pyridine mixture was kept for several hours, DCC removed by extraction with ether and the acetyl group removed by keeping the mixture in concentrated ammonia at room temperature for 15 min. The total products were separated by preparative paper chromatography in Solvent A. Three bands were obtained: the main product was thymidylyl-(3′→5′)-thymidine (R_f 0.42); there was a weak band with R_f 0.35, the major new product having R_f 0.21. Examination of the last mentioned material on DEAE-cellulose paper (development with 0.2 *M* triethylammonium bicarbonate) gave three spots: one with R_f 0.79, which was identical with the material (λmax. 261 mμ) in peak 1 (Table V) of the preceding experiment; a weak spot with R_f 0.34, which was identical with the unidentified peak 5 (Table V) of the preceding experiment and another weak spot with R_f 0.18. The total material of R_f 0.21 from paper chromatography was applied to a DEAE-cellulose (carbonate) column (12 cm. × 1 cm. dia.). The water wash contained the above-mentioned material which had R_f 0.79 on DEAE-cellulose (carbonate) paper. This substance (designated XX) showed λmax. at 261–262 mμ and a shoulder at 265 mμ in acid, neutral and alkaline *p*H. Treatment of the solution in the quartz cell with sodium borohydride gave an ultraviolet spectrum typical of thymidylyl-(3′→5′)-thymidine. Five optical density units of the above substance were incubated with snake venom phosphodiesterase. The products were thymidine-5′ phosphate and a second product with R_f 0.33 in Solvent A. The latter product moved towards the cathode on paper electrophoresis at *p*H 7.5 and showed an ultraviolet absorption composite of that of thymidine and N-methylpyridinium cation.

The identity of the weak band with R_f = 0.35 remains unknown.

Phosphorylation of 5′-*O*-Tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine with a **Mixture of β-Cyanoethyl Phosphate and DCC.**—Pyridinium 5′-*O*-trityl-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine (3.3 μmole) was kept in 0.5 ml. of anhydrous pyridine with a mixture of pyridinium β-cyanoethyl phosphate (from 16 mg. of the barium salt) and DCC (42 mg.). After 2 days at room temperature, water (5 ml.) was added and the reaction mixture left overnight. It was then evaporated and to the residue were added 3 ml. of 1 *N* sodium hydroxide and the mixture kept at 100° for 30 min. The trityl group was removed by passing the mixture through a Dowex 50 (H^+) column and keeping the acidic solution for 8 hr. at room temperature. The solution then was neutralized with ammonia and applied to a 9 inch wide strip of Whatman 40 paper. After chromatography in Solvent C, the slowest band corresponded to the desired thymidine tetranucleotide and was eluted. The yield as estimated spectrophotometrically was 43%. Rechromatography on DEAE-cellulose paper showed a small amount (about 5%) of impurity (R_f 0.49) in the main product (R_f 0.12) which had the same mobility as the tetranucleotide previously characterized.[5a] The impurity was removed by applying the total material on a DEAE-cellulose column (15 cm. long × 1 cm. dia.) and eluting with a linear salt gradient, 2 l. of water in the mixing vessel and 2 l. of 0.25 *M* triethylammonium bicarbonate in the reservoir. About 5-ml. fractions were collected at 10 min. intervals. Pure tetranucleotide was eluted in fractions 398–452.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN, AND THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, BRITISH COLUMBIA]

Studies on Polynucleotides. XIV.[1] Specific Synthesis of the $C_{3'}$–$C_{5'}$ Interribonucleotide Linkage. Syntheses of Uridylyl-(3′ → 5′)-Uridine and Uridylyl-(3′ → 5′)-Adenosine[2]

BY M. SMITH,[3] D. H. RAMMLER,[4] I. H. GOLDBERG AND H. G. KHORANA

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Uridine-3′,5′ cyclic phosphate (I) on reaction, under its own catalysis, with 2,3-dihydropyran gives quantitatively 2′-*O*-tetrahydropyranyluridine-3′,5′ cyclic phosphate (II). Treatment of II with barium hydroxide affords a mixture of 2′-*O*-tetrahydropyranyluridine-3′ phosphate (III) and the isomeric -5′ phosphate (IV) in the ratio 5:1. On treatment of the mixture of III and IV with trityl chloride in pyridine only IV reacts and the resulting 5′-*O*-trityl ether (V; R = R′ = R″ = H) is separated from III by anion exchange chromatography on a DEAE-cellulose column. 5′-*O*-*p*-anisyldiphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (V; R = R′ = H; R″ = OCH_3) and 5′-*O*-di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (V; R = H; R′ = R″ = OCH_3) were prepared by careful treatment of the mixture of III and IV with, respectively, *p*-anisyldiphenylmethyl chloride and di-*p*-anisylphenylmethyl chloride followed by chromatography. The preparation of 2′,3′-*O*-*p*-anisylideneuridine, 5′-*O*-*p*-anisyldiphenylmethyluridine, 5′-*O*-di-*p*-anisylphenylmethyluridine, 5′-*O*-tri-*p*-anisylmethyluridine and 5′-*O*-tri-*p*-anisylmethyladenosine is described. The rates of hydrolysis in acid of these new protected derivatives of uridine and uridine-3′ and -5′ phosphates were determined. The introduction of each *p*-methoxy group in the trityl group increased the rate of hydrolysis approximately by a factor of ten. Careful treatment of 5′-*O*-di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate with cold acetic acid gave quantitatively III, a suitable starting material for polymerization to form C_3′–C_5′ linked uridine polynucleotides. Condensation of compounds of the type V with 2′,3′-di-*O*-acetyluridine in the presence of dicyclohexylcarbodiimide followed by ammoniacal and then acetic acid treatment (4 hr. at room temperature) gave pure uridylyl-(3′ → 5′)-uridine (50%) which was fully characterized by analytical and enzymic methods. Prolonged treatment with acetic acid during work-up of the above condensation product increased the yield of uridylyl-uridine to 70%, but a trace (1%) contamination by the rearranged product, uridylyl-(2′ → 5′)-uridine, was then detected. N,N,2′,3′-tetrabenzoyladenosine (XII) was prepared in excellent yield by benzoylation of 5′-*O*-trityladenosine or 5′-*O*-tri-*p*-anisylmethyladenosine with an excess of benzoyl chloride followed by removal of the 5′-*O*-protecting group. Condensation of derivatives of the type V with XII followed by removal of the protecting groups gave 50–70% yield of uridylyl-(3′ → 5′)-adenosine, the results being analogous to those obtained in the synthesis of uridylyl-(3′ → 5′)-uridine.

In the deoxyribopolynucleotide field, progress has been made in recent years both in the area of the stepwise synthesis of C_3′–C_5′ linked oligonucleotides as well as on the polymerization of suitably protected deoxyribomononucleotides.[1,5–7] In

(1) Paper XIII, G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 419 (1962) (preceding paper).

(2) This work has been supported by grants from the National Cancer Research Institute of the National Institutes of Health, U. S. Public Health Service.

(3) Technological Station, Fisheries Research Board of Canada, Vancouver 8, B. C., Canada.

(4) U. S. Public Health Service Post-doctoral Research Fellow, 1959–1961.

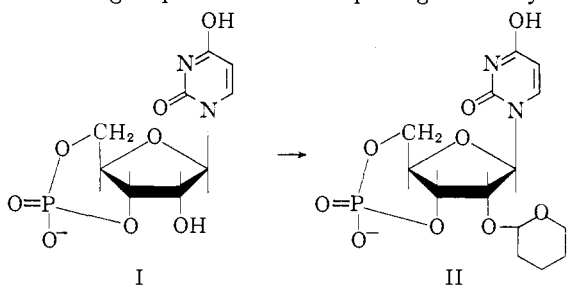
(5) H. G. Khorana, in E. Chargaff and J. N. Davidson, eds., "The Nucleic Acids," Vol. III, Academic Press, Inc., New York, N. Y., 1960, p. 105.

(6) H. G. Khorana, *Federation Proc.*, **19**, 931 (1960).

(7) 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.

ribopolynucleotides, the presence of the 2′-hydroxyl group in the ribose moiety of the ribonucleosides profoundly influences the chemistry of the ribonucleic acids[8] and complicates by far the problem of the synthesis of the C_3'–C_5' linked ribopolynucleotides. Syntheses so far have been reported only of complex mixtures of C_2'–C_5' and C_3'–C_5' linked riboöligonucleotides.[9,10] No specific synthesis of the naturally occurring C_3'–C_5' inter-ribonucleotidic linkage has been recorded.[11] As a part of the program of synthetic work in the polynucleotide field which has been in progress in this Laboratory,[5–7] attention has been focussed on the development of methods for the specific synthesis of C_3'–C_5' linked ribopolynucleotides. In the present communication we report on an approach which has been used successfully in the synthesis of uridylyl-(3′→5′)-uridine[5,6,12] and uridylyl-(3′→5′)-adenosine.[7] The present paper also describes the use of a series of new protecting groups,[13] which, when taken together with the basic approach developed, makes feasible the stepwise synthesis of ribopolynucleotides as well as the polymerization of suitably protected ribomononucleotides to form C_3'–C_5' linked ribopolynucleotides.

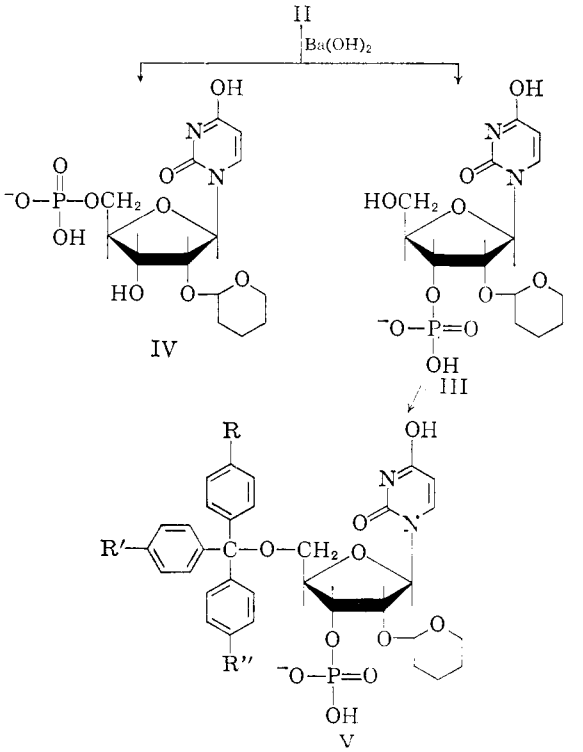
Protected Derivatives of Uridine and Uridine Nucleotides.—In recent papers, the preparation of deoxyribo- and ribonucleoside-3′,5′ cyclic phosphates[14,15] in high yield from the corresponding nucleoside-5′ phosphates has been reported. The method is general and efficient[15] and the resulting nucleoside cyclic phosphates have been thoroughly characterised. Uridine-3′,5′ cyclic phosphate (I), thus prepared, served as the key starting material in the present work. The aim was to protect the 2′-hydroxyl function in this compound by a group which would not migrate to the 3′-hydroxyl group when this group was freed on opening of the cyclic



I II

phosphate ring. A second requirement of the protecting group was that it should be removed at the final step under conditions which are safe for the sensitive inter-ribonucleotide bonds. In early experiments, the preparation of the 2′-*O*-benzyl ether of I was attempted, using benzyl chloride and potassium *t*-butoxide in a homogeneous medium.[16] However, the monobenzylated product obtained was found to carry the benzyl group on the pyrimidine ring, the 2′-hydroxyl group being free. This result is at variance with the earlier work on the preparation of *O*-benzyl ethers of nucleosides by Todd and co-workers[17] and is probably to be ascribed to the fact that in our experiments the reaction mixture was homogeneous whereas the earlier workers[17] used the heterogeneous medium involving benzyl chloride and potassium hydroxide. Although the preparation of the 2′-*O*-benzyl ether of I probably could be realized, this line of investigation was not pursued when the alternative, more attractive approach described below became available.

The cyclic phosphate (I) as the free acid was quantitatively converted under its own catalysis on treatment with dihydrohydropyran[18] in dioxane for 1 hr. at room temperature to the tetrahydropyranyl ether (II). Hydrolysis with barium hydroxide[15,19] at 100° gave a mixture of 2′-*O*-tetrahydropyranyluridine-3′ phosphate (III) and the −5′ phosphate (IV) in the ratio 5:1.[20]



Efforts to separate III and IV either by partition or by ion exchange chromatography were fruitless.[21]

(8) D. M. Brown and A. R. Todd, in E. Chargaff and J. N. Davidson, eds., "The Nucleic Acids," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 409.

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

(10) A. M. Michelson, *J. Chem. Soc.*, 1371, 3655 (1959).

(11) Using suitably protected derivatives the synthesis of adenylyl-(2′ → 5′)-uridine has been reported. A. M. Michelson, L. Szabo and A. R. Todd, *ibid.*, 1546 (1956).

(12) A brief announcement of a part of this work was first made in 1959; M. Smith and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 2911 (1959).

(13) For a brief announcement of a part of this work see in D. H. Rammler and H. G. Khorana, *Federation Proc.*, **19**, 349 (1960).

(14) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6224 (1958).

(15) M. Smith, G. I. Drummond and H. G. Khorana, *ibid.*, **83**, 693 (1961).

(16) *Cf.* D. Lipkin and P. T. Talbert, *Chem. and Ind. (London)*, 143 (1955).

(17) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 3459 (1956); B. E. Griffin and A. R. Todd, *ibid.*, 1389 (1958).

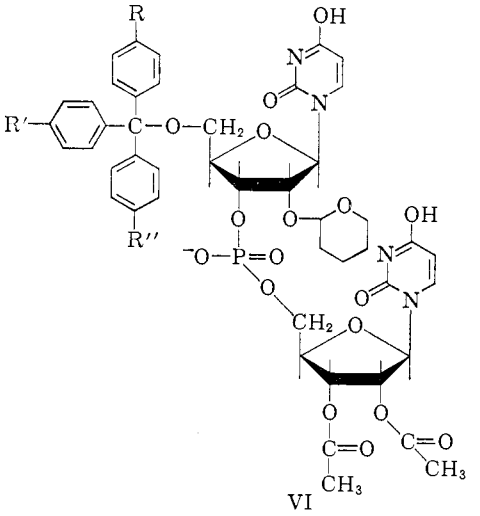
(18) G. F. Woods and D. N. Kramer, *J. Am. Chem. Soc.*, **69**, 2246 (1947).

(19) D. Lipkin, W. H. Cook and R. Markham, *ibid.*, **81**, 6198 (1959).

(20) Barium hydroxide-catalyzed hydrolysis of uridine-3′,5′ cyclic phosphate gives uridine-3′ and -5′ phosphates in the same proportions.

(21) Under the conditions tried, uridine-3′ and -5′ phosphates readily separated on a Dowex-1 (8% cross-linked) ion exchange column.

The mixture of the two nucleotides was therefore treated with triphenylmethylchloride in pyridine at 25°. The reaction occurred specifically with III to give quantitatively the 5′-*O*-trityl ether (V; R = R′ = R″ = H). The latter then was readily separated from the unaffected IV either by partition chromatography on cellulose or preferably by ion exchange chromatography on a diethylaminoethyl (DEAE)-cellulose column (*cf.* ref. 1). Derivatives of the type IV and V are suitable starting materials for internucleotide bond synthesis by the methods that have previously been developed in the deoxyribonucleotide field.[5-7,22] The synthesis of uridylyl-(3′→5′)-uridine briefly reported[12] (see also below) was accomplished by the condensation of V (R = R′ = R″ = H) with 2′,3′-di-*O*-acetyluridine followed by removal of the protecting groups from the initially formed product VI.

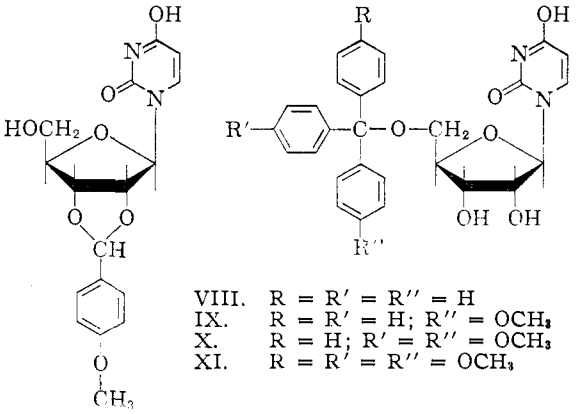


VI

In extending this work to the stepwise synthesis of ribopolynucleotides containing $C_{3'}$–$C_{5'}$ internucleotide bonds, the selective removal of the 5′-*O*-trityl group in VI would be necessary. The chain then could be extended in a manner analogous to that in the deoxyribopolynucleotide series.[1] For polymerization to form the $C_{3'}$–$C_{5'}$ linked ribopolynucleotides compounds of the type III would be required (cf. polymerization of thymidine-3′ phosphate).[23] Ideally, the removal of 5′-*O*-trityl groups from V (R = R′ = R″ = H) should be affected by catalytic hydrogenolysis. Although this approach has in some cases proved satisfactory, hydrogenation of cytosine nucleotides was concomitantly observed.[24] In general, hydrogenolysis of trityl derivatives of nucleosides and carbohydrates is sluggish and unsatisfactory.[25,26]

Removal of the trityl groups in the above-mentioned derivatives by acidic catalysis would, therefore, be preferable and was studied. The rates of acid-catalyzed hydrolysis of the 5′-*O*-trityl groups in nucleotides varied greatly from those of corresponding groups in nucleosides and the observations made point to the participation of a phosphoryl group in the reaction. For example, the trityl group in 5′-*O*-tritylthymidine-3′ phosphate[1,23] was hydrolyzed about ten times faster than that in 5′-*O*-trityluridine. The selective removal of the 5′-*O*-trityl group in V (R = R′ = R″ = H) without affecting the acid-labile tetrahydropyranyl group did not prove possible. In fact, a paper chromatographic study showed that the latter group was removed at a faster rate than the trityl group. The requirement therefore appeared to be for a protecting group which would be specific for the 5′-hydroxyl function in III and which could be removed without affecting the tetrahydropyranyl group.

In a related search for new protecting groups for the 2′,3′ hydroxyl groups of ribonucleosides, 2′,3′-*O*-*p*-anisylideneuridine (VII) was prepared and the anisylidene group was found to be about ten times more labile to acid than the customary isopropylidene or benzylidene group. Seeking analogy with the labilizing effect of the methoxy group in VII, the *p*-methoxy-substituted derivatives of the trityl group[27,28] were investigated. The three derivatives 5′-*O*-*p*-anisyldiphenylmethyl-5′-*O*-*p*-dianisylphenylmethyl- and 5′-*O*-tri-*p*-anisylmethyl-uridine (IX-XI, respectively) were prepared and their stabilities compared with that of the



VIII. R = R′ = R″ = H
IX. R = R′ = H; R″ = OCH_3
X. R = H; R′ = R″ = OCH_3
XI. R = R′ = R″ = OCH_3

parent 5′-*O*-trityluridine (VIII). Introduction of each *p*-methoxy group increased the rate of hydrolysis in 80% acetic acid at room temperature by a factor of approximately ten. Thus the times required for complete hydrolysis to uridine were: 5′-*O*-trityluridine (VIII), 48 hr.; 5′-*O*-*p*-anisyldiphenylmethyluridine (IX), 2 hr.; 5′-*O*-di-*p*-anisylphenylmethyluridine (X), 15 min.; 5′-*O*-tri-*p*-anisylmethyl-uridine (XI), 1 min. These attributes of high acid-lability are also obviously desirable for work in the deoxyribonucleoside field and their use in that series will be reported subsequently.[29] (The

(22) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958).

(23) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

(24) P. T. Gilham and H. G. Khorana, *ibid.*, **81**, 4647 (1959).

(25) W. Andersen, D. H. Hayes, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 1882 (1954).

(26) D. L. MacDonald and H. G. Fletcher, *J. Am. Chem. Soc.*, **81**, 3719 (1959).

(27) H. Lund, *ibid.*, **49**, 1346 (1927), estimates that the ease of ionization of *p*-methoxy-substituted triphenylcarbinols increases in the order: triphenylcarbinol, 1; *p*-anisyldiphenylcarbinol, 6.3; di-*p*-anisylphenylcarbinol, 34; tri-*p*-anisylcarbinol, 286.

(28) We are indebted to Drs. R. B. Moodie and R. Stewart, Chemistry Dept., University of British Columbia, B. C., Canada, for helpful discussions on the stabilities of substituted triphenylcarbinols and for a sample of *p*-anisyldiphenylmethyl chloride.

(29) B. Lerch, H. Schaller, G. Weimann and H. G. Khorana, unpublished work.

use of 5′-*O*-tri-*p*-anisyl-methyladenosine in the synthesis of N^1,N^6,2′,3′-tetrabenzoyladenosine is described below.)

With the above information, the use of substituted trityl groups in protecting the 5′-hydroxyl group in 2′-*O*-tetrahydropyranyluridine-3′ phosphate (III) was investigated. The reaction of the mixture of III and IV, as obtained from the barium hydroxide hydrolysis of II, with *p*-anisyldiphenylmethyl chloride in pyridine was studied using different amounts of the reagent and different reaction times. The introduction of the *p*-methoxy group in the trityl chlorides increased the rate of reaction with the hydroxyl functions in a manner comparable to the increase in rates of hydrolysis of the resulting ethers. The increased reactivity of the trityl chlorides also increased the rate of reaction of the reagents with the secondary hydroxyl functions.[30] Although in the case of the tetrahydropyranyl derivatives (IV) the presence of the tetrahydropyranyl group probably offers additional steric hindrance to the entry of the trityl group in the adjoining 3′-hydroxyl group, using an excess[31] of reagent (*p*-anisyldiphenylmethyl chloride) over a prolonged period (48 hr.) a small amount of reaction with 2′-*O*-tetrahydropyranyluridine-5 phosphate (IV) was observed. When a shorter time (24 hr.) was used, the 2′-*O*-tetrahydropyranyluridine-5′ phosphate (IV) isolated was contaminated by a trace of 2′-*O*-tetrahydropyranyluridine-3′ phosphate (III) but pure 2′-*O*-tetrahydropyranyl-5′-*O*-*p*-anisyldiphenylmethyluridine-3′ phosphate (V; R = R′ = H; R″ = OCH_3) was obtained after chromatography on a DEAE-cellulose column. A parallel study of the reaction of the mixture of III and IV with an excess of di-*p*-anisylphenylmethyl chloride showed that in 0.5 hr. practically all of III had reacted to form 2′-*O*-tetrahydropyranyl-5′-*O*-di-*p*-anisylphenylmethyluridine-3′ phosphate (V; R,R′ = OCH_3; R″ = H), only a trace of III being left to contaminate IV. In longer reaction periods a slow reaction of di-*p*-anisylphenylmethyl chloride with IV also was observed; for example, in a 5-hr. run the contamination of the uridine-3′ phosphate derivative (V) by the corresponding -5′ phosphate derivative amounted to 4%.

Both 2′-*O*-tetrahydropyranyl-5′-*O*-*p*-anisyldiphenylmethyluridine-3′ phosphate (V; R = R′ = H; R″ = OCH_3) and 2′-*O*-tetrahydropyranyl-5′-*O*-di-*p*-anisylphenylmethyluridine-3′ phosphate (V; R = H; R′ = R″ = OCH_3) having been isolated pure, their behavior towards 80% acetic acid at 0° and at room temperature was studied. While the former lost the *p*-anisyldiphenylmethyl group faster than the tetrahydropyranyl group,[32] the difference in rate did not permit the selective removal of the protecting group on the 5′-hydroxyl function. Treatment of 5′-*O*-di-*p*-anisylphenylmethyluridine-3′ phosphate (V; R = H; R′ = R″ = OCH_3) with 80% acetic acid at 0° for 6 hr. gave an excellent yield of 2′-*O*-tetrahydropyranyluridine-3′ phosphate[32] (III). The polymerization of this nucleotide and 2′-*O*-tetrahydropyranyluridine-5′ phosphate is being actively investigated.

Protected Derivatives of Adenosine.—In undertaking the synthesis of uridylyl-(3′→5′)-adenosine from the protected uridine-3′ phosphate derivatives described above, it was considered advisable to use an adenosine derivative containing a suitably protected N^6-amino group.[33] Acetylation of 5′-*O*-tritylladenosine followed by acidic removal of the 5′-*O*-trityl group from the resulting N,2′,3′-triacetyladenosine gives 2′-3′-*O*-diacetyladenosine.[34] In the present work, attempts were first made to prepare N, 2′,3′-triacetyladenosine *via* acetylation of 5′-*O*-tri-*p*-anisylmethyladenosine, the latter compound being prepared in good yield from adenosine and tri-*p*-anisylmethyl chloride. Even the extremely brief (1 minute at room temperature) treatment necessary for the removal of the 5′-*O*-tri-*p*-anisylmethyl group caused partial removal of the N-acetyl group as judged by the shift in the ultraviolet absorption spectrum. The use of the more stable benzoyl groups then was investigated. Benzoylation of 5′-*O*-tritylladenosine and of 5′-*O*-tri-*p*-anisylmethyladenosine with an excess of benzoyl chloride at room temperature readily gave the corresponding tetrabenzoyl derivatives, two benzoyl groups having entered the adenine ring.[35,36] (Benzoylation of adenosine itself with an excess of benzoyl chloride under mild conditions gave the crystalline pentabenzoyl derivative.[35]) Brief acid treatment of 5′-*O*-tri-*p*-anisylmethyl-tetrabenzoyladenosine gave the crystalline N,N,2′,3′-tetrabenzoyladenosine[36] (XII) in high yield. The same compound also was prepared by careful treatment of 5′-*O*-tritytetrabenzoyladenosine with hydrogen bromide in acetic acid followed by chromatography on a silicic acid column. It may be added that when the benzoylation of 5′-*O*-tritylladenosine was carried out using four moles of the reagent (conditions which have earlier been used by Levene and Tipson[34]) a mixture of 5′-*O*-trityltribenzoyl- and 5′-*O*-trityltetraben-

(30) This has been evident in the several cases studied (present work) and also in unpublished work in the deoxyribonucleoside field.

(31) Ideally, for the selective reaction with the 5′-hydroxyl group in 2′-*O*-tetrahydropyranyluridine-3′ phosphate, a stoichiometric amount of the reagent should be used. The use of this amount as well as a 100% excess gave only a small yield of the 5′-*O*-*p*-anisyldiphenylmethyl derivative. This presumably implies a reaction of the reagent with the phosphate group resulting in the consumption of one mole of the reagent.

(32) The catalytic effect of the 3′-phosphate group on the hydrolysis of 5′-*O*-*p*-anisyldiphenylmethyl group (V; R=R′=H; R″=OCH_3) and di-*p*-anisylphenylmethyl (V; R=H; R′=R″=OCH_3) is also apparent. It is also interesting to note that 2′-*O*-tetrahydropyranyluridine-3′ phosphate, in acetic acid loses the tetrahydropyranyl group twice as fast as the 5′-phosphate derivative.

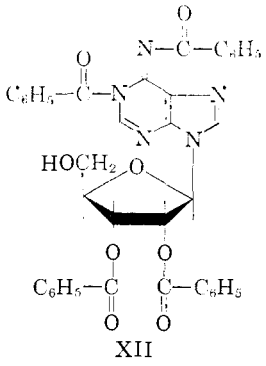
(33) Although the phosphorylation of N^6-amino group evidently does not occur when dibenzylphosphorochloridate is used to phosphorylate 2′,3′-*O*-isopropylidene adenosine (J. Baddiley and A. R. Todd, *J. Chem. Soc.*, 648 (1947)), phosphorylation with phosphorus oxychloride apparently gives an N,5′-O-diphosphate.[34] Phosphorylation by the powerful phosphorylating agent provided by a mixture of a mononucleotide and dicyclohexylcarbodiimide may be expected to result in some N-phosphorylation. Further work on the extent of N-phosphorylation during synthesis of internucleotide bonds will be reported subsequently.

(34) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **121**, 131 (1937).

(35) That two benzoyl groups can go in the adenine ring has been shown previously by H. R. Bentley, K. G. Cunningham and F. S. Spring, *J. Chem. Soc.*, 2301 (1951); F. Weygand and F. Wirth, *Chem. Ber.*, **85**, 1000 (1952). However, the conditions used by both groups of workers involved high temperature in place of the room temperature used in the present work.

(36) Of the two benzoyl groups on the adenine ring, one is most probably located on the N^6-amino group. The second group may be tentatively placed on the N-1 position, in analogy with the work on the polybenzoyl derivatives of cytidine [D. M. Brown, A. R. Todd and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956)].

zoyl- adenosine was obtained. The mixture was separable by chromatography but neither of the products could be obtained in crystalline form. The earlier workers[34] apparently obtained only the amorphous tribenzoyl derivative after benzoylation of 5′-*O*-trityladenosine. Furthermore, while in the present work, detritylation could be effected without loss of N-benzoyl groups, Levene and Tipson[34] obtained only 2′,3′-di-*O*-benzoyladenosine from 5′-*O*-trityl N, 2′,3′-tribenoyladenosine



XII

Synthesis and Characterization of Uridylyl-(3′ → 5′)-uridine and Uridylyl-(3′ → 5′)-adenosine.—The condensation of 5′-*O*-di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyl-uridine-3′ phosphate (V; R = H; R′ R″ = OCH_3) with 2′, 3′-di-*O*-acetyluridine[37] in the presence of dicyclohexylcarbodiimide according to the standard method[1,22] gave, presumably, VI (R = H; R′ = R″ = OCH_3) which was directly treated with ammonia to remove the acetyl groups and then with 80% acetic acid at room temperature to remove the 5′-*O*-di-*p*-anisylphenylmethyl and tetrahydropyranyl groups. Separate experiments with enzymatically synthesized uridylyl-(3′→5′)-uridine[38] showed that it was necessary to impose a time limit on the duration of the acidic treatment since after 30 hr. at room temperature a small amount of hydrolysis and concomitant formation of a ribonuclease-resistant product, presumably uridylyl-(2′→5′)-uridine,[39] was observed. When the treatment of the synthetic reaction mixture with 80% acetic acid was carried out for 4 hr. and the products isolated by preparative paper chromatography, uridylyl-(3′→5′)-uridine was obtained in 50% yield. This product was identical both on paper chromatograms and on paper electrophoresis with the enzymatically synthesized sample of the same compound. It was completely degraded to uridine-3′ phosphate and uridine by a spleen phosphodiesterase[40–42] preparation and by pancreatic ribonuclease.[42] In the ribonuclease-catalysed degradation, uridine-2′,3′ cyclic phosphate was, as expected, an intermediate.[42] A preparation of snake venom phosphodiesterase[42,43] degraded the dinucleoside phosphate to uridine-5′ phosphate and uridine.

In the above experiment while the yield of uridylyl-(3′→5′)-uridine was 50%, the recovered unreacted uridine-3′ phosphate amounted to somewhat less than 10%. It was, therefore, clear that removal of the protecting groups from the synthetic product did not go to completion. Treatment with acetic acid for 10 hr. at room temperature increased the yield of uridylyl-(3′→5′)-uridine to 69%, but the product then showed a trace contamination (about 1%) by a ribonuclease-resistant material, presumably uridylyl-(2′→5′)-uridine.

When in the synthesis of uridylyl-(3′→5′)-uridine, 2′,3′-*O*-anisylideneuridine (VII) was used in place of 2′,3′-di-*O*-acetyluridine, the work-up involved only a single step, namely, the acetic acid treatment, to remove all of the protecting groups. The yield of pure uridylyl-(3′→5′)-uridine (4 hr. acetic acid treatment) was, however, only about 25%, indicating insufficient acidic lability of the anisylidene group in the initially formed protected dinucleoside phosphate.

For the synthesis of uridylyl-(3′→5′)-adenosine the condensation of 5′-*O*-di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (V; R = H; R′ = R″ = OCH_3) with N,N,2′,3′-tetrabenzoyladenosine (XII) was carried out in a manner analogous to that described above, and the product was treated first with concentrated ammonia for about two days to remove all of the benzoyl groups[44] and then with 80% acetic acid at room temperature. The yield of uridylyl-(3′→5′)-adenosine after 4 hr. acidic treatment was 54.5%, while after a total of 11 hr. acidic treatment the yield was 70.2%. The product isolated after 4 hr. treatment was fully characterised by the analytical and enzymic methods described above and shown to be pure $C_{3'}$–$C_{5'}$ linked product, whereas the sample obtained after 11 hr. acidic treatment again showed a slight contamination (a maximum of 1.5%) of ribonuclease-resistant material.

When in the synthesis of uridylyl-(3′→5′)-adenosine 5′-*O*-trityl 2′-*O*-tetrahydropyranyluridine-3′ phosphate (V; R = R′ = R″ = H) was used in condensation with the tetrabenzoyladenosine (XII), the yield of the dinucleoside phosphate was 57%, there being only a faint trace of the ribonuclease-resistant product.

Concluding Remarks.—The need for a variety of specific and suitable protecting groups in the deoxyribopolynucleotide synthesis has been emphasized previously[7,24] and the requirements in the specific synthesis of $C_{3'}$–$C_{5'}$ linked ribopolynucleotides are even greater. The present work has described the first synthesis of $C_{3'}$–$C_{5'}$ inter-ribonucleotide bonds using an approach which promises to be general and fairly flexible. Clearly, the new protecting groups devised make feasible the extension

(37) G. W. Kenner, A. R. Todd, R. F. Webb and F. J. Weymouth, *J. Chem. Soc.*, 2288 (1954).

(38) Prepared by the treatment of uridine and uridine-2′,3′ cyclic phosphate with pancreatic ribonuclease according to the general method of L. A. Heppel, P. R. Whitfeld and R. Markham, *Biochem. J.*, **60**, 8 (1955).

(39) D. M. Brown, D. I. Magrath, A. H. Neilson and A. R. Todd (*Nature*, **177**, 1124 (1956)) have previously reported on the partial isomerization (C_3'–C_5' → C_2'–C_5') of the internucleotidic linkage during acidic treatment.

(40) R. J. Hilmoe, *J. Biol. Chem.*, **235**, 2117 (1960).

(41) W. E. Razzell and H. G. Khorana, *ibid.*, **236**, 1144 (1961).

(42) H. G. Khorana, in "The Enzymes," Vol. V, 2nd Ed., eds. P. D. Boyer, H. A. Lardy and K. Myrbäck, Academic Press, Inc., New York, N. Y., 1961, p. 79.

(43) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

(44) *Cf.* R. K. Ralph and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 2926 (1961).

of this work to the stepwise synthesis of ribooligonucleotides and to the polymerization of suitably protected ribomononucleotides so as to yield homologous series of polynucleotides. Work along both these lines will be reported in forthcoming papers.

Experimental

General Methods.—Reagent grade pyridine was dried over calcium hydride. All evaporations were carried out using a rotary evaporator at about 10 mm. pressure, with a bath temperature of less than 40°. During evaporations, constant vigilance and the liberal use of an anti-foaming agent (*n*-octanol) was necessary because of the detergent property of many of the protected nucleotide derivatives. Phosphorus analyses were carried out by the method of King[45] or Chen, *et al.*[46]

Paper chromatography was performed using the descending technique on double acid-washed Whatman 40 paper. Nucleotides were detected by viewing under an ultraviolet lamp equipped with a short-wave ultraviolet filter.[47] A fluorescent screen further increased the sensitivity of the technique for the detection of trace amounts of nucleotides.[48] Phosphorus-containing compounds were detected by the molybdate-perchloric acid spray.[49] This same spray, by virtue of the perchloric acid, with trityl derivatives gives on heating to 100° a yellow color which fades on cooling. *p*-Anisyldiphenylmethyl derivatives give a yellow color immediately at 25°, while di-*p*-anisylphenylmethyl and tri-*p*-anisylmethyl derivatives give immediately an orange and a reddish-orange color, respectively. The solvent systems used were: Solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2); Solvent B, isopropyl alcohol–concentrated ammonia–0.1 *M* boric acid (7:1:2).

Uridine-3′,5′ Cyclic Phosphate.—Disodium uridine-5′ phosphate (4.04 g., 10 mmole) in water was passed through a column of Dowex 50W (H^+ form) resin and the column washed with water until the effluent was neutral. After removal of the water by evaporation, the free nucleotide was dried by repeated (3 times) co-evaporation with dry pyridine. Finally the nucleotide was dissolved in dry pyridine (900 ml.) containing 4-morpholine-N,N′-dicyclohexylcarboxamidine[50] (2.93 g., 10 mmole) and the solution run dropwise into a boiling solution of dicyclohexylcarbodiimide (6.18 g., 30 mmole) in pyridine (900 ml.). The addition was completed in 2.5 hr. and heating under reflux was continued for a further 1 hr. After being cooled to 25°, the solution was diluted with water (100 ml.) and kept at 25° for 12 hr. before being concentrated to dryness. Water (250 ml.) and petroleum ether, b.p. 30–80° (250 ml.), were added to the residue and the mixture shaken vigorously. The insoluble dicyclohexylurea was removed by filtration. The aqueous layer, containing the nucleotide, was concentrated to 100 ml. volume and passed onto a column (55 cm. × 6.5 cm.) of diethylaminoethyl (DEAE) cellulose (carbonate form). After a water wash (6 l.), elution was carried out using a linear salt gradient: 0.005 *M* triethylammonium bicarbonate[51] (4 l.) in the mixing chamber and 0.025 *M* triethylammonium bicarbonate (4 l.) in the reservoir. The flow-rate was 4 ml./min., 20 ml. fractions being collected. The elution of nucleotides was followed spectrophotometrically at 261 mμ. After this volume of salt solution (8 l.) had passed through the column elution was continued with 0.03 *M* triethylammonium bicarbonate (2 l.). Uridine-3′,5′ cyclic phosphate was eluted in fractions 250–500. These were combined, concentrated to dryness and the residual gum containing some triethylammonium bicarbonate crystallized from ethanol–acetone to give, after washing with acetone and drying over P_2O_5, 2.7 g. (65%) of triethylammonium uridine-3′,5′ cyclic phosphate. The cyclic nucleotide was homogeneous when examined chromatographically in Solvent A.

TABLE I

PAPER CHROMATOGRAPHY OF NUCLEOTIDES AND DERIVATIVES

Compound	R_f in solvent A
Uracil	0.48
Uridine	.42
Uridine-2′(3′) phosphate	.09
Uridine-5′ phosphate	.04
Uridine-2′,3′ cyclic phosphate	.31
Uridine-3′,5′ cyclic phosphate	.28
2′-*O*-Tetrahydropyranyluridine-3′,5′ cyclic phosphate	.54
2′-*O*-Tetrahydropyranyluridine-3′ phosphate	.24
2′-*O*-Tetrahydropyranyluridine-5′ phosphate	.21
5′-*O*-Trityl-2′-*O*-tetrahydropyranyluridine-3′ phosphate	.58
5′-*O*-*p*-Anisyldiphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate	.58
5′-*O*-Di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate	.58
5′-*O*-Trityluridine	.80
5′-*O*-*p*-Anisyldiphenylmethyluridine	.80
5′-*O*-Di-*p*-anisylphenylmethyluridine	.80
5′-*O*-Tri-*p*-anisylmethyluridine	.80
2′,3′-*O*-*p*-Anisylidine uridine	.73
Uridylyl-(3′ → 5′)-uridine	.16
Uridylyl-(3′ → 5′)-adenosine	.18

Benzyl Uridine-3′,5′ Cyclic Phosphate.—To a solution of pyridinium uridine-3′,5′ cyclic phosphate (1.0 mmole) in redistilled formamide (5 ml.) were added benzyl chloride (1 ml.) and then *M* potassium *t*-butoxide in *t*-butanol (5 ml.). The solution was heated under reflux and potassium chloride separated immediately. After 1 hr. benzyl chloride (0.6 ml.) and *M* potassium *t*-butoxide solution (5 ml.) were added and heating under reflux continued for 1 hr. further. After being cooled to 25°, the mixture was filtered and the filtrate concentrated under reduced pressure to remove *t*-butanol. The residue was diluted with 80% acetic acid (25 ml.) and heated under reflux for 15 min. Acetic acid was removed under reduced pressure, the residue diluted with water (200 ml.) and the solution washed twice with ether. Nucleotides were absorbed from the aqueous solution onto acid washed charcoal (6 g.). After a water wash (500 ml.), the nucleotides were eluted with 50% ethanol containing 2% concentrated ammonia. The eluate was concentrated to a small volume, traces of charcoal were removed by centrifugation and the clear solution applied to a sheet (45 cm. wide) of Whatman 3 MM paper. Chromatography in Solvent A gave two ultraviolet-absorbing phosphorus-containing bands; R_f 0.33 (uridine-3′,5′ cyclic phosphate) and R_f 0.7 (benzyl uridine-3′,5′ cyclic phosphate). The benzyl derivative was isolated as its barium salt (135 mg.); ultraviolet absorption: λmax. 262 mμ (ε 9,600) at *p*H 7.0 and at *p*H 12.

Anal. Calcd. for $C_{16}H_{16}N_2O_8P\cdot{}^1/_2Ba\cdot 4H_2O$: P, 5.77. Found: P, 5.69.

Treatment of the above product with crude *crotalus adamenteus* venom gave a nucleoside, R_f 0,73 in Solvent A, which was oxidized by periodic acid.[52] All attempts to

(45) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(46) P. S. Chen, T. Y. Toribara and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

(47) Black Light Eastern Corp., 4 Manhasset Avenue, Port Washington, L. I., New York.

(48) Paper chromatography as applied for testing the purity of various compounds reported in this paper has been carried out by applying 10–50 optical density units (1–5 μmoles) of the compounds on a single spot. The technique at this level of application of compounds is the most sensitive analytical tool in this field.

(49) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949); R. S. Bandursky and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(50) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **82**, 649 (1961).

(51) A stock solution of *M* triethylammonium bicarbonate is prepared by adding redistilled triethylamine (140 ml.) dropwise (60 min.) to water (500 ml.) cooled in ice through which carbon dioxide is bubbling vigorously. When the *p*H drops to 7.5, addition of carbon dioxide is stopped and the solution made up to 1 l.

(52) The periodate-benzidine spray (M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955)) was used to detect vicinal hydroxyl groups on paper chromatograms.

recover uridine-3′,5′ cyclic phosphate by hydrogenolysis of the benzyl-nucleotide in either ethanol or acetic acid with palladium black, palladium on barium suphate[53] or Raney nickel were unsuccessful.

2′-*O*-Tetrahydropyranyluridine-3′,5′ Cyclic Phosphate.—Triethylammonium uridine-3′,5′ cyclic phosphate (2.7 g., 6.65 mmole) in water was passed slowly through a column of Dowex 50W (H^+ form) resin. The total acidic effluent was concentrated to an oil *in vacuo* with an oil pump, the bath temperature being 10°. Dry dioxane (25 ml.) was added and the solution concentrated to dryness as above. After two further co-evaporations with dry dioxane, the anhydrous nucleotide was obtained as a finely divided white powder. To this was added dry dioxane (50 ml.) and redistilled dihydropyran (10 ml.). Vigorous shaking at 25° gave a clear solution in 5 min. After 60 min. at 25°, concentrated ammonia (10 ml.) was added to the solution, resulting in the separation of a white precipitate. Evaporation of the solvent gave a sweet-smelling oil to which was added water and chloroform. The aqueous layer was twice washed with chloroform and then concentrated to dryness to give the ammonium salt of 2′-*O*-tetrahydropyranyluridine-3′,5′ cyclic phosphate as a hygroscopic gum (6.65 mmole as estimated spectrophotometrically). The product was homogeneous on chromatography in Solvent A. Treatment of an aliquot with 80% acetic acid at 25° for 4 hr. gave uridine-3′,5′ cyclic phosphate. Ultraviolet absorption: λmax 261 mμ (ε 10,956) at *p*H 7.0; λmax. 261 (ε 7,900) at *p*H 12.0. A sample of the nucleotide was converted to its calcium salt for analysis.

Anal. Calcd. for $C_{14}H_{19}N_2O_9P.{}^1/_2Ca.2H_2O$; C, 37.69; H, 5.20; N, 6.28; P, 6.36. Found: C, 37.55; H, 4.71; N, 6.15; P, 6.38.

2′-*O*-Tetrahydropyranyluridine-3′ and -5′ Phosphates.—A solution of ammonium 2′-*O*-tetrahydropyranyluridine-3′,5′ cyclic phosphate (1.2 mmole) and barium hydroxide octahydrate (8.0 g.) in water (60 ml.) was heated at 100° for 30 min. in a polyethylene tube. After cooling to 25°, the mixture was treated with an excess of Dowex 50W resin (ammonium form). After removal of the resin by filtration, the combined solution and water wash was concentrated to a small volume and passed onto a column (35 cm. × 3.5 cm. dia.) of DEAE cellulose (carbonate form). The column was washed with water (1 l.) and then eluted using a linear salt gradient: 0.002 *M* triethylammonium bicarbonate (2 l.) in the mixing vessel and 0.1 *M* triethylammonium bicarbonate (2 l.) in the reservoir. A flow-rate of 2 ml./min. was maintained, 20 ml. fractions being collected. Unreacted 3′,5′-cyclic phosphate was eluted in fractions 60–70 and the mixture of 2′-*O*-tetrahydropyranyluridine-3′ and -5′ phosphates appeared in fractions 90–140. These were concentrated to a small volume (100 ml.) and the residual solution lyophilized to give the triethylammonium salt of the nucleotide mixture as a white powder (580 mg.). The nucleotide mixture was chromatographically homogeneous in Solvent A. Treatment of a sample with 80% acetic acid at 25° for 4 hours, followed by examination of the resultant mixture of uridine-5′ and uridine-3′ phoshates in Solvent B, showed that the proportions of the -3′ phosphate and the -5′ phosphate were 83% and 17%, respectively. Ultraviolet absorption: λmax 262 mμ (ε 10,700) at *p*H 7.0, λmax 261 mμ (ε 7,900) at *p*H 12.0.

Anal. Calcd. for $C_{14}H_{21}N_2O_{10}P.2C_6H_{15}N.1H_2O$: C, 49.8; H, 8.53; P, 5.5. Found: C, 49.7; H, 8.53; P, 5.24.

Tri-*p*-anisylmethyl Chloride.—Diethyl carbonate (35.4 g.) in dry benzene (100 ml.) was added dropwise (30 min.) to a stirred solution of *p*-anisylmagnesium bromide (from 187 g. of *p*-bromoanisole and 24.3 g. of magnesium) in dry ether (350 ml.). The mixture was heated under reflux for 30 min., after which the organo-metallic complex was decomposed by addition of the mixture to a solution of ammonium chloride (50 g.) in water (250 ml.). The crude tri-*p*-anisylcarbinol was extracted into benzene (1 l.) and obtained as a reddish oil on removal of the solvent. The oil was dissolved in benzene (35 ml.) and acetyl chloride (50 ml.) was added. Tri-*p*-anisylmethyl chloride hydrochloride[54] crystallized as dark red needles. These were washed with a little dry benzene and dried over potassium hydroxide pellets to yield tri-*p*-anisylmethyl chloride as a pale orange powder (15.7 g.); m.p. 149–152°.

Di-*p*-anisylphenylmethyl chloride[54] was prepared by an analogous procedure using *p*-anisylmagnesium bromide and methyl benzoate. The product when crystallized from cyclohexane–acetyl chloride melted at 114°.

p-Anisyldiphenylmethyl chloride[55] was obtained *via* benzophenone and *p*-anisylmagnesium bromide. The chloride was crystallized from cyclohexane–acetyl chloride and has m.p. 118–124°.

5′-*O*-Tri-*p*-anisylmethyluridine.—A solution of dry uridine (244 mg., 1.0 mmole) and tri-*p*-anisylmethyl chloride (553 mg., 1.5 mmole) in dry pyridine (5 ml.) was kept at 25° for 2 hr. After removal of pyridine *in vacuo*, the product was dissolved in ethyl acetate, washed with water and dried (Na_2SO_4). The solution was concentrated to dryness and the residue dissolved in benzene (10 ml.). 5′-*O*-Tri-*p*-anisylmethyluridine (481 mg.) crystallized almost immediately. Recrystallization from benzene-ethyl acetate gave colorless prisms; m.p. 137–139° (decomp.); ultraviolet absorption (in 95% ethanol): *p*H 7.0, λmax. 231 mμ (ε 24,700), 268 mμ (ε 10,150), inflex 282 mμ (ε 5,240); *p*H 12.0, λmax. 229 mμ (ε 29,750), 268 mμ (ε 8,480), inflex 282 mμ (ε 4,830).

Anal. Calcd. for $C_{31}H_{32}O_9N_2$: C, 64.57; H, 5.59; N, 4.86. Found: C, 63.44; H, 5.76; N, 4.89.

5′-*O*-*p*-Anisyldiphenylmethyluridine.—Treatment of uridine (244 mg., 1.0 mmole) with *p*-anisyldiphenylmethyl chloride (463 mg., 1.5 mmole) in dry pyridine (5 ml.) for 48 hr. at 25° followed by work-up as described for 5′-*O*-tri-*p*-anisylmethyluridine and crystallization from benzene gave 5′-*O*-*p*-anisyldiphenylmethyluridine (600 mg., 95%) as prisms, m.p. 103–105° (gas evolved); ultraviolet absorption (in 95% ethanol): λmax. 232 mμ (ε 18,000), 263 mμ (ε 10,400), inflex 281 mμ (ε 4,750) at *p*H 7.0, λmax. 230 mμ (ε 22,000), 262 mμ (ε 8,500), inflex 281 mμ (ε 3,750) at *p*H 12.0.

Anal. Calcd. for $C_{29}H_{28}O_7N_2.1.5C_6H_6$: C, 72.02; H, 5.88; N, 4.42. Found: C, 71.82; H, 5.94; N, 4.28.

5′-*O*-Di-*p*-anisylphenylmethyluridine.—Uridine (0.967 g.; 4.0 mmole) was added to a solution of di-*p*-anisylphenylmethyl chloride (1.4 g.; 4.1 mmole) in 7 ml. of dry pyridine and the mixture kept at room temperature for 12 hr. It then was poured into 50 ml. of ice-water with rapid stirring. The precipitate was collected by filtration, washed with ice-water, dried and then dissolved in 10 ml. of chloroform containing a drop of pyridine. The solution was chromatographed on a column (30 cm. × 2.5 cm. dia.) of silicic acid. The column was washed with three bed volumes of chloroform and then with chloroform containing 3% methyl alcohol to elute 5′-*O*-di-*p*-anisylphenylmethyluridine. The product was crystallised from ethyl acetate–cyclohexane containing a drop of pyridine. The total yield obtained in three crops was 1.725 g. (78%); m.p. 123°–124°; ultraviolet absorption in 95% ethyl alcohol, λmax. 235 mμ (ε 20,000), 266 mμ (ε 10,180), inflex 281 mμ (ε 5,460) at *p*H 7.0; λmax. 232 mμ (ε 23,300), 266 mμ (ε 8,740), inflex 281 mμ (ε 4,550) at *p*H 12.0.

Anal. Calcd. for $C_{30}H_{30}O_8N_2$ (546.73): C, 66.0; H, 5.55; N, 5.14. Found: C, 66.06; H, 5.82; N, 5.15.

2′,3′-*O*-*p*-Anisylidineuridine.—Uridine (1 g.) and anhydrous zinc chloride (2 g.) were suspended in *p*-anisaldehyde (5 ml.) and the mixture shaken for 4 days at 25°. The semi-solid product was extracted with ether (100 ml.) and water (100 ml.). The insoluble residue was crystallized from hot water containing a little ethanol to give 2′,3′-*O*-*p*-anisylidineuridine (1.1 g., 78%) as plates, m.p. 207–208°; ultraviolet absorption (in 95% ethanol): λmax. 227 mμ (ε 14,500), 261 mμ (ε 11,350) inflex 278 mμ (ε 5,100) at *p*H 7.0, λmax. 227 mμ (ε 20,200), 260 mμ (ε 8,100), inflex 278 mμ (ε 3,450).

Anal. Calcd. for $C_{17}H_{18}O_7N_2$: C, 56.35; H, 5.01; N, 7.73. Found: C, 56.09; H, 5.21; N, 7.69.

Pentabenzoyladenosine.—This was prepared by the method of Weygand and Wirth[35] with the modification that

(53) R. Kuhn and H. J. Haas, *Angew. Chem.*, **67**, 785 (1955).

(54) M. Gomberg and L. H. Cone, *Ann.*, **370**, 142 (1909); A. Bayer and V. Villiger, *Ber.*, **35**, 1189 (1902); **36**, 2774 (1903).

(55) C. S. Marvel, J. Whitson and H. W. Johnston, *J. Am. Chem. Soc.*, **66**, 415 (1944).

the reaction was carried out at room temperature. The yield of the analytically pure pentabenzoyladenosine was 84%; m.p., 185–187°, previously reported[35] m.p., 187°. Benzoylation of adenosine also was carried out at 5° using a solution of dry adenosine (2.67 g.; 10 mmole) in 10 ml. of anhydrous pyridine and 45 mmole (5.4 ml.) of freshly distilled benzoyl chloride. On working up after 18 hr. crystalline pentabenzoyladenosine (m.p. 187°) was obtained in 55% yield (4.3 g.).

5′-*O*-Trityladenosine.—The substance was prepared by a modification of the procedure described in literature.[34,56] To a refluxing solution of adenosine[57] (5.34 g.; 20 mmole) in dry pyridine (100 ml.) was added dropwise a solution of trityl chloride (7.0 g.; 25 mmole) in 50 ml. of dry pyridine. The addition was completed in 2.5 hr. with exclusion of moisture. The straw-colored solution was allowed to attain room temperature and then was poured into 1 l. of ice-water under rapid stirring. After 1 hr. the product was extracted with 200 ml. of chloroform and the chloroform solution extracted back with salt water and then dried over anhydrous sodium sulfate. Evaporation of the solvent *in vacuo* gave a light tan powder which was triturated with heptane. The insoluble material was crystallized from a mixture of pyridine and ethyl alcohol. The yield was 6.0 g. (58%); m.p. 253°; that reported in literature[56] is 259°. Additional 5′-*O*-trityladenosine could be recovered from the mother liquors.

Tribenzoyl-5′-*O*-trityl- and Tetrabenzoyl-5′-*O*-trityl-adenosine.[34]—A solution of 5′-*O*-trityladenosine (1.5 g.; 2.93 mmole) in 20 ml. of dry pyridine was cooled and freshly distilled benzoyl chloride (1.4 ml.; 12.0 mmole) was added. After 18 hr. at room temperature the solution was cooled and a small amount of methyl alcohol was added. The solution was evaporated to a small volume and 100 vol. of chloroform was added. The chloroform solution was washed with water (25 ml. × 5), dried over sodium sulfate and then evaporated. The residual oil resisted attempts at crystallization and was applied in chloroform (8 ml.) to the top of a silicic acid (40 g.) column (25 cm. × 2 cm. dia.). Elution was carried out by a linear gradient technique with 500 ml. of chloroform in the mixing vessel and 475 ml. of chloroform + 25 ml. of methyl alcohol in the reservoir. A flow rate of 1 ml./min. was maintained, 12 ml. fractions being collected. The major peak (1.3 g.) was obtained at a methyl alcohol concentration of 0.5%. A second less distinct peak (0.357 g.) was eluted at 1.4% methyl alcohol concentration. There was partial overlapping between the two peaks and 0.7 g. of material was obtained in this region. Neither of the two peaks could be crystallized but they corresponded respectively to 5′-*O*-trityl-tetrabenzoyladenosine and 5′-*O*-tribenzoyladenosine.

Anal. Peak 1 Calcd. for $C_{57}H_{43}O_8N_5$ (925.95): C, 74.0; H, 4.68; N, 7.62. Found: C, 73.51; H, 4.19; N, 7.64.

Anal. Peak 2 Calcd. for $C_{50}H_{39}O_7N_5$ (821.85): C, 73.05; H, 4.79; N, 8.50. Found: C, 73.53; H, 4.98; N, 9.21.

Removal of trityl group from the material of peak 1 using the method described later gave N^1,N^6-2′,3′-tetrabenzoyladenosine, while the second peak after similar treatment gave a small amount of tetrabenzoyladenosine and, presumably, N, 2′,3′-*O*-tribenzoyladenosine, which was not investigated further.

5′-*O*-Tri-*p*-anisylmethyladenosine.—Dry adenosine (267 mg.; 1 mmole) was dissolved in 25 ml. of dry pyridine by warming and the solution was cooled. Tri-*p*-anisylmethyl chloride (367 mg.; 1 mmole) was added and the sealed clear solution was kept at room temperature. After 12 hr. it was concentrated to about 5 ml. under reduced pressure and the concentrate diluted with 50 ml. of water. The product was extracted with ethyl acetate and the extract washed twice with water. The solvent then was removed and the residue applied to a column of Alumina (Woelm No. V) (about 40 g.). Elution with chloroform gave 193 mg. of a product, presumably N,5′-*O*-di-(tri-*p*-anisylmethyl)-adenosine. Subsequent elution with chloroform containing 2% methyl alcohol gave 363 mg. (60%) of the desired 5′-*O*-tri-*p*-anisylmethyladenosine. Continued elution with chloroform containing 5% methyl alcohol followed by methyl alcohol alone gave only traces of material. 5′-*O*-Tri-*p*-anisylmethyladenosine could not be obtained in crystalline form and the amorphous powder melted at 142° with decomposition. Its ultraviolet absorption spectrum showed λmax. at 235 mμ and a broad maximum at 262–265 mμ, the ratio of the extinction at 235 mμ to that at 265 mμ being 1.7. These spectral characteristics were close to those expected for the composite spectrum of tri-*p*-anisylmethyl chloride and adenosine.

Anal. Calcd. for: $C_{32}H_{33}N_5O_7$ (599.62): C, 64.1; H, 5.55; N, 11.7. Found: C, 63.77; H, 5.86; N, 11.6.

The product eluted with chloroform and presumed to be N,5′-*O*-di-(tri-*p*-anisylmethyl)-adenosine had λmax. at 275 mμ with inflection at 282–283 mμ and λmin. at 255–256 mμ.

In another run on a 4 mmole scale, the yield of the desired 5′-*O*-tri-*p*-anisylmethyladenosine was 52%. When larger proportions of tri-*p*-anisylmethyl chloride were used, the yield of the N,5′-*O*-di-tris-*p*-anisylmethyladenosine increased at the expense of 5′-*O*-tri-*p*-anisylmethyladenosine.

5′-*O*-Tri-*p*-anisylmethyl-N^1,N^6,2′,3′-*O*-tetrabenzoyladenosine.—5′-*O*-tri-*p*-anisylmethyladenosine (0.5 g.; 0.83 mmole) was dissolved in 10 ml. of anhydrous pyridine and to the solution was added 2 ml. (17.5 mmole) of freshly distilled benzoyl chloride. The sealed reaction mixture was kept at room temperature for 18 hr. and then poured into 150 ml. of ice-water. After 2 hr. the product was extracted with ethyl acetate and the extract washed three times with sodium bicarbonate solution and then twice with water. After being dried over sodium sulfate, the ethyl acetate solution was evaporated to dryness. The residue (661 mg.) was dissolved in 95% ethyl alcohol, and the solution deposited 575 mg. of a white solid with m.p. 112–114°.

Anal. Calcd. for $C_{60}H_{49}O_{11}N_5$ (1016.03): C, 71.40; H, 4.86; N, 6.92. Found: C, 71.47; H, 5.05; N, 7.01.

N^1,N^6,2′,3′-*O*-Tetrabenzoyladenosine.—(a) From 5′-*O*-tri-*p*-anisylmethyl- N^1,N^6,2′,3′-*O*- tetrabenzoyladenosine. The total product obtained in the foregoing experiment was dissolved in 5 ml. of glacial acetic acid, and the solution was diluted with 1 ml. of water. After 15 min. at room temperature, solid sodium bicarbonate was added until evolution of carbon dioxide ceased. Five ml. of water was added and the mixture extracted with ethyl acetate. After two washings with water, the extract was dried over sodium sulfate and then evaporated to dryness. The residue was dissolved in benzene and the solution gave the crystalline tetrabenzoate with m.p. 179–181°. The yield was 372 mg. (66% over-all from 5′-*O*-tri-*p*-anisylmethyladenosine).

Anal. Calcd. for $C_{88}H_{29}O_9N_7$ (683.65): C, 66.9; H, 4.28; N, 10.23. Found: C, 66.26; H, 4.23; N, 10.23.

(b) From 5′-*O*-trityl,N^1,N^6,2′,3′-*O*-tetrabenzoyladenosine. To a solution of 5′-*O*-trityladenosine (2.6 g., 5.1 mmole) in 10 ml. of dry pyridine was added 5 ml. (42 mmole) of benzoyl chloride. After 18 hr. at room temperature, the solution was poured into 500 ml. of ice-water and the mixture stirred for 2 hr. The product which precipitated was extracted into 100 ml. of chloroform. The chloroform solution was washed with water and dried over anhydrous sodium sulfate. Subsequent evaporation *in vacuo* yielded a light brown residue. This was dissolved in 10 ml. of chloroform and to the solution 1 ml. of hydrobromic acid in acetic acid[58] (48% by weight) was added. After 1 min. at room temperature, the chloroform solution was evaporated *in vacuo* at room temperature. The residual oil was further sucked under a high vacuum. The residue, still smelling slightly of acetic acid, was dissolved in a small volume (10 ml.) of chloroform and was chromatographed on a silicic acid[59] column (27 × 2 cm.). Elution was carried out using a linear gradient of methyl alcohol in chloroform. The mixing vessel contained 1 l. of chloroform and the reservoir a solution of 100 ml. of methyl alcohol in 900 ml. of chloroform. A flow rate of 1 ml./min. was maintained, 10 ml. fractions being collected. The peak obtained at a concentration of 1.5% methyl alcohol in the eluent was processed by removal of chloroform and trituration of the residue with cold ethyl alcohol. The tetrabenzoyladenosine thus obtained (3.1 g.; 95%) had a m.p. of 172°. (Small amounts of other benzoyl derivatives of adenosine were

(56) H. Bredereck, *Ber.*, **B66**, 198 (1933).

(57) Adenosine was first taken up in pyridine and the solution filtered from a small amount of insoluble residue. The clear solution was diluted with water and the diluted solution lyophilized to give a finely divided powder. This was dried over phosphorus pentoxide and then used in the above experiment.

(58) British Drug Houses Ltd., Poole, England.

(59) Mallinckrodt Silicic Acid, 100 mesh, analytical reagent.

obtained subsequently from the column at higher methyl alcohol concentration.) Two grams of the tetrabenzoyladenosine were rechromatographed as described above but using a 5% methyl alcohol–chloroform solution in the reservoir. Tetrabenzoyladenosine (1.9 g.) was eluted at a concentration of 1.2% methyl alcohol in chloroform. The analytical sample as crystallized from ethyl alcohol had m.p. 185° λ_{max} 275, 238 mμ λ_{min} 263. mμ (ethyl alcohol).

Anal. Calcd. for $C_{38}H_{29}O_8N_5$ (683.65): C, 66.9; H, 4.28; N, 10.23. Found: C, 67.16; H, 4.25; N, 10.26.

The substance showed in ethyl alcohol λmax. at 275 and 238 mμ and $\lambda_{min.}$ at 263 mμ.

2′-*O*-Tetrahydropyranyluridine-5′ Phosphate and 5′-*O*-Di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ Phosphate.—(a) The mixture of triethylammonium 2′-*O*-tetrahydropyranyluridine-3′ and -5′ phosphate (0.65 mmole) as obtained from the barium hydroxide ring opening of 2′-*O*-tetrahydropyranyluridine-3′,5′ cyclic phosphate (see above) was dissolved in 15 ml. of dry pyridine. Di-*p*-anisylphenylmethyl chloride (2.2 g.; 6.5 mmole) was added and the clear solution kept at 25° for 5 hr. Two hundred ml. of 0.1 *M* ammonium hydroxide solution was added and the mixture extracted several times with 50 ml. portions of ether. The colorless aqueous layer was concentrated to 100 ml. *in vacuo* at low temperature and the resulting cloudy solution clarified by the addition of a little ethyl alcohol. The clear solution was applied to the top of a DEAE-cellulose (carbonate form) column (35 cm. × 3.5 cm. dia.). The column was successively washed with 20% ethanol (1 l.) and water (1 l.) after which 2′-*O*-tetrahydropyranyluridine-5′ phosphate and chloride ion were eluted with 0.1 *M* triethylammonium bicarbonate (1 l.). The nucleotide was precipitated as its calcium salt from 95% ethanol (40 mg.); ultraviolet absorption: λmax. 261 mμ (ε 9,750), at *p*H 7.0, $\lambda_{max.}$ 261 mμ (ε 7,260) at *p*H 12.0.

Anal. Calcd. for $C_{14}H_{19}N_2O_{10}P.Ca.9H_2O$; P, 5.08. Found: P, 5.06.

That this product was completely free from the isomeric 2′-*O*-tetrahydropyranyluridine-3′ phosphate was shown as follows. The tetrahydropyranyl group was removed by treatment with 80% acetic acid at room temperature for 4 hr., and the product was examined on a paper chromatogram in Solvent B, as well as on a Dowex 1 (formate) column.[60] Only uridine-5′ phosphate was present.

Further elution of the DEAE cellulose column with 0.2 *M* triethylammonium bicarbonate in 20% ethanol (2 l.) gave 5′-*O*-di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate, which was isolated as its triethylammonium salt (480 mg.) by lyophilization after concentration of the eluate to a small (100 ml.) volume. For testing the purity of this nucleotide derivative, a portion was treated with 80% acetic acid at room temperature to remove the tetrahydropyranyl and di-*p*-anisylphenylmethyl groups. The resulting nucleotide was analyzed on a standardized Dowex-1 (formate) column.[60] This analysis showed contamination of uridine-3′ phosphate by uridine-5′ phosphate to the extent of 4%.

(b) The reaction of 2′-*O*-tetrahydropyranyluridine-3′ and -5′ phosphates with di-*p*-anisylphenylmethyl chloride was carried out exactly as described under (a) but the time of reaction was 3.5 hr. at room temperature. After separation of the products on the standard DEAE cellulose column as described above, 2′-*O*-tetrahydropyranyl-5′-*O*-di-*p*-anisyl phenylmethyluridine-3′ phosphate was analyzed by acidic treatment and chromatography on a standardized Dowex-1 (formate) column. The uridine-3′ phosphate thus obtained was contaminated by uridine-5′ phosphate to the extent of 1%. Reduction of the time of reaction with di-*p*-anisylphenylmethyl chloride to 2 hr. showed that the 2′-*O*-tetrahydropyranyl-5′-*O*-di-*p*-anisylphenylmethyluridine-3′ phosphate was pure but that 2′-*O*-tetrahydropyranyluridine-5′ phosphate was contaminated by 2′-*O*-tetrahydropyranyluridine-3′ phosphate.

(c) For the preparation of the pure 2′-*O*-tetrahydropyranyl-5′-*O*-di-*p*-anisylphenylmethyluridine-3′ phosphate used in the following synthesis of uridylyl-(3′→5′) uridine and uridylyl-(3′→5′)-adenosine, the reaction described under (a) was carried out for 0.5 hr. at room temperature. The protected 3′ phosphate was obtained from the standard DEAE-cellulose column in fractions 90–170 in a total volume of 1200 ml.; total optical density at 260 mμ of the peak was 5340 units. For recovery of the protected-3′ phosphate in pure state, extreme care was necessary in evaporation and handling of the nucleotide. The triethylammonium bicarbonate eluate was evaporated at low temperature on a rotary evaporator using Dry-Ice–acetone in the condensing bath to about 10 ml. Pyridine (20 ml.) was added and the solution re-evaporated. The process was repeated several times to remove all the triethylammonium bicarbonate. A pyridine solution of the residue then was passed slowly through a Dowex 50 (pyridinium) column (1.5 cm. × 20 cm.). The column was washed with a mixture of pyridine–water–ethyl alcohol (2:1:7). The total effluent (300 ml.) was concentrated at 5° to about 10 ml. and the solution made up to 25 ml. with pyridine. The concentration of this stock solution was determined by removing a suitable aliquot, evaporating off the solvent, hydrolysing for 5 hr. with 80% acetic acid and chromatographing known aliquots in triplicate in Solvent A. The free nucleotide thus produced was estimated spectrophotometrically. The ultraviolet absorption characteristics of 2′-*O*-tetrahydropyranyl-5′-*O*-di-*p*-anisylphenylmethyluridine-3′ phosphate were: λmax. 234 mμ (ε 19,100), 264 mμ (ε 13,350) at *p*H 7.0; inflex. 229 mμ (ε 25,600), λmax. 264 mμ (ε 9,400) at *p*H 12.0.

Anal. Calcd. for $C_{35}H_{39}N_2O_{12}P.C_6H_{15}N$: C, 60.6; H, 6.7; N, 5.2. Found (on a sample dried in a high vacuum at room temperature). C, 60.63; H, 7.24; N, 5.7.

2′-*O*-Tetrahydropyranyluridine-3′ Phosphate.—5′-*O*-Di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (0.8 mmole of the triethylammonium salt) was dissolved in 80% acetic acid (20 ml.) at 0° and the solution was kept at 0° for 6 hr. The solvent then was removed by lyophilization and the residue dissolved in water (100 ml.) adjusted to *p*H 7.0 with *M* ammonia. After an ether wash, the solution was passed onto a DEAE cellulose (carbonate form) column (35 cm. × 3.5 cm.). The column was washed with 10% ethanol (1 l.) followed by water (1 l.) and then was eluted using a linear salt gradient; 0.002 *M* triethylammonium bicarbonate (2 l.) in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (2 l.) in the reservoir. The flow rate was 2 ml./min., 20-ml. fractions being collected. 2′-*O*-Tetrahydropyranyluridine-3′ phosphate (0.55 mmole) was eluted in fractions 110–145 followed by a small amount of uridine-3′ phosphate in fractions 146–155. Unreacted nucleotide was eluted by 0.2 *M* triethylammonium bicarbonate in 20% ethanol. Fractions 110 145 were combined, concentrated to a small volume and then lyophilized to yield the triethylammonium salt of 2′-*O*-tetrahydropyranyluridine-3′ phosphate as a white powder; ultraviolet absorption: λmax. 262 (ε 10,300) at *p*H 7.0, λmax. 261 (ε 7,500) at *p*H 12.0. A sample of the nucleotide was treated with 80% acetic acid for 2 hr. at 25° to remove the tetrahydropyranyl group. Examination of the product on a standardized Dowex 1 (formate form) column showed that it was pure uridine-3′ phosphate.

Anal. Calcd. for $C_{14}H_{21}N_2O_{10}P.C_6H_{15}N.1H_2O$: C, 46.5; H, 7.42. Found on a sample dried in a high vacuum, C, 46.59; H, 7.76.

5′-*O*-*p*-Anisyldiphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ Phosphate.—The mixture of 2′-*O*-tetrahydropyranyluridine-3′ and -5′ phosphates (0.65 mmole) was rendered anhydrous by repeated evaporation of pyridine solution and then taken up in 15 ml. of dry pyridine. *p*-Anisyldiphenylmethyl chloride (2.3 g.) was added and the reaction mixture kept at room temperature for varying lengths of time. The work-up, isolation of 2′-*O*-tetrahydropyranyluridine-5′ phosphate and 5′-*O*-*p*-anisyldiphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate, and analysis of their purity was as described above for the preparation of 5′-*O*-di-*p*-anisylphenylmethyl derivatives. After a 24-hr. reaction period, the isolated 2′-*O*-tetrahydropyranyluridine-5′ phosphate was contaminated by a trace of the —3′ phosphate. On the other hand, 5′-*O*-*p*-anisyldiphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate was pure.[61] After a 48-hr. reaction period, the latter product was found to be contaminated by the -5′ phosphate to the extent of 3.5%. Ultraviolet absorption characteristics of 5′-*O*-*p*-

(60) W. E. Cohn and J. X. Khym in "Biochemical Preparations," Vol. V, ed. D. Shemin, John Wiley and Sons, Inc., New York, N. Y., p. 40.

(61) For the preparation of pure 5′-*O*-*p*-anisyldiphenylmethyl-2′-*O*-

anisyldiphenylmethyl-2'-*O*-tetrahydropyranyluridine-3' phosphate were: λmax. 232 mμ (ε 16,320), 262 mμ (ε 10,880) at *p*H 7.0; λinflex. 228 mμ (ε 21,300), λmax. 263 mμ (ε 8,700) at *p*H 12.0.

Anal. Calcd. for $C_{34}H_{37}N_2O_{11}P.C_6H_{15}N.1H_2O$: C, 60.1; H, 6.8; N, 5.27. Found on a sample dried in a high vacuum at room temperature, C, 60.33; H, 7.04; N, 5.81.

5'-*O*-Trityl-2'-*O*-tetrahydropyranyluridine-3' Phosphate.—The mixture of 2'-*O*-tetrahydropyranyluridine-3' and -5' phosphates (0.64 mmole) in dry pyridine (10 ml.) was treated with trityl chloride (2 g., 6.4 mmole) for 10 days at 25°. Work-up and ion-exchange chromatography as above yielded 5'-*O*-trityl-2'-*O*-tetrahydropyranyluridine-3' phosphate (0.39 mmole) which was precipitated from 95% ethanol as its calcium salt; ultraviolet adsorption: λmax. 262 mμ (ε 10,580) at *p*H 7.0; λmax. 261 mμ (ε 8,480) at *p*H 12.0.

Anal. Calcd. for $C_{33}H_{33}N_2O_{10}P.Ca$. P, 4.5%. Found: P, 3.29.[62]

Acid Catalyzed Hydrolysis of Nucleoside and Nucleotide Derivatives.—A solution (0.1 *M*) of the nucleoside or nucleotide derivative in 80% acetic acid was kept at 25° and the hydrolysis to the parent nucleoside or nucelotide was followed chromatographically in Solvent A (10 μl. of solution in each spot). The time required for complete hydrolysis is given below: 5'-*O*-trityluridine[63] 48 hr.; 5'-*O*-*p*-anisyldiphenyluridine, 2 hr.; 5'-*O*-di-*p*-anisylphenylmethyluridine, 15 min.; 5'-*O*-tri-*p*-anisylmethyluridine, <1 min.; 5'-*O*-tritylthymidine-3' phosphate, 4 hr.; 2'-*O*-tetrahydropyranyluridine-5' phosphate, 2 hr.; 2'-*O*-tetrahydropyranyluridine-3' phosphate, 1 hr.; 5'-*O*-trityl-2'-*O*-tetrahydropyranyluridine-3' phosphate, 4 hr. 5'-*O*-*p*-anisyldiphenylmethyl-2'-*O*-tetrahydropyranyluridine-3' phosphate was completely converted to a mixture of uridine-3' phosphte and 2'-*O*-tetrahydropyranyluridine-3' phosphate after 30 min. 5'-*O*-Di-*p*-anisylphenylmethyl-2'-*O*-tetrahydropyranyluridine-3' phosphate was converted to mainly 2'-*O*-tetrahydropyranyluridine-3' phosphate and a trace of uridine-3' phosphate in 2 min. 2',3'-*O*-Anisylidineuridine was completely hydrolyzed to uridine after 10 hr. After 20 hr. both 2',3'-*O*-benzylidineuridine[64] and 2',3'-*O*-isopropylidineuridine[65] were only 20% hydrolyzed to uridine.

Enzymically synthesized uridylyl-(3'→5')-uridine on treatment with 80% acetic acid at 25° for 30 hr. was hydrolyzed to uridine and uridine-2' (3') phosphate to a slight extent. The residual dinucleoside phosphate contained material which was resistant to pancreatic ribonuclease and, presumably, was uridylyl-(2'→5')-uridine.

Enzymic Synthesis of Uridylyl-(3'→5')-uridine.[38]—A solution of uridine (98 mg., 0.4 mmole), the ammonium salt of uridine-2',3' cyclic phosphate (39.3 mg., 0.1 mmole) and bovine pancreatic ribonuclease (0.1 mg.) in 0.015 *M* tris-(hydroxymethyl)-aminomethane buffer (5.75 ml., *p*H 7.5) was incubated at 37° for 75 min. The enzyme was inactivated by shaking the solution with a mixture of isoamyl alcohol (0.6 ml.) and chloroform (0.15 ml.), then the separated aqueous solution was applied to a sheet of Whatman 40 paper (45 cm. wide) and the chromatogram developed in Solvent A. The four products, uridine-3' phosphate, uridylyl-(3'→5')-uridine, uridine-2',3' cyclic phosphate and uridine were completely resolved by this system. The dinucleoside phosphate was eluted with water and stored at −20°.

Enzymic Assays of Synthetic Uridylyl-(3'→5')-uridine and Uridylyl-(3'→5')-adenosine.—(a) Using pancreatic ribonuclease. The incubation mixture consisted of the dinucleoside phosphate (*ca.* 1.0 μmole), crystalline pancreatic ribonuclease (50 μg.) and tris-hydroxymethylaminomethane buffer (*p*H 7.5) (5 μmole) in a total volume of 0.1 ml. The incubation was carried out at 37° for 1 hr., and the products were determined by chromatography in Solvent A. For detection of uridine-2',3' cyclic phosphate as the intermediate the enzyme concentration was reduced to 5 μg.

(b) Using snake venom phosphodiesterase.[43] The phosphodiesterase preparation used had a specific activity of 1135 μmole/hr./mg. protein for hydrolysis of *p*-nitrophenyl thymidine-5' phosphate.[43] The incubation mixture contained the dinucleoside phosphate (*ca.* 1 μmole), tris-hydroxy methylaminomethane buffer (*p*H 8.9) (2 μmole) and the phosphodiesterase (22 μg protein) in a total volume of 0.2 ml. The incubation was carried out at 37° for 1 hr., and the products were determined by paper chromatography in Solvents A and B.

(c) Using spleen phosphodiesterase.[41] The phosphodiesterase preparation used had a specific activity of 123 μmole/hr./mg. protein for hydrolysis of *p*-nitrophenyl thymidine-3' phosphate.[41] The incubation mixture contained the dinucleoside phosphate (*ca.* 1 μmole) in 0.2 ml. of 0.5 *M* ammonium acetate buffer (*p*H 6.5) and 99 μg. of the phosphodiesterase in 0.1 ml. of 0.01 *M* pyrophosphate buffer (*p*H 6.5). The incubation was carried out at 37° and the products were characterized in Solvent A.

Uridylyl-(3'→5')-uridine.—(a) A mixture of 5'-*O*-di-*p*-anisylphenylmethyl-2'-*O*-tetrahydropyranyluridine-3' phosphate (0.0147 mmole) and 2',3'-di-*O*-acetyluridine (0.061 mmole) was rendered anhydrous by repeated dissolution in pyridine and evaporation of the solution *in vacuo*. The resulting gum was taken up in 0.5 ml. of dry pyridine and dicyclohexylcarbodiimide (0.125 g.) was added. The sealed reaction mixture was kept at room temperature for 3 days, after which an additional amount (0.05 g.) of the carbodiimide was added. After another day, water (1 ml.) was added and the mixture kept for 12 hr. Dioxane (25 ml.) and conc. ammonia (25 ml.) then were added and, after keeping for 2 hr., 75 ml. more of concn. ammonia was added. After 18 hr. the total mixture was evaporated to dryness at room temperature. To the residue ethyl alcohol was added and the solution re-evaporated. Twenty ml. of 80% acetic acid then was added and the solution kept at room temperature. One half of the total solution was concentrated after 4 hr. and chromatographed on a sheet of Whatman 44 paper in Solvent A. The dinucleoside phosphate band was eluted with water. The yield as determined spectrophotometrically was 3.5 μmole (49.9% of theoretical). Enzymatic assay with pancreatic ribonuclease showed the product to be completely degraded to uridine-3' phosphate and uridine. The remaining half of acetic acid solution was chromatographed after 10 hr. at room temperature. The yield of the dinucleoside phosphate was 4.6 μmole (69%). The product thus obtained contained a trace (about 1%) of ribonuclease-resistant material, presumably uridylyl-(2'→5')-uridine. The amount of the unreacted nucleotide, recovered as uridine-3' phosphate, in the above synthesis was estimated to be 77%.

(b) In another experiment carried out on a 0.16 mmole scale of the protected nucleotide, 2',3'-*O*-anisylideneuridine was used in place of 2',3'-di-*O*-acetyluridine. The reaction mixture was worked up by treatment with water, complete removal of the solvent (aqueous pyridine) and treatment with 80% acetic acid at room temperature for 4 hr. The products were again separated by preparative paper chromatography in Solvent A. The yield of the pure dinucleoside phosphate was about 25%. The low yield was due to the lack of complete removal of the anisylidene group.

(c) In earlier experiments, 5'-*O*-trityl-2'-*O*-tetrahydropyranyluridine-3' phosphate was used as the protected nucleotide. The work-up and results were similar to those described in the synthesis of uridylyl-(3'→5')-adenosine (b).

Uridylyl-(3'→5')-adenosine.—(a) Using 5'-*O*-di-*p*-anisylphenylmethyl-2'-*O*-tetrahydropyranyluridine-3' phosphate. A stock pyridine solution of 5'-*O*-di-*p*-anisylphenylmethyl-2'-*O*-tetrahydropyranyluridine-3' phosphate[66] (0.062 mmole) and N^1,N^6,2',3'-*O*-tetrabenzoyladenosine (0.138 g.; 0.2 mmole) was evaporated to dryness *in vacuo*. The resi-

tetrahydropyranyluridine-3' phosphate even a shorter time than 24 hr. is recommended.

(62) The low phosphorus content is presumably due to a very high degree of hydration of the calcium salt. There is little doubt of the purity of the substance. It was homomgeneous by paper chromatography,[44] and gave after acidic treatment only uridine-2' (or 3') phosphate and no uridine-5' phosphate. When the substance was used in condensation with 2',3'-di-*O*-acetyluridine, the uridylyl-(3' → 5')-uridine isolated was pure as tested by careful enzymic methods.

(63) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **104**, 385 (1934).

(64) J. M. Gulland and H. Smith, *J. Chem. Soc.*, 338 (1947); *cf.* D. M. Brown, L. J. Haynes and A. R. Todd, *ibid.*, 643 (1950).

(65) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934).

(66) Just before use, it was ascertained by paper chromatography (at least at 1 μmole level) in Solvent A that this starting material was completely pure.

due was rendered anhydrous by redissolution in dry pyridine and evaporation *in vacuo*, the process being repeated three times. Pyridine (0.5 ml.) and dicyclohexylcarbodiimide (0.103 g.; 0.5 mmole) were added and the solution was kept sealed at room temperature for 4 days. Water (0.5 ml.) was added and after 12 hr. at room temperature, a small amount of dioxane (1 ml.) and concentrated ammonium hydroxide (3 ml.) were added. When the solution had cleared, additional concd. ammonium hydroxide (9 ml.) was added and the solution was shaken for 2 days. The ammonium hydroxide was carefully removed by evaporation, and 80% aqueous acetic acid (2 ml.) was added to the residual solid. After 4 hr. at room temperature, one-half of the solution was applied on a sheet of Whatman 44 paper and the chromatogram developed in Solvent A. The bands corresponding to uridylyl-(3′→5′)-adenosine and uridine-3′ phosphate were eluted with water and estimated spectrophotometrically. The free nucleotide recovered corresponded to 3.75 μmole (11.3%). The dinucleoside phosphate corresponded to 415 optical density units at 260 mμ. Assuming an εmax. of 23,000 for this compound, the yield was 18.05 μmole (54.5%). This product was shown to be pure uridylyl-(3′→5′)-adenosine by the enzymatic tests specified above. The second half of acetic acid solution was chromatographed after 11 hr. at room temperature. The yield of the dinucleoside phosphate thus isolated was 532 optical density units at 260 mμ (23.2 μmole; 70.2%). This sample was found to contain a maximum of 1.5% ribonuclease-resistant material. Analysis for phosphorus showed ϵ/P at 260 mμ to be 23,000. Ultraviolet absorption characteristics, λmax. 259 mμ at *p*H 7.5; λmax. 259 mμ in alkali; λmax. 257 mμ in acid.

(b) **Using 5′-*O*-trityl-2′-*O*-tetrahydropyranyluridine-3′ phosphate.** An anhydrous pyridine solution (3 ml.) of 5′-*O*-trityl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (0.057 mmole) and N^1,N^6,2′,3′-*O*-tetrabenzoyladenosine (0.146 g.; 0.22 mmole) was prepared by the technique described above under (a). Dicyclohexylcarbodiimide (0.103 g.; 0.5 mmole) was added and the sealed reaction mixture kept at room temperature for 4 days. The volume of the solution then was reduced to one-half and a further amount (0.05 g.) of the carbodiimide was added. After another day, water (1 ml.) was added and the mixture kept for 12 hr. Concentrated ammonia was added until opalescence and when the solution cleared, more conc. ammonia was added until the final volume was 50 ml. After a total of 50 hr. at room temperature, the insoluble precipitate was removed by filtration and the clear filtrate and washings were evaporated to dryness *in vacuo*. The residue was dissolved in 50 ml. of 80% acetic acid and the solution kept at room temperature for 18 hr. The acetic acid was removed *in vacuo* and the residue applied, after adjusting *p*H to 8, to the top of a DEAE-cellulose (carbonate) column (50 cm. × 2 cm. dia.). Elution was carried out using a 0.10 *M* triethylammonium bicarbonate gradient. The dinucleoside phosphate was eluted as a broad peak at 0.045 *M* triethylammonium bicarbonate concentration. Assuming an εmax. (260 mμ) of 23,000, the yield was 57%. Assay with spleen phosphodiesterase and pancreatic ribonuclease showed only a trace of material resistant to these enzymes. Using 7.5 optical density units of the dinucleoside phosphate and applying the total on a paper chromatogram, the optical density of the resistant material was too small to be measured spectrophotometrically.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Spectroscopic Studies of α-Chymotrypsin Catalyzed Reactions. II. Spectral Changes at 290 mμ

BY JOHN F. WOOTTON[1] AND GEORGE P. HESS

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Absorbancy changes of an enzyme, consisting of amino acids only, have been observed during the formation of an enzyme–substrate complex. Evidence is presented that the ultraviolet difference spectrum of acyl-chymotrypsin *versus* chymotrypsin, with a principal peak at 290 mμ, is intimately related to the acylation of the active site of the enzyme. Both chemical and spectroscopic studies indicate that the origin of the spectral changes is an interaction with a tryptophyl residue in acyl-chymotrypsin. This interaction is not found in chymotrypsin. At the present, one of two different types of interaction with a tryptophyl residue in acyl-chymotrypsin is consistent with the data and considered most likely to account for the observed difference spectrum. These are: (1) an internal cross linkage between an amino acid residue and the indole ring of a tryptophyl residue; or (2) the envelopment of one or possibly more tryptophyl residues in a hydrophobic region of the molecule. This latter possibility requires a reversible change in conformation of the enzyme during the formation of the enzyme–substrate complex.

During the course of investigations of the α-chymotrypsin (CT) catalyzed hydrolysis of *p*-nitrophenyl acetate[2] changes in absorption of the enzyme at 290 mμ,[3] which accompany the formation of the enzyme–substrate complex, were observed. These absorption changes have so far been observed in the catalytic reaction of CT with *p*-nitrophenyl acetate, or with diisopropylphosphorofluoridate (DFP), and in the reaction of trypsin with DFP. The difference spectra of acyl-CT *versus* CT appear to be the same whether the acyl group is acetyl or diisopropylphosphoryl (DIP). The characterization and location of the origin of the difference spectrum could be of considerable significance in terms of the elaboration of the mechanism of action of the enzyme and determination of the structure of the active site of the molecule.

Since DIP-CT is stable over a wide *p*H range the difference spectrum of DIP-CT *versus* CT is characterized in these studies. A preliminary report of a part of these investigations has appeared.[4]

The Relation of the Difference Spectra to the Specific Acylation of the Active Site of CT. A. Difference Spectrum of DIP-CT *versus* CT.—The difference spectrum of DIP-CT *versus* CT at *p*H 6.9 is illustrated in Fig. 1. In this experiment approximately three times the stoichiometric amount of DFP was added directly to the sample cuvette. An equivalent aliquot of dilute 2-propanol was added to the reference solution. The characteristic component of this difference spec-

(1) This work is part of a thesis submitted by J. F. Wootton to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address, Department of Chemistry, University College London, England.

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