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# THE PHOSPHORYLATION OF NUCLEOSIDES WITH *o*-PHENYLENE PHOSPHOROCHLORIDATE AND *o*-PHENYLENE PHOSPHATE

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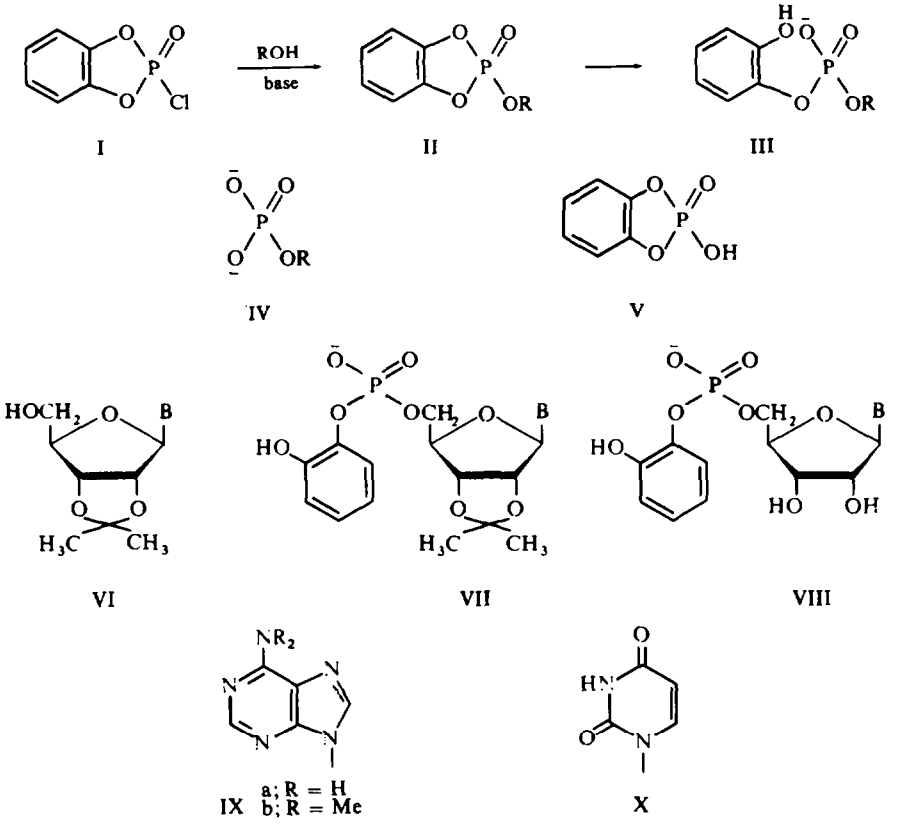
**Abstract**—Reaction between *o*-phenylene phosphorochloridate (I) and the 2',3'-O-isopropylidene derivatives of adenosine, $N^6$,$N^6$-dimethyladenosine and uridine gave, after aqueous work-up, the corresponding 5'-*o*-hydroxyphenyl phosphate esters (VII) in good yields. Acidic hydrolysis followed by $Br_2$-aqueous bicarbonate oxidation of the adenosine derivatives (VII; B = IXa and IXb) gave the corresponding 5'-nucleotides; in the case of the uridine derivative (VII: B = X), two additional steps were necessary. Thymidine 5'-*o*-hydroxyphenyl phosphate (XII) has also been prepared.

The reactions between the anion of *o*-phenylene phosphate (V) and the 2',3'-O-isopropylidene derivatives of adenosine and uridine have been investigated. The main products obtained by heating the 5'-*o*-hydroxyphenyl phosphates of adenosine and uridine (VIII; B = IXa and X, respectively) in anhydrous pyridine solution have been identified.

THE use of *o*-phenylene phosphorochloridate (I) in the conversion of alcohols into their monophosphate esters was first suggested by Reich.[1] However, the claim that hydrolysis of the intermediate phosphotriesters (II) gave the monophosphate esters (IV) directly was later proved to be incorrect by Lora Tomayo and Calderón.[2] These workers showed[2] that hydrolysis of alkyl *o*-phenylene phosphates (II) gave the corresponding *o*-hydroxyphenyl phosphates (III), and Calderón later showed[3] that the latter compounds could be converted into monoalkyl phosphates (IV) by catalytic hydrogenolysis. We found[4,5] that the *o*-hydroxyphenyl group could be removed very rapidly at room temperature by oxidative means. Thus monoalkyl phosphates (IV) were obtained[4,5] in high yields by treating *o*-hydroxyphenyl alkyl phosphates (III) in neutral buffer solution with an excess of bromine water. Very recently, we have found[5] that the conversion of III into IV can also be effected readily with periodic acid, and that alkaline hydrolysis of the lead tetraacetate oxidation products of *o*-hydroxyphenyl phosphate esters (III) also leads to monoalkyl phosphates (IV) in satisfactory yields.

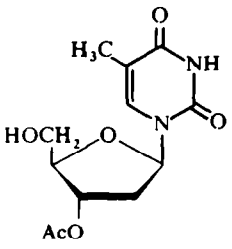
The use of *o*-phenylene phosphate (V) as a phosphorylating agent has also been demonstrated. Thus Nagasawa[6] obtained *o*-hydroxyphenyl phosphate esters (III) by allowing primary and secondary alcohols to react with *o*-phenylene phosphate (V). In most cases phosphorylation was carried out by heating a solution of V in an excess of the alcohol, under reflux.[6] In this paper we wish to describe the reactions between some nucleoside derivatives and both *o*-phenylene phosphorochloridate (I) and *o*-phenylene phosphate (V). A preliminary account of some of this work has already been published.[4]

The phosphorylation of 2',3'-O-isopropylidene ribonucleosides (VI) was readily effected at room temperature with one molecular equivalent or a slight excess of

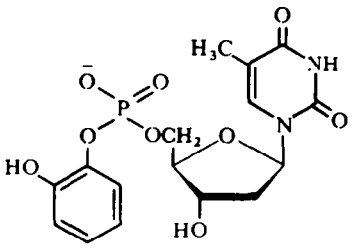



*o*-phenylene phosphorochloridate (I) in acetonitrile solution in the presence of 2,6-lutidine. After a reaction time of 3 hr, water was added to hydrolyze the intermediate phosphotriesters and the 2′,3′-O-isopropylidene ribonucleoside 5′-*o*-hydroxyphenyl phosphates (VII) were obtained in good yields. Thus the adenosine derivative (VII: B = IXa) was isolated as the free acid in 70% yield and the uridine derivative (VII: B = X) as its barium salt in 91% yield. As ribonucleoside 5′-*o*-hydroxyphenyl phosphates (VIII) were stable under the necessary acidic conditions (see below), they could be readily prepared by hydrolysis of the corresponding isopropylidene derivatives (VII). Thus both adenosine and $N^6$,$N^6$-dimethyladenosine 5′-*o*-hydroxyphenyl phosphates (VIII; B = IXa and IXb) were obtained as crystalline free acids in overall yields* of 75 and 70%, respectively. Barium uridine 5′-*o*-hydroxyphenyl phosphate (VII; B = X) was also obtained in good overall yield from 2′,3′-O-isopropylideneuridine (VI; B = X). The phosphorylation of a 2′-deoxyribonucleoside derivative was examined in one case: reaction between 3′-O-acetylthymidine (XI) and a slight excess of *o*-phenylene phosphorochloridate (I), work-up under the usual conditions and deacetylation of the product with methanolic ammonia gave thymidine 5′-*o*-hydroxyphenyl phosphate (XII). The latter compound was isolated as its crystalline ammonium salt in 70% overall yield.

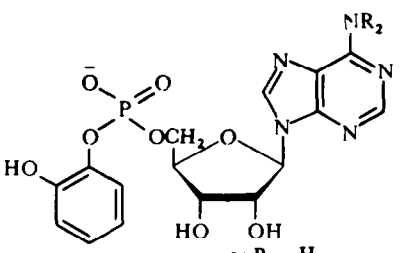
* Based on 2′,3′-O-isopropylidene ribonucleosides (VI) as starting materials.



XI

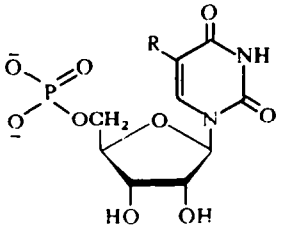


XII



XIII a; R = H b; R = Me

When a solution of adenosine 5′-*o*-hydroxyphenyl phosphate (VIII: B = IXa) in aqueous bicarbonate buffer was treated with a sixfold excess of bromine water at room temperature, a rapid reaction occurred and adenosine 5′-phosphate (XIIIa) was obtained as the sole nucleotide product.* When the experiment was carried out on a preparative scale, crystalline adenosine 5′-phosphate was isolated from the products in 78% yield, based on 2′,3′-O-isopropylideneadenosine (VI: B = IXa) as starting material. In the same way $N^6$,$N^6$-dimethyladenosine 5′-phosphate (XIIIb) was obtained by oxidation of the corresponding *o*-hydroxyphenyl ester (VIII: B = IXb): in this case, the yield of isolated crystalline material (XIIIb) was 43%, based on the 2′,3′-O-isopropylidene derivative (VI: B = IXb) as starting material.

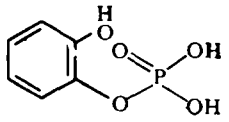


XIV a; R = Br b; R = H

Treatment of uridine 5′-*o*-hydroxyphenyl phosphate (VIII: B = X) with an excess of bromine water resulted in a loss of ultraviolet absorption in the region of 260 nm. However, when the oxidation products were heated in ethanol solution, ultraviolet absorption ($\lambda_{max}$ 273 nm) was restored. As expected from reported studies[7] on the bromination of uridine, the products consisted largely of 5-bromouridine 5′-phosphate (XIVa). When this material was shaken with hydrogen in the presence of palladium catalyst, uridine 5′-phosphate (XIVb) was obtained as the sole nucleotide product in 80% yield. Thus although phosphorylation with *o*-phenylene phosphorochloridate (I), followed by oxidation with bromine water proved to be a convenient procedure for the preparation of adenosine 5′-phosphate (XIIIa) and its $N^6$,$N^6$-dimethyl derivative (XIIIb), this method is rather less satisfactory for the synthesis of uridine 5′-phosphate (XIVb). However, it would be possible to convert uridine 5′-*o*-hydroxy-

* We also found that adenosine 5′-*o*-hydroxyphenyl phosphate underwent quantitative conversion to adenosine 5′-phosphate in the presence of *Crotalus adamanteus* snake venom phosphodiesterase.

phenyl phosphate (VIII; B = X) directly into uridine 5'-phosphate (XIVb) by oxidation with periodic acid.[5]

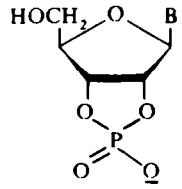


XV

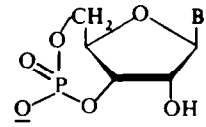
When it was established that *o*-phenylene phosphorochloridate (I) was a useful phosphorylating agent in the nucleotide field, the possible value of *o*-phenylene phosphate (V) in this respect was considered. 2',3'-O-Isopropylideneadenosine (VI; B = IXa) was heated under reflux with an excess of the 1-methylpyridinium salt of *o*-phenylene phosphate[5] in pyridine solution for 6 hr. After the products had been submitted to acidic hydrolysis to remove the isopropylidene group, paper chromatography and electrophoresis revealed the presence of unphosphorylated nucleoside, adenosine 5'-*o*-hydroxyphenyl phosphate (VIII; B = IXa), adenosine 5'-phosphate (XIIIa), *o*-hydroxyphenyl phosphate (XV) and other components. Adenosine 5'-*o*-hydroxyphenyl and adenosine 5'-phosphates were isolated by anion-exchange chromatography and their yields estimated spectrophotometrically to be 35 and 20%, respectively. The yield of unphosphorylated nucleoside was estimated to be approximately 40%. The reaction between 2',3'-O-isopropylideneuridine (VI; B = X) and 1-methylpyridinium *o*-phenylene phosphate followed the same course and led to comparable yields of uridine 5'-*o*-hydroxyphenyl and uridine 5'-phosphates (VIII; B = X and XIVb, respectively).

The above results demonstrate that even under comparatively drastic reaction conditions with an excess of 1-methylpyridinium *o*-phenylene phosphate, phosphorylation was incomplete and a mixture of products was obtained. Thus *o*-phenylene phosphate (V) does not appear to be a useful phosphorylating agent in the nucleotide field. It is noteworthy that free 5'-nucleotide was isolated from the acid hydrolysate of the products in each of the above experiments. When 2',3'-O-isopropylideneadenosine 5'-*o*-hydroxyphenyl phosphate (VII; B = IXa) was heated alone in pyridine solution for 6 hr and the products submitted to acidic hydrolysis, adenosine 5'-phosphate (XIIIa) was obtained. The mechanism of removal of the *o*-hydroxyphenyl group has not yet been elucidated.

The stability of adenosine and uridine 5'-*o*-hydroxyphenyl phosphates (VIII; B = IXa and X, respectively) in acidic and basic media was then investigated. As indicated above, the latter compounds were stable enough in acidic solution to make possible their preparation from the corresponding 2',3'-O-isopropylidene derivatives (VII; B = IXa and X, respectively); thus they were both unchanged after they had been kept at 50° in N-hydrochloric acid solution for 1 hr. The adenosine derivative (VIII; B = IXa) was contaminated with a trace (~1%) of adenosine after it had been in solution in 30% formic acid at 20° for 58 hr, while the uridine derivative (VIII; B = X) underwent *ca* 50% hydrolysis to uridine and *o*-hydroxyphenyl phosphate (XV) when it was heated in N-hydrochloric acid solution at 100° for 8 min. Adenosine 5'-*o*-hydroxyphenyl phosphate (VIII; B = IXa) was contaminated with a trace amount of adenosine after it had been kept either in saturated methanolic ammonia at 20° for 6 hr or in aqueous ammonia solution (pH 11) at 20° for 16 hr.

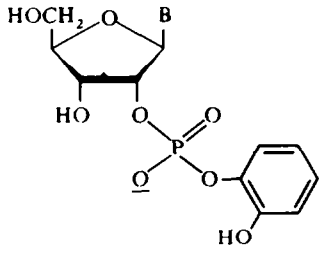


a; B = adenine
XVI b; B = uracil

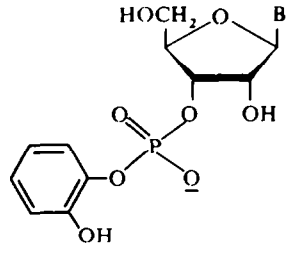


XVII

Finally, the behaviour of both adenosine and uridine 5′-*o*-hydroxyphenyl phosphates (VIII; B = IXa and X) in anhydrous pyridine solution was investigated. After the adenosine derivative (VIII; B = IXa) had been heated under reflux in pyridine solution for 6 hr, no starting material remained but a major product with the same paper electrophoretic mobility as adenosine 2′,3′- and 3′,5′- cyclic phosphates (XVIa and XVIIa) was obtained. The yield of this material was estimated spectrophotometrically to be 64%. On mechanistic grounds, it seemed likely that this product was adenosine 3′,5′-cyclic phosphate[8] (XVIIa). However, this proved not to be the case: that the product was indeed adenosine 2′,3′-cyclic phosphate[9] (XVIa) followed from its alkaline hydrolysis properties. When XVIa was treated with aqueous baryta, it was readily converted into a mixture of adenosine 2′- and 3′-phosphates. No adenosine 5′-phosphate could be detected in the products. In the same way, when uridine 5′-*o*-hydroxyphenyl phosphate (VIII; B = X) was heated under reflux in pyridine solution, uridine 2′,3′-cyclic phosphate (XVIb) was obtained. The latter compound was characterized on the basis of its alkaline hydrolysis products (uridine 2′- and 3′-phosphates) and of its pancreatic ribonuclease catalyzed hydrolysis to uridine 3′-phosphate.



XVIII



XIX

A possible mechanism for this reaction involves the pyridine-promoted dephosphorylation of a nucleoside 5′-*o*-hydroxyphenyl phosphate (VIII) to give the corresponding nucleoside and the pyridinium salt of *o*-phenylene phosphate (V). The nucleoside could then be phosphorylated by *o*-phenylene phosphate on its 2′- or 3′- hydroxyl group to give a mixture of 2′- and 3′-*o*-hydroxyphenyl phosphates (XVIII and XIX, respectively). In the presence of pyridine, the latter compounds could both be converted into the corresponding nucleoside 2′,3′-cyclic phosphate (XVI) and catechol. The conversion of 1,2-diol systems into the corresponding cyclic phosphates by reaction with *o*-phenylene phosphate has been reported by Ukita and Nagasawa.[10]

## EXPERIMENTAL

UV absorption spectra were measured with a Cary recording spectrophotometer, model 14M-50; spectrophotometric assays were carried out with a Unicam SP 500 spectrophotometer. Paper electrophoresis on Whatman No 4 paper (No 3 paper for preparative work) was conducted at 2 kv in a $CCl_4$-cooled apparatus in the following buffers: I, 0·05 M triethylammonium bicarbonate (pH 7·5): II, 0·05 M sodium borate (pH 7·94): III, 0·05 M sodium phosphate (pH 7·3): IV, 0·025 M sodium acetate (pH 4·6). The following solvent systems were used for ascending paper chromatography: A, butan-1-ol-acetic acid-water (5:2:3): B, propan-2-ol-aqueous $NH_3$ (*d* 0·88)-water (7:1:2): C, propan-2-ol-aqueous $NH_3$ (*d* 0·88)-0·1 M aqueous boric acid (7:1:2): D, saturated aqueous $(NH_4)_2SO_4$-M aqueous $NH_4OAc$-propan-2-ol (80:18:2); E, isobutyric acid-N aqueous $NH_3$-0·1M aqueous EDTA (100:60:1·6). Pyridine was dried by heating with $CaH_2$, under reflux, and then redistilled before use.

*2',3'-O-Isopropylideneadenosine 5'-o-hydroxyphenyl hydrogen phosphate*

A soln of *o*-phenylene phosphorochloridate[5,11] (0·662 g, 3·3 mmole) in acetonitrile (8 ml) was added to a stirred soln of 2',3'-O-isopropylideneadenosine[12] (0·921 g, 3 mmole) and 2,6-lutidine (1·38 ml, 12 mmole) in acetonitrile (6 ml) at 20°. After 3 hr, water (3 ml) was added and, after a further 10 min, the products were concentrated under reduced pressure (bath temp 40°). The residual gum was dissolved in water (5 ml), and the soln applied to a column (12 cm × 2·5 $cm^2$) of Dowex-1 × 2 (formate) anion-exchange resin. The column was eluted first with water and then with 0·1M-formic acid. The acidic eluate was immediately concentrated under reduced pressure (bath temp < 40°) to a gum. Trituration of this material with a solution of EtOH (10 ml) and ether (30 ml) gave 2',3'-*O-isopropylideneadenosine 5'-o-hydroxyphenyl hydrogen phosphate* [Found: P, 7·1. $C_{19}H_{22}NO_8P$ requires: P, 6·5%] as a crystalline solid (0·91 g, 70%). This material was paper chromatographically (systems A, B, E) and electrophoretically (buffer I) homogeneous.

*Adenosine 5'-o-hydroxyphenyl hydrogen phosphate*

A soln of *o*-phenylene phosphorochloridate[5,11] (0·438 g, 2·2 mmole) in acetonitrile (3 ml) was added to a stirred soln of 2',3'-O-isopropylideneadenosine (0·614 g, 2 mmole) and 2,6-lutidine (0·92 ml, 8 mmole) in acetonitrile (5 ml) at 20°. After 3 hr, water (1 ml) was added and, after a further 10 min, the products were concentrated as above and redissolved in 30% formic acid (5 ml). This soln was allowed to stand at 20° for 48 hr and then filtered. The residue and filtrate were both retained.

Recrystallization of the residue (0·405 g) from aqueous EtOH gave *adenosine 5'-o-hydroxyphenyl hydrogen phosphate* [Found, in material dried *in vacuo* over $P_2O_5$ at 120° for 5 hr: C, 43·25; H, 4·1; N, 15·8; P, 7·2. $C_{16}H_{18}N_5O_8P$ requires: C, 43·4; H, 4·1; N, 15·8; P, 7·0%] as colourless crystals, m.p. 207–208°; UV absorption (water, pH 2): $\lambda_{max}$ 258 ($\varepsilon$ 13,800), $\lambda_{min}$ 230 nm ($\varepsilon$ 3,800).

The filtrate was treated with alkali (to pH 9), concentrated under reduced pressure (to *ca* 3 ml) and applied to a column (14 cm × 2·5 $cm^2$) of Dowex-1 (formate) anion-exchange resin. The column was eluted first with water (975 ml) and then with 0·1M-formic acid. The fractions containing adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate, which were eluted with formic acid, were combined and concentrated under reduced pressure (bath temp < 40°). The gum, so obtained, was twice evaporated from EtOH soln (5 ml) and then dissolved in water (3 ml). A crystalline ppt (0·253 g) of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate was obtained, total yield, 0·658 g (75%).

*Barium 2',3'-O-isopropylideneuridine 5'-o-hydroxyphenyl phosphate*

A soln of this gum in water (5 ml) was passed through an Amberlite-IR 120 ($Ba^{++}$) cation-exchange stirred soln of 2',3'-O-isopropylideneuridine[12] (2·00 g, 7 mmole) and 2,6-lutidine (3·22 ml, 28 mmole) in acetonitrile (10 ml) at 20°. After 3 hr, the products were filtered and the residue washed with acetonitrile (3 ml). The combined filtrate and washings were treated with water (3 ml) and, after 10 min, concentrated under reduced pressure to a gum which was shown by paper chromatography (system A) to consist of a major UV-absorbing product ($R_f$ 0·67) and a trace of 2',3'-O-isopropylideneuridine ($R_f$ 0·77).

A soln of this gum in water (5 ml) was passed through an Amberlite-IR 120 ($Ba^{++}$) cation-exchange column, which was then eluted with water. The combined UV-absorbing fractions were concentrated under reduced pressure, and the residue twice evaporated from EtOH soln. Further trituration of this material with EtOH gave barium 2',3'-O-*isopropylideneuridine 5'-o-hydroxyphenyl phosphate* [Found, in material dried *in vacuo* over $P_2O_5$ at 100° for 5 hr: P, 5·8. $C_{18}H_{20}N_2O_{10}PBa_{\frac{1}{2}}$ requires: P, 5·9%] as a white powder: yield, 3·01 g (91%). This product was paper chromatographically (systems A, B, E) and electrophoretically (buffer I) homogeneous.

*Barium uridine 5'-o-hydroxyphenyl phosphate*

The phosphorylation of 2',3'-O-isopropylideneuridine was carried out as above but on one-half the scale. The gum obtained after aqueous treatment and evaporation was dissolved in 30% formic acid (15 ml) and allowed to stand at 20°. After 48 hr, the products were concentrated under reduced pressure, the residue kept *in vacuo* over KOH for 16 hr, and then dissolved in water (3 ml). When saturated alcoholic $BaBr_2$ (100 ml) was added to this soln, a ppt of *barium uridine 5'-o-hydroxyphenyl phosphate* (1·38 g, 78%) was obtained. This material, which was hygroscopic, was washed first with EtOH, then with ether and was finally dried [Found: C, 35·5; H, 4·55; N, 5·5; P, 6·2. $C_{15}H_{16}N_2O_{10}PBa_{\frac{1}{2}}$, $H_2O$ requires: C, 35·9; H, 3·6; N, 5·6; P, 6·2%], UV absorption (water): $\lambda_{max}$ 263 (ε 12,300), $\lambda_{min}$ 231 nm (ε 1,950).

*Ammonium thymidine 5'-o-hydroxyphenyl phosphate*

A soln of *o*-phenylene phosphorochloridate[5, 11] (0·209 g, 1·1 mmole) in acetonitrile (2 ml) was added to a stirred soln of 3'-O-acetylthymidine[13] (0·285 g, 1·0 mmole) and 2,6-lutidine (0·46 ml, 4 mmole) in acetonitrile (2 ml) at 20°. After 5 hr, water (0·5 ml) was added, and the products concentrated under reduced pressure. The resultant gum was evaporated from EtOH soln (2 × 5 ml), and then dissolved in $NH_3$/MeOH (half-saturated at 0°: 10 ml) at 20°. After 16 hr, the products were evaporated, dissolved in water (3 ml), and the soln passed through a column of Amberlite-IR 120 ($H^+$) cation-exchange resin. The column was eluted with water until the optical density of the eluate was low. The total eluate was neutralized with aqueous $NH_3$, evaporated under reduced pressure, and the residual gum crystallized from EtOH to give *ammonium thymidine 5'-o-hydroxyphenyl phosphate* [Found, in material dried *in vacuo* over $P_2O_5$ at 85° for 5 hr: C, 44·7; H, 5·5; N, 9·85; P, 7·5. $C_{16}H_{22}N_3O_9P$ requires: C, 44·5; H, 5·1; N, 9·7; P, 7·2%], yield, 0·303 g (70%): UV absorption (water): $\lambda_{max}$ 268 (ε 10,200), $\lambda_{min}$ 235 nm (ε 2,200).

$N^6$,$N^6$-*Dimethyladenosine 5'-o-hydroxyphenyl hydrogen phosphate*

A soln of *o*-phenylene phosphorochloridate[5, 11] (0·248 g, 1·3 mmole) in acetonitrile (4 ml) was added to a stirred soln of $N^6$,$N^6$-dimethyl-2',3'-O-isopropylidineadenosine* (0·336 g, 1·0 mmole) and 2,6-lutidine (0·46 ml, 4 mmole) in acetonitrile (7 ml) at 20°. After 4 hr, water (0·5 ml) was added and, after a further 10 min, the products were concentrated under reduced pressure. The residue obtained was dissolved in 30% formic acid (5ml), the soln allowed to stand at 20° for 72 hr and then evaporated, under reduced pressure, to give a gum. This material was dissolved in a small volume of dilute aqueous ammonia (pH 9), and the soln applied to a column (9 cm × 1 $cm^2$) of Dowex-1 × 2 (formate) anion-exchange resin. The column was eluted first with water (900 ml) and then with 0·1M formic acid. All the acid-eluted, UV-absorbing fractions were combined and concentrated under reduced pressure. When the residual gum was triturated with EtOH, crystalline $N^6$,$N^6$-dimethyladenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·328 g, 70%) was obtained: m.p. 184°, UV absorption (water, pH 2): $\lambda_{max}$ 267 (ε 17,800), $\lambda_{min}$ 234 nm (ε 3,300).

*Acidic hydrolysis of nucleoside 5'-o-hydroxyphenyl phosphates*

(a) A soln of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·002 g) in N HCl (0·5 ml) was maintained at 50° for 1 hr, and then examined by paper chromatography (system A) and electrophoresis (buffer II). Only unchanged starting material was detected.

(b) A soln of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·003 g) in 30% formic acid (3 ml) was allowed to stand at 20°. After 58 hr, paper chromatography (system A) revealed starting material contaminated with a trace (~1%) of adenosine.

(c) A soln of barium uridine 5'-*o*-hydroxyphenyl phosphate (0·003 g) in N HCl (0·5 ml) was (i) maintained at 50° for 1 hr and (ii) heated on a boiling water-bath. In experiment (i), only unchanged starting material could be detected by paper chromatography (system A) and electrophoresis (buffer II); in experiment (ii), 50% hydrolysis to *o*-hydroxyphenyl phosphate, as indicated by paper electrophoresis (buffer II), had occurred after 8 min, and no unchanged starting material could be detected after 1 hr.

*Action of ammonia on adenosine 5'-o-hydroxyphenyl phosphate*

(a) A soln of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·005 g) in saturated $NH_3$/MeOH (1 ml) was allowed to stand at 20°. After 60 hr, paper chromatography (system A) and electrophoresis (buffer III) revealed unchanged starting material, contaminated with a trace (~1%) of adenosine.

(b) A soln of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·002 g) in dil aqueous $NH_3$ (pH 11;

* We thank Dr. B. E. Griffin for a gift of this compound.

0·5 ml) was allowed to stand at 20°. After 16 hr, paper chromatography (system A) revealed unchanged starting material, contaminated with *ca* 2% of adenosine.

*Enzymatic hydrolysis of adenosine 5'-o-hydroxyphenyl phosphate*

A soln of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·005 g) and *Crotalus adamanteus* snake venom phosphodiesterase (500 μg) in 0·05M sodium borate buffer [0·3M with respect to magnesium acetate] (pH 9·2; 1 ml) was incubated at 37°. After 5 hr, paper chromatography (system B) and electrophoresis (buffer II) of the products revealed a sole component corresponding to adenosine 5'-phosphate. Under the same conditions the control solution remained completely unchanged.

*Preparation of adenosine 5'-phosphate*

(a) A soln of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·0044 g, 0·01 mmole) in 0·2M aqueous triethylammonium bicarbonate (pH 7·5; 0·5 ml) was treated with 2% bromine water (0·5 ml, 0·06 mmole) at 20°. After 5 min, paper chromatography (system B) and electrophoresis (buffer I) revealed adenosine 5'-phosphate as the sole phosphorus-containing product.

(b) 2',3'-O-Isopropylideneadenosine[12] (0·307 g, 1 mmole) was phosphorylated as above with *o*-phenylene phosphorochloridate[5,11] (0·238 g, 1·25 mmole) in the presence of 2,6-lutidine (0·46 ml, 4 mmole). After aqueous work-up and evaporation (see above), the residue was dissolved in water (3 ml) and the soln applied to a column (5 cm × 2·5 $cm^2$) of Dowex-50 ($H^+$) cation-exchange resin. The column was eluted with water, the combined u.v.-absorbing fractions concentrated to *ca* 20 ml, and then acidified to pH 1 with formic acid. After 48 hr, the products were concentrated under reduced pressure, the residue dissolved in 0·2M triethylammonium bicarbonate (pH 7·5, 50 ml) and the soln treated with 2% aqueous bromine (50 ml, 6·25 mmole). The products were shaken at 20° for 10 min and then extracted with ether until the ethereal extracts were colourless. After air had been bubbled through the aqueous layer for 10 min, it was neutralized and concentrated under reduced pressure to *ca* 8 ml.

The concentrated soln, which was found by paper chromatography (system A) to contain adenosine 5'-phosphate as its sole UV-absorbing component, was applied to a column (14 cm × 5 $cm^2$) of Dowex-1 × 2 (formate) anion-exchange resin. The column was eluted first with water (625 ml) and then with 0·05M formic acid. The acidic, UV-absorbing eluate was concentrated under reduced pressure to a gum which was first dried *in vacuo* over KOH and then crystallized from EtOH to give adenosine 5'-phosphate [Found, in material dried *in vacuo* over $P_2O_5$ at 80° for 6 hr: C, 34·55; H, 4·6; N, 20·8; P, 8·8. Calc. for $C_{10}H_{14}N_5O_7P$: C, 34·6; H, 4·1; N, 20·2; P, 8·9%] as colourless crystals (0·27 g, 78%), UV absorption (water, pH 2): $\lambda_{max}$ 257 (ε 13,500), $\lambda_{min}$ 229 nm (ε 2,630).

*Preparation of $N^6$,$N^6$-dimethyladenosine 5'-phosphate*

$N^6N^6$-Dimethyl-2',3'-O-isopropylideneadenosine (0·336 g, 1 mmole) was phosphorylated, as described above, with *o*-phenylene phosphorochloridate[5,11] (0·248 g, 1·3 mmole) in the presence of 2,6-lutidine (0·46 ml, 4 mmole). After aqueous work-up and concentration, the products were passed through a column of Dowex-50 ($H^+$) cation-exchange resin, the UV-absorbing eluate concentrated and acidified to pH 1 with formic acid, according to the procedure described above in the preparation of adenosine 5'-phosphate. After 48 hr at 20°, paper chromatography of the products revealed a principal UV-absorbing component [$R_f$ 0·56 (system A), 0·71 (system B)], corresponding to the *o*-hydroxyphenyl ester of the desired product.

The products were concentrated under reduced pressure, the residue dissolved in 0·2M triethylammonium bicarbonate (pH 7·5, 50 ml), and treated with 2% bromine water (55 ml, 6·9 mmole) as in the above preparation of adenosine 5'-phosphate. The products were then worked-up and chromatographed on Dowex-1 × 2 (formate) anion-exchange resin in the same way. The acidic eluate containing the desired product (59% yield, based on $\varepsilon_{267}$ = 18,600) was concentrated under reduced pressure to a gum, which crystallized from aqueous ethanol to give $N^6$,$N^6$-dimethyladenosine 5'-phosphate, pentahydrate [Found, in material dried *in vacuo* over $P_2O_5$ at 60° for 6 hr: C, 31·4; H, 6·55; N, 15·3; P, 6·4. Calc. for $C_{12}H_{18}N_5O_7P$, $5H_2O$: C, 31·0; H, 6·0; N, 15·05; P, 6·2%] as colourless crystals (0·159 g, 43%), UV absorption (water, pH 2); $\lambda_{max}$ 267 (ε 18,600), $\lambda_{min}$ 232 nm (ε 1,900).

*Conversion of uridine 5'-o-hydroxyphenyl phosphate into uridine 5'-phosphate*

2% Bromine water (22 ml, 2·8 mmole) was added to a solution of barium uridine 5'-*o*-hydroxyphenyl phosphate (0·10 g, 0·20 mmole) in water (1 ml) at 20°. After 10 min, the solution was extracted several times with ether until the ethereal layer was colourless. The aqueous layer, which showed no UV absorption at 260 nm, was concentrated to a gum. This material was redissolved in EtOH (5 ml) and the solution re-

evaporated. After this process had been repeated, the residue was dissolved in ethanol (20 ml) and the soln heated under reflux. After 20 min, water (*ca* 0·2 ml) was added to dissolve the crystalline ppt, and heating was continued for a further 10 min. The soln then showed strong UV absorption ($\lambda_{max}$ 273, $\lambda_{min}$ 240 nm). Paper electrophoresis (buffer I) of the products revealed one component with a mobility corresponding to that of uridine 5'-phosphate. However, paper chromatography (system A) revealed only 5–10% of uridine 5'-phosphate ($R_f$ 0·27) and 90–95% of another component ($R_f$ 0·34).

The alcohol soln of the products was concentrated to 15 ml, diluted with water (15 ml) and shaken with $H_2$ at atmospheric temp and pressure in the presence of Pd black (0·10 g) and 10% Pd-C (0·30 g). After 20 hr, the products were filtered and examined by paper chromatography: the sole component detected corresponded with uridine 5'-phosphate [$R_f$ 0·26 (system A), 0·08 (system B)]; UV spectrum: $\lambda_{max}$ 262, $\lambda_{min}$ 232 nm. The yield of uridine 5'-phosphate was estimated spectrophotometrically to be 0·052 g (80%).

*Reaction between 2',3'-O-isopropylideneadenosine and o-phenylene phosphate*

A suspension of 2',3'-*o*-isopropylideneadenosine[12] (0·8 g, 2·6 mmole) and 1-methylpyridinium *o*-phenylene phosphate[5] (1·037 g, 5·2 mmole) in pyridine (20 ml) was heated, under reflux, with the exclusion of moisture. After 2 hr and 4 hr, additional quantities (each of 1·037 g) of 1-methylpyridinium *o*-phenylene phosphate were added and, after a total reaction period of 6 hr, the products were cooled to 20° and evaporated under reduced pressure. The residual gum was kept *in vacuo* over $P_2O_5$ for 4 hr and then dissolved in 30% formic acid (20 ml) at 20°. After 48 hr, the products were evaporated under reduced pressure and redissolved in water (3 ml). Paper chromatography (system A) revealed seven UV-absorbing components, four of which were phosphorus-containing: three of the latter components corresponded to adenosine 5'-phosphate ($R_f$ 0·27), adenosine 5'-*o*-hydroxyphenyl phosphate ($R_f$ 0·44) and *o*-hydroxyphenyl phosphate ($R_f$ 0·51). Paper electrophoresis (buffer III) confirmed the presence of the latter compounds, and revealed that *ca* 40% of the UV-absorbing material was unphosphorylated.

The products were applied to a column (8 cm × 7 $cm^2$) of Dowex-1 × 2 (formate) anion-exchange resin, which was eluted first with water (500 ml) and then with formic acid (linear gradient from 0·05–0·1M over 1,000 ml). Adenosine 5'-phosphate (spectroscopically estimated yield, 20%) and adenosine 5'-*o*-hydroxyphenyl phosphate (spectroscopically estimated yield, 35%) were eluted separately with formic acid, and in the order indicated.

The fractions containing adenosine 5'-phosphate were combined, concentrated under reduced pressure and the gum crystallized from water. [Found, in recrystallized (from aqueous EtOH) material dried *in vacuo* over $P_2O_5$ at 80° for 5 hr: C, 34·7; H, 4·0; N, 20·3; P, 9·0. Calc. for $C_{10}H_{14}N_5O_7P$: C, 34·6; H, 4·1; N, 20·2; P, 8·9%], UV absorption (water, pH 2): $\lambda_{max}$ 256 (ε 13,500), $\lambda_{min}$ 228 nm (ε 2,880).

The fractions containing adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate were combined, concentrated under reduced pressure and the residual gum crystallized from water (10 ml). [Found, in material dried *in vacuo* over $P_2O_5$ at 100° for 6 hr: C, 43·2; H, 4·4; N, 15·5; P, 7·5. Calc. for $C_{10}H_{14}N_5O_7P$: C, 43·4; H, 4·1; N, 15·8; P, 7·0%], UV absorption (water): $\lambda_{max}$ 259 (ε 14,100), $\lambda_{min}$ 227 nm (ε 3,390).

*Reaction between 2',3'-O-isopropylideneuridine and o-phenylene phosphate*

A soln of 2',3'-O-isopropylideneuridine[12] (0·8 g, 2·8 mmole) in pyridine (20 ml) was heated, under reflux, with 1-methylpyridinium *o*-phenylene phosphate[5] (3 × 1·59 g, 17·3 mmole) over a total period of 6 hr, as in the corresponding experiment with 2',3'-O-isopropylideneadenosine. The products were worked-up in the same way and treated with 30% formic acid at 20° for 48 hr. Paper chromatography (system A) and electrophoresis (buffer III) of the products revealed the presence of uridine 5'-phosphate and uridine 5'-*o*-hydroxyphenyl phosphate together with *ca* 40–50% of unphosphorylated material.

The products were chromatographed on a column (17 cm × 5 $cm^2$) of Dowex-1 × 2 (formate) anion-exchange resin. The column was eluted with (a) water, (b) 0·2M formic acid and (c) M formic acid. Eluate (b) contained uridine 5'-phosphate (spectroscopically estimated yield, 11%) as its sole UV-absorbing constituent; it was identified by paper chromatography (systems A, B) and electrophoresis (buffers III, IV). Eluate (c) was found to contain a mixture of uridine 5'-*o*-hydroxyphenyl phosphate and *o*-hydroxyphenyl phosphate.

*Decomposition of adenosine 5'-o-hydroxyphenyl phosphate in boiling pyridine solution*

(a) A suspension of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·50 g) in pyridine (100 ml) was heated under reflux, with the exclusion of moisture, for 6 hr. Paper electrophoresis (buffer III) of the products revealed only two UV-absorbing spots: one non-mobile (corresponding to adenosine) and one with a mobility corresponding to that of adenosine 3',5'- or 2',3'-phosphate.

The cooled pyridine soln was decanted from the insoluble residue, which was then washed with pyridine (5 ml). The combined soln and washings were evaporated under reduced pressure (bath temp $<30°$), and the residual gum kept *in vacuo* over $P_2O_5$ at 20° for 16 hr. A soln of this gum in water (3 ml) was applied to a column (16 cm × 1·2 $cm^2$) of Dowex-1 × 2 (formate) anion-exchange resin. The column was eluted first with water (600 ml) and then with 0·04M formic acid. The fractions containing the major component eluted with formic acid were combined and evaporated under reduced pressure (bath temp $<30°$) to a gum which was first kept *in vacuo* over KOH and then dissolved in water (3 ml). This soln was carefully neutralized with dil NaOH aq, then lyophilized and the residue triturated with EtOH. The colourless solid obtained was identified (see below) as the sodium salt of adenosine 2',3'-cyclic phosphate, contaminated with adenosine 2'- and 3'- phosphates.

(b) A suspension of adenosine 5'-*o*-hydroxyphenyl hydrogen phosphate (0·01 g) in pyridine (2 ml) was heated under reflux for 6 hr, and then evaporated under reduced pressure as above. The residue was kept *in vacuo* over $P_2O_5$ at 20° for 16 hr and then dissolved in water (0·1 ml). The soln was applied to a paper electrophoretogram, which was run in buffer I. The band corresponding to adenosine 2',3'-cyclic phosphate was cut out and eluted with water; yield (estimated spectrophotometrically, based on $\varepsilon_{max}$ = 14,700), 64%.

*Identification of adenosine 2',3'-cyclic phosphate*

The above material was found to have the same paper chromatographic (systems A,B,C,D) and electrophoretic (buffers II, III, IV) properties as authentic adenosine 2',3'- and 3',5'-cyclic phosphates.[8,9]

A soln of the above material (0·003 g, 0·01 mmole) and $Ba(OH)_2$, $8H_2O$ (0·032 g, 0·1 mmole) in water (0·5 ml), contained in a stoppered polythene tube, was heated in a boiling water-bath for 20 min. The products were cooled and treated with Amberlite-IR 120 ($H^+$, 2 ml) cation-exchange resin. After 2 min, the resin was collected by filtration and washed with water (0·5 ml). The combined filtrate and washings were examined by paper chromatography (system D): two components were revealed ($R_f$'s 0·17 and 0·28, respectively) corresponding to adenosine 3'- and 2'- phosphates. No adenosine 5'-phosphate ($R_f$ 0·34) could be detected.

When authentic adenosine 2',3'-cyclic phosphate[9] was treated with aqueous baryta, as above, precisely the same result was obtained. However, when authentic adenosine 3',5'-cyclic phosphate[8] was heated under the same conditions in aqueous baryta soln, and the products worked-up as above, paper chromatography (system D) revealed three components ($R_f$'s 0·11, 0·17 and 0·34, respectively), corresponding to starting material, adenosine 3'-phosphate and adenosine 5'-phosphate.

*Decomposition of uridine 5'-o-hydroxyphenyl phosphate in boiling pyridine solution*

A soln of barium uridine 5'-*o*-hydroxyphenyl phosphate (0·04 g, 0·08 mmole) in water (0·5 ml) was applied to a column (18 cm × 1·1 $cm^2$) of Amberlite-IR 120 ($H^+$) cation-exchange resin. The column was eluted with water until the eluate had negligible UV-absorption. The total eluate was concentrated under reduced pressure, the residual gum dissolved in anhydrous pyridine (5 ml), and the soln re-evaporated. After two further evaporations from pyridine (5 ml) soln, the residue was dissolved in pyridine (8 ml), and the soln heated under reflux for 190 min. Paper electrophoresis (buffer III) revealed two UV-absorbing spots with mobilities corresponding to those of uridine and uridine 2',3' (or 3',5')-cyclic phosphate.[8,9]

The products were concentrated under reduced pressure (below 30°) and kept *in vacuo* over $P_2O_5$. The residue obtained was dissolved in water (0·1 ml) and the soln applied to a paper electrophoretogram, which was run in buffer I. The band corresponding to uridine 2',3'-cyclic phosphate (for identification, see below) was cut out and eluted with water; yield (estimated spectrophotometrically, based on $\varepsilon_{max}$ = 9,900), 45%.

*Identification of uridine 2',3'-cyclic phosphate*

(a) Hydrolysis of the above material with aqueous baryta under the conditions described for adenosine cyclic phosphates (see above) gave uridine 2'(3')-phosphates, as identified by paper electrophoresis (buffer II) and chromatography (system C). No uridine 5'-phosphate could be detected.

(b) A soln of the above material (*ca* 0·01 mmole) and pancreatic ribonuclease (50 μg) in 0·1M tris hydrochloride buffer (pH 7·5, 0·1 ml) was incubated at 37° for 1 hr. Paper chromatography (system B) revealed that the starting material had been quantitatively converted into a product ($R_f$ 0·08), corresponding to uridine 3'-phosphate. The control soln had remained completely unchanged under the same conditions.

*Decomposition of 2',3'-O-isopropylideneadenosine 5'-o-hydroxyphenyl phosphate in boiling pyridine solution.*

A soln of 2',3'-O-isopropylideneadenosine 5'-*o*-hydroxyphenyl phosphate (0·005 g) in pyridine (1 ml) was heated under reflux for 6 hr and then concentrated under reduced pressure. The residue was dissolved

in 30% formic acid (2 ml) and the soln allowed to stand at 20° for 48 hr. Paper chromatographic (system A) and electrophoretic (buffer III) examination of the products revealed the presence of adenosine 5'-phosphate.

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