaporated under reduced pressure and the gum redissolved in 5 ml. of dry pyridine. The process of evaporation and dissolution in pyridine was repeated several times, using a Dry Ice–acetone trap and admitting dry air into the system each time. The residual gum was dissolved in 0.5 ml. of dry pyridine and a solution of dicyclohexylcarbodiimide (300 mg., 1.46 mmoles) in 0.5 ml. of dry pyridine was added rapidly under exclusion of moisture from a pressure equalizing funnel. The sealed reaction mixture was shaken vigorously first manually and then mechanically in the dark. Except for the insoluble crystalline dicyclohexylurea, a clear solution resulted in about 24 hr. A further amount of dicyclohexylcarbodiimide (300 mg., 1.46 mmoles)[33] was then added and this again caused the separation of two phases. A clear solution again resulted on shaking for a further 48 hr. After a total of 5 days, aqueous sodium hydroxide (5 ml. of water +3 ml. of 1 *N* sodium hydroxide) was added and the mixture kept at room temperature for 1 hr. After three extractions with ether and filtration to remove dicyclohexylurea, the clear aqueous solution was passed through a column of Amberlite IR-120 ion exchange resin in the ammonium form. The total eluate and washings were evaporated to dryness *in vacuo* and the residue kept in concentrated ammonia (20 ml.) for 48 hr. at room temperature. The solution was evaporated to dryness and the residue dissolved in 3 ml. of water. The insoluble material (benzamide) was removed by centrifugation and the sediment washed twice with water. The combined aqueous solution was adjusted to *p*H 8–9 with ammonia for column chromatography.

Chromatography of Polymeric Mixture on a DEAE-cellulose (Bicarbonate) Column.—The solution of the polymeric products as obtained above from 1 mmole of the mononucleotide was applied at *p*H 8.5–9 on a DEAE-cellulose column (bicarbonate form)[8] (32 cm. × 4 cm. dia.) and the column was washed with water (1 l.). Elution was carried out using a linear salt gradient technique, the mixing vessel contained 6 l. of water and the salt vessel an equal volume of 0.45 *M* triethylammonium bicarbonate buffer (*p*H 7.5).[34] Approximately 18-ml. fractions were collected at 10-min. intervals. The elution pattern obtained in one of the typical runs is shown in Fig. 1. The distribution of nucleotidic material in different peaks is shown in Table I. The pooled fractions containing different peaks were evaporated to a gum under reduced pressure and temperature. In order to remove the residual triethylammonium bicarbonate, the residue was dissolved in water and the solution reevaporated, the process being repeated several times with the peaks corresponding to larger polymers. Frequently, for example for paper chromatography, the residual triethylammonium salts were passed through small columns of Amberlite IR-120 resin in the ammonium form to obtain the products as their ammonium salt.

Further Purification of Linear Polynucleotides on DEAE-cellulose (Chloride Form)Columns.—A part of the material (300–400 optical density units at 260 mμ) from the first peaks and essentially all of the material from the later peaks corresponding to linear polynucleotides was applied on top of DEAE-cellulose (chloride form) columns (19 cm. × 2.5 cm. dia.) and the columns were washed with about four bed volumes of water. Elution was carried out using lithium acetate (*p*H 5.5) buffer and a linear chloride ion gradient. The concentrations of salt and volumes of eluent used for rechromatography of different peaks are shown in Table II.

The polynucleotides contained in different peaks were recovered by adsorption onto the minimum amount of acid-washed Darco G-60 charcoal and subsequent elution with 50% aqueous ethyl alcohol containing 5% pyridine. The eluted materials were converted to the ammonium salts after removal of the solvent by passage through ammonium Dowex-50 ion exchange resin columns.

Hydrolysis of *N*,3′-*O*-Diacetyldeoxyadenosine-5′ Phosphate in Lithium Carbonate (By Dr. P. T. Gilham).—Diacetyldeoxyadenosine-5′ phosphate (about 0.01 mmole) purified by preparative chromatography in solvent D was kept in 10 ml. of saturated lithium carbonate solution (*p*H around 11.3) (excess solid lithium carbonate present) for 1 hour at 25°. Pyridinium Amberlite IR-120 resin was then added to remove all of the lithium, and after removing the resin by filtration the total aqueous solution and washings were concentrated *in vacuo* and the total material applied on a Whatman 3MM sheet and chromatographed in solvent D. Bands corresponding to the diacetyldeoxyadenosine-5′ phosphate (R_f 0.57), monoacetyldeoxyadenosine-5′ phosphate (R_f 0.43) and deoxyadenosine-5′ phosphate (R_f 0.28) were present. Their amounts were determined by elution, hydrolysis (2 hours) with 50% concentrated ammonia and spectrophotometric estimation at 260 mμ of the resulting deoxyadenosine nucleotide. The amounts were found to be: unchanged diacetyldeoxyadenosine-5′ phosphate, 16%; monoacetyldeoxyadenosine-5′ phosphate, 62%; and deoxyadenosine-5′ phosphate, 22%. Separately, band 2 corresponding to monoacetyldeoxyadenosine-5′ phosphate was eluted from another paper strip and subjected to paper electrophoresis at *p*H 3 using ammonium acetate buffer. Separation into two bands resulted, the faster-moving corresponding to *N*-acetyldeoxyadenosine-5′ phosphate and the slow-traveling corresponding to 3′-*O*-acetyldeoxyadenosine-5′ phosphate. The latter had the same mobility as deoxyadenosine-5′ phosphate. The ratios of the *N*- and 3′-*O*-monoacetyl derivatives were again determined after ammoniacal removal of the acetyl groups.

Of the total of 62% of monoacetyl derivatives formed, 50% thus was shown to be *N*-acetyldeoxyadenosine-5′ phosphate and 10% 3′-*O*-acetyldeoxyadenosine-5′ phosphate.

(32) *N*-Benzoyldeoxyadenosine compounds are rather poorly visible under ultraviolet light. Observation by reflected light is better than by transmitted light. The compounds appear as weak blue fluorescent spots.

(33) In a subsequent experiment, 2 mmoles of dicyclohexylcarbodiimide was added at the start and a further 1 mmole was added after 30 hr.

(34) A standard 1 *M* solution of the buffer is prepared by suspending 141 ml. of distilled triethylamine in ice-cold water and bubbling in carbon dioxide until the *p*H drops to 7.5. It is important to have the aqueous triethylamine suspension completely immersed in an ice-bath in order to avoid appreciable loss of the volatile amine and not to prolong the passage of carbon dioxide. The solution is then made up to 1 l. with water.

Determination of the Rate of Debenzoylation of *N*-Benzoyldeoxyadenosine-5′ Phosphate.—A solution of the substance in 1 *N* sodium hydroxide was prepared so as to give an optical density reading at 300 mμ of about 1 in a 1-cm. path cell. The rate was followed by the decrease in the optical density at the above wave length. The half-life was thus found to be 52 hr. at room temperature. In a similar experiment using saturated lithium carbonate (*p*H 11.3) the half-life was about 100 hr. at room temperature.

Degradation of Cyclic Deoxyadenosine Trinucleotide to Deoxyadenylyl-(3′→5′)-deoxyadenosine.—The cyclic trinucleotide (about 200 optical density units at 260 mμ) was dissolved in 1 ml. of 0.1 *N* hydrochloric acid at 50° for 20 min. Concentrated ammonia (0.1 ml.) then was added and the solution kept at 37° for 4 hr. The products were chromatographed in solvent C and the band at the origin was cut out and eluted with dilute ammonia. The eluted material (20 optical density units) was digested with 0.04 ml. of the prostatic phosphomonoesterase preparation for 6 hr. The digest was chromatographed in solvent C together with a sample of deoxyadenylyl-(3′→5′)-deoxyadenosine prepared from the corresponding dinucleotide by phosphomonoesterase treatment. In addition to the dinucleoside phosphate, spots with R_f values corresponding to those of adenine, adenosine and those traveling slower than deoxyadenosine-5′ phosphate were observed. Retreatment of the slow-traveling spots with phosphomonoesterase again gave a similar picture. (The adenine spot arose because of a contaminating enzyme in the phosphomonoesterase preparation which caused the glycosidic cleavage of deoxyadenosine.)

[CONTRIBUTION FROM THE BOUND BROOK LABORATORIES, AMERICAN CYANAMID CO., BOUND BROOK, N. J.]

The 1,2-Dithiolium Cation. A New Pseudoaromatic System. I. Preparation and Properties of 3-Phenyl- and 4-Phenyl-1,2-dithiolium Salts

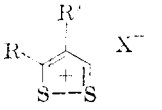
BY ERWIN KLINGSBERG

RECEIVED FEBRUARY 28, 1961

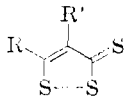
3-Phenyl- and 4-phenyl-1,2-dithiolium salts (IV and V) are prepared by peracetic acid oxidation of the corresponding phenyl-1,2-dithiole-3-thiones (II and III): the 4-phenyl nitrates *para;* the 3-phenyl, *meta* and *para*. Phenyldithiolium salts react with hydrazines to give pyrazoles. Spectral and other properties are discussed.

Two recently discovered reactions have given rise to the first known compounds of unequivocal 1,2-dithiolium (I) structure. Leaver and Robertson[1] have shown that hydrogen disulfide condenses with 1,3-diketones to give 3,5-disubstituted dithiolium salts, provided that aryl groups are present. The peracetic acid oxidation of 1,2-dithiole-3-thiones has been used to prepare both aryl derivatives of the system and the parent 1,2-dithiolium cation itself.[2]

I



IV, R = Ph, R′ = H
IVa, X = HSO_4
b, X = I
c, X = ClO_4

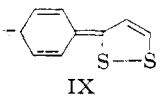


V, R′ = Ph, R = H
Va, X = HSO_4
b, X = Br
c, X = I
d, X = SCN
e, X = ClO_4

II, R = Ph, R′ = H

III, R′ = Ph, R = H

VI, R′ = *p*-$NO_2C_6H_4$, R = H
VIa, X = HSO_4
b, X = Br



IX

VII, R = *m*-$NO_2C_6H_4$, R′ = H
VIIa, X = HSO_4

VIII, R = *p*-$NO_2C_6H_4$, R′ = H
VIIIa, X = Br

When an acetone solution of either isomeric phenyl-1,2-dithiole-3-thione (II, III) is treated with three mole equivalents of peracetic acid, an 85–90% yield of the acid sulfate (IVa, Va) of the corresponding phenyl-1,2-dithiolium salt rapidly crystallizes out of solution. Little can be asserted at this time about the mechanism of this reaction in which the extracyclic sulfur is eliminated as sulfate, but analogies may be noted in the formation of thiazoles[3] and imidazoles[4,5] from their 2-mercapto derivatives. Thiopyridones are reported to behave similarly,[6] although Schmidt and Gieselmann[7] were unable to effect oxidative desulfuration directly to the pyridine derivative. The success of the present reaction probably depends upon the insolubility of the dithiolium sulfates in acetone, since the dithiolethione system is known to be destroyed by hydrogen peroxide oxidation.[8]

The acid sulfates (IVa, Va) are freely soluble in water to a pale yellow solution, stable in the absence of alkali, which precipitates barium sulfate on addition of barium chloride. The water-insoluble perchlorates, bromides, iodides and thiocyanates separate instantly on addition of the respective anion.

The 1,2-dithiolium cation I, which is isosteric with the tropylium cation, may be regarded as a resonance hybrid of two equivalent sulfonium and two equivalent carbonium ions. The symmetry of the cation V is indicated by n.m.r. spectroscopy. In Va the hetero ring is represented by a single peak, showing the equivalence of its two protons, in the expected ratio of 2:5 against the phenyl protons. In the unsymmetrical isomer IVa, the protons on the hetero ring are represented by two doublets, showing their non-equivalence.

These structures are also in apparent agreement

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(7) U. Schmidt and G. Gieselmann, *Chem. Ber.*, **93**, 1500 (1960).

(8) B. Bötteher and A. Lüttringhaus, *Ann.*, **557**, 89 (1947).