Uridine Oligonucleotide Synthesis by the Reaction of Uridine with Tri-(imidazol-1-yl)phosphine

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Uridine oligonucleotides have been synthesized using tri-(imidazol-1-yl)phosphine. The procedure involves two steps: (i) the reaction of uridine with tri-(imidazol-1-yl)phosphine (< 60 min) and (ii) the *in situ* oxidation of the resulting phosphite with iodine and water (10 min). Uridine oligonucleotides up to a hexamer were obtained in high yield at a tri-(imidazol-1-yl)phosphine to uridine ratio of 1.5 at −78 °C. The inter-ribonucleotidic linkage of the oligomer consists of 2′–5′- and 3′–5′-linkages, formed in the oxidation process. Additives such as metal cations and polynucleotides in the oxidation process showed a significant effect on the formation of the inter-nucleotidic linkage.

OLIGORIBONUCLEOTIDE synthesis by the method of chemical polymerization, first performed by Khorana *et al.*,[1] has been developed by several researchers.[2] The procedure generally involves activation of the phosphate moiety of the suitably protected nucleotides in the polymerization. Sawai *et al.* have recently reported the metal ion-promoted oligomerization of a nucleoside 5′-phosphoroimidazolide.[3] However, these procedures inadvertently need long reaction times and yield many side-products, *e.g.* pyrophosphates and cyclic oligonucleotides.

To overcome these problems, we have explored new approaches to the synthesis of oligoribonucleotides by the polycondensation method. Recently, phosphorylating reagents such as dichlorophosphites [4] and phosphoryltris-(triazole) [5] were shown to be very useful for the rapid preparation of the triester intermediates in the oligonucleotide synthesis. In this paper, we describe tri-(imidazol-1-yl)phosphine as a new sort of phosphorylating reagent and demonstrate its utility in the synthesis of oligoribonucleotides starting from unprotected ribonucleosides.

This approach is based on the observations that many active phosphorus compounds readily react with *cis*-glycols, that the resulting cyclic phosphite having a P–X (X = Cl or amine) group can react rapidly with hydroxy-groups,[6] and that phosphite triesters can be rapidly converted into the corresponding phosphates by iodine oxidation.[4] A preliminary report of this work has already appeared.[7]

RESULTS AND DISCUSSION

The reaction of uridine with tri-(imidazol-1-yl)-phosphine was carried out in pyridine–tetrahydrofuran solution at −78 °C and the subsequent oxidation of the resulting phosphite employed iodine and water. The time–conversion curves for uridine oligonucleotides are shown in Figure 1. During the initial 60 min of the reaction, the amount of uridine and UpU decreased, while the higher oligonucleotide materials were formed. This result shows that an ensuing reaction takes place in this stage. After 60 min, the amount of oligonucleotide material formed did not significantly increase, showing that the polymerization reaction was almost complete after this time. The products were separated on a DEAE–cellulose column (hydrogencarbonate form). The elution pattern is shown in Figure 2.

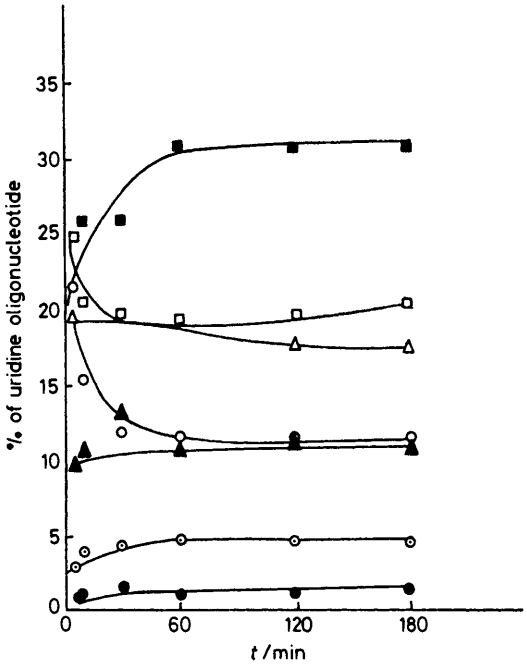

FIGURE 1 Formation of uridine oligonucleotide in the reactions of uridine with tri-(imidazol-1-yl)phosphine. ○, uridine; □, UpU; △, $(Up)_2U$; ■, $(Up)_3U$, $(Up)_2$; ▲, $(Up)_4U$, $(Up)_3$; ○, $(Up)_5U$, $(Up)_4$; ●, $(Up)_5$

The polymerization products were classified into two series. One series consists of uridine oligonucleotides with a free 5′-OH end, $(Up)_n$, and the other consists of uridine oligonucleotides with free 5′-OH and 3′-OH ends, $(Up)_nU$. However, uridine monophosphate and uridine 2′,3′-(cyclic)phosphate were not found in the products.

The type of the phosphodiester linkage and the degree of oligomerization were investigated by degradation of the products, *viz.* degradations by snake venom phosphodiesterase, by spleen phosphodiesterase, and by alkali (KOH). The products, oligonucleotides, were completely hydrolysed by both snake venom phosphodi-

esterase and KOH. It is noteworthy that the phosphodiester linkage in the oligonucleotides is composed of both 3′–5′- and 2′–5′-linkages but not 3′–3′- and 5′–5′-linkages. That 32% of UpU was hydrolysed by spleen phosphodiesterase shows the ratio of the 3′–5′- to the 2′–5′-linkage in UpU to be 1 : 2 (mol/mol).

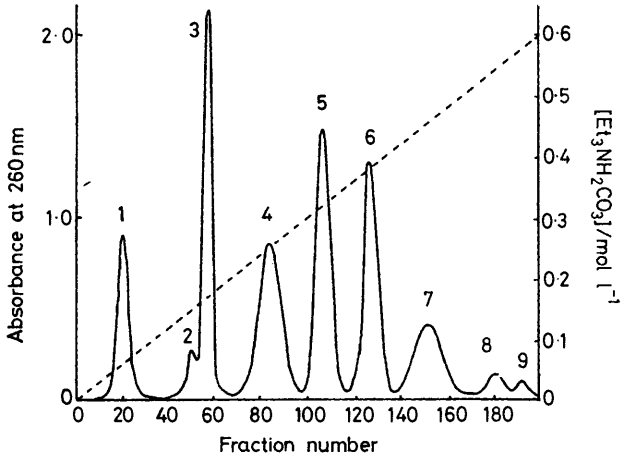

FIGURE 2 Chromatography [DEAE–cellulose (carbonate) column (3.4 × 28.0 cm)] of the polymerization products from the reaction of uridine with tri-(imidazol-1-yl)phosphine. A linear gradient of triethylammonium hydrogencarbonate, pH 7.5, 0.001—0.6M, was used for elution and 15-ml fractions (each collected over 15 min) were collected. % Yields: peak 1, uridine, 11.3; peak 2, unidentified, 3.2; peak 3, UpU, 16.1; peak 4, $(Up)_2U$, 20.0; peak 5, $(Up)_3U$, 9.2; $(Up)_2$, 13.6; peak 6, $(Up)_4U$, 7.6; $(Up)_3$, 8.5; peak 7, $(Up)_5U$, 5.5; $(Up)_4$, 2.7; peak 8, $(Up)_5$, 1.5; peak 9, $(Up)_6$, 0.8

We conclude, therefore, that tri-(imidazol-1-yl)-phosphine does not attack the 5′-OH group of unprotected uridine in the first phosphorylation step. The polymerization reaction proceeds *via* the uridine 2′,3′-(cyclic)phosphorimidazole (2) which is formed by selective attack of tri-(imidazol-1-yl)phosphine at the 2′- and 3′-OH groups of uridine. Generally, the reaction of phosphorus compound with *cis*-glycols gives only the cyclic phosphite ester.[6] This observation supports the intermediacy of the cyclic compound (2). A similar consideration for a nucleoside 2′,3′-(cyclic)phosphite ester intermediate has been made for the reaction of a ribonucleoside with triethylphosphine.[8] In the present study, neither uridine 2′(3′)-phosphate nor uridine 2′,3′-(cyclic)phosphate, resulting from oxidation of uridine 2′,3′-(cyclic)phosphorimidazole (2), were found in the products, indicating that the intermediate uridine 2′,3′-(cyclic)phosphorimidazole (2) is highly reactive and reacts rapidly with the 5′-OH group of (1) or (2) soon after the formation of (2).

Uridine oligonucleotides (4) were easily obtained by the *in situ* oxidation of the oligomer (3) with iodine and water. This process involves oxidation of the cyclic phosphite triester and ring-opening of the resulting cyclic phosphate. The type of the phosphodiester linkage results from cleavage of either the 3′-O–P or the 2′-O–P bond in (3) at the ring-opening step. That no cleavage of the 5′-O–P bond occurred was shown by the absence of uridine monophosphate in the products.

The yield of uridine oligonucleotide was affected by the molar ratio of tri-(imidazol-1-yl)phosphine to uridine (Table 1). Even when this ratio was greater than 1, unchanged uridine still remained, indicating that parallel reactions, namely phosphorylation of uridine with tri-(imidazol-1-yl)phosphine and its deactivation with the pyridine solvent, take place. With increase of tri-(imidazol-1-yl)phosphine, the amount of unchanged

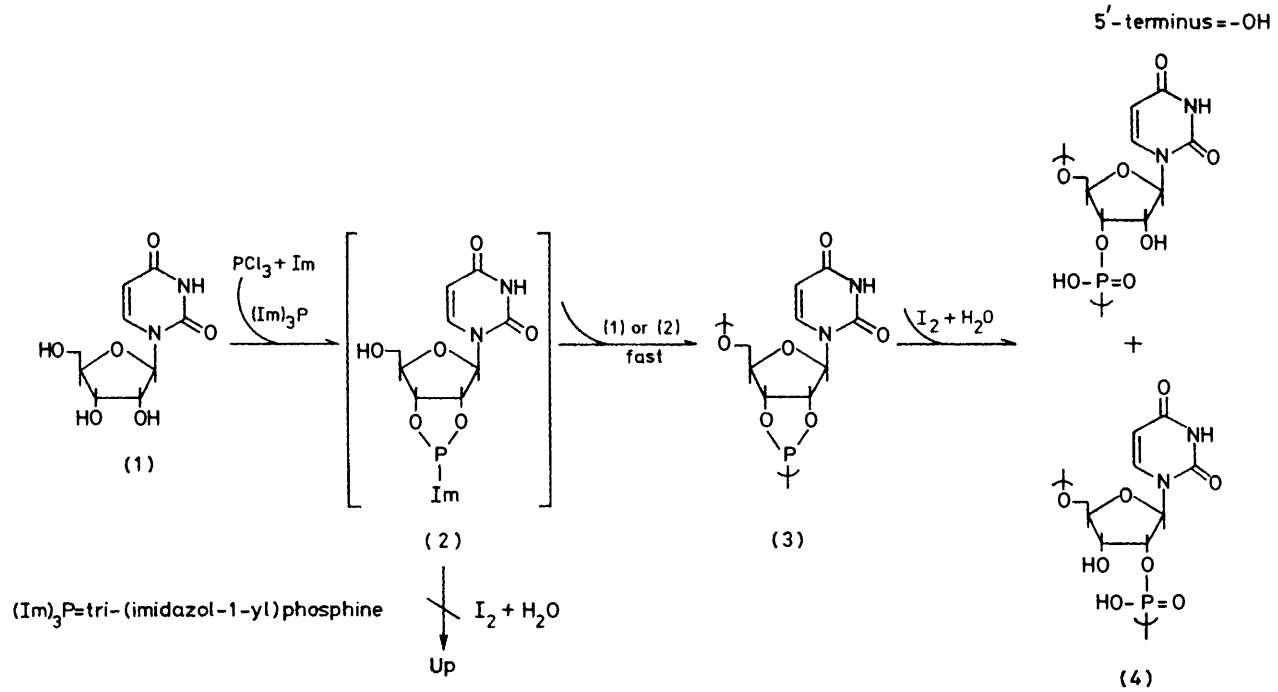

TABLE 1

Effect of the molar ratio of tri-(imidazol-1-yl)phosphine to uridine on the yield of uridine oligonucleotide [a]

Molar ratio $(Im)_3P$: Urd [c]	Yield (%) [b] Uridine	UpU	Unidentified	$(Up)_2U$	$(Up)_3U$, $(Up)_2$	$(Up)_4U$, $(Up)_3$	$(Up)_5U$, $(Up)_4$	$(Up)_5$
0.43	38.2	41.1		14.2	5.5	0.9		
0.64	28.3	36.3		18.8	12.8	3.2	0.6	
0.85	24.7	34.1		19.7	15.8	4.7	0.9	
1.06	16.6	24.0	1.2	22.9	28.0	12.0	2.4	0.9
1.28	4.9	21.1	2.5	22.7	32.8	11.3	3.5	1.2
1.49	2.7	13.5	3.3	19.7	36.8	15.7	6.6	1.8
1.70	1.7	13.2	7.3	26.1	29.8	16.1	4.8	1.0

[a] The reaction of uridine with tri-(imidazol-1-yl)phosphine was carried out at −78 °C for 5 h. Oxidation with iodine and water was performed at 0 °C for 10 min. [b] Based on the number of O.D. units in each h.p.l.c. peak. [c] $(Im)_3P$ = tri-(imidazol-1-yl)-phosphine; Urd = uridine.

uridine decreased, while oligonucleotides of a high degree of polymerization were formed. The optimum yield for the high-molecular-weight oligonucleotides was obtained at a ratio of tri-(imidazol-1-yl)phosphine to uridine of 1.5. The maximum chain length of uridine oligonucleotide obtainable under the present conditions was six.

The effect of temperature on the yield of uridine oligonucleotide is shown in Table 2. Reactions were complete, even at −78 °C, within 60 min. Yields slightly decreased as the temperature was raised from −78 to −50 °C but remained constant at temperatures higher than −50 °C. On the other hand, as the temperature increased so did the yield of UpU, while that of the high-molecular-weight oligonucleotides decreased. Thus, a low reaction temperature is suitable for the preparation of uridine oligonucleotides having a high degree of polymerization.

TABLE 2

Effect of temperature on the yields of uridine oligonucleotide obtained from the reaction of uridine with tri-(imidazol-1-yl)phosphine.[a]

T/°C	Yield (%) [b] Uridine	UpU	$(Up)_2U$	$(Up)_3U$, $(Up)_2$	$(Up)_4U$, $(Up)_3$	$(Up)_5U$, $(Up)_4$	$(Up)_5$
−78	22.1	22.8	20.0	21.3	6.7	2.5	0.6
−50	27.8	24.6	15.8	18.6	6.9	2.1	0.5
−30	27.8	27.5	16.4	16.2	6.1	1.5	0.5
0	27.7	34.8	15.7	13.9	3.5	0.9	

[a] The reaction of uridine with tri-(imidazol-1-yl)phosphine was carried out in pyridine–THF solution for 2 h. Oxidation with iodine and water was performed at 0° for 10 min. [b] Based on the number of O.D. units in each h.p.l.c. peak.

The type of the inter-ribonucleotidic linkage was controlled by the oxidative ring-opening reaction of the oligomer (3) with iodine and water in the presence of divalent metal cations or polynucleotides. The results are shown in Table 3. Of the uridine oligonucleotides obtained from the control iodine–water oxidation system 32% contained the 3′–5′-phosphodiester linkage. However, the naturally occurring percentage (*ca.* 50%) was obtained when the oxidations were carried out in the presence of Poly A, Mn^{2+}, or Co^{2+}. Poly U also affected the reaction producing oligonucleotide of which only 12% contained the 3′–5′-linkage. We consider that complex formation between the polynucleotides and the substrate plays an important role in the formation of the phosphodiester linkage, chelation of metal cations to the phosphate and base moieties having a considerable influence.

Our approach still leaves unsolved the synthesis of oligoribonucleotides containing exclusively 3′–5′-, exclusively 2′–5′-, or definite controlled linkages. To solve this problem, further refinement of the oxidative ring-opening reaction is needed. Although the problem has not yet been fully solved, our present approach has some merits as a rapid and convenient method for preparation of short-chain homo-oligoribonucleotides from unprotected ribonucleosides. Further research to evaluate the potential of this method for the synthesis of homo-oligoribonucleotides containing different nucleotides is in progress.

TABLE 3

Influence of polynucleotides and metal ions on the oxidation reaction [a]

	3′–5′-linkage	2′–5′-linkage
	Mol fraction in UpU	
None	0.32	0.68
Poly A	0.52	0.48
Poly U	0.12	0.88
$MnCl_2$	0.45	0.55
$Co(NO_3)_2$	0.43	0.57
$CaCl_2$	0.27	0.73
$Pb(NO_3)_2$	0.28	0.72
$MgCl_2$	0.35	0.65
$ZnCl_2$	0.25	0.75
$CdCl_2$	0.32	0.68
$NiCl_2$	0.29	0.71
$HgCl_2$	0.32	0.68
$CuCl_2$	0.28	0.72

[a] Polynucleotides were used in two-fold excess of the uracil moiety. Metal cations were used in stoicheiometric amounts to the phosphine.

EXPERIMENTAL

Paper chromatography was carried out by the descending technique on Whatman 3 MM paper. Solvent systems employed were: A, isopropyl alcohol–concentrated ammonium hydroxide–water (7 : 1 : 2, v/v); B, propan-1-ol–concentrated ammonium hydroxide–water (55 : 10 : 35, v/v). Paper electrophoresis was performed using the same paper at 35 V cm^{-1} for 60 min; buffer solutions were controlled at pH 7.5 with triethylammonium hydrogencarbonate and at pH 7.0 with 0.1M-KH_2PO_4–0.1M-Na_2HPO_4 (1 : 2, v/v). High-pressure liquid chromatography was performed on a Shimadzu–Dupont LC 830 instrument using Permaphase AAX column; the exponential gradient was 0.002M-KH_2PO_4 (pH 3.4) to 0.5M-KH_2PO_4 (pH 4.5) at 70 atm. Ultraviolet spectra were obtained on a Union SM 401 recording spectrophotometer.

Reagent grade pyridine was distilled from toluene-*p*-sulphonyl chloride, redistilled from calcium hydroxide, and stored over 4A molecular sieves. Tetrahydrofuran was distilled from sodium metal, dried by refluxing in a Soxhlet apparatus filled with Type 5A molecular sieves, redistilled, and stored over 5A molecular sieves. Phosphorus trichloride was distilled and stored in a brown ampoule. Uridine and imidazole were dried *in vacuo* over phosphorus pentaoxide for 24 h before use. Alkaline phosphatase suspended in 65% saturated aqueous ammonium sulphate (1 mg ml^{-1}) was obtained from Sigma Chemical Co. Lyophilized snake venom phosphodiesterase (5 mg) (Sigma Chemical Co.) and spleen phosphodiesterase (14.5 units) (P. L. Biochemical Inc.) were dissolved in water (1 ml). These enzyme stock solutions were used for the enzyme assays. Metal chlorides and metal nitrates were obtained from Nakarai Chemical Co. Polynucleotides (Sigma Chemical Co.) were obtained commercially.

Tri-(imidazol-1-yl)phosphine.—Tri-(imidazol-1-yl)phosphine was prepared by a modification of the reported method.[9] The reaction of phosphorus trichloride (200 μl, 2.3 mmol) with imidazole (940 mg, 13.8 mmol) was carried out in anhydrous tetrahydrofuran (THF) (16 ml) at 0 °C under nitrogen for 20 min. The supernatant separated from imidazolium hydrochloride was directly used as a phosphorylating reagent. That the supernatant did not contain chloride ion after the treatment with water showed that phosphorus trichloride had been completely converted into the desired material. The phosphine content in this supernatant was determined by the chelatometric method.[10]

Uridine Oligonucleotides.—Generally, the reaction of uridine with tri-(imidazol-1-yl)phosphine was carried out in pyridine–THF solution; the reaction mixtures consisted of uridine (35.1 mg, 0.14 mmol) in 5 : 3 (v/v) pyridine–THF (0.5 ml) and tri-(imidazol-1-yl)phosphine (approximately equimolar amount to uridine) in THF (1.25 mmol) and were prepared immediately before use. The mixtures were stirred at −78 °C for 5, 10, 30, 60, 120, and 180 min. After these time intervals they were added to a solution of iodine (43.2 mg, 0.17 mmol) in 2 : 1 (v/v) THF–water (1.25 ml) at 0 °C and stirred for 10 min. After treatment with freshly prepared aqueous sodium hydrogensulphite (5%), the solvent was removed *in vacuo* and the residues were dissolved in water (1 ml). The resulting products were analysed by h.p.l.c. and paper chromatography.

The solution obtained from the reaction for 180 min was adjusted to pH 8 with aqueous ammonium hydroxide and then applied on the top of a DEAE–cellulose column (3.4 × 28.0 cm; in HCO_3^- form). The column was washed with water (100 ml) and then eluted with a linear gradient with 0.001M-triethylammonium hydrogencarbonate (1 l, pH 7.5) in the mixing vessel and 1 l of 0.6M-triethylammonium hydrogencarbonate (pH 7.5) in the reservoir. Fractions (15 ml each) were collected at 15-min time intervals. The fractions were evaporated to a gum under reduced pressure at ≤30 °C. To remove the residual triethylammonium hydrogencarbonate, the residue was dissolved in water and the solution evaporated repeatedly. The remaining materials were lyophilized.

Effect of the Molar Ratio of Tri-(imidazol-1-yl)phosphine to Uridine on the Polymerization.—The reaction mixtures consisted of uridine (35.1 mg, 0.14 mmol) in 5 : 3 (v/v) pyridine–THF (0.5 ml) and tri-(imidazol-1-yl)phosphine (an appropriate amount) in THF (2 ml). The reactions were carried out at −78 °C for 5 h. The oxidations in which iodine was used in the equimolar amount to the phosphine were done in the similar manner described above. The products were analysed by h.p.l.c. The amount of the phosphine used in this experiment was determined to be 0.06mM, 0.09mM, 0.12mM, 0.15mM, 0.18mM, 0.21mM, and 0.24mM by the chelatometric method.[10]

Effect of Temperature on the Polymerization.—To uridine (35.1 mg, 0.14 mmol), dissolved in 5 : 3 (v/v) pyridine–THF (0.5 ml), was added tri-(imidazol-1-yl)phosphine (1 mol equiv.) solution (1.2 ml) at −78, −50, −30, and 0 °C, and the mixture were stirred for 2 h. After the oxidation with iodine and water, the products were analysed by h.p.l.c.

Effect of Metal Ions and Polynucleotides on the Oxidation.—The reaction of uridine (35.1 mg, 0.14 mmol) with tri-(imidazol-1-yl)phosphine (1 mol equiv.) was carried out at −78 °C for 2 h. The oxidations with iodine (1 mol equiv.) and water were carried out in the presence of various metal cations (1 mol equiv.) and polynucleotides (two fold excess of the uracil moiety) at 0 °C for 10 min. The products were separated by paper chromatography (solvent A) and paper electrophoresis (triethylammonium hydrogencarbonate buffer). The effect of metal ions and polynucleotides on the oxidation reaction was investigated for the phosphodiester linkage in the produced UpU.

Degradation of Products.—(a) *Alkaline phosphatase.* Digestion was carried out in a mixture of substrate (5—10 optical density units), 0.1M-tris-HCl, pH 8.2 (40 μl), and enzyme suspension (20 μl). After the incubation at 37 °C for 30 min, the products were analysed by h.p.l.c.

(b) *Snake venom phosphodiesterase.* Digestion was carried out in a mixture of substrate (1 O.D. unit), 0.1M-$MgCl_2$ (10 μl), 0.1M tris-acetate, pH 8.8 (10 μl), and enzyme stock solution (2 μl). After incubation at 37 °C for 30 min, the products were analysed by paper chromatography in solvent A and h.p.l.c.

(c) *Spleen phosphodiesterase.* Digestion was carried out in a mixture of substrate (1 O.D. unit), 0.3M-ammonium acetate pH 6.0 (10 μl), and the enzyme solution (5 μl). After incubation at 37 °C for 60 min, the products were analysed as for snake venom phosphodiesterase.

(d) *Potassium hydroxide hydrolysis.* Potassium hydroxide hydrolysis was carried out in a mixture of substrate (2 O.D. units) and 0.3M-potassium hydroxide (10 μl) at 37 °C for 18 h. The products were analysed as for snake venom phosphodiesterase.

Characterization of the Polymerization Products.—(a) *Evidence for the absence of cyclic oligonucleotides and oligonucleotides with a 5′-phosphate end.* Cyclic oligonucleotides

are resistant to mild alkali.[11] Oligonucleotides with 5′-phosphate ends are degraded by KOH to pUp, Up, and U [from p(Up)$_n$U] or pUp and Up [from p(Up)$_n$]. However, all the oligonucleotides obtained here were completely degraded by KOH to Up and U (pUp was not detected), showing that there were no cyclic oligomers and oligonucleotides with a 5′-phosphate end in in the products.

(b) *Characterisation of all peaks except peak* 2 *in Figure* 2. Peaks 1, 3, and 4 in Figure 2 each gave only one spot on paper chromatography and paper electrophoresis and one peak on h.p.l.c. Peak 1 had the same R_F as uridine (R_F 0.58 in solvent A, 0.62 in solvent B). Therefore, the material of this peak was deemed to be uridine. Peak 3 had the same R_F as UpU (R_F 0.28 in solvent A, 0.53 in solvent B). The material of peak 3 was completely degraded by KOH to give a Up : U ratio of 1.05 and completely degraded by snake venom phosphodiesterase to give a pU : U ratio of 1.01.

Therefore, the material of this peak represented UpU containing the 2′–5′ and 3′–5′ phosphodiester linkages. 32% of the UpU obtained from the usual oxidation with iodine and water was degraded by spleen phosphodiesterase. Peak 4 was completely degraded by KOH to give a Up : U ratio of 1.80 and completely degraded by snake venom phosphodiesterase to give a pU : U ratio of 2.10, showing the material of this peak to be $(Up)_2U$. The material of peak 5 gave one peak on h.p.l.c.; after treatment with alkaline

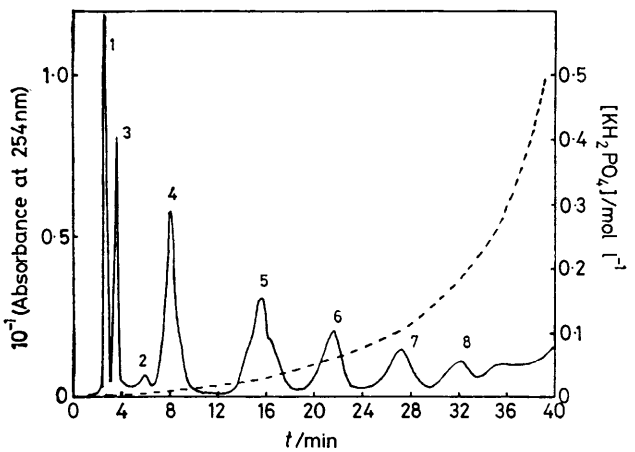

FIGURE 3 H.p.l.c. (Permaphase AAX column) of the reaction mixture obtained from the reaction of uridine with tri-(imidazol-1-yl)phosphine. The broken line shows the KH_2PO_4 gradient (0.002—0.05M, pH 3.4)

phosphatase it gave two peaks. The first peak had the same retention time as UpU and the same R_F as UpU, and the second peak had the same retention time as the original sample; thus peak 5 was due to $(Up)_2$ and $(Up)_3U$. Similar analysis for peaks 6 and 7 showed that these peaks consisted of two series of uridine oligonucleotides, $(Up)_n$ and $(Up)_nU$. Peaks 8 and 9 showed one peak on h.p.l.c. and the material was degraded by KOH to Up only, showing the materials of these peaks to be $(Up)_n$.

(c) *Presence of unidentified peak* 2. Peak 2 had an R_F value of 0.90 relative to uridine in solvent A and a relative mobility of 0.45 based on Up in electrophoresis at pH 7.0, clearly distinguishable from UpU and uridine 2′,3′-(cyclic)-phosphate. Judging from these observations and the elution position on DEAD–cellulose column chromatography, the material of this peak was thought to have a single negative charge and may be uridine monophosphite.

(d) *Identification of all h.p.l.c. peaks.* Each component from separation on a DEAE–cellulose column was subjected to h.p.l.c. The results are shown in Figure 3. The number of each peak agrees with the number shown in Figure 2. Thus, the yields of uridine oligonucleotides in Figure 1 and Tables 1 and 2 could be determined from the optical densities for each component separated by h.p.l.c.

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