The Preparation of ^{32}P -Labelled Adenosine Polyphosphates

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This paper describes the preparation by chemical methods of adenosine polyphosphates† labelled with radioactive phosphorus.

Several biochemical methods of preparing P^* -labelled adenine nucleotides with specific activities which are of practical use have been described. The method of Hems & Bartley (1953) for preparing APP^*P^* utilizes respiring sheep-heart suspensions as the phosphorylating agent. The specific activity of the product is relatively high, but the yield in terms of P^* incorporated is low. A similar method, which involves the use of rat-liver mitochondria, has been described by Derache & Lowy (1955), and another method, which involves the use of purified enzyme systems, has been described by Rosenberg (1955). The method of Eggleston (1954) employs a yeast extract for the preparation of AP^* . The specific activity of the product and the yield, in terms of P^* incorporated, are high.

A variety of chemical methods of synthesizing nucleoside polyphosphates have been used by Todd (1955) and his co-workers. The method which is described here is based on that introduced by Khorana & Todd (1953) for the preparation of symmetrical di- and tetra-esters of pyrophosphoric acid. It has been used by Khorana (1954) and by Hall & Khorana (1954) for the synthesis of adenosine and uridine polyphosphates. The basis of the method is the reaction of DCC with acids. Equation (1) shows the reaction for monoesters of phosphoric acid.

When $\text{R} = \text{R}'$, the product is a symmetrical diester of pyrophosphoric acid. When R and R' are different, at least three products are possible. If,

† Adenosine mono-, di-, tri-, and tetra-phosphate are represented by AP , APP , APPP and APPPP respectively, and orthophosphate and pyrophosphate by P and PP respectively. Radioactive phosphate is indicated by a suffixed asterisk, thus P^* . The phosphorus atoms of the polyphosphate chain are differentiated by the symbols α , β , γ and δ , where α is the position of the phosphorus atom adjoining the sugar. Similar representations have been employed by Knivett (1954) and by Hems & Bartley (1953). Other abbreviations used are APPA , *s*-di(adenosine 5')-pyrophosphate, and DCC , *NN'*-dicyclohexylcarbodiimide.

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however, one of the reactants is unsubstituted orthophosphate ($\text{R} = -\text{OH}$) then the building up of a polyphosphate chain becomes possible, e.g. when $\text{R} = -\text{OH}$ and $\text{R}' = \text{adenosyl}$, the products include APP , APPP and probably higher polyphosphates (Khorana, 1954).

In his preparation of adenosine polyphosphates, Khorana (1954) considered it desirable to use an excess of P over AP in order to minimize the formation of the symmetrical APPA . Accordingly, P and AP were used in a molar ratio of about 15:1. Thus when the object is a high yield of adenosine polyphosphates in terms of AP used as starting material, the use of an excess of phosphoric acid is indicated.

In the preparation of labelled nucleoside polyphosphates, the object becomes one of maximum yield in terms of P^* , rather than of yield in terms of AP . Another object is to obtain a product possessing a high specific activity. These considerations suggest that the reaction should be tested with successively smaller ratios of $\text{P}^*:\text{AP}$. The smallest $\text{P}^*:\text{AP}$ ratios, as well as the highest specific activity possible, are attained if carrier-free P^* is used. (The latter, as supplied by the Radiochemical Centre, Amersham, has a specific activity of about $1 \text{ mc}/\mu\text{g.}$ of phosphorus.)

Preliminary tests of the reaction revealed that a condensation of carrier-free P^* with AP does indeed take place. Analyses showed the main radioactive product to consist of APP^* .

When the starting materials were APP and carrier-free P^* , the main radioactive products consisted of APPP^* and APP^*P^* . When the starting materials were APPP and carrier-free P^* , the main product was radioactive APPPP . The distribution of P^* in this product was not studied.

When the starting materials were AP and P^*P^* the main reaction product was APP^*P^* .

The identification of the purified products of the reactions described in this paper has depended upon comparisons with reference compounds. The first comparison consists of cochromatography on filter paper. The second comparison is an extension of the principle of cochromatography applied to radio-

active compounds. The radioactive reaction product and the reference compound are cochromatographed, the latter being present in excess. The shape of the radioactive spot is determined by autoradiography, that of the reference spot by some other means (in the work described here by ultraviolet photography). The compounds can be considered to be identical only if the irregularities in the shapes of the spots recorded by these independent methods of detection coincide accurately. The method has been discussed fully by Roberts, Abelson, Cowie, Bolton & Britten (1955). It is particularly well suited to the characterization of trace amounts of radioactive substances, and its reliability compares with the method of isotope dilution.

EXPERIMENTAL

Materials and reagents

Nucleotides. The sodium salts of APP, APPP and APPPP were products of the Sigma Chemical Co., St Louis. The free acid of AP was obtained from the Pabst Laboratories, Milwaukee.

The pyridine salt of APP was prepared by mixing 15 mg. of the barium salt of APP (Schwarz Laboratories, Mount Vernon, N.Y.) with an aqueous suspension (approx. 1:1, v/v) of the pyridine salt of Dowex-50 (0.15 g. dry wt.). The barium salt dissolved on stirring, and the solution was separated on a microfilter. The resin was washed with 2 × 1 ml. of water, and the combined filtrate and washings were freeze-dried. The resulting powder was used as such.

Radioactive phosphate (P*). Carrier-free ³²P-orthophosphate in dilute HCl was obtained from The Radiochemical Centre, Amersham, Bucks. It was evaporated to dryness in the reaction vessel by means of a stream of cold air, before other reagents were added. This procedure removes HCl, which interferes with the condensation reaction.

Radioactive pyrophosphate (P*P*). This was prepared after the method of Bell (1950). To 0.1 mc of carrier-free P*, evaporated to dryness as described above, was added 0.05 ml. of 0.020M-Na₂HPO₄ (1 μmole of Na₂HPO₄). The solution was again evaporated to dryness. The material was then heated at 290° for 24 hr.

After cooling it was dissolved in 0.05 ml. of water and chromatographed with solvent system 3 (see below). The paper was dried in a current of cold air. The exact locations of the spots on the chromatogram were best found by autoradiography. An exposure of 2 min. sufficed, owing to the high radioactivity of the material. The area of the paper containing the P*P* spot was cut out and eluted by the slow addition of water to the top edge of the suspended piece of paper. The first 0.2 ml. collected contained most of the radioactivity, yield 76%. The eluted P*P* was rechromatographed with solvent system 3, and was found to contain 99.4% of P*P* and 0.3% of P*. The material was eluted and rechromatographed a second time, and was found to contain 99.5% of P*P* and 0.1% of P*. After elution with water the twice-rechromatographed material was neutralized with pyridine.

NN'-Dicyclohexylthiourea. The preparation of this compound was based on the method of Skita & Rolfe (1920). *cyclo*Hexylamine (200 g., 2 moles) was mixed with 650 ml. of

ethanol. Carbon disulphide (150 ml., 2.5 moles) was added slowly with stirring over a period of 30 min. The reaction mixture became very warm after about 1 min. Boiling was prevented by regulating the rate of addition of CS₂ and by adequate stirring. The mixture was cooled to 5°. The precipitate was filtered off, transferred to a 2 l. flask, and suspended in 1 l. of ethanol. The mixture was refluxed for 20 hr., during which time the precipitate dissolved and H₂S was evolved. The solution was cooled to 5°. The precipitate was filtered off, recrystallized from 2.5 l. of boiling methanol and dried *in vacuo* over anhydrous CaCl₂. Yield 145 g. (60%); m.p. 178–180°.

NN'-Dicyclohexylcarbodiimide (DCC). This was prepared by a modification of the method of Schmidt, Seefelder, Jennen, Striowsky & Martius (1951). Sodium hydroxide (120 g., 3 moles) was dissolved in 1 l. of water in a 2 l. round-bottomed flask. The solution was cooled to 0°, and 71 g. of Cl₂ (1 mole) was passed into the solution. This yielded a solution containing 1 mole of NaOCl, 1 mole of NaOH, and 1 mole of NaCl in 1 l. of water. Methylene chloride (225 ml.) was added, followed by 60 g. of *N,N'*-dicyclohexylthiourea (0.25 mole). The mixture was stirred at room temp. for 5 hr., a reflux condenser being fitted to prevent the loss of methylene chloride. The methylene chloride layer was separated and was then stored at –20° for 24 hr. Fine crystals of ice formed on the surface of the solution, and large crystals of sulphur formed on the bottom of the flask. The former were removed by filtration, the latter by decantation. (The dried sulphur crystals weighed 2.7 g.)

The methylene chloride was distilled off under reduced pressure. The residue was distilled from a 150 ml. flask immersed in a heated-oil bath. The first fraction of distillate, which included residual methylene chloride, was collected until the distillation temperature was 119° at 1 mm. Hg approx. It weighed 4.5 g. The main fraction was collected until the distillation temp. had risen to 125°. It consisted of the product, DCC, which was colourless and odourless, and weighed 33 g. The residue in the distillation flask (4.5 g.) was discarded. The product was redistilled. The main fraction weighed 27.5 g. Yield 53%.

Analytical methods

Chromatography. The *isopropyl* ether–90% formic acid solvent (3:2, v/v) of Krebs & Hems (1953) and Zetterström & Ljunggren (1951) was used for ascending chromatograms (solvent system 1). The *isobutyric* acid–*n*-NH₃ soln.–0.1M ethylenediaminetetraacetic acid solvent (60:36:1, by vol.) of Krebs & Hems (1953) was used for descending chromatograms (solvent system 2). When these solvent systems were used consecutively according to the method of Krebs & Hems (1953), they are referred to as solvent system 1–2. After chromatography in solvent system 1, the paper carrying P and PP was cut off. This left insufficient paper to form a wick for chromatography of the nucleotides in solvent system 2. A piece of filter paper was therefore sewn on for this purpose with a toy sewing machine.

The *tert*.-butanol–90% formic acid–water solvent (80:5:20, by vol.) of Crowther (1954) was used for descending chromatograms (solvent system 3).

Potato apyrase. This enzyme catalyses the hydrolysis of the γ-phosphate of APPP at 0°, and the consecutive hydrolysis of the γ- and β-phosphates of APPP at 40° (Lee & Eiler, 1951; Thoai, Roche & An, 1954). The preparation used

for the work in this report was made and described by Whittam, Bartley & Weber (1955). It was used to determine the relative amounts of radioactivity in the γ and β positions of APPP.

Other methods. Phosphate-containing compounds on chromatograms were detected by the spraying method of Hanes & Isherwood (1949) or by autoradiography. Orthophosphate was estimated by the method of Berenblum & Chain (1938). Radioactive phosphorus was measured in a liquid counter (Veall, 1948). Ultraviolet photographs were made according to the method of Markham & Smith (1949). Autoradiographs of chromatograms were prepared with Kodak Blue Brand X-ray film. When the chromatograms contained labile compounds of low radioactivity the exposures were made at -20° .

PREPARATIONS AND RESULTS

Preparation of APP.* A 3 ml. glass-stoppered flask was used as reaction vessel. A volume (0.3 ml.) of carrier-free ³²P-orthophosphate solution, containing 1 mc/10 ml. was measured into the reaction flask and evaporated to dryness. Next, 10 mg. of AP and 0.50 ml. of pyridine-water (10:1, v/v) were added to the flask, followed by 0.20 g. of DCC. A glass bead was added, the flask was stoppered, and the stopper was secured by metal springs. The whole was shaken vigorously on a mechanical shaker. Complete solution occurred within 3 min. and a precipitate (probably dicyclohexylurea) began to separate after about 1 hr. Shaking was stopped after 24 hr. Water (1 ml.) was added, and the mixture was shaken rapidly for 1 min. The flask was centrifuged slowly to separate the aqueous solution, which was then transferred to a clean, glass-stoppered tube by means of a drawn-out dropper. Free pyridine was removed by extracting the aqueous solution with 2×7 ml. of ether. A sample (5 μ l.) of the aqueous extract was chromatographed on Whatman no. 1 paper with solvent system 1-2. Results of analyses performed on the chromatogram are shown in Table 1a. The remainder of the solution

was applied to Whatman no. 3 MM paper as a band 20 cm. wide and was chromatographed with solvent system 1-2. The chromatogram was extracted by washing it in ether. The area corresponding to APP* was detected by autoradiography, and was

Table 1b. *Identification and analysis of purified APP**

The solution of APP* contained 110 000 counts/min. and 0.31 μ g. of phosphorus/ml. A sample of the solution containing about 500 counts/min. was chromatographed in triplicate with solvent system 1-2. One sample (A) was run without additions. With the second sample (B) were cochromatographed 0.2 μ mole each of P, AP, APP and APPP. With the third sample (C) was cochromatographed 0.5 μ mole of APP. The irregularities of the APP* spot on the chromatogram of sample (C), which were revealed by autoradiography, coincided in every detail with those revealed by ultraviolet-light photography. A further sample (D) was heated with an equal volume of N-HCl at 100° for 10 min. before being chromatographed.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample A	Sample B	Sample D
P	0	0	100
AP	0	0	0
APP	100	100	0
APPP	0	0	0
Rest of paper	0	0	0

Fig. 1. Incorporation of P* into adenosine polyphosphates. The reaction mixture, which was shaken rapidly, contained 30 μ c of carrier-free P*, 10 mg. of AP, and 0.2 g. of DCC in 0.5 ml. of pyridine-water (10:1, v/v). Samples (2.5 μ l.) were withdrawn at the times indicated and placed on chromatograms. The latter were developed immediately with solvent system 1. The solvent front reached the start line about 3 min. after withdrawing the sample. (The reaction was found to continue on the paper if chromatography was not started immediately.) The developed chromatogram was analysed for radioactivity of adenosine polyphosphates and P.

Table 1a. *Formation of APP**

The aqueous extract of the reaction products contained 1742 000 counts/min., and the combined ether extracts, which were discarded, contained 148 000 counts/min. A volume (5 μ l.) of the aqueous extract was cochromatographed with 0.2 μ mole each of AP, APP and APPP, and 0.02 μ mole each of P and PP, with solvent system 1-2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	3150	21
PP	810	5
AP	570	4
APP	8460	57
APPP	1250	9
Rest of paper	520	4

cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic analyses shown in Table 1*b*. These show the solution to have contained essentially carrier-free APP*. The solution was stored frozen.

Similar reaction conditions were employed in a time-course experiment, the results of which are shown in Fig. 1.

Preparation of radioactive APPP (from APP plus P)*. Carrier-free ^{32}P -orthophosphate solution (0.3 ml.) containing 1 mc/10 ml. was measured into a 3 ml. glass-stoppered reaction flask and evaporated to dryness. An amount (10 mg.) of the sodium salt of APP and 0.5 ml. of pyridine-water (10:1,

v/v) were added to the flask, followed by 0.20 g. of DCC. A glass bead was added, the flask was stoppered, and the stopper secured by metal springs. The whole was shaken rapidly on a mechanical shaker for 6 hr. A small amount of solid remained in suspension. The aqueous extract of the reaction mixture was prepared and analysed as described for the preparation of APP*. Results are shown in Table 2*a*. The remainder of the aqueous extract was chromatographed on Whatman no. 3 MM paper with solvent system 1-2. The chromatogram was extracted by washing it in ether. The area corresponding to radioactive APPP was detected by autoradiography, and was cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic analyses shown in Table 2*b*. These show the solution to have contained radioactive APPP of high specific activity. An analysis of the distribution of P* in the β and γ positions of this compound by means of the action of potato apyrase is shown in Fig. 2. It shows two-thirds of the radioactivity in the γ -phosphate, and one-third in the β -phosphate of the APPP. The solution was stored frozen.

Similar results were obtained when the sodium salt of APP was replaced by the pyridine salt of APP.

*Preparation of radioactive APPP (from AP plus P*P*)*. An aqueous solution of P*P*, neutralized with pyridine and containing 2.29×10^6 counts/min., was evaporated to dryness in a 3 ml. glass-stoppered

Table 2*a*. *Formation of radioactive APPP (from APP plus P*)*

The aqueous extract of the reaction products contained 1348000 counts/min., and the combined ether extracts, which were discarded, contained 150 000 counts/min. A volume (5 μl .) of the aqueous extract of the reaction products was cochromatographed with 0.2 μmole each of AP, APP, APPP and APPPP, and 0.02 μmole each of P and PP, with solvent system 1-2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	2820	24
PP	1140	10
AP	120	1
APP	1310	11
APPP	4270	36
APPPP	1010	9
Rest of paper	1170	10

Table 2*b*. *Identification and analysis of purified radioactive APPP*

The solution of APPP contained 132 400 counts/min. and 4.6 μg . of phosphorus/ml. A sample containing about 500 counts/min. was chromatographed in triplicate with solvent system 1-2. One sample (*A*) was run without additions. With the second sample (*B*) were cochromatographed 0.2 μmole each of AP, APP, APPP and APPPP, and 0.02 μmole each of P and PP. With the third sample (*C*) was cochromatographed 0.5 μmole of APPP. The irregularities of the radioactive APPP spot on the chromatogram of sample (*C*), which were revealed by autoradiography, coincided in every detail with those revealed by ultraviolet-light photography. A further sample (*D*) was heated with an equal volume of N-HCl at 100° for 10 min. before being chromatographed.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample A	Sample B	Sample D
P	3	0	100
PP	0	0	0
AP	0	0	0
APP	7	6	0
APPP	89	93	0
APPPP	1	1	0

Fig. 2. Action of potato apyrase on the product of the reaction of APP and P*. For ease of comparison the scales of the phosphate estimations (O) and of the radioactivity assays (●) have been adjusted so that the first plateau of each curve coincides. The incubation mixture consisted of 0.2 ml. of M sodium succinate, pH 6.8; 0.2 ml. of 0.1 M - CaCl_2 ; 0.6 ml. of 0.01 M APPP; 0.1 ml. of a solution of the reaction product containing 1000 counts/min.; 0.2 ml. of enzyme (0.2 mg. of protein); and 0.9 ml. of water. Temp., 0° during first 80 min., thereafter 38° . Samples (0.2 ml.) were withdrawn for analysis at the times indicated, and pipetted into 3 ml. of ice-cold trichloroacetic acid (5%, w/v).

reaction flask. Next, 10 mg. of AP and 0.20 g. of DCC were added, followed by 0.5 ml. of pyridine-water (10:1, v/v), and a glass bead. The whole was shaken rapidly for 6 hr. An aqueous extract of the reaction mixture was prepared and analysed as described for the preparation of APP*. Results are shown in Table 3a. The remainder of the aqueous extract was chromatographed on Whatman no. 3 MM paper with solvent system 1-2. A slight modification of the chromatographic method was adopted. After chromatography with solvent system 1, the paper was dried in a current of cold air, and an autoradiograph was prepared. The latter was

Table 3a. Formation of radioactive APPP (from AP plus P*P*)

The aqueous extract of the reaction products contained 1 750 000 counts/min., and the combined ether extracts, which were discarded, contained 338 000 counts/min. A volume (5 μ l.) of the aqueous extract was cochromatographed with 0.2 μ mole each of AP, APP, APPP and APPPP, and 0.02 μ mole each of P and PP, with solvent system 1-2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	380	3
PP	5660	39
AP	110	1
APP	1320	9
APPP	5750	39
APPPP	570	4
Rest of paper	820	6

Table 3b. Identification and analysis of purified APP*P*

The solution of APP*P* contained 586 500 counts/min. and 5.5 μ g. of phosphorus/ml. A sample containing about 5000 counts/min. was chromatographed in duplicate with solvent system 1-2. One sample (A) was run without additions. With the second sample (B) were cochromatographed 0.2 μ mole each of AP, APP, APPP and APPPP, and 0.02 μ mole each of P and PP. A third sample (C) was cochromatographed with 0.5 μ mole of APPP with solvent system 1-2. The irregular outline of the APP*P* spot on the chromatogram of sample (C), which was revealed by autoradiography, coincided in every detail with that revealed by ultraviolet-light photography. A further sample (D) was heated with an equal volume of N-HCl, at 100° for 15 min. before being chromatographed.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample A	Sample B	Sample D
P	1	1	100
PP	1	2	—
AP	0	1	—
APP	6	7	—
APPP	88	82	—
APPPP	3	4	—
Rest of paper	3	3	0

used as a guide for cutting the chromatogram between the APP*P* and P*P* areas. Chromatography with solvent system 2 was then carried out as described previously. The area corresponding to APP*P* was detected by autoradiography and was cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic analyses shown in Table 3b. These show the solution to have contained radioactive APPP of very high specific activity. An analysis of the distribution of P* in the β and γ positions of this compound by means of the action of potato apyrase is shown in Fig. 3. It shows that the radioactivity was equally divided between the β - and γ -phosphates of the APPP. The solution was stored frozen.

Preparation of radioactive APPPP. Carrier-free ³²P-orthophosphate solution (0.03 ml.) containing 1 mc/ml. was measured into a 3 ml. glass-stoppered reaction flask and evaporated to dryness. Next, 9 mg. of the sodium salt of APPP and 0.5 ml. of pyridine-water (10:1, v/v) was added to the flask, followed by 0.20 g. of DCC. A glass bead was added, the flask was stoppered securely, and shaken rapidly for 6 hr. The aqueous extract of the reaction mixture was prepared and analysed as described for the preparation of APP*. Results are shown in Table 4a. The remainder of the aqueous extract was chromatographed on Whatman no. 3 MM paper with solvent system 1-2. The chromatogram was extracted by washing it in ether. The area on the chromatogram corresponding to radioactive APPPP was detected by autoradiography, and was cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic

Fig. 3. Action of potato apyrase on the product of the reaction of AP and P*P*. For ease of comparison the scales of the phosphate estimations (O) and the radioactivity assays (●) have been adjusted so that the first plateau of each curve coincides. The test solution and conditions were similar to those described in Fig. 2, except for the solution of the reaction product, of which 0.1 ml. of a solution containing 11 500 counts/min. was added.

analyses shown in Table 4b. These show the solution to have contained mainly radioactive APPPP. The solution was stored frozen.

DISCUSSION

The methods of preparing labelled adenosine polyphosphates which are described in this paper are simple and quick, and the yields in terms of P* used are high.

Table 4a. *Formation of radioactive APPPP*

The aqueous extract of the reaction products contained 3700000 counts/min., and the combined ether extracts, which were discarded, contained 612 000 counts/min. A volume (5 μ l.) of the aqueous extract of the reaction products was cochromatographed with 0.2 μ mole each of AP, APP, APPP, and APPPP and 0.02 μ mole each of P and PP, with solvent system 1-2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	18 060	66
PP	2 210	8
AP	20	0
APP	340	1
APPP	1 640	6
APPPP	3 070	11
Rest of paper	1 980†	7

† Autoradiography showed this radioactivity to be mainly concentrated in an area just above APPPP.

Table 4b. *Identification and analysis of purified radioactive APPPP*

The solution of radioactive APPPP contained 114 600 counts/min. and 31 μ g. of phosphorus/ml. A sample containing about 500 counts/min. was chromatographed in duplicate with solvent system 1-2. One sample (A) was run without additions. With the second sample (B) were cochromatographed 0.2 μ mole each of AP, APP, APPP and APPPP, and 0.02 μ mole each of P and PP. A third sample (C) was cochromatographed with 0.5 μ mole of APPPP with solvent system 2. The irregular outline of the radioactive APPPP spot on the chromatogram of sample (C), which was revealed by autoradiography, coincided in every detail with that revealed by ultraviolet-light photography. A further sample (D) was heated with an equal volume of N-HCl, at 95° for 5 min., before it was chromatographed with solvent system 1-2.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample A	Sample B	Sample D
P	2	2	82
PP	0	2	11
AP	0	0	0
APP	0	2	1
APPP	8	12	1
APPPP	84	76	2
Rest of paper	6	7	3

The preparation of APP* has presented the greatest practical difficulties in the past. It was obtained as a by-product in the preparation of APP*P*, or it was prepared by the selective enzymic cleavage of APP*P*, which had in turn to be prepared enzymically (e.g. Webster & Varner, 1954; Lieberman, 1956). With AP and P* as starting materials, the method which is reported in this paper yields APP* of very high specific activity, in high yield in terms of P* added. The time-course of the reaction for the experimental conditions which were employed is shown in Fig. 1. The rate of condensation of P* with AP is rapid initially, but it decreases sharply after about 6 hr. The data of Table 1b demonstrate that the radioactivity of the product is found entirely in the orthophosphate released by hydrolysis in 0.5 N-HCl at 100° for 10 min. The ribose 5-phosphate ester linkage is not hydrolysed under these conditions (Eggleston, 1954). Since there is no radioactivity in the AP or in ribose 5-phosphate, this shows clearly that the radioactivity is confined to the β -phosphate of APP.

When the starting materials are APP and P*, the main radioactive products are a mixture of APPP* and APP*P*. In the experiment described above these were formed in equal amounts, as is indicated by the γ - to β -labelling ratio of 2:1 (see Fig. 2). The manner in which the β -phosphate becomes labelled is probably as follows. During the reaction some breakdown to AP occurs, and some APP* is formed. The latter then gives rise to APP*P*. The labelling of the β position can probably be reduced, if not abolished, by varying the reaction conditions; for example, by using a shorter reaction time. Similar considerations apply when the starting materials are APPP and P* and the main radioactive product is APPPP. Here, too, some labelling of the γ position, and possibly also of the β position, is to be expected. That some P is released when APP or APPP are used as starting materials is indicated by the lower specific activities of the products obtained from these compounds.

When the starting materials are AP and P*P*, the method yields APP*P* of high specific activity, in high yields in terms of P*P* added. The APP*P* so formed was labelled in the γ and β positions in the ratio of 1:1, as is to be expected (see Fig. 3). This is the first successful use of PP in the preparation of polyphosphates with DCC as condensing agent.

The formation of symmetrical dinucleotides such as, for example, APPA was not observed during these preparations. A possible explanation is that in the solvents used, the symmetrical dinucleotides have R_f values similar to those of the corresponding mononucleotides from which they would be expected to arise, and that they thus escape detection.

The method is capable of extension to the preparation of other nucleoside polyphosphates labelled with radioactive phosphorus.

SUMMARY

1. The reaction of orthophosphate with adenosine 5'-monophosphate, diphosphate and triphosphate in the presence of *NN'*-dicyclohexylcarbodiimide has been used for the preparation of labelled adenosine polyphosphates.

2. The adenosine diphosphate so prepared is labelled exclusively in the terminal phosphate group. The adenosine triphosphate and adenosine tetraphosphate so prepared are labelled in the terminal phosphate group, and to a lesser extent in the penultimate phosphate group.

3. A feature of the method is that carrier-free labelled orthophosphate can be used without prior dilution with unlabelled orthophosphate.

4. The reaction of pyrophosphate with adenosine 5'-monophosphate in the presence of *NN'*-dicyclohexylcarbodiimide has been used for the preparation of labelled adenosine triphosphate.

5. The adenosine triphosphate so prepared is labelled in the β - and γ -phosphate groups to an equal extent.

6. An improved method for the preparation of *NN'*-dicyclohexylcarbodiimide is described.

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Properties of Limpet β -Glucuronidase

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The common limpet (*Patella vulgata*) was found to contain an α -glucuronidase (Conchie, Levvy & Marsh, 1956). This mollusc, like many others, was already known to possess β -glucuronidase activity (Dodgson, Lewis & Spencer, 1953), and may be one of the best sources of this enzyme for use in the hydrolysis of steroid glucuronides in urine. It was necessary to compare the properties of the two enzymes, and the present paper is concerned with

the β -glucuronidase. Most of the experiments were done with phenolphthalein β -glucuronide, which has come to be the standard substrate for this enzyme. The opportunity was also taken to examine the fluorimetric method of Mead, Smith & Williams (1955), 4-methylumbelliferone β -glucuronide being used as substrate. Among other possible advantages is the fact that this substrate can be synthesized (Marsh & Levvy, 1956a).