2'-O-Acetyl-N-benzoyladenylyl-(3' → 5')-2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoylidine (VIII).—Pyridinium 2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (0.1 mmole) and pyridinium N-benzoyl-2'-O-acetyl-5'-O-monomethoxytrityladenosine-3' phosphate (0.5 mmole) (see accompanying paper[11]) were rendered anhydrous by repeated evaporation of their solution in dry pyridine. To the anhydrous gum was added dry pyridine (2 ml.) followed by pyridinium Dowex-50 ion exchange resin (100 mg.) and DCC (2.5 mmoles). The reaction mixture was kept sealed at room temperature for 3 days. Water (1 ml.) and pyridine (2 ml.) were then added and the mixture kept at room temperature for 6 hr. Dicyclohexylurea was then removed by filtration and the unreacted DCC extracted with petroleum ether. The aqueous pyridine solution was coevaporated with pyridine to a small volume (10 ml.) and the pyridine solution added dropwise to an excess (250 ml.) of dry ether. The precipitate was collected by centrifugation and dried under vacuum over phosphorus pentoxide. The weight of the dry powder was 910 mg.[21]

The dry powder (100 mg.) was dissolved in 2 ml. of 50% aqueous ethyl alcohol and the apparent pH of this solution was adjusted to 2.5 by gradual addition of Dowex-50 (H^+) ion exchange resin. (The addition of about 1 ml. of ethyl alcohol was necessary after the addition of the resin to redissolve the insoluble material which separated.) The solution was maintained at pH 2.5 for 3 hr. at room temperature, then treated with pyridine and filtered from the resin, the latter being washed with aqueous ethyl alcohol. The total solution was placed on a DEAE-cellulose (carbonate) column (32 × 2 cm.) in a 2° room. The column was washed with water containing 20% ethyl alcohol (1 l.) and elution was carried out with a linear gradient of triethylammonium bicarbonate (pH 7.5), the reservoir containing 0.5 *M* triethylammonium bicarbonate in 20% ethyl alcohol (3 l.) and the mixing vessel containing water–20% ethyl alcohol (3 l.). About 15-ml. fractions were collected every 10 min. The first peak, a minor one which appeared in fractions 40–50, has not been identified. The second peak (fractions 51–68) corresponded to N-benzoyl-2'-O-acetyladenosine-3' phosphate. Third peak (fractions 75–105) corresponded to P^1,P^2-(2'-O-acetyl-N-benzoyladenosine-3') pyrophosphate (X, R = N-benzoyladenine). The fourth peak (fractions 113–140, 117 optical density units at 260 mμ) contained 2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (IV) and the last peak (fractions 160–260) contained 2'-O-acetyl-N-benzoyladenylyl-(3' → 5')-2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (VIII) (396 optical density units). The yield of the desired product thus was about 72% as based on 2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine used.

P^1,P^2-(2'-O-Acetyluridine-3') pyrophosphate (X, R = uracil) had R_f 0.78 in solvent B. Treatment with 9 *N* ammonium hydroxide at room temperature gave a mixture of uridine-2',3' cyclic phosphate and uridine-3' phosphate as judged by chromatography in solvent A. The decomposition to these products was found to be complete in the first aliquot (after 2 hr.) applied on paper chromatogram in solvent A.

Uridylyl-(3' → 5')-adenylyl-(3' → 5')-uridylyl-(3' → 5')-uridine (IX).—Pyridinium 2'-O-acetyl-N-benzoyladenylyl-(3' → 5')-2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (350 optical density units, about 10 μmoles) and pyridinium 2',3'-di-O-acetyluridine-3' phosphate (0.08 mmole) were rendered anhydrous by repeated evaporation of their solution in dry pyridine. To the anhydrous gum was added dry pyridine (0.5 ml.), pyridinium Dowex-50 ion exchange resin (100 mg.), and DCC (150 mg.) and the sealed reaction mixture kept for 3 days at room temperature. Water (1 ml.) and pyridine (2 ml.) were then added and after a further 15 hr. at room temperature dicyclohexylurea was removed by filtration and any unreacted DCC extracted with petroleum ether. The total aqueous pyridine solution was evaporated and the residue kept in 9 *N* ammonium hydroxide at room temperature for 24 hr. After removal of the solvent, one-fifth of the total products was placed on a DEAE-cellulose (carbonate) column (37 × 2 cm.) which was washed first with water (500 ml.) and then eluted with a linear gradient of triethylammonium bicarbonate (pH 7.5). The reservoir contained 3 l. of 0.4 *M* triethylammonium bicarbonate and the mixing vessel an equal volume of water. The first peak (fractions 90–110) contained uridine-3' phosphate. Adenylyl-(3' → 5')-uridylyl-(3' → 5')-uridine (12 optical density units at 260 mμ) appeared in fractions 131–145 and the last peak (fractions 210–235) contained the desired uridylyl-(3' → 5')-adenylyl-(3' → 5')-uridylyl-(3' → 5')-uridine (45 optical density units at 260 mμ). The yield as based on adenylyl-uridylyl-uridine recovered was 75%. The R_f's of the product are shown in Tables I and II. Degradation with the *Lactobacillus acidophilus* phosphodiesterase under the standard conditions using 8.5 optical density units of the product showed complete disappearance of the starting material. The resulting products as separated by paper chromatography in solvent B were adenosine-3' phosphate, uridine-3' phosphate, and uridine. Their concentrations in optical density units as determined after elution were: 1.53:2.2:1. The molar proportions of these products thus found were: 1.02:2.2:1.

(21) The total weight is in excess of the theoretical nucleotidic material used in the reaction mixture. It is certain that residual dicyclohexylurea is responsible for the excess weight. The total recovery of ultraviolet-absorbing material from the 100-mg. portion work-up described checks with the total nucleotidic material expected to be present.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, THE UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

Studies on Polynucleotides. XXIX.[1] The Specific Synthesis of $C_{3'}$–$C_{5'}$-Linked Ribooligonucleotides(5).[2] Homologous Adenine Oligonucleotides[3]

BY Y. LAPIDOT AND H. G. KHORANA

RECEIVED JULY 23, 1963

The reaction of pyridinium adenosine-3' phosphate with mono- and di-*p*-methoxytrityl chlorides gave the corresponding 5'-O-methoxytrityl derivatives in good yields. Pyridinium adenosine-3' phosphate with an excess of benzoic anhydride at 50° in the presence of tetraethylammonium benzoate gave after work-up inclusive of an acetic anhydride–pyridine treatment N,2',5'-tribenzoyladenosine-3' phosphate. Alkaline treatment of the latter gave N-benzoyladenosine-3' phosphate. Reaction of the latter with monomethoxytrityl chloride followed by column chromatography gave 5'-O-monomethoxytrityl-N-benzoyladenosine-3' phosphate. Acetylation of the latter by the procedure previously developed followed by an acidic treatment gave N-benzoyl-2'-O-acetyladenosine-3' phosphate. Treatment of a mixture of the latter compound and pyridinium N,2',5'-triacetyladenosine-3' phosphate in dry pyridine with dicyclohexylcarbodiimide followed by successive acetic anhydride–pyridine and ammoniacal treatments gave homologous adenine oligonucleotides. Members up to the pentanucleotide, ApApApApAp, were isolated pure and fully characterized. The presence of only $C_{3'}$–$C_{5'}$ interribonucleotidic linkages in the products was demonstrated.

Methods for the chemical polymerization of deoxyribonucleotides and for the separation and characterization of the resulting homologous deoxyribopolynucleotides have been reported in a number of previous publications from this Laboratory.[4-6] Similar work in the ribopolynucleotide field was not undertaken[7] until

(1) Paper XXVIII: Y. Lapidot and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3852 (1963).

(2) The previous papers which deal directly with the present topic: (a) ref. 1; (b) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962); (c) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (d) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963).

(3) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, the National Science Foundation, Washington, D. C., and the Life Insurance Medical Research Fund, New York, N. Y.

(4) 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, Chapter 5.

(5) (a) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6224 (1958); (b) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959); (c) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961); (d) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961); (e) H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, *ibid.*, **84**, 414 (1962); (f) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).

(6) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961).

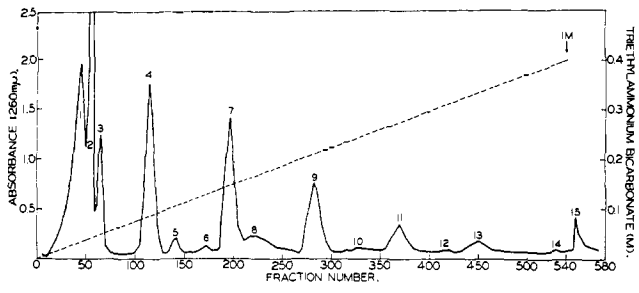
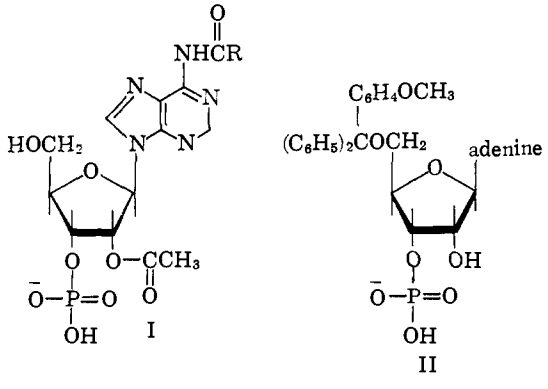


Fig. 1.—Chromatography of polymeric mixture containing adenine oligonucleotides (0.15 mmole) on a DEAE-cellulose (carbonate) column. For details of the salt gradient see text. The distribution of nucleotidic material in different peaks is given in Table IV. The peaks 1 and 2 contain mainly nonnucleotidic material formed by interaction of acetic anhydride and pyridine.

methods were available for the specific synthesis of the $C_{3'}$–$C_{5'}$ interribonucleotidic linkage. With a satisfactory solution to this key problem now in hand, attention is being focused on the synthesis of $C_{3'}$–$C_{5'}$ linked ribopolynucleotides by the chemical polymerization of protected ribomononucleotides.[2d] The present communication deals with the preparation of suitably protected derivatives of adenosine-3′ phosphate and the use of these derivatives in the synthesis of homologous adenine oligonucleotides.

Protected Derivatives of Adenosine-3′ Phosphates.—The protected derivative desired for polymerization was of the type I. The discovery, which proved useful



in this and the work described in the earlier papers,[1,2d] was made at the start of this work that the direct reaction of a pyridinium ribonucleoside-3′ phosphate with mono- or dimethoxytrityl chloride[2b] yields the corresponding 5′-O-tritylribonucleoside-3′ phosphate. Thus, in the case of adenosine-3′ phosphate, 5′-O-monomethoxytrityladenosine-3′ phosphate (II) was obtained in good yield; by chromatographic analysis of the parent nucleotide regenerated from this product after acidic treatment it was established that the 3′-phosphoryl group had not undergone any migration.

In view of the ready availability of compounds of the type II, the direct acetylation of the latter to form the N,2′-O-diacetyl derivative was first considered, but a study of the simplier N,2′,5′-triacetyladenosine-3′ phosphate[1] showed that the N-acetyl group was too acid labile and therefore the selective removal of a methoxytrityl group was not possible (*cf.* ref. 2b). It was therefore decided to use a benzoyl group for protecting the adenine amino group.[2b,6]

Pyridinium adenosine-3′ phosphate (III) was converted quantitatively in 45 min. at room temperature[8] to 2′,5′-di-O-acetyladenosine-3′ phosphate (IV) by the method described previously.[1,2d] Benzoylation in pyridine with benzoyl chloride, presumably, gave V, in analogy with the previous work (ref. 6 and the earlier references cited therein). When V was treated with alkali with a view to prepare VI, some (about 15%) migration of the phosphoryl group occurred. It therefore was clear that the 2′-O-acetyl group was removed by alkali before all of the benzoyl phosphate (mixed anhydride) linkage in V had hydrolyzed. To circumvent this migration, V was first treated with an excess of acetic anhydride–pyridine so as to cause an anhydride exchange reaction to give the highly labile acetyl phosphate. The latter was hydrolyzed with aqueous pyridine and the product on subsequent alkaline treatment gave pure N-benzoyladenosine-3′ phosphate (VI).[9]

A more direct route to N-benzoyladenosine-3′ phosphate (VI) was subsequently made possible by the discovery that the benzoylation of adenosine-3′ phosphate with benzoic anhydride in the presence of an excess of benzoate ions proceeds quantitatively to give, presumably, VII, which by treatment with acetic anhydride–pyridine may be converted to N,2′,5′-tribenzoyladenosine-3′ phosphate. Treatment of the latter compound with alkali under the conditions previously developed[6] gave VI, in an over-all yield of better than 90% from adenosine-3′ phosphate.

The reaction of pyridinium N-benzoyladenosine-3′ phosphate with monomethoxytrityl chloride gave the 5′-O-substituted derivative VIII in 70% isolated yield. The latter was converted by the standard procedure to the 2′-O-acetyl derivative IX. The use of this fully protected derivative in stepwise synthesis of ribooligonucleotides is described in the preceding paper.[1] For the present purpose of polymerization, it was converted by acidic treatment to N-benzoyl-2′-O-acetyladenosine-3′ phosphate (I), isolated as the pyridine salt.

Adenine Oligonucleotides.—For polymerization, a mixture of pyridinium N-benzoyl-2′-O-acetyladenosine-3′ phosphate and pyridinium N,2′,5′-triacetyladenosine-3′ phosphate was treated in anhydrous pyridine with dicyclohexylcarbodiimide for 6 days. Before removal of the protecting groups from the polymeric products, an acetic anhydride–pyridine treatment[5e,10] was given to cleave any pyrophosphate bonds which linked the oligonucleotides through the terminal 3′-phosphomonoester groups.[1] After subsequent ammoniacal treatment, the total mixture was separated on a DEAE-cellulose column. The elution pattern thus obtained is shown in Fig. 1 and the distribution of the ultraviolet-absorbing material is given in Table IV.

Peak 4 contained the mononucleotide while the subsequent major peaks 7, 9, 11, and 13 contained pure homologous oligonucleotides of the general structure X (n = 0–3). These were characterized for purity and size by paper chromatography, both before and after enzymic removal of the 3′-phosphomonoester groups (R_f's in Table III). Further confirmation of structure was afforded by direct comparison with samples of the oligonucleotides prepared by digestion of enzymatically prepared polyadenylic acid with, respectively, the pork liver nuclei endonuclease and the micrococcal nuclease. The former gave compounds

(7) The formation of polymeric products containing random mixtures of $C_{2'}$–$C_{5'}$ and $C_{3'}$–$C_{5'}$ interribonucleotidic linkages has previously been recorded by a number of workers: M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958); A. M. Michelson, *J. Chem. Soc.*, 1371 (1959); G. Schramm, H. Grotsch, and W. Pollmann, *Angew. Chem.*, **74**, 53 (1962).

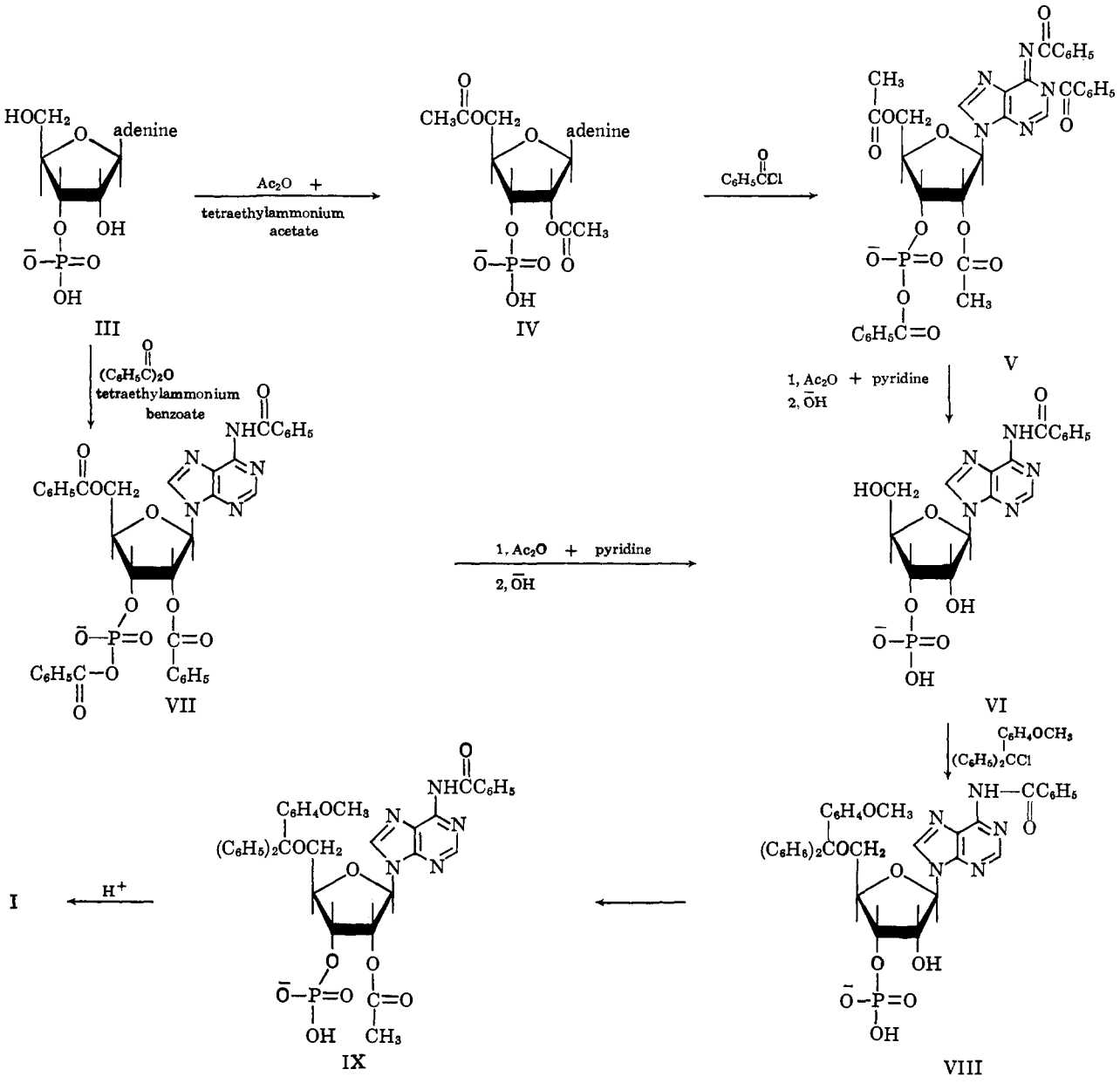
(8) Acetylation of the ring amino group requires several days.

(9) The purity was tested by ammoniacal conversion to adenosine-3′ phosphate followed by paper chromatography in solvent E (Experimental).

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

CHART I

PREPARATION OF PYRIDINIUM N-BENZOYL-2′-O-ACETYLADENOSINE-3′ PHOSPHATE (I, R = C_6H_5)



bearing the 5′-phosphate end groups while the latter enzyme gave homologous oligonucleotides bearing 3′-phosphate end groups. The chemically synthesized compounds were identical on paper chromatography with the appropriate sized members of the latter class of oligonucleotides. Compounds lacking the terminal phosphomonoester groups were also prepared from the enzymically prepared oligonucleotides. These again corresponded exactly in chromatographic mobilities with the corresponding compounds prepared from the synthetic oligonucleotides.

Finally, the exclusive presence of the $C_{3'}$–$C_{5'}$ internucleotidic linkages in the synthetic products was checked specifically in the tetranucleotide X (n = 2). The compound ApApApA prepared from it was tested at a level of 0.7 μmole of mononucleotide with the *Lactobacillus acidophilus R-26* phosphodiesterase.[11] Complete degradation occurred to form adenosine-3′ phosphate and adenosine and the ratio of the two products provided further confirmation of the size of the oligonucleotide.

(11) W. Fiers and H. G. Khorana, *J. Biol. Chem.*, **238**, 2781 (1963).

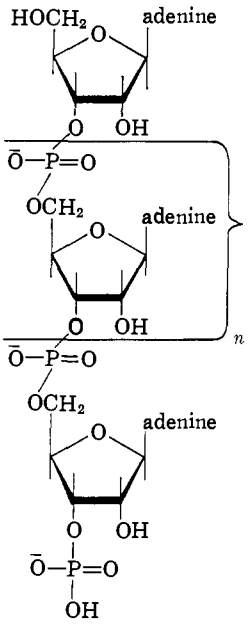
The material in peak 3 (Fig. 1) was identified as adenosine-3′,5′ cyclic phosphate[12] (XI), clearly different from adenosine-2′,3′ cyclic phosphate as ascertained by the rate of alkaline hydrolysis. Peak 5 contained the cyclic dinucleotide XII, whereas the higher homolog, cyclic trinucleotide was present in peak 8. The properties of these compounds and their positions of elution were as expected from previous experience.[2d,5c–5f]

Another product present in peak 1 was identified as the pyridinium nucleotide XIII. The derivation for the structure followed from the properties described earlier for this class of compounds.[2d,5c–5f]

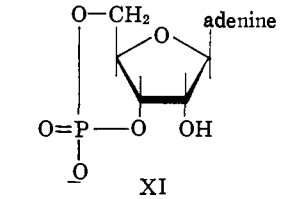
Experimental

Methods.—Most of the paper chromatographic and enzymic methods used in this work have already been described in the preceding paper. The solvent systems A–D were as described therein. In addition, the solvent saturated aqueous ammonium sulfate–1 *M* sodium acetate–isopropyl alcohol (79:19:2) (solvent E) was used to separate adenosine-2′ and adenosine-3′ phosphates.

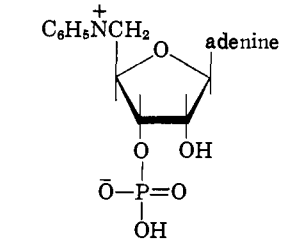
(12) M. Smith, G. I. Drummond, and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 698 (1961).



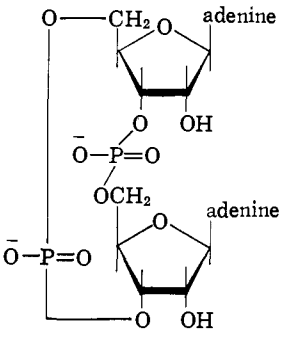
X, $n = 0-3$



XI



XIII



XII

Materials.—Adenosine-3′ phosphoric acid was a commerically available sample[13] which was checked for purity and freedom from adenosine-2′ phosphoric acid at 1 μmole/spot level by paper chromatography in solvent E.

Adenosine oligonucleotides bearing 5′-phosphomonoester end groups, pApA, pApApA, and homologs, were prepared by degradation of the commercially available polyadenylic acid with the pork liver nuclei enzyme[14] as described elsewhere.[15] The corresponding oligonucleotides bearing 3′-phosphate end groups, ApApAp, ApApApAp, and homologs, were prepared by degradation of polyadenylic acid with the micrococcal nuclease, a highly purified preparation of which was given to us by Dr. C. A. Dekker of the University of California. The oligonucleotides were isolated by paper chromatography of the products in solvent D by the descending technique for 2 days. Removal of the terminal phosphomonoester groups was carried out by the bacterial alkaline phosphomonoesterase,[16] a preparation of which was kindly provided by Dr. L. A. Heppel of the National Institutes of Health.

Ammonium 5′-O-Di-*p*-methoxytrityladenosine-3′ Phosphate.—To a suspension of lyophilized adenosine-3′ phosphoric acid (17.2 mg., 0.05 mmole) in 3 ml. of dry pyridine was added di-*p*-methoxytrityl chloride (85 mg., 0.25 mmole) and the sealed reaction mixture shaken at room temperature for 30 min. To the resulting clear solution, ammonium hydroxide (10 ml. of 0.1 *N*) was added and after concentration under reduced pressure, the products were separated on 4 strips (9 in. wide) of Whatman 3 MM paper using solvent A. The bands corresponding to 5′-O-di-*p*-methoxytrityladenosine-3′ phosphate (R_f 0.53) were cut out without allowing the chromatographic papers to dry and eluted with an excess of solvent A. The pure compound thus obtained was recovered by evaporation under reduced pressure with frequent addition of pyridine. The yield as estimated spectrophotometrically corresponded to about 70%. The spectral properties were λ_{max} 258 and 232 mμ. The following tests were performed on this sample.

(a).—To a solution of an aliquot (28 optical density units at 260 mμ) in pyridine (5 ml.) was added water (0.10 ml.) and then DCC (50 mg.). After 6 hr. at room temperature paper chromatography in solvent A showed only a single ultraviolet-absorbing spot (R_f 0.85) traveling faster than the starting material (R_f 0.53). The product corresponded, presumably, to 5′-O-dimethoxy trityladenosine-2′,3′ cyclic phosphate.

(b).—Treatment of an aliquot with 80% acetic acid at room temperature for 20 min. followed by paper chromatography in solvent E showed the presence of only a single nucleotidic spot, corresponding to adenosine-3′ phosphate.

Pyridinium 5′-O-Monomethoxytrityladenosine-3′ Phosphate.—To a suspension of pyridinium adenosine-3′ phosphate (0.5 mmole) in dry pyridine (15 ml.) was added mono-*p*-methoxytrityl chloride (460 mg., 1.5 mmoles) and the sealed reaction mixture shaken at room temperature for 6 hr. Ammonium hydroxide (100 ml. of 0.1 *M*) was then added and the mixture extracted with ether (4 × 50 ml.) to remove methoxytritanol. The aqueous solution was evaporated at low temperature and reduced pressure to a small volume (20 ml.) and some ethyl alcohol added to redissolve the cloudiness which appeared. The solution was placed on a DEAE-cellulose (carbonate) column (45 × 3.2 cm. diam.) which was then washed with 1 l. of water containing 20% ethyl alcohol. Subsequent washing with aqueous 0.1 *M* triethylammonium bicarbonate (2 l.) removed unreacted adenosine-3′ phosphate and chloride ions. 5′-O-Monomethoxytrityladenosine-3′ phosphate was then eluted with 0.2 *M* triethylammonium bicarbonate containing 20% ethyl alcohol (2 l.). Further elution with 0.5 *M* triethylammonium bicarbonate in 20% ethyl alcohol gave bis-monomethoxytrityladenosine-3′ phosphate. The pooled fractions containing 5′-O-monomethoxytrityladenosine-3′ phosphate were evaporated at 10° bath temperature under reduced pressure, with pyridine frequently added in the later stages of evaporation. The evaporation from pyridine was repeated several times to ensure complete removal of triethylammonium bicarbonate. The product thus obtained was homogeneous by paper chromatography (R_f in Table I). The yield as determined spectrophotometrically was 65% by the use of the value of 16,300 for ϵ_{max} at 260 mμ.

Pyridinium 2′,5′-Di-O-acetyladenosine-3′ Phosphate.—Pyridinium adenosine-3′ phosphate (1 mmole) and tetraethylammonium acetate (10 mmoles) were rendered anhydrous by repeated evaporation of dry pyridine. During the last evaporation the suction under vacuum was prolonged to obtain a gum which had lost essentially all of pyridine. Acetic anhydride (1 ml., 10 mmoles) was added and the sealed reaction mixture was kept at room temperature for 45 min. Methyl alcohol was then added to destroy excess of acetic anhydride, and after 15 min. at room temperature the solution was evaporated under an oil pump. The residual sirupy solution was treated with pyridine (1 ml.) and then water (10 ml.). This solution was kept for 1 hr. at room temperature to hydrolyze the mixed anhydride–acetyl phosphate linkage. The total solution was then passed through a column of Dowex-50 ion exchange resin (pyridinium) (50 ml. of wet resin). The total effluent and washings were evaporated with pyridine several times at low temperature (bath temperature 10–20°) and and the final pyridine solution (10 ml.) was added dropwise to ether (250 ml.). The fine precipitate which resulted was collected by centrifugation, washed with ether, and dried in a vacuum desiccator. The yield of the dry powder was 469 mg. (92% of theory, as calculated for monopyridine salt). The product was essentially homogeneous (R_f in Table I) there being a trace of the N-acetylated product. The product showed ultraviolet absorption spectrum characteristic of adenosine-3′ phosphate and different from that of N-acetyladenosine.

Treatment of N,2′,5′-Triacetyladenosine-3′ Phosphate with 80% Acetic Acid.—Pyridinium N,2′,5′-triacetyladenosine-3′ phosphate (10 mg.) was dissolved in 0.5 ml. of 80% acetic acid and the solution kept at room temperature. Aliquots were removed at different intervals and chromatographed in solvent B. Spots corresponding to that of the starting material and that of 2′,5′-di-O-acetyladenosine-3′ phosphate were detected (R_f's in the table). The spots were eluted and their intensities determined spectrophotometrically. The extent of formation of 2′,5′-di-O-acetyladenosine-3′ phosphate in 1 hr. was estimated to be around 14%.

Pyridinium N,2′,5′-Tribenzoyladenosine-3′ Phosphate.—Pyridinium adenosine-3′ phosphate (0.1 mmole) and tetraethylammonium benzoate (1 mmole) were rendered anhydrous by repeated evaporation of their solution in dry pyridine. During the last evaporation the suction under vacuum was continued until a hard gum remained and essentially all of pyridine had been removed. Benzoic anhydride (452 mg., 2 mmoles) was added and the homogeneous solution which resulted on shaking was kept sealed at 50°[17] for 24 hr. A mixture of ethyl alcohol and water (2:1) was added and the solution passed through a column of Dowex-50 (pyridinium) ion exchange resin (10 ml. of wet resin). The column was washed with aqueous ethyl alcohol. The total effluent and washings were evaporated with frequent addition of pyridine and the final gum dissolved in 2 ml. of pyridine. This solution was added dropwise to ether (100 ml.) and the resulting white precipitate collected by centrifugation and washed with

(13) Schwarz Bioresearch, Inc.

(14) The enzyme preparation was made in the laboratory of Dr. L. A. Heppel at the National Institutes of Health, Bethesda, Md. We are grateful to Dr. Heppel for instruction in the use of this enzyme.

(15) R. K. Ralph, R. J. Young, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 2002 (1963).

(16) A. Garen and C. Levinthal, *Biochim. Biophys. Acta*, **38**, 670 (1960).

(17) An earlier experiment at room temperature showed that N-benzoylation was not complete in under 5 days.

TABLE I

PAPER CHROMATOGRAPHY OF DIFFERENT COMPOUNDS

Compound	R_f (solvents) A	B	C	E
Adenosine-3′ phosphate	0.13	0.21	0.19	0.12
Adenosine-2′ phosphate	.13	.21	.19	0.25
Adenosine-2′,3′ cyclic phosphate	.43	.49	.20	
5′-O-Monomethoxytrityladenosine-3′ phosphate	.56			
5′-O-Dimethoxytrityladenosine-3′ phosphate	.53			
2′,5′-Di-O-acetyladenosine-3′ phosphate		0.49	0.36	
N,2′,5′-Triacetyladenosine-3′ phosphate		.59	.38	
N-Benzoyladenosine-3′ phosphate		.48	.35	
5′-O-Monomethoxytrityl-N-benzoyladenosine-3′ phosphate	0.65	.74		
2′-O-Acetyl-N-benzoyl-5′-O-monomethoxytrityladenosine-3′ phosphate		.84		
N-Benzoyl-2′-O-acetyladenosine-3′ phosphate		.65	.51	
N,2′,5′-Tribenzoyladenosine-3′ phosphate		.79	.88	

TABLE II

PAPER CHROMATOGRAPHY OF ADENINE OLIGONUCLEOTIDES

Compound[a]	R_f solvent D (relative to Ap)
Oligonucleotides with 3′-phosphate end groups	
Ap	1.00
ApAp	0.60
ApApAp	.29
ApApApAp	.20
ApApApApAp	.07
Oligonucleotides after removal of the phosphomonoester groups	
Ap	1.00
ApA	1.35
ApApA	0.82
ApApApA	.38
ApApApApA	.20
Adenosine-3′,5′ cyclic phosphate	1.85
Cyclo-adenylyl-(3′ → 5′)-adenylyl-(3′ → 5′)	1.48
5′-Pyridinium adenosine-3′ phosphate (XIII)	0.70

[a] Abbreviations used as in previous papers and as in current usage in *J. Biol. Chem.*

TABLE III

PAPER ELECTROPHORETIC MOBILITIES OF DIFFERENT COMPOUNDS

The buffer used was phosphate (0.03 *M*), pH 7.10; values recorded are relative to the mobility of adenosine-3′ phosphate

Adenosine-3′ phosphate	1.0
N-Benzoyladenosine-3′ phosphate	0.84
N,2′,5′-Tribenzoyladenosine-3′ phosphate	.57
N,2′,5′-Triacetyladenosine-3′ phosphate	.73
5′-Pyridinium adenosine-3′ phosphate	.43
5′-Pyridinium adenosine	−.88[a]
N-Benzoyl-2′-O-acetyladenosine-3′ phosphate	.68
Adenosine-3′,5′ cyclic phosphate	.56
Cyclo-adenylyl-(3′ → 5′)-adenylyl-(3′ → 5′)	.67

[a] The negative sign implies migration in the opposite direction (toward cathode).

ether. The yield of the dry powder was 68 mg. (80%) as calculated on the basis of the mixed benzoic acid anhydride with N,2′,5′-tribenzoyladenosine-3′ phosphate. This product had essentially zero mobility on paper electrophoresis in contrast with N,2′,5′-tribenzoyladenosine-3′ phosphate obtained as follows.

The powder was dissolved in dry pyridine (3 ml.) and acetic anhydride (0.5 ml., 5 mmoles) added. The sealed reaction mixture was kept at room temperature overnight and then treated with methyl alcohol (3 ml.). After 15 min. the excess of methyl alcohol and methyl acetate were evaporated. The residual sirup was treated with 2 ml. of 50% aqueous pyridine and the solution kept at room temperature for 3 hr. The solution was then coevaporated with pyridine several times and the anhydrous residue finally taken up in 2 ml. of dry pyridine. The solution was added dropwise to ether (100 ml.) and the resulting precipitate of pyridinium N,2′,5′-tribenzoyladenosine-3′ phosphate was collected by centrifugation and washed with ether. After drying in a vacuum over phosphorus pentoxide, the yield was 52 mg. (72% as calculated for the monopyridinium salt). This product was homogeneous by paper chromatography (Table I) and by paper electrophoresis (Table III). Treatment with 9 *N* ammonium hydroxide at room temperature for 20 hr. followed by paper chromatography (6 optical density units at 260 mμ) in solvent E showed only a single spot corresponding to adenosine-3′ phosphate.

N-Benzoyladenosine-3′ Phosphate. (a) *Via* **N,2′,5′-Tribenzoyladenosine-3′ Phosphate.**—Pyridinium N,2′,5′-tribenzoyladenosine-3′ phosphate (5 mmoles) was dissolved in a mixture of pyridine (100 ml.) and water (50 ml.). To the solution was added 2 *N* sodium hydroxide (150 ml.) and the clear solution was kept at room temperature for 10 min. An excess of Dowex-50 (pyridinium) ion exchange resin was added rapidly to bring the pH down to neutrality. 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 of a fresh batch of the same resin. The total effluent and washings were evaporated with frequent addition of pyridine and the product dissolved in 50 ml. of pyridine. The solution was added dropwise to ether (2 l.). The resulting fine precipitate of pyridinium N-benzoyladenosine-3′ phosphate was collected by centrifugation, washed with ether, and dried. The yield was 2.41 g. (91%). The product was homogeneous by paper chromatography and paper electrophoresis when tested at 10 optical density level per spot, and gave only adenosine-3′ phosphate (solvent E) after ammoniacal treatment.

(b) *Via* **Pyridinium 2′,5′-Di-O-acetyladenosine-3′ Phosphate.**—To a solution of pyridinium 2′,5′-di-O-acetyladenosine-3′ phosphate (510 mg., 1 mmole) in dry pyridine (20 ml.), benzoyl chloride (2.5 ml., 20 mmoles) was added. The clear solution was kept at room temperature in the dark for 1 hr. and then poured into a cold mixture of water (50 ml.) and chloroform (50 ml.). After about 15 min. the chloroform layer was separated and the aqueous layer extracted again with chloroform (2 × 25 ml.). The combined chloroform extracts were washed with water (25 ml.), then evaporated to a small volume with addition of pyridine. The pyridine solution (10–20 ml.) was added dropwise to ether. The yield of the resulting powder which was collected by centrifugation was 593 mg. (72% as based on pyridine salt of the mixed benzoic acid anhydride of N-benzoyl-2′,5′-di-O-acetyladenosine-3′ phosphate). The total product was dissolved in dry pyridine (10 ml.) and acetic anhydride (2 ml., 20 mmoles). After keeping the sealed solution overnight, methyl alcohol (5 ml.) was added and after 15 min. the total solution was evaporated *in vacuo*. Pyridine (10 ml.) and water (10 ml.) were added to the residual gum and after 2 hr. at room temperature the solution was coevaporated with pyridine to a gum. The gum was dissolved in a cold mixture of pyridine (20 ml.) and water (10 ml.) and to the solution was added 30 ml. of 2 *N* sodium hydroxide under agitation. After 5 min. at about 10° an excess of pyridinium Dowex-50 ion exchange resin was added to remove the sodium ions rapidly. To ensure complete removal of the metal ions the solution was further passed through a column of fresh pyridinium resin. The total effluent and washings were evaporated with frequent addition of pyridine and dissolved in 10 ml. of dry pyridine. The solution was added dropwise to ether (200 ml.). The resulting fine precipitate of pyridinium N-benzoyladenosine-3′ phosphate was collected by centrifugation, washed with ether, and dried. The yield was 334 mg.[18] (63% as calculated in monopyridinium salt). This product had spectral properties (λ_{max} 282 mμ at pH 7) characteristic of N-benzoyladenosine and distinct from that of adenosine. It traveled as a single spot on paper chromatograms (Table I) and on paper electrophoresis (Table III). Treatment with 9 *N* ammonia under the standard conditions gave adenosine-3′ phosphate; no adenosine-2′ phosphate was detected (solvent E).

Pyridinium 5′-O-Monomethoxytrityl-N-benzoyladenosine-3′ Phosphate.—To a solution of pyridinium N-benzoyladenosine-3′ phosphate (1062 mg., 2 mmoles) in dry pyridine (30 ml.) was added monomethoxytrityl chloride (6 mmoles) and the reaction mixture was shaken at room temperature. After 4 hr. the solution was added dropwise to ether (500 ml.) and the fine precipitate thus obtained was collected by centrifugation and after drying *in vacuo* was dissolved in a mixture of ethyl alcohol (10 ml.) and water (10 ml.). The pH of the solution was adjusted to 7.5 by adding triethylammonium bicarbonate and the solution was placed on a DEAE-cellulose (carbonate) column (45 × 3.7 cm. diam.). Elution was carried out in a 2° cold room using a

(18) This product has also been obtained in a crystalline form from aqueous ethyl alcohol although the recovery during crystallization is not satisfactory.

linear gradient of triethylammonium bicarbonate, the reservoir containing 0.5 *M* triethylammonium bicarbonate in 20% ethyl alcohol (4 l.) and the mixing chamber containing water (4 l.). About 16-ml. fractions were collected every 10 min. A small amount of N-benzoyladenosine-3′ phosphate appeared first (fractions 115–160) and was followed by the main broad peak corresponding to 5′-O-monomethoxytrityl-N-benzoyladenosine-3′ phosphate (fractions 120–640). These fractions were pooled and evaporated at 10° using Dry Ice–alcohol in the condenser trap, *n*-octyl alcohol being added to prevent foaming. Finally, evaporation was repeated several times by addition of pyridine in order to remove completely triethylammonium bicarbonate. The pyridine solution was added dropwise to an excess (600 ml.) of ether and the resulting fine precipitate was collected by centrifugation and dried in a vacuum desiccator. The yield of the dry powder was 965 mg. (60% as calculated on the basis of monopyridinium salt). This product had the expected ultraviolet absorption characteristics (λ_{max} 281 and 235 mμ at pH 7) and was homogeneous on paper chromatography in two solvents (R_f's in Table I).

Pyridinium N-Benzoyl-5′-O-monomethoxytrityl-2′-O-acetyladenosine-3′ Phosphate.—Pyridinium 5′-O-monomethoxytrityl-N-benzoyladenosine-3′ phosphate (0.5 mmole) and tetraethylammonium acetate (5 mmoles) were rendered anhydrous by repeated evaporation of pyridine and the final residue freed from most of pyridine by continued suction. Acetic anhydride (0.5 ml., 5 mmoles) was added and the sealed reaction mixture was kept at room temperature for 2 hr. Methyl alcohol (2 ml.) was added and after 15 min. at room temperature the solution was evaporated under vacuum. The residual solution was treated with 20 ml. of 50% aqueous pyridine. After keeping the solution for 2 hr. at room temperature, the solution was slowly passed through a column of Dowex-50 (pyridinium form) ion exchange resin (25 ml. of wet resin). The total effluent and aqueous pyridine washings were evaporated repeatedly with pyridine and the final pyridine solution (about 5 ml.) was added dropwise to anhydrous ether (200 ml.). The resulting fine precipitate was collected by centrifugation, washed with ether, and dried under vacuum. The yield of the dry powder was 378 mg. (calculated on the basis of monopyridinium salt the yield was 89%). The product which was homogeneous by paper chromatography (Table I) was stored in the dry state in a vacuum desiccator. The spectral properties were λ_{max} 282 and 235 mμ.

Pyridinium 2′-O-Acetyl-N-benzoyladenosine-3′ Phosphate.—Pyridinium 2′-O-acetyl-5′-O-monomethoxytrityl-N-benzoyladenosine-3′ phosphate (340 mg., 0.4 mmole) was dissolved in aqueous ethyl alcohol (5 ml. of 66%) and Dowex-50 (H^+) ion exchange resin was added to bring the pH down to 2.5. The mixture was maintained at this pH for 2 hr. at room temperature and then treated with pyridine. The resin was removed by filtration and washed with aqueous pyridine. The combined filtrate and washings were evaporated with pyridine several times and the residue finally dissolved in pyridine. The solution (about 5 ml.) was added dropwise to ether (200 ml.). The fine precipitate of pyridinium 2′-O-acetyl-N-benzoyladenosine-3′ phosphate was collected by centrifugation, washed with ether, and dried under vacuum. The yield was 205 mg. (90% as based on the monopyridinium salt). The product was shown to be homogeneous by paper chromatography and paper electrophoresis (Tables I and III).

Polymerization of N-Benzoyl-2′-O-acetyladenosine-3′ Phosphate.—A mixture of pyridinium 2′-O-acetyl-N-benzoyladenosine-3′ phosphate (260 mg., 0.42 mmole) and pyridinium N,2′,5′-triacetyladenosine-3′ phosphate (83 mg., 0.15 mmole) was rendered anhydrous by several evaporations from dry pyridine. The residual gum was dissolved in dry pyridine (0.5 ml.) and a solution of dicyclohexylcarbodiimide (400 mg., about 2 mmoles) in dry pyridine (0.5 ml.) was added rapidly from a pressure equalizing dropping funnel under exclusion of moisture. The resulting clear solution was vigorously shaken and after some 5 min. about half of the pyridine was removed under vacuum when a gum separated. The heterogeneous mixture was kept sealed in the dark for 6 days at room temperature. A mixture of pyridine and water (10 ml. of each) was added. After shaking for 6 hr. at room temperature the excess of DCC was extracted with petroleum ether (3 × 10 ml.) and the insoluble dicyclohexylurea was removed by filtration. The aqueous pyridine solution was evaporated and the residue rendered anhydrous by coevaporation with dry pyridine several times. The residue was dissolved in 5 ml. of dry pyridine and acetic anhydride (2 ml.) added. After 4 days at room temperature in the dark methyl alcohol (5 ml.) was added; after 15 min. the excess of methyl alcohol and methyl acetate was removed by evaporation. A mixture of pyridine and water (1:1, 18 ml.) was added and the solution kept overnight at room temperature and then stored at −15°.

A portion (4 ml. out of 18 ml.) of the above solution was treated with an equal volume of concentrated ammonium hydroxide for 20 hr. at room temperature. Subsequently, the ammonia was removed by evaporation and the residual solution (about 5 ml.) was placed on a DEAE-cellulose (carbonate) column (31 × 2 cm. diam.). The column was washed with water (300 ml.) and elution begun with a linear gradient of triethylammonium bicarbonate. The mixing vessel contained 4 l. of water and the reservoir an equal volume of 0.4 *M* triethylammonium bicarbonate. About 14 ml. fractions were collected at 6-min. intervals. The elution pattern is shown in Fig. 1 and the manner of pooling the different fractions and the distribution of nucleotidic material in the different peaks is shown in Table IV. The pooled fractions were evaporated to a small volume under reduced pressure and then coevaporated with pyridine several times to remove completely the triethylammonium bicarbonate.

TABLE IV

ADENINE OLIGONUCLEOTIDES: DISTRIBUTION OF NUCLEOTIDIC MATERIAL IN DIFFERENT PEAKS OF FIG. 1

Peak	Fractions pooled	% of total nucleotidic material	Identification remarks
1	20–49	..	Nonnucleotidic + pyridinium nucleotide (see text)
2	51–58	..	Nonnucleotidic
3	61–70	9.2	Adenosine-3′,5′ cyclic phosphate
4	100–127	26.0	Adenosine-3′ phosphate
5	134–147	3.0	Cyclo-adenylyl-(3′ → 5′)-adenylyl-(3′ → 5′)
6	161–176	2.2	Unidentified
7	181–205	20.3	Dinucleotide ApAp
8	213–255	7.0	Cyclic trinucleotide
9	270–298	12.0	Trinucleotide, ApApAp
10	315–345	3.1	Unidentified
11	355–385	7.0	Tetranucleotide, ApApApAp
12	410–423	1.2	Unidentified
13	435–465	4.2	Pentanucleotide, $Ap(Ap)_3Ap$
14	521–534	1.1	Unidentified
15	1 *M* fraction (548–572)	3.8	Higher polynucleotides