# A NEW METHOD FOR THE SEQUENCE ANALYSIS OF OLIGODEOXYNUCLEOTIDES+

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Limited degradation of an oligodeoxynucleotide (I) with snake venom phosphodiesterase leads to a population of oligodeoxynucleotides of successively smaller chain lengths. Labelling of these oligonucleotides at the 3'-ends with [$^{32}$P]-riboadenylic acid residues followed by separation according to their chain lengths and degradation with spleen phosphodiesterase allows the sequence determination of the original oligodeoxynucleotide (I) except for the two nucleotide residues towards the 5'-end.

The enzyme terminal deoxynucleotidyl transferase from calf thymus gland catalyzes the addition of one or two riboadenylic acid residue(s) to a given oligodeoxynucleotide primer when riboadenosine triphosphate is used as substrate (1). This observation has recently led to the development of a new end group labelling technique for the 3'-ends of oligodeoxynucleotides (2).

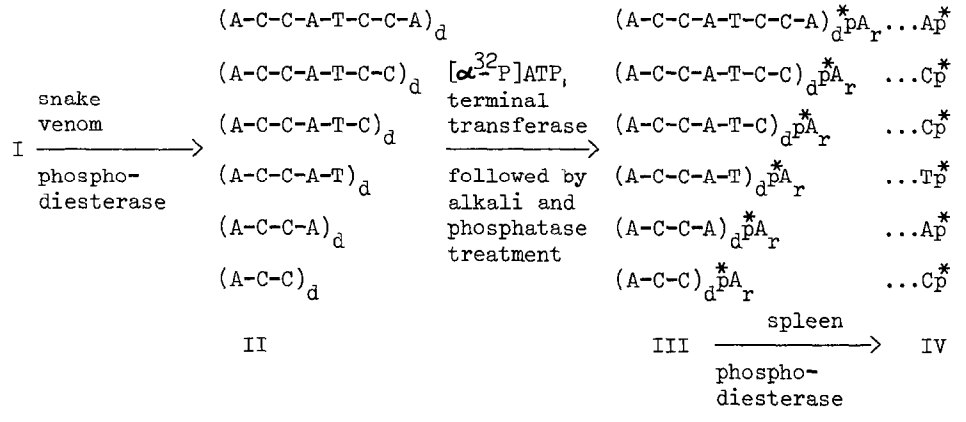
In this communication we want to describe the application of this method for the partial sequence determination of the oligodeoxynucleotide $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C\text{-}C\text{-}A)_d$ (I). The whole reaction sequence consists of the following steps (see also the scheme I):

1. Limited digestion of the oligodeoxynucleotide (I) with snake venom phosphodiesterase to yield oligodeoxynucleotide population of successively smaller chain lengths (II),
2. terminal addition of one [$^{32}$P]-riboadenylic acid residue to the 3'-end of each species of the population (III),
3. separation of the riboadenylic acid terminated radioactive species on the basis of their chain lengths followed by complete digestion of the

+The abbreviations and symbols in this paper follow recommendations of IUPAC-IUB Commission on Biochemical Nomenclature, published in Eur. J. Biochem. 15, 203 (1970), except for $\overset{*}{p}$, which symbolizes [$^{32}$P]-phosphate groups in contrast to the nonradioactive phosphate groups which are represented by hyphens.

individual species with spleen phosphodiesterase. The radioactive label from the $[^{32}P]pA_r$ residues thus will be transferred to their nearest neighbours (IV), which are identical with the nucleotide residues originally present at the 3'-terminus of the respective oligodeoxynucleotide species.

Scheme I




## Materials

Terminal deoxynucleotidyl transferase, isolated according to Yoneda and Bollum (3) had a specific activity of 25.000 units per mg protein as tested with $(pT_d)_6$ as primer in the cacodylate buffer system. $[\alpha\text{-}^{32}P]$rATP was obtained from Radiochemical Center, Amersham.

E.coli alkaline phosphatase and calf spleen phosphodiesterase were products of Worthington. Snake venom phosphodiesterase (1.5 units/mg tested with bis-p-nitrophenylphosphate as substrate) was obtained from Boehringer. The deoxyoctanucleotide A-C-C-A-T-C-C-A, a fragment of the DNA minus strand of the phage fd (4), was prepared by organic chemical synthesis (5) using dinucleotide blocks as intermediates (6). A marker of $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C)_d\text{-}[^{14}C]A_r$ was prepared by the single terminal addition of $[^{14}C]$ATP to $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C)_d$ as described previously with $[\alpha\text{-}^{32}P]$ATP (2).

## Results

It is evident from figure 1 that the $[^{32}P]$-radioactivity of peak 5

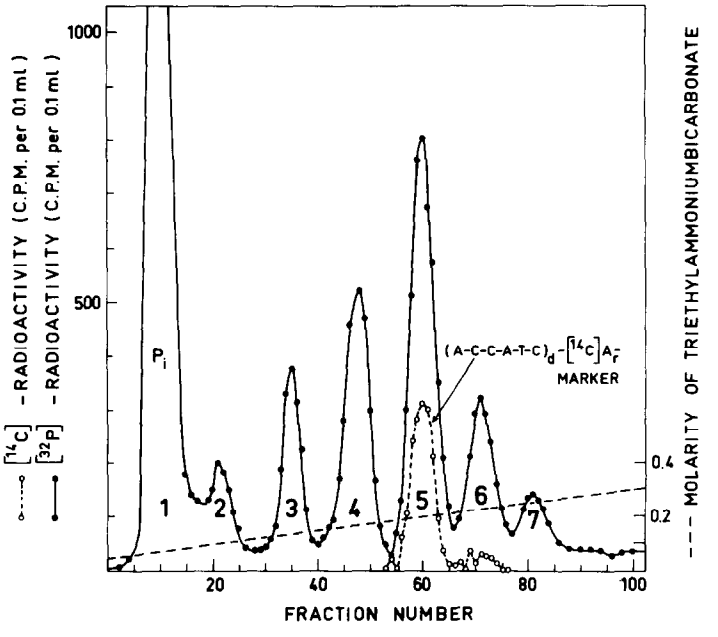



Figure 1. Separation of the oligonucleotide mixture resulting from partial digestion of $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C\text{-}C\text{-}A)_d$ with snake venom phosphodiesterase and subsequent labelling of the resulting oligonucleotide species with [$^{32}$P]-riboadenylic acid residues.

0.8 $A_{260}$-unit of the octanucleotide was incubated for 5 minutes at 37° in 30 mM potassium cacodylate (pH 6.8), 6 mM $MgCl_2$, 0.3 mM dithiothreitol and 1 µg of venom phosphodiesterase in a final volume of 170 µl. Phosphodiesterase was then inactivated by heating the incubation mixture in a boiling water bath for 3 minutes. The reaction mixture after cooling to 0° was adjusted to 40 mM potassium cacodylate, 8 mM $MgCl_2$, 0.5 mM dithiothreitol and 1 mM [$\alpha$-$^{32}$P]rATP (20 µc per umole). After addition of 32 µg of terminal transferase (final volume 400 µl) incubation was carried out at 37° for 4 hours. Subsequent addition of 100 µl of 2 N NaOH was followed by incubation of the resulting mixture for 21 hours at 37° (2). Neutralization was then achieved by passing the whole incubation mixture through a 0.5 ml column of pyridinium Dowex 50 in the presence of 33 % pyridine.

The radioactive material after evaporation of the solvent was dissolved in 100 mM Tris-HCl (pH 8.1) and incubated with 1.2 units of alkaline phosphatase in 400 µl for a period of 12 hours at 37°.

The incubation mixture together with $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C)_d$-[$^{14}$C]$A_r$ as internal marker finally was diluted tenfold with buffer containing 0.05 M triethylammonium bicarbonate (pH 8.0) and 7 M urea, loaded onto a DEAE-cellulose column (0.3 $cm^2$ x 25 cm) preequilibrated with the same buffer and eluted with a linear gradient using 100 ml of 0.05 M triethylammonium bicarbonate, 7 M urea in the mixing vessel and 100 ml of 0.4 M triethylammonium bicarbonate, 7 M urea in the reservoir at room temperature. Twenty drop fractions (approx. 1.4 ml) were collected about every 12 minutes. Aliquots (100 µl) from each fraction were placed on filter paper discs, air dried and counted for radioactivity.

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cochromatographes with the internal oligonucleotide $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C)_d$p[$^{14}$C]$A_r$ marker carrying 6 negative charges. Therefore, the [$^{32}$P]-radioactivity of

the peaks 2-7 must correspond to the oligodeoxynucleotides carrying 3-8 negative charges respectively, with the last peak (peak 7) representing the undigested octanucleotide to which one [$^{32}$P]rAMP has been added. The excess of [$^{32}$P]rATP unutilized during the terminal addition reaction is completely degraded to inorganic [$^{32}$P]-phosphate (peak 1 of figure 1) and nonradioactive adenosine by the phosphatase treatment.

As shown in figure 2, the radioactivity from the individual peaks

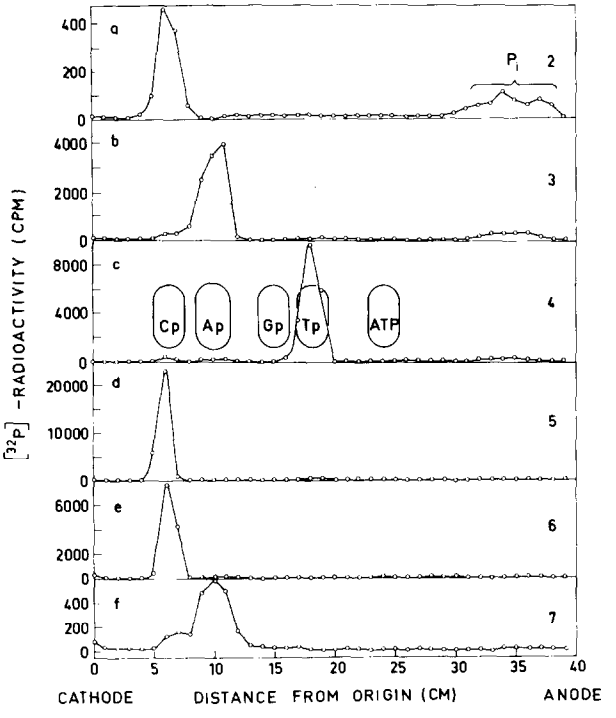


Figure 2. Electrophoretic separation of the deoxynucleoside-3'-monophosphates obtained from [$^{32}$P]-riboadenylic acid terminated oligodeoxynucleotides by spleen phosphodiesterase treatment.

For desalting, the peak fractions of figure 1 were pooled, diluted with 3 volumes of water and loaded separately onto small DEAE-cellulose columns (bed volume 1 ml). After washing the columns with 20 ml of water the materials were eluted with 1 M triethylammonium bicarbonate buffer (6 ml). The material was freed from the buffer by evaporation in the presence of excess pyridine. Traces of pyridine were removed by addition of 0.1 N $NH_4OH$ (1 ml) and re-evaporation. The dried residues from each peak were dissolved and incubated in the buffer system described by Wu (7) with 1.2 units of spleen phosphodiesterase in final volumes of 200 µl for 2-4 hours at 37°. The incubation mixtures were then subjected to paper electrophoresis in the ammonium acetate system (pH 3.5) at 2000 volts for 120 minutes as described previously (2). The numbers at the right side of each diagram refer to the peak numbers (2-7) of figure 1. Paper strips (1 cm) were cut serially and counted.

2, 3, 4, 5, 6 and 7 of figure 1, after spleen phosphodiesterase treatment appears with the mononucleotides dCp, dAp, dTp, dCp, dCp and dAp respectively. From this the base sequences at the 3'-terminus of the original octanucleotide can be deduced directly to be $(C\text{-}A\text{-}T\text{-}C\text{-}C\text{-}A)_d$.

A small amount of cross contamination from the neighbouring peaks is observed with materials from peak 2 and peak 7 of figure 1. In these cases all the fractions belonging to the respective peaks had to be pooled for greater recovery of the radioactive material, which resulted in contamination of peak 2 by some inorganic [$^{32}$P]-phosphate from peak 1, and in trailing of $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C\text{-}C)_d\overset{*}{p}A_r$ from peak 6 into peak 7; this is evident from the presence of a small amount of $^{32}P_i$ in figure 2a and from some [$^{32}$P]-labelled dCp in figure 2f, where the bulk of radioactivity [as expected after spleen phosphodiesterase treatment of $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C\text{-}C\text{-}A)_d\overset{*}{p}A_r$] is located in dAp.

Analogous results to figures 1 and 2 were obtained when the entire reaction sequence was applied to the hexanucleotide $(A\text{-}C\text{-}C\text{-}A\text{-}T\text{-}C)_d$ (not shown).

## Discussion

One crucial point of the method consists in meeting the proper conditions for the partial digestion of the oligonucleotide with snake venom phosphodiesterase. In fact a few previous unsuccessful experiments had to be encountered which apparently were due to overdigestion of the material. Since on the other hand the priming activity necessary for the terminal addition reaction with rATP decrases with the chain length of the primer (1), the ideal digestion condition would be the one in which more molecules of smaller oligonucleotides are produced with very little undigested molecules remaining. Thus the extent of digestion has to be balanced carefully.

For practical usefulness, the method should be workable with small amounts of oligonucleotide material. According to the present results less than 1 $A_{260}$-unit of oligonucleotide is sufficient for the sequence

determination of a given oligonucleotide excluding the two nucleotide residues towards the 5'-terminus. It seems, however, possible to further reduce this amount by using [$\alpha$-$^{32}$P]ATP of higher specific activity and by application of the nucleotide finger print method, developed by Sanger and his coworkers (8).

A complimentary method based on the partial degradation of an oligodeoxynucleotide with spleen phosphodiesterase followed by labelling of the resulting oligonucleotide species with polynucleotide kinase at the 5'-ends was proposed recently (8). This method would allow the sequence determination of the 5'-end of a given oligodeoxynucleotide, which remains undetermined by the method described in this paper. Thus by a combination of both the methods the complete sequence analysis of deoxyoligonucleotides seems possible.

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