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Some studies on the thermic phosphorylation of activated nucleoside by phosphate anion and nucleotide anion

Phosphorylation reactions involving unprotected nucleosides and inorganic phosphate have been reported by several laboratories[1–4]. The first study about the formation of internucleotide linkage using a halogenated nucleoside at the 5′-position and a nucleotide anion has been made by ELMORE AND TODD[5]. Since then a few studies on the SN2 reaction of the activated 5′ alcoholic carbon with nucleotide anion have been reported[6,7].

In the present paper some experiments on the displacement reaction with phosphate and nucleotide anion using a new type of activation at the 5′-position of the nucleoside are described. In particular the phosphorylation of activated uridine using ^{32}P-labeled tri-*n*-butyl ammonium phosphate has been studied under various conditions. Also reported is the formation of thymidylyl-(3′ → 5′)-thymidine using 5′-*O*-methane sulfonyl thymidine and thymidine 3′-phosphate.

The phosphorylation reactions with ^{32}P-labeled phosphoric acid were performed as follows: $10 \cdot 10^6$ counts/min of the labeled acid (NEN, carrier free), 12 μmoles unlabeled phosphoric acid, 24 μmoles tri-*n*-butylamine and from 1 to 3 equiv (36–110 μmoles) of 5′-*O*-*p*-toluene sulfonyl(5′-*O*-methane sulfonyl) uridine or 2′,3′-*O*-isopropylidene-5′-*O*-*p*-toluene sulfonyl uridine were dissolved in dimethyl formamide and extensively dried. The reaction mixture was concentrated to a small volume, and then the bulb-shaped reaction vessel was sealed under nitrogen. The phosphorylation reaction was followed at time intervals of 6, 12, 24 and 36 h and at temperatures ranging from 75 to 110°. The reaction mixtures were analyzed by electrophoresis using ammonium formate buffer, pH 3.5 or 7.0. The incorporation of the radioactivity into the reaction products was determined with a paper strip scanner (Nuclear Chicago).

The following results were obtained: In a series of experiments with 5′-*O*-*p*-toluene sulfonyl or 5′-*O*-methane sulfonyl uridine at reaction temperatures ranging from 75 to 110°, it was found that from 10 to 30% of the radioactivity was incorporated into uridine 5′-phosphate, 5 to 25% into uridylyl uridine and 1 to 5% into the triester. The products were identified by comparison of their electrophoretic mobility with reference compounds and by paper chromatography in different solvent systems. The reactions were generally completed after 24 h or more, in which about 60% of the phosphate had reacted. Thin-layer chromatographic analysis of the reaction mixtures revealed that after a heating process of more than 30 h at 90° most of the activated nucleoside had been decomposed. The overall yield for uridine 5′-phosphate and uridylyl uridine could be even further increased by raising the temperature to 110° and by using the 2′,3′-*O*-isopropylidene-5′-*O*-*p*-toluene sulfonyl uridine. Yields of 46% uridine 5′-phosphate, 31% uridylyl uridine and 1–5% triester were obtained after a reaction time of 15 h. On the other hand, however, when the activated uridine without the blocking group in the 2′- and 3′-position was heated with the phosphate at 110°, the major product was the uridine 2′,3′-cyclic phosphate (50%), which was identified by its electrophoretic mobility and by acid hydrolysis. The yields for uridine 5′-phosphate and uridylyl uridine were 21% and 18% (Fig. 1). After a heating

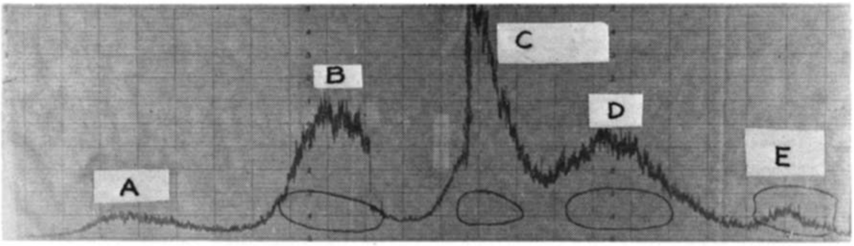

Fig. 1. Distribution of the radioactivity on a chromatogram of a reaction mixture obtained by heating 5′-*O*-*p*-toluene (5′-*O*-methane) sulfonyl uridine with ^{32}P-labeled tri-*n*-butyl ammonium phosphate. Whatman No. 3 paper was used for the electrophoretic separation, buffer: ammonium formate, pH 7. A, inorganic phosphate; B, uridine 5′-phosphate; C, cyclic phosphate ester; D, uridylyl uridine; E, triester + nucleoside.

period of 24 h the radioactive phosphate had reacted to 96%. The formation of the cyclic phosphate is in accordance with earlier findings of Škoda and Moravek[2], who determined this compound as the major product in the thermal phosphorylation of unprotected uridine with inorganic phosphate at 160°.

The cyclic phosphate decomposed on prolonged heating (34 h) to 29%, yielding inorganic phosphate (17%) and the isomers uridine 2′(3′)-phosphate (14%). This phenomenon might be explained by an interaction of *p*-toluene sulfonic acid, which was formed during the reaction.

An interesting observation was made when the phosphorylation was performed in the presence of a large excess of base (triethylamine, tri-*n*-butylamine, tri-*n*-octylamine, pyridine). The formation of a positively charged species was observed on electrophoresis, and only little amounts of phosphate esters were obtained. Several characteristics of the unknown indicated an interaction of the bases with the activated nucleoside: (1) the compound[4] showed the ultraviolet spectrum of uridine, (2) the amount of product formed was dependent on the base nucleophilicity, and (3) the electrophoretic mobility was that expected for a particular base, *e.g.* least with tri-*n*-octylamine. The structure of the unknown remains to be determined.

The phosphorylation of 2′,3′-isopropylidene-N^3, 5′-cycloadenosine under the same conditions as for uridine revealed only one product, the 2′,3′-isopropylidene adenosine 5′-phosphate (λ_{max} water, 265 nm) after a reaction time of 12 h at 110°. The phosphate had reacted almost quantitatively.

In the reaction of 5′-*O*-methane sulfonyl thymidine (30 μmoles) and thymidine 3′-phosphate (10 μmoles) as the tri-*n*-butyl ammonium salt in dimethyl sulfoxide, heated between 90 and 110°, the thymidylyl-(3′ → 5′)-thymidine was obtained in yields of 45–65%. These were determined by eluting the product and the unreacted mononucleotide from the paper and measuring the relative absorbance units. The dinucleotide was characterized by its electrophoretic mobility and by cleavage with snake venom phosphodiesterase. The ultraviolet spectrum showed the following values: λ_{max} HCl, 266 nm; λ_{max} NaOH, 265 nm; λ_{min} HCl, 237 nm; λ_{min} NaOH, 246 nm (Fig. 2).

The phosphorylation of an activated nucleoside at the 5′-position with the phosphate anion is efficient and proceeds by an SN2 reaction mechanism, if the reaction temperature does not exceed 90° (in the ribo series) or if fully protected nucleosides are used at temperatures above 100°. However, if the nucleosides with the free hydroxyl groups in the 2'- and 3'-positions are reacted with phosphate at high temperatures, both a displacement reaction and an esterification take place, with a predominance of the latter.

Besides its theoretical interest, the phosphorylation reactions with radioactive phosphate which are studied here are thought to be a general, rapid and inexpensive means of making radioactive-labeled oligo- and polynucleotides (labeled at the terminal position), which are often desired substrates for biochemical assay experiments.

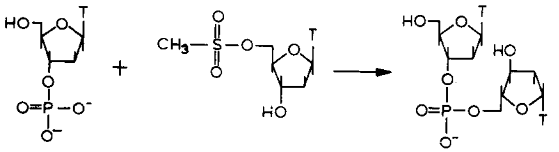

Fig. 2. Reaction of 5'-*O*-methane sulfonyl thymidine and thymidine 3'-phosphate to yield thymidylyl-(3'-5')-thymidine, T = thymine.

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