enzyme was found. It is not clear at present whether the lack of a catalytic effect exercised by carboxypeptidase is due to the absence from O-acetylbenzilic acid of a proton on the carbon atom at the alkoxide link or else to a steric factor.

The stereochemistry of the substrate has received relatively little attention as yet in the study of the specificity of carboxypeptidase as an esterase. A preliminary examination of the hydrolysis of O-acetyl-D-mandelate in the presence of 10^{-5} M enzyme at pH 7.5 reveals no enzymatic catalysis. The stringency of the stereochemical requirements of carboxypeptidase are being investigated further at present, and the results of this work should be of importance to the development of mechanistic hypotheses for the action of the enzyme.[29]

In conclusion, although considerable information on the kinetic behavior of carboxypeptidase A as an esterase is now available, the mechanism of its action clearly remains to be elucidated. Work on many mechanistic aspects of carboxypeptidase action is underway in our laboratory and will be reported in due course.

(29) The variations in the structures of the substrates discussed so far have been in the alcoholic residue of the ester. Studies have also been started on other esters such as O-chloroacetylmandelic acid in which the acyl part of the ester is changed. A comparison of the rates of the carboxypeptidase-catalyzed hydrolysis of O-chloroacetyl-DL-mandelate and O-acetyl-DL-mandelate at reactant concentrations of 0.02 M shows that the chloroacetyl compound reacts about forty times faster than the acetyl ester at pH 7.5. Should this rate enhancement for the chloroacetyl compound still hold true when V_{max} values are compared, it will suggest that nucleophilic attack on the ester carbonyl group is involved in a kinetically important step of the ester hydrolyses. We are now in the process of unraveling the kinetic parameters for the hydrolysis of O-chloroacetylmandelate.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH AND THE DEPARTMENT OF BIOCHEMISTRY, THE UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN]

Studies on Polynucleotides. XXXI.[1] The Specific Synthesis of $C_{3'}$–$C_{5'}$-Linked Ribopolynucleotides (6).[2] A Further Study of the Synthesis of Uridine Polynucleotides[3]

BY C. COUTSOGEORGOPOULOS AND H. G. KHORANA

RECEIVED MARCH 20, 1964

Acetylation of 5′-O-monomethoxytrityluridine 3′-phosphate followed by an acidic treatment gave 2′-O-acetyluridine 3′-phosphate in practically quantitative yield. Treatment of a mixture of pyridinium 2′,5′-di-O-acetyluridine 3′-phosphate and 2′-O-acetyluridine 3′-phosphate with dicyclohexylcarbodiimide in dry pyridine followed by a work-up involving successive acetic anhydride–pyridine and ammoniacal treatments gave homologous uridine polynucleotides. Members up to the hexanucleotide were isolated pure and characterized. Higher homologs up to the decanucleotide were also demonstrated to be present in the polymeric mixture. Several of the side products encountered were also characterized.

In an earlier paper the synthesis of uridine oligonucleotides containing exclusively the $C_{3'}$–$C_{5'}$ internucleotidic linkages was reported.[2c] At that time, however, 2′-O-acetyluridine 3′-phosphate, the key intermediate, was available in limited quantities, and the study of the polymerization reaction was necessarily of a preliminary nature with only the di- and trinucleotides being characterized definitively as the products. Further work on the synthesis of uridine polynucleotides is reported in this paper. Added interest in these polymers accrued from the fact that at the time the present work was undertaken, enzymatically synthesized high molecular weight polyuridylic acid was the only homopolymer known to stimulate the formation of polyphenylalanine in a cell-free amino acid incorporation system.[4] It was hoped that the availability of the short-chain uridine polynucleotides of known size and end groups would facilitate further study of the polypeptide-synthesizing system and perhaps indicate direction of further work on the synthesis of specific ribopolynucleotides of interest in the study of the amino acid code.[5]

The method used for the preparation of pyridinium 2′-O-acetyluridine 3′-phosphate (V) is illustrated in the structures I–V. 5′-O-Monomethoxytrityluridine 3′-phosphate (I) may be prepared directly from uridine 3′-phosphate in good yield.[2d] Treatment with acetic anhydride in the presence of an excess of tetraethylammonium acetate results in the quantitative acetylation of the 2′-hydroxyl group,[2d] the initial product in this reaction being the mixed anhydride II. The decomposition of anhydrides of this type is routinely effected by a short aqueous pyridine treatment of the acetylation mixture.[6] However, II was found to have rather high stability in aqueous pyridine, the hydrolysis being apparently inhibited by the presence of the bulky monomethoxytrityl group[7] and by the large excess of acetate ions.[8] Because of the restriction on the duration of the aqueous pyridine treatment of II imposed by the lability of the 2′-O-acetyl group,[9] the procedure

(1) Paper XXX: T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964).

(2) Earlier papers which deal directly with this topic: (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *ibid.*, **84**, 430 (1962); (b) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (c) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); (d) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3852 (1963); (e) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3857 (1963).

(3) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, the National Science Foundation, and the Life Insurance Medical Research Fund, Rosemont, Pa.

(4) (a) M. W. Nirenberg and J. H. Matthaei, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1588 (1961); (b) P. Lengyel, J. F. Speyer, and S. Ochoa, *ibid.*, **47**, 1936 (1961).

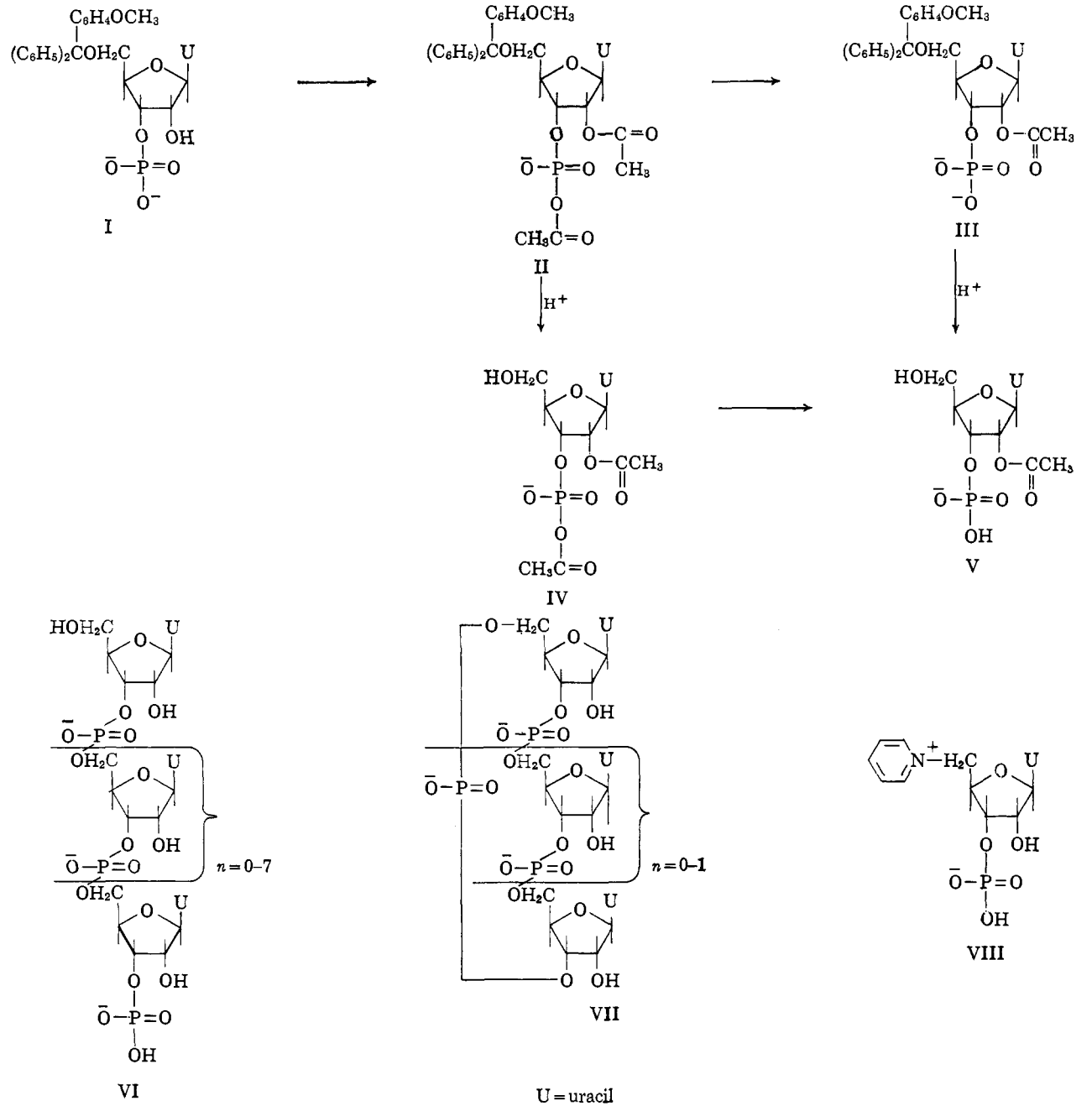
(5) F. H. C. Crick in "Progress in Nucleic Acid Research," Vol. I, J. N. Davidson and W. E. Cohn, Ed., Academic Press, Inc., New York, N. Y., 1963, p. 163.

(6) (a) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958); (b) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).

(7) Thus the mixed anhydride linkage in II is more stable than that in 2′,5′-di-O-acetyluridine 3′-phosphate.[2c]

(8) A similar case is the inhibition of the hydrolysis of acetic anhydride by pyridine in buffers containing acetate ions [A. R. Butler and V. Gold, *J. Chem. Soc.*, 4362 (1961)].

(9) The group owes its lability to the presence of the adjacent phosphomonoester group. The 2′-O-acetyl groups in nucleosides involved in 3′→5′-phosphodiester linkage are much more stable.



developed for the preparation of 2′-O-acetyluridine 3′-phosphate (V) involved the stripping of the methoxytrityl group from the mixture of II and III without delay, the removal of the bulk of the acetate ions, and a short subsequent aqueous pyridine treatment to complete the breakdown of the mixed anhydride linkage in IV.

The polymerization of pyridinium 2′-O-acetyluridine 3′-phosphate was carried out in a concentrated dry pyridine solution using dicyclohexylcarbodiimide (DCC) as the reagent. In analogy with the work in the deoxyribopolynucleotide series[6b,10] some (20%) of the fully protected derivative pyridinium 2′,5′-di-O-acetyluridine 3′-phosphate[2c] was included in the polymerization mixture. The addition of the latter derivative appeared to be even more desirable than in the polymerization work in the deoxynucleotide series where nucleoside 5′-phosphates are used as the starting materials. Thus there is much greater tendency in nucleoside 3′-phosphate derivatives than in the nucleoside 5′-phosphate derivatives to undergo the intramolecular 3′,5′-cyclic phosphate formation[11] and, analogously, side products would be expected to be formed more readily in the case of the free 5′-hydroxyl end groups by virtue of the reaction of the latter groups with the adjoining activated phosphodiester bonds.[12] The incorporation of 2′,5′-di-O-acetyluridine 3′-phosphate would reduce the side reactions by promoting the formation

(10) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 5.

(11) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

(12) Support for these conclusions is provided by the results previously recorded in the present series of papers. Thus, the treatment of thymidylyl-(3′→5′)-3′-O-acetylthymidine with DCC in pyridine gave a number of side products, one of which was characterized as the 5′-C-pyridinium nucleoside derivative [G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962)]. Further, the polymerization of thymidine 3′-phosphate by the standard method gave a much higher proportion of side products than that encountered in thymidine 5′-phosphate polymerization [A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959)].

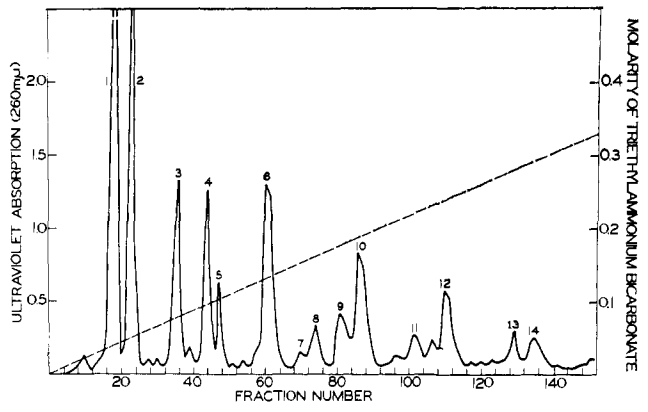


Fig. 1.—Chromatographic analysis of the polymerization products of a mixture of 2′-O-acetyluridine 3′-phosphate and 2′,5′-di-O-acetyluridine 3′-phosphate. Five-hundred optical density units at 260 mμ were fractionated on a DEAE–cellulose (carbonate) column (40 cm. × 1.2 cm.). A linear gradient of triethylammonium bicarbonate, pH 7.5, from 0.0 (1 l. of water) to 0.4 *M* (1 l.) was used for elution and 14 ml. fractions per 10 min. were collected. The characterization of the several peaks is given in Table I. After tube number 148 (0.32 *M* buffer) the rest of the ultraviolet-absorbing material was eluted with 1 *M* triethylammonium bicarbonate, pH 7.5, and rechromatographed (see Experimental).

of oligonucleotide chains bearing the 5′-*O*-acetyl end groups.

Following the polymerization reaction, the mixture was given a short[13] aqueous pyridine treatment before being reacted with an excess of acetic anhydride in dry pyridine to break the surviving pyrophosphate bonds linking the oligonucleotides through the terminal 3′-phosphomonoester groups. After a final ammoniacal treatment the products were separated on a DEAE–cellulose anion exchanger column. The pattern obtained on chromatography of a portion of the mixture is shown in Fig. 1, the percentages and the composition of the different peaks being given in Table I.

Uridine oligonucleotides up to the hexanucleotides (general structure VI) were pure as obtained after a single column chromatography (Fig. 1), and by refractionation of the higher polynucleotidic material eluted with 1 *M* salt concentration members up to the decanucleotide were obtained. The linear homologous oligonucleotides were thoroughly characterized by paper-chromatographic (Table II), paper-electrophoretic (Table III), and enzymatic tests. Table IV shows the results of the determination of the chain length by degradation to uridine 3′-phosphate and uridine by the reaction sequence illustrated below for the hexanucleotide.[14]

$$\underset{\text{(hexanucleotide)}}{\text{UpUpUpUpUpUp}} \xrightarrow[\text{alkaline phosphatase}]{\text{bacterial}} \text{UpUpUpUpUpU} \xrightarrow[\text{ribonuclease}]{\text{pancreatic}} \begin{matrix}\text{5 uridine 3′-phosphate} \\ + \\ \text{1 uridine}\end{matrix}$$

It should be emphasized that the degradation with pancreatic ribonuclease went to completion as ascertained by paper chromatography of the products de-

(13) The duration of this treatment, which is considered necessary to hydrolyze the fully protected pyrophosphate bonds, was restricted so as to avoid the hydrolysis of any of the 2′-O-acetyl groups adjoining the terminal 3′-phosphate end groups. Exposure of the 2′-hydroxyl groups would enable 2′,3′-cyclic phosphate formation to occur during the subsequent acetic anhydride–pyridine treatment.

(14) Shorthand formulations as used in previous papers and explained in ref. 10.

TABLE I

DISTRIBUTION OF NUCLEOTIDIC MATERIAL IN THE PEAKS OF FIG. I

Peak	Fractions pooled	% of total nucleotidic material	Molarity of salt of peaks	Composition of peaks
1	17–19	5.0[a]	0.04	5′-C-pyridinium uridine 3′-phosphate, 12% of the peak
2	22–24	21.0	.05	Pure uridine 3′,5′-cyclic phosphate
3	34–37	8.3	.07	Pure uridine 3′-phosphate
4	43–45	6.7	.09	Cyclic uridine dinucleotide
5	47–48	1.4	.10	Unidentified
6	60–63	8.3	.13	UpUp
7	69–71	0.8		Unidentified
8	73–75	1.5		Unidentified
9	80–82	5.0	.18	Cyclic trinucleotide (57%), UpUpUp (38%)
10	85–89	5.5[b]	.19	Pure UpUpUp
11	100–104	0.9		Unidentified
12	109–112	4.7	.24	Pure UpUpUpUp
13	127–129	2.3	.28	Pure UpUpUpUpUp
14	134–136	1.1	.30	Pure UpUpUpUpUpUp
1 *M* salt		3.2	1.0	Rechromatographed

[a] This is the amount of the ultraviolet-absorbing material corresponding to 5′-C-pyridinium uridine 3′-phosphate. There is also present in this peak a large amount of ultraviolet-absorbing material (λ_{max} 294 mμ, R_f 0.76, solvent B, and 0.85, solvent G) arising from the reaction of acetic anhydride with pyridine alone. [b] Including the amount of UpUpUp in peak no. 9, the total yield of the trinucleotide is 7.4%.

rived from 10 optical density units (260 mμ) of the oligonucleotide. This series of tests thus proved conclusively that the internucleotidic linkages formed were exclusively $C_{3'}$–$C_{5'}$ in the synthetic compounds. Finally, the homologous members were compared directly with samples of the same members prepared by limited degradation of high molecular weight polyuridylic acid with the micrococcal nuclease. (For details of this experiment see Experimental.)

An appreciable (5%) amount of uridine 3′,5′-cyclic phosphate was isolated from the polymeric mixture. Uridine cyclic di- and trinucleotides (VII) were present in peaks 4 and 8, respectively, of Fig. 1. Their characterization was accomplished as described in previous papers.[2c,6b] Under the standard conditions of incubation with pancreatic RNase the cyclic mononucleotide (uridine 3′,5′-cyclic phosphate) gave only 5% decomposition to uridine 3′-phosphate while the cyclic dinucleotide (VII, $n = 0$) gave 40% decomposition to uridine 3′-phosphate and the cyclic trinucleotide (VII, $n = 1$) was completely degraded to uridine 3′-phosphate. These results indicate that the resistance of cyclic pyrimidine ribonucleotides towards pancreatic RNase is gradually diminished as the number of the ring members is increased Another side product characterized was 5′-C-pyridinium uridine 3′-phosphate (VIII), which also has been encountered previously.[2c]

A large number of side peaks present in the elution pattern shown in Fig. 1 remain unidentified. They are likely to contain in the main two series of compounds: the first are homologous oligonucleotides with 2′,3′-

TABLE II

R_f'S OF THE DIFFERENT COMPOUNDS ON PAPER CHROMATOGRAMS

Compound	Solvents A	B	C	D	E	F	G	H
Uridine 3'-phosphate	0.034	0.36	0.26	0.24	0.55	0.38	0.30	0.21
Uridine		.52						
2'-O-Acetyluridine 3'-phosphate				.39[a]	.67	.56		.30
2'-O-Acetyl-3'-uridylyl acetate			(.77)	.71	.74	.68		.45
5'-O-Monomethoxytrityluridine 3'-phosphate	.36		.70	.82				
Monomethoxytritanol				.91				
5'-C-Pyridinium uridine 3'-phosphate		.34					.57	
Uridine 3',5'-cyclic phosphate		.54					.33	
Cyclic dinucleotide		.32	.34				.17	
UpUp		.25					.15	
UpU		.40						
Cyclic trinucleotide		.22						
UpUpUp		.18	.06				.07	
UpUpU		.28						
UpUpUpUp		.12					.04	
UpUpUpU		.20						
UpUpUpUpUp		.11						
UpUpUpUpU		.14						
UpUpUpUpUpUp		.07						
UpUpUpUpUpU		.10						
U(pU)$_6$p		.31[b]						
U(pU)$_7$p		.23[b]						
U(pU)$_8$p		.17[b]						
U(pU)$_9$p		.10[b]						

[a] Pyridinium 2'-O-acetyluridine 3'-phosphate spotted (10 O.D. units at 260 mμ) on paper from pyridine solutions gives elongated spots with R_f 0.5–0.6, while if spotted from solutions in solvent F the spots are round with R_f 0.39. [b] R relative to Up.

TABLE III

PAPER-ELECTROPHORETIC MOBILITIES RELATIVE TO URIDINE 3'-PHOSPHATE

	pH 7.1	pH 3.8
Uridine 3'-phosphate	1.00[a]	1.00[a]
2'-O-Acetyluridine 3'-phosphate	0.84	
2'-O-Acetyl-3'-uridylyl acetate	.48	
C_5'-Pyridinium Up	.42	0.00
Uridine 3',5'-cyclic phosphate	.61	1.08
Cyclic dinucleotide	.82	1.41
UpUp	1.00	1.24
Cyclic trinucleotide	0.92	1.60
UpUpUp	1.05	1.49
UpUpUpUp	1.04	1.51
UpUpUpUpUp	1.03	1.56
UpUpUpUpUpUp	1.03	1.52

15–17 cm. run for uridine 3'-phosphate.

TABLE IV

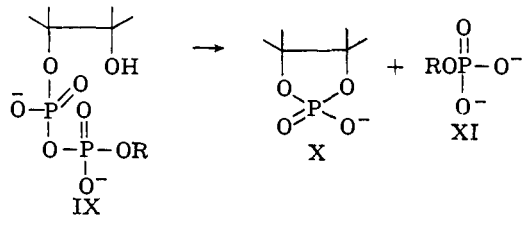
DETERMINATION OF THE CHAIN LENGTH OF THE LINEAR OLIGONUCLEOTIDES (FOR DETAILS, SEE TEXT)

Peak no. of Fig. 1	Compound obtained after phosphomonoesterase	Products of enzymatic degradation: Uridine, O.D. units at 260 mμ	Up, O.D. units at 260 mμ	Ratio of Up/uridine: Found	Theory
6	UpU	2.70	2.74	1.01	1
10	UpUpU	2.22	4.50	2.02	2
12	UpUpUpU	1.50	4.77	3.18	3
13	UpUpUpUpU	1.15	4.50	3.92	4
14	UpUpUpUpUpU	1.32	6.60	5.0	5

cyclic phosphate end groups[15] and the second may contain 3'-phosphate or 2',3'-cyclic groups at one end and a C-5'-pyridinium group at the opposite end. The presence of the large number of side products in the polymeric mixture emphasizes the added complications of working in the ribopolynucleotide series. It is clear that further studies on improving the techniques of chemical polymerization, especially in the ribopolynucleotide series, are necessary.

In preliminary experiments typified by the hexanucleotide, chemically synthesized as well as enzymatically prepared hexauridylic acid bearing a 3'-phosphate terminal group, in a mixture with highly polymerized polyU, was found to inhibit by 30% the template activity of the latter in directing polyphenylalanine synthesis in a cell-free system from *E. coli* prepared according to Nirenberg, *et al.*[4a] A similar inhibition but with oligouridylic acids bearing a 5'-phosphomonoester group was recently reported by Jones, *et al.*[16] We further found that when either the chemically synthesized hexauridylic acid or the enzymatically prepared one was treated with DCC under conditions that would cyclize the terminal 3'-phosphomonoester group to 2',3'-cyclic phosphodiester, this inhibition was abolished. This interesting cyclization effect requires further study.

(15) The formation of these would occur upon removal of the 2'-O-acetyl group. Compounds of this type would be formed if pyrophosphate linkages linking oligonucleotides through the terminal 3'-phosphate groups survived partially after the acetic anhydride–pyridine treatment. During the subsequent removal of the 2'-O-acetyl groups rapid decomposition of the pyrophosphates would occur as shown simply in the partial formulae (IX–XI).



IX X XI

Experimental

Materials and Methods.—Disodium uridine 3'-phosphate was obtained by recrystallizing a batch of disodium uridine-2'(3')-

(16) O. W. Jones, E. Townsend, H. A. Sober, and L. Heppel, *Biochemistry*, **3**, 238 (1964).

phosphate purchased from Schwartz Bioresearch Inc., which contained only 12% of the 2′ isomer. The mixture (15.0 g.) was dissolved in water (45 ml.) and 96% ethanol (30 ml.) was added. Crystallization began immediately and the crystals were filtered off after 24 hr. at 5° and washed with a cold mixture of ethanol–water of the same composition. The recrystallized product obtained in a 70% yield was found to be pure 3′ isomer when examined chromatographically.[17] DEAE-cellulose Selectacel Type 40, Brown Co., Berlin, N. H., was converted to the carbonate form as described previously.[18] A standard 1 *M* solution of triethylammonium bicarbonate (pH 7.5) was prepared as described previously.[18] Tetraethylammonium hydroxide (aqueous 10% solution), purchased from Eastman-Kodak Co., was neutralized to pH 7.5 with acetic acid to give an approximately 0.6 *M* solution of tetraethylammonium acetate which was used in the acetylation experiments. Polyuridylic acid of high molecular weight was purchased from Miles Laboratories. Crystalline pancreatic ribonuclease was purchased from Worthington Biochemical Corp. Purified *E. coli* alkaline phosphomonoesterase free of phosphodiesterase activity was a gift from Dr. J. Schwartz of the Rockefeller Institute. Micrococcal nuclease was a gift of Dr. C. A Dekker of the University of California. Reagent grade pyridine was once distilled before use. To obtain anhydrous pyridine, reagent grade pyridine was refluxed over barium oxide, distilled, and kept over a molecular sieve (Type 4A, Linde Molecular Sieves), for several days before use.

Paper chromatography was carried out using a descending technique on Whatman No. 1 or 3 MM paper. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v./v.); solvent B, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v./v.); solvent C, ethyl alcohol–1 *M* ammonium acetate, pH 7.5, (7:3, v./v.); solvent D, ethyl alcohol–1 *M* sodium acetate, pH 7.5 (7:3, v./v.); solvent E, ethyl alcohol–0.5 *M* ammonium acetate, pH 3.8 (7:3, v./v.); solvent F, ethyl alcohol–0.5 *M* sodium acetate, pH 3.8 (7:3, v./v.); solvent G, isobutyric acid–concentrated ammonia–water (66:1:33), pH 3.7; solvent H, *n*-butyl alcohol–acetic acid–water (5:2:3, v./v.); R_f's of different compounds are given in Table II.

Paper electrophoresis was carried out in an apparatus similar to that of Markham and Smith[19] or in a commercially available apparatus designed on the same principle and capable of giving a potential of 5000 v. The buffers used were: potassium phosphate, 0.03 *M*, pH 7.1; ammonium formate–formic acid buffer, pH 3.6, 5×10^{-2} *M*. Electrophoretic mobilities relative to uridine 3′-phosphate are given in Table III.

Evaporations were carried out under reduced pressure using a rotary evaporator and a bath temperature not exceeding 25°. "Evaporations in the presence of pyridine" of aqueous solutions of pyridinium salts of nucleotides were carried out with frequent additions of pyridine so that the water content of the evaporating solution was always less than 50% of the total volume. The monomethoxytrityl-containing compounds were made visible on paper chromatograms by spraying with 10% perchloric acid and drying in a stream of warm air. The monomethoxytrityl-containing compounds appeared orange-yellow.

Degradations by alkaline phosphomonoesterase were carried out by incubating 0.7–1.0 μmole of nucleotidic material in 5 μmoles of Tris chloride buffer, pH 8.0, and an appropriate volume (usually 0.01 ml.) of alkaline phosphatase in a final volume of 0.1 ml., at 37° for 2 hr. The mixture was spotted directly on Whatman No. 1 paper and developed in solvent B.

Degradations by pancreatic RNase were carried out by incubating a mixture of 0.7–1.0 μmole of nucleotidic material, 5 μmoles of Tris chloride buffer, pH 7.5, and 50 μg. of pancreatic RNase in a total volume of 0.1 ml., at 37° for 4 hr. The mixture was directly applied on Whatman No. 1 paper and developed in solvent B. The degradation products of the cyclic dinucleotide were examined in solvent G.

Pyridinium 2′-O-Acetyluridine 3′-Phosphate.—Triethylammonium 5′-O-monomethoxytrityluridine 3′-phosphate was prepared from pyridinium uridine 3′-phosphate and monomethoxytrityl chloride as described previously.[2d] The same preparation could be scaled up to 10-mmole nucleotide quantities. In this case, the desired product was chromatographed on a DEAE-cellulose column (65 cm. × 8 cm.) with a linear gradient of triethylammonium bicarbonate (pH 7.5) consisting of 20 l. of water in the mixing vessel and 20 l. of 0.4 *M* triethylammonium bicarbonate in 20% ethyl alcohol in the reservoir. A flow rate of 3–4 ml./min. was maintained. The yield of 5′-O-monomethoxytrityluridine 3′-phosphate on this scale was 45–50%, using the value of 12,000 for ϵ_{max} at 260 mμ.

Anhydrous pyridinium 5′-O-monomethoxytrityluridine 3′-phosphate (0.1 mmole) and tetraethylammonium acetate (1 mmole) was prepared and treated with acetic anhydride (1.0 mmole) as described previously.[2d] After 2 hr. at room temperature methyl alcohol (0.5 ml.) and anhydrous pyridine (0.5 ml.) were added and the sealed mixture was kept further for 10 min. The solvents were then removed by evaporation. The residual gum was treated with 10 ml. of a mixture of pyridine–water–ethanol (10:70:20, v./v.) and the solution freed from tetraethylammonium ions by passage through a column (12 cm. × 1 cm. i.d.) of (pyridinium) ion-exchange resin prewashed with the same solvent mixture. The total effluent and washings (two 10-ml. portions) were evaporated to a viscous residue which was dissolved in 6 ml. of 60% ethyl alcohol. Dowex-50 (H^+) ion-exchange resin was added until the apparent pH of the solution dropped to 2.5. The mixture was kept at room temperature for 3–4 hr., at the end of which period the removal of the monomethoxytrityl group was complete (paper chromatography in solvent E). The resin was removed by filtration and washed with 20% aqueous pyridine. The filtrate and washings were evaporated in the presence of pyridine. The residue was dissolved in anhydrous pyridine (7 ml.) and the solution added dropwise to an excess (300 ml.) of anhydrous ether.[20] The precipitate was collected, washed with anhydrous ether, and redissolved in 50% aqueous pyridine (25 ml.). The solution was kept at room temperature for 3–4 hr., during which time the mixed acetyl phosphoric anhydride completely disappeared (paper chromatography at 0.5–1 μmole level of the nucleotide in solvent D or paper electrophoresis at pH 7.1. The solvent was then removed by evaporation and pyridinium 2′-O-acetyluridine 3′-phosphate was stored in dry pyridine at −15°. The yield of the chromatographically pure product was 90% as based on the amount of 5′-O-monomethoxytrityluridine 3′-phosphate.

In the above preparation, when the acetylation mixture was kept at 50% aqueous pyridine without removal of the tetraethylammonium acetate, the decomposition of the acetyl phosphoric anhydride was much slower and the concomitant removal of the 2′-O-acetyl group became appreciable. This was shown by removal of aliquots at intervals from the aqueous pyridine solution, removal of the methoxytrityl group by treatment with Dowex-50 (H^+) resin, and paper chromatography in solvent D. The products uridine 3′-phosphate, 2′-O-acetyluridine 3′-phosphate, and the acetyl phosphoric anhydride of the latter were clearly separated (Table II).

2′-O-Acetyluridine 3′-phosphate was found to be stable in anhydrous pyridine solutions (2.2×10^{-3} *M*) at −15° for at least 2 weeks. At room temperature no decomposition to uridine 3′-phosphate occurs over a period of 24 hr. In 50% aqueous pyridine solutions (1×10^{-3} *M*), decomposition to uridine 3′-phosphate occurs at room temperature (7% in 5 hr., 25% in 20 hr.). In aqueous ethanol solutions it is particularly stable when sodium acetate or acetic acid is present. In aqueous or 10% aqueous ethanol solutions, above pH 6.0 it is decomposed at room temperature, especially when acetate ions are at a low concentration. In 70% aqueous ethanol solution (4×10^{-3} *M*) in the presence of sodium acetate, pH 7.5 (0.25 *M*), it was found stable at room temperature for a period up to 4 hr. In 70% aqueous ethanol solution (4.4×10^{-3} *M*) in the presence of ammonium acetate, pH 7.5 (0.3 *M*), at room temperature, it shows 10% decomposition to uridine 3′-phosphate in 2 hr. Thus the compound was found less stable in ammonium acetate aqueous–ethanolic solutions than in sodium acetate aqueous–ethanolic solutions under similar conditions. This result agrees with the fact that the mixed anhydride IV could not be found during paper chromatography in solvent C (10 optical density units at 260 mμ of a mixture of 70% of V and 30% of IV applied on No. 1 Whatman paper) while it could be found under the same conditions in solvent D. Solutions of 2′-O-acetyluridine 3′-phosphate in solvent F (2.3×10^{-3} *M*) are remarkably stable at room temperature for up to 48 hr. If such solutions are treated with excess of Dowex-

(17) W. Cohn in *Biochem. Prepn.*, **5**, 40 (1957).
(18) H. G. Khorana and W. J. Connors, *ibid.*, in press.
(19) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).
(20) This precipitation serves to remove monomethoxytritanol and pyridinium acetate. This method for the removal of the latter is preferred over the alternative one of repeated evaporation with added solvent.
(21) Prompt use is recommended in order to avoid any decomposition.

50 (H^+) resin at room temperature and the resin is filtered off, the filtrate has an apparent pH of 3.0. By passing a stream of nitrogen gas near the surface of the solution, its volume is reduced to half and the apparent pH is 2.5. At this condition, 2′-O-acetyluridine 3′-phosphate in the mixture of ethanol–acetic acid–water is stable at room temperature for periods up to 5 hr. without any decomposition to uridine 3′-phosphate.

2′,5′-Di-O-acetyluridine 3′-Phosphate.—Pyridinium uridine 3′-phosphate was treated with acetic anhydride in the presence of tetraethylammonium acetate as described previously.[2d] After 2 hr. at room temperature the acetylation mixture was worked up as described above in the acetylation of 5′-O-monomethoxytrityluridine 3′-phosphate. After the precipitation of pyridinium 2′,5′-di-O-acetyluridine 3′-phosphate in ether the precipitate was dissolved in 50% aqueous pyridine (250 ml. per mmole of uridine 3′-phosphate) and left at room temperature until (2–3 hr.) the decomposition of the mixed acetyl phosphoric anhydride was complete (paper electrophoresis at 1 mmole level at pH 7.1). The solvent was then removed by evaporation. Pyridinium 2′,5′-di-O-acetyluridine 3′-phosphate was stored in dry pyridine at −15° or reprecipitated in anhydrous ether and kept as a powder in a vacuum desiccator over phosphorus pentoxide.

Preparation of Uridine Polynucleotides.—A mixture of pyridinium 2′-O-acetyluridine 3′-phosphate (2.0 mmoles) and pyridinium 2′,5′-di-O-acetyluridine 3′-phosphate (0.5 mmole) was rendered anhydrous by repeated evaporations with anhydrous pyridine (three 5-ml. portions). The mixture was dissolved in dry pyridine (5.0 ml.) and dry pyridinium Dowex-50 resin (300 mg.) was added. Solid DCC (1.25 mmoles) was added. A clear solution first resulted but within a few minutes a crystalline precipitate of dicyclohexylurea appeared. More DCC (5.0 mmoles)[22] was added, the contents of the flask being agitated vigorously. By the time that most of the DCC had dissolved a gum began to separate at the bottom of the flask. Fifteen minutes after the addition of the second portion of DCC, the gum that had separated had hardened to a semisolid deposit. At this time about 2.5 ml. of the solvent was evaporated carefully under anhydrous conditions and the residual mixture was sealed and shaken in the dark for 1 week. At the end of this period, water (3.0 ml.) was added, the gum was broken with the aid of a glass rod, and the mixture was extracted with cyclohexane several times (four 1-ml. portions). The aqueous solution containing the insoluble dicyclohexylurea was left at room temperature for 2 hr. and the urea was removed by filtration and washed with 5% aqueous pyridine. The filtrate and the washings were evaporated with pyridine and the residue was rendered anhydrous by repeated evaporations with anhydrous pyridine. The resulting viscous gum was dissolved in 30 ml. of dry pyridine and an excess of distilled acetic anhydride (9.0 ml.) was added. In a few minutes a homogeneous solution resulted which was left in the dark for 3 days. Then the mixture was cooled in ice and cold water (60 ml.) was added. The solution was kept at room temperature for 4 hr. and evaporated to a sirup. The latter was subjected to suction under high vacuum to remove most of the pyridinium acetate. The residual gum was dissolved in 9 *N* ammonia (100 ml.) and kept at room temperature for 7 hr. After evaporation, the residue was dissolved in water containing some ethanol (less than 15% of total volume).

A small portion (500 optical density units at 260 mμ) of the total polymeric mixture was applied to the top of a DEAE–cellulose (carbonate) column (40 cm. × 1.2 cm.). After a water wash (one bed volume), elution was carried out with a linear salt gradient with water (1 l.) in the mixing vessel and 1 l. of 0.4 *M* triethylammonium bicarbonate (pH 7.5) in the reservoir. Fourteen-milliliter fractions were collected every 10 min. The elution pattern is shown in Fig. 1 and the distribution of the nucleotidic material in different peaks is shown in Table I. The bulk of the material (2 mmoles of the nucleotide) was chromatographed on a larger DEAE–cellulose column (100 cm. × 4.5 cm.) with a linear gradient as above, there being 20 l. of water in the mixing vessel and 20 l. of 0.4 *M* triethylammonium bicarbonate in the reservoir. The elution pattern was similar to that in Fig. 1.

The various fractions were pooled so as to avoid contamination from the material in the neighboring peaks (Table I). The pooled fractions were evaporated under reduced pressure, the excess of triethylammonium bicarbonate being removed by repeated evaporation after addition of water.

(22) Thus, a total of 6.25 mmoles of DCC was used in this experiment. If, under similar conditions, increasing amounts of DCC were used, a decrease in the yield of polymers was observed.

Determination of the Chain Length of the Linear Oligonucleotides.—The linear oligonucleotide (1 μmole) was incubated with bacterial alkaline phosphatase according to the conditions given under Methods. The incubation mixture after chromatography in solvent B gave only one, faster moving, spot which was eluted with water. The eluate was evaporated to a small volume and incubated with RNase as mentioned under Methods. The new incubation mixture was applied on Whatman No. 1 paper and developed in solvent B along with uridine and uridine 3′-phosphate markers. The degradation to uridine and uridine 3′- phosphate was quantitative. The concentrations of the nucleoside and nucleotide were determined spectrophotometrically after appropriate elution of the spots. The results are given in Table IV.

Higher Polynucleotides.—The fraction after the hexanucleotide eluted with 1 *M* triethylammonium bicarbonate, pH 7.5, from a big preparative column was evaporated with water until triethylamine had been completely removed; it was then (420 optical density units at 260 mμ) placed on a DEAE–cellulose (carbonate) column (40 cm. × 1.2 cm. i.d.) preequilibrated with 0.2 *M* triethylammonium bicarbonate, pH 7.5. Washing with 0.2 *M* triethylammonium bicarbonate was continued until no ultraviolet-absorbing material emerged for 1 column volume. Then a linear gradient consisting of 1 l. of 0.2 *M* triethylammonium bicarbonate in the mixing flask and 1 l. of 0.8 *M* of the same buffer in the reservoir was applied. Eight to nine milliliter fractions per 15 min. were collected. Four major peaks could be identified.

Peak A (fraction 56–72) constituted 33% of the material (based on optical density units at 260 mμ) applied. The peak was eluted at a concentration of 0.38 *M* of triethylammonium bicarbonate. After evaporation and removal of the triethylamine the residue was spotted on No. 1 paper and developed in solvent B for 60 hr. The slowest moving band constituted 80% of the material applied on the paper and was identified as the linear heptanucleotide. Comparison with enzymatically prepared U+pU+$_6$p on paper chromatography in solvent B proved them to be identical. Degradation by RNase gave 90% decomposition to uridine 3′-phosphate.

Peak B (fractions 73–88) was eluted at a concentration of 0.42 *M* of triethylammonium bicarbonate and accounted for 24% of the applied material. By the same criteria applied for peak A, 80% of this peak, was identified as the linear octanucleotide U(pU)$_7$p.

Peak C (fractions 89–102) was eluted at a concentration of 0.46 *M* triethylammonium bicarbonate and constituted 2.5% of the applied material. Examined as above, it showed U(pU)$_8$P as its major constituent; 70% of the material applied on a paper chromatogram moved as a band moving slower than U(pU)$_7$p in solvent B, R_f 0.17, relative to Up.

Peak D (fractions 104–120) was eluted at a concentration of 0.5 *M* triethylammonium bicarbonate and constituted 2% of the applied material. Its major constituent was U(pU)$_9$p; 65% of the material in the peak moved as a band moving slower than (U(pU)$_8$p in solvent B, R_f 0.10, relative to Up.

About 10% of the applied material was eluted before peak A as a minor peak. The material beyond peak D (28% of the applied material) was not examined.

Enzymatic Preparation of Uridine Oligonucleotides.—The preparation of uridine oligonucleotides bearing 3′-phosphate end groups was accomplished by limited degradation of commercially available polyuridylic acid with micrococcal nuclease, and subsequent chromatography on DEAE–cellulose under the conditions used for the fractionation of the chemically synthesized uridine polynucleotides. In a typical experiment polyuridylic acid (16 mg.) dissolved in water (0.88 ml.) was neutralized to pH 8.5 with 1 *N* sodium hydroxide. Sodium hydroxide–glycine buffer, pH 8.6 (160 μmoles), calcium chloride (16 μmoles), and the preparation of micrococcal nuclease (15 λ)[23] in a total volume of 1.5 ml. were added. After incubation at 37° for 1 hr. the mixture was applied on a 3 MM paper (14 cm. wide strip) and then developed in solvent B until a uridine 3′-phosphate marker had travelled close to the front of the paper (60 hr., 40 cm. run for the monomer). Oligonucleotides up to the pentanucleotide were clearly separated and could be recovered by elution. The broad paper band between the pentamer and the origin was eluted with 0.01 *M* ammonium hydroxide. A small amount of ultraviolet-absorbing material did not leave the paper. The eluate was evap-

(23) The amount of the enzyme required for obtaining the desired extent of degradation was determined as in a prior small scale experiment.

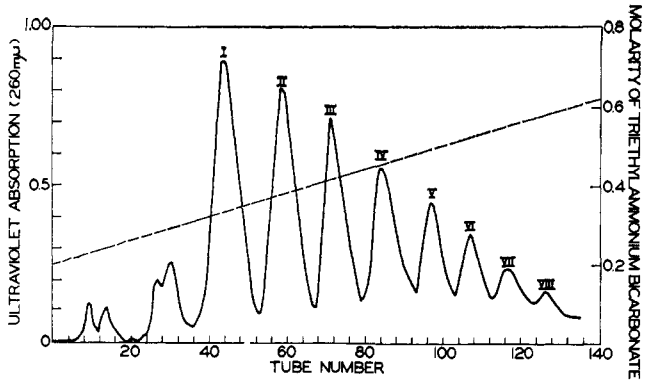


Fig. 2.—Fractionation of the degradation products of polyuridylic acid with micrococcal nuclease. The lower oligonucleotides up to the pentamer were removed by paper chromatography in solvent B. The rest of the degradation products (233 optical density units at 260 mμ) were fractionated on a DEAE-cellulose (carbonate) column (58 cm. × 0.6 cm. i.d.). A linear gradient of triethylammonium bicarbonate, pH 7.5, from 0.2 *M* (500 ml.) to 0.8 *M* (500 ml.) was used for elution and 5 ml./30 min. fractions were collected. Peak I consisted of pure UpUpUpUpUpUp, peak II of pure U(pU)$_6$p, peak III of pure U(pU)$_7$p, peak IV of pure U(pU)$_8$p, and peak V of pure U(pU)$_9$p. For further details see Experimental.

orated to a small volume and the residue (233 optical density units at 260 mμ) dissolved in water (1.0 ml.) was applied on a DEAE-cellulose (carbonate) column (58 cm. × 0.6 cm. i.d.) preequilibrated with 0.2 *M* triethylammonium bicarbonate, pH 7.5. The elution (3 ml. per 15 min.) with 0.2 *M* triethylammonium bicarbonate was continued until no ultraviolet-absorbing material appeared for a period of 4 fractions (approximately 100 ml. of buffer). A linear gradient was then applied, consisting of 500 ml. of 0.2 *M* triethylammonium bicarbonate, pH 7.5, in the mixing vessel and 500 ml. of 0.8 *M* of the same buffer in the reservoir. Uridine polynucleotides up to the tridecanucleotide were thus obtained (Fig. 2). Of these members, up to the decanucleotide were characterized with respect to chain length.

Experiments with Cell-Free Systems.—Extracts from *E. coli* B cultures, "washed ribosomes," and "supernatant" were prepared according to Nirenberg, *et al.*[4a] The incubation mixture (0.5 ml.) contained: 50 μmoles of Tris chloride buffer, pH 7.8; 25 μmoles of ammonium chloride; 5 μmoles of magnesium acetate; 5 μmoles of mercaptoethanol; 15 mμmoles of GTP; 0.5 μmole of ATP; 2.5 μmoles of trisodium phosphoenolpyruvate; 20 μg. of pyruvate kinase; 40 μg. of commercial polyuridylic acid, 0.4–2.0 optical density units at 260 mμ of oligouridylic acid (terminally cyclized or not); 15 mμmoles of C^{14}-phenylalanine (no additional amino acids were added), 20 μc. per μmole; 0.8 mg. of ribosomal protein; and 1.6 mg. of "supernatant" protein.[24] The radioactive amino acid was added last in the incubation mixture and the ribosomes and "supernatant" before it in that order. Incubations were carried out at 37° for 1 hr. An aliquot (0.1 ml.) of the incubation mixture was applied on a 3 MM paper disk[25] (2.3 cm. diameter). The paper disks were air-dried and then successively washed by soaking in 7% perchloric acid for 15 min., 3.5% perchloric acid for 5 min., and again in 3.5% perchloric acid which was heated in a boiling-water bath for 15 min. Volumes of 200 ml. of acid per 10–15 paper disks were used. Finally the paper disks were transferred in 3.5% perchloric acid for 15 min., ethanol–ether (2:1) for 1 min., ethanol–ether (1:2) for 1 min., and ether for 1 min. After the disks had been dried in the air they were transferred in separate scintillation vials containing 5.0 ml. of phosphor[26] and counted in a Packard Tricarb liquid scintillation counter. Typical results are given in Table V. No crosscontamination between the paper disks during the washing procedures could be detected. The amount of enzyme protein and the amount of polyuridylic acid needed for optimal incorporation are those mentioned in the incubation mixture.

TABLE V

EFFECT OF OLIGONUCLEOTIDES ON THE TEMPLATE ACTIVITY OF HIGH MOLECULAR WEIGHT POLYURIDYLIC ACID

Oligonucleotide additions	O.D. units at 260 mμ of oligonucleotide added	μμmoles of C^{14}-L-phenylalanine incorporated[a]: In the absence of high molecular weight polyuridylic acid	In the presence of 40 μg. of high molecular weight polyuridylic acid
No oligonucleotide added	0	2.9	95.3
UpUpUpUpUpUp[b]	0.4	3.8	66.5
UpUpUpUpUpUp[b]	1.2	2.9	66.0
UpUpUpUpUpU-cyclic-p[c]	0.4	3.5	96.5
UpUpUpUpUpU-cyclic-p[c]	1.2	3.8	113.0
UpUpUpUpUpUp[d]	0.4	3.5	65.0
UpUpUpUpUpUp[d]	1.2	2.5	66.0
UpUpUpUpUpUp-cyclic-p[e]	0.4	2.9	100.0
UpUpUpUpUpUp-cyclic-p[e]	1.2	3.2	96.0

[a] Per 0.16 mg. of "ribosomal" and 0.32 mg. of "supernatant" protein; incubation at 37° for 1 hr. [b] Enzymatically prepared. [c] From enzymatically prepared oligonucleotide. [d] Chemically synthesized. [e] From chemically synthesized oligonucleotide.

For cyclization of the 3′-terminal phosphate group, the oligonucleotide (10 optical density units at 260 mμ) in methanol solution (0.5 ml.) was treated with tri-*n*-butylamine (3 μmoles) and DCC (100 μmoles) for 6 hr. at room temperature.[27] Methanol was removed by blowing nitrogen gas near the surface of the solution. Water (1.0 ml.) was added and the excess of DCC was extracted with cyclohexane (three 0.5-ml. portions). The water phase was evaporated as above to the desired volume. This solution was used directly in the incubation experiments.

(24) The protein was determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(25) R. J. Mans and G. D. Novelli, *Arch. Biochem. Biophys.*, **94**, 48 (1961); F. J. Bollum, *J. Biol. Chem.*, **234**, 2733 (1959).

(26) The phosphor consisted of 4 g. of 2,5-diphenyloxazole (PPO) and 100 mg. of 1,4-*bis*-2-(5-phenyloxazolyl)benzene (POPOP) per liter of reagent grade toluene.

(27) H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4657 (1959).

COMMUNICATIONS TO THE EDITOR

Excited State p*K*'s. I. Azobenzene and Azoxybenzene

Sir:

Since the discovery by Förster[1] and Weller[2] that fluorescence spectra in buffered solution permit the determination of the p*K*'s of acids and bases in excited singlet states, a number of such p*K**'s have been reported.[1-3] In almost every case, the p*K** has been substantially different than the ground state p*K*, usually by some five units.

Also, since the beginning of the investigations into p*K**'s, it has been recognized that an estimate of Δp*K* can be made from the shift in transition energy of a given absorption or fluorescence band in going from

(1) T. Förster, *Z. Elektrochem.*, **54**, 42 (1950).

(2) A. Weller in "Progress in Reaction Kinetics," Vol. 1, Pergamon Press, London, 1961, p. 187.

(3) W. Bartok, P. J. Lucchesi, and N. S. Snider, *J. Am. Chem. Soc.*, **84**, 1842 (1962); J. C. Haylock, S. F. Mason, and B. E. Smith, *J. Chem. Soc.*, 4897 (1963).