The Synthesis of Isotopically Labelled Adenosine-2′ Phosphate and Adenosine-3′ Phosphate.

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A method is described which is suitable for the preparation of adenosine-2′ phosphate and adenosine-3′ phosphate labelled either in the nucleoside or the phosphate residue. Details are given of a technique used for the phosphorylation of adenosine with [^{32}P]phosphorus oxychloride.

Isotopically labelled nucleotides have so far been obtained only biosynthetically (Roll, Weinfeld, and Brown, *Biochim. Biophys. Acta*, 1954, **13**, 141, and references therein). Biosynthetical methods, however, necessarily involve low recoveries and, except when micro-organisms are used, considerable dilution of the isotope. Furthermore, in the preparation of ^{32}P-labelled material the maximum activity which can be used may be determined by the sensitivity of the organism to radiation damage. Attempts have therefore been made to overcome these limitations and to find a method suitable for the chemical synthesis of isotopically labelled adenosine-2′ phosphate and adenosine-3′ phosphate.

A synthesis of these nucleotides previously reported (Brown and Todd, *J.*, 1952, 44) involves phosphorylation of 5′-*O*-trityladenosine with dibenzyl phosphochloridate. This method has two disadvantages for the present purpose. First, the use of dibenzyl phosphochloridate necessitates a lengthy preparation of the phosphorylating agent which would be inconvenient with isotopic phosphorus. Secondly, the low yield of 5′-*O*-trityladenosine obtainable by Levene and Tipson's method (*J. Biol. Chem.*, 1937, **121**, 131) make this an unsuitable starting material for the phosphorylation of labelled adenosine. The experiments now reported have been designed to overcome these two difficulties.

In the tritylation of adenosine with 1 mol. of triphenylchloromethane, some N^6 : $O^{5'}$-ditrityladenosine is formed. This fact, besides resulting in a reduced yield of monotrityl compound, means that some adenosine remains unchanged and it would be undesirable to attempt phosphorylation without isolation of the trityladenosine. Tritylation of adenosine with 2 mol. of reagent gave a considerable quantity of the monotrityl compound as well as the ditrityl compound. It is clear that in these circumstances, some triphenylchloromethane is left unchanged and it was therefore unlikely that any adenosine would remain. This assumption is probably correct since no adenosine-5′ phosphate was obtained by phosphorylating the crude mixture of tritylated products.

It was decided to use the commercially available [^{32}P]phosphorus oxychloride for phosphorylation. Earlier attempts (Jachimowicz, *Biochem. Z.*, 1937, **292**, 356; Gulland and Barker, *J.*, 1942, 231) to use this reagent for the phosphorylation of unprotected

adenosine resulted in very poor yields, probably owing partly to the inadequacy of the methods then available for isolation of the products. Furthermore, greater success with this reagent has been achieved more recently (Forrest and Todd, *J.*, 1950, 3295) by carrying out the reaction in the presence of water. The mode of attack of this reagent on adenosine will be discussed in a future communication. Experiment showed that this method could be used successfully for the phosphorylation both of N^6 : $O^{5'}$-ditrityladenosine and of the crude mixture obtained in the tritylation of adenosine. The initial product was obtained by precipitation with water and, after removal of trityl groups with 80% acetic acid, ion-exchange chromatography yielded fractions containing adenosine, adenosine-2′ phosphate, and adenosine-3′ phosphate. The two nucleotides were obtained in an overall yield of 40% from adenosine. Brown and Todd (*loc. cit.*) report a yield of 49% from 5′-*O*-trityl-adenosine which is prepared in 50% yield from adenosine (Levene and Tipson, *loc. cit.*). It is clear, therefore, that the method now described is suitable for the phosphorylation of isotopically labelled adenosine. The method also has the advantage that unchanged adenosine is readily recovered.

On the basis of the phosphorus oxychloride used, approximately 18% of the phosphorus was recovered as total nucleotides. Brown and Todd's results (*loc. cit.*) show a recovery as nucleotides of approximately 10% of the phosphorus of phosphorus trichloride, a 57% yield of the phosphorylating agent (Atherton, Openshaw, and Todd, *J.*, 1945, 382) being assumed. It is thus evident that the method is suitable for preparation of [^{32}P]nucleotides. An additional advantage is that the operations up to the stage of ion-exchange chromatography can be completed within 24 hours. The success of the phosphorylation, however, depends on the purity of the phosphorus oxychloride and a technique for its purification is described which has been used successfully with radioactive material.

Experimental

Tritylation of Adenosine.—To a hot solution of adenosine (1·03 g.) in dry pyridine (30 c.c.) triphenylchloromethane (2·14 g.) was added. The solution was kept for 3 hr. at 95—100° with exclusion of moisture and was then cooled to 0°. In subsequent experiments, this solution (*A*) was treated directly with phosphorus oxychloride. For the isolation of 5′-*O*-trityl- and N^6 : $O^{5'}$-ditrityl-adenosine, the solution was poured into water (400 c.c.) at 0° with vigorous stirring, and the precipitate was collected by filtration, washed with water until free from pyridine, and dried at room temperature. The material (3 g.) was heated on the steam-bath with benzene (50 c.c.) for 45 min. and the 5-*O*-trityladenosine remaining undissolved was collected by filtration of the hot solution, washed with a little benzene, and dried (yield, 0·75 g.). It had m. p. 260° with sintering from 254°. The combined filtrate and washings were concentrated under reduced pressure to 10 c.c. and seeded with N^6 : $O^{5'}$-ditrityladenosine. The product (0·88 g.), after being collected by filtration and dried, had m. p. 200—202°.

Phosphorylation Procedure.—Solution *A* (above) or the corresponding quantity of N^6 : $O^{5'}$-ditrityladenosine in pyridine, was allowed to cool to room temperature and a solution in dry pyridine (5 c.c.) of phosphorus oxychloride (0·75 c.c.) (distilled twice immediately before use, see below) followed by pyridine (2 c.c.) containing water (0·075 c.c.) was added dropwise with stirring. The solution was set aside overnight at room temperature and then cooled to 0° ; 50% aqueous pyridine (10 c.c.) at 0° was added with stirring and the solution was poured into water (400 c.c.). Saturated aqueous barium chloride was added dropwise to coagulate the precipitate which was collected by centrifuging and washed twice with water. The precipitate was boiled under reflux with 80% aqueous acetic acid (70 c.c.) for ½ hr., and the resulting solution was cooled, filtered, and poured into stirred water (500 c.c.). The precipitated triphenylmethanol was filtered off on charcoal and the pH of the filtrate (*B*, see below) brought to 7·5 with hot saturated aqueous barium hydroxide. The solution was diluted to 10 l. and percolated through Dowex-1 ion-exchange resin (10 × 4·5 cm.; mesh size 200—400) in the formate form. The column was washed with water until the effluent was free from barium ions and was eluted first with 0·02N-formic acid (1 l.) and then with 0·15N-formic acid. Fractions (100 c.c.) were collected automatically every 15 min. and the optical density at 260 mμ was determined. Fractions containing adenosine-2′ phosphate were united, concentrated below 40° to 100 c.c., and freeze-dried. The product (0·203 g.) had m. p. 187° (decomp.) and gave one spot (R_F 0·74) on a paper chromatogram developed with a mixture of 5% aqueous disodium hydrogen phosphate

and *iso*amyl alcohol (Carter, *J. Amer. Chem. Soc.*, 1950, **72**, 1466) (Found: C, 34·5; H, 4·5; N, 19·1. Calc. for $C_{10}H_{14}O_7N_5P$: C, 34·6; H, 4·1; N, 20·2%). Fractions containing adenosine-3′ phosphate were combined and the product was isolated in a similar way. The material (0·132 g.) had m. p. 195° (decomp.) and was chromatographically homogeneous, having R_P 0·67 in the same solvent system as above (Found: C, 34·4; H, 4·3; N, 19·0. Calc. for $C_{10}H_{14}O_7N_5P$: C, 34·6; H, 4·1; N, 20·2%).

A portion of the filtrate *B* (above) was adjusted to pH 7·5, diluted to 250 c.c. (optical density of this solution was 0·6 at 260 mμ), and percolated through Dowex-1 ion-exchange resin (30 × 1 cm.) as described above. The column was eluted as previously described and fractions (6 c.c.) were collected every 45 min. The first 64 fractions had zero optical density at 260 mμ; adenosine-2′ phosphate was collected in 16 fractions; a further 47 fractions failed to show absorption and then adenosine-3′ phosphate was collected in 30 fractions. No absorbing material other than these two nucleotides could be detected.

Manipulation of Radioactive Phosphorus Oxychloride.—In using non-radioactive material, distillation of the phosphorus oxychloride at atmospheric pressure in an all-glass apparatus was found to be satisfactory, providing that the first fraction which contains dissolved hydrogen chloride was rejected. Radioactive material was distilled as follows, in the vacuum-apparatus shown in Fig. 1. Silicone grease was used for all taps and joints; trap *A* was cooled in liquid

FIG. 1.

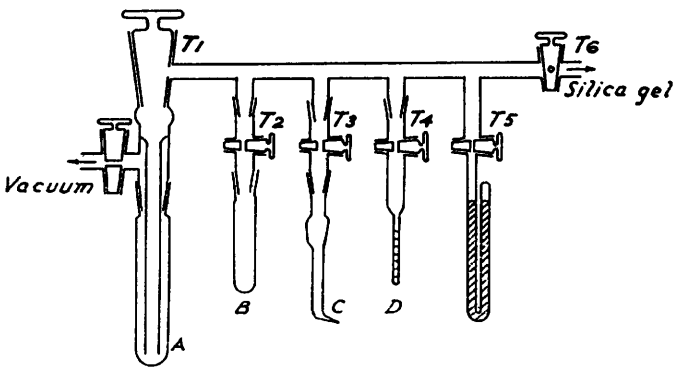
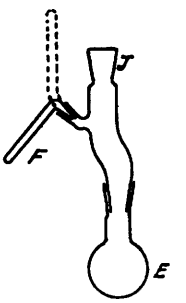


FIG. 2.



nitrogen. An ampoule containing [^{32}P]phosphorus oxychloride (approx. 1 c.c.) was broken and quickly placed in tube *B*, which was then cooled by acetone–solid carbon dioxide and, with taps T_1, T_2, and T_5 open and T_3, T_4, and T_6 closed, the system was evacuated. Tap T_2 was closed, *B* was allowed to attain room temperature, the cooling mixture was replaced, and T_2 was again opened. This process was repeated twice. T_1 and T_5 were then closed, T_4 was opened, the graduated tube *D* was cooled in liquid nitrogen and the cooling mixture was removed from *B*. Periodically, T_4 was closed and the phosphorus oxychloride in *D* was thawed until the required volume had been distilled; T_2 and T_4 were then closed. Pyridine (5 c.c.) was placed in *C* which was cooled in liquid nitrogen and evacuated by opening T_1 and T_3. T_1 was again closed, T_4 was opened, and the phosphorus oxychloride was allowed to distil into *C*. T_3 was then closed and after *C* had reached room temperature, condensed moisture was removed from the outside of the tube. The apparatus shown in Fig. 2 was then attached to *C* by means of joint *J*. Flask *E* contained solution *A* (above) and *F* contained pyridine (2 c.c.) containing the appropriate quantity of water. T_3 and T_6 were opened and, by rotating the apparatus about joint *J*, the capillary at the end of *C* was broken and the contents of *E* were stirred magnetically as the pyridine and phosphorus oxychloride were allowed to enter *E*. When this was complete, *F* was rotated into the position shown by the dotted line. Stirring was continued for a further 5 min., T_3 was closed, and the apparatus left overnight. For subsequent processes, flask *E* was removed and normal techniques were used, with the usual precautions.

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