mer. More than one configuration of the initiator–addase complex may be formed, only one of which is competent to initiate. The enzyme conformation for proper binding should be favored by high phosphate molarity, low temperature, and high addase specific activity. Other considerations affecting competency are the length and base composition of the initiator.

Acknowledgment

The authors wish to express their gratitude to Mrs. E. H. Lilly for phosphorus analyses, to D. E. Hoard and V. N. Kerr for supplying some of the oligonucleotides, and to C. H. Blomquist and D. G. Ott for very helpful discussions.

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Nucleoside Phosphonic Acids. I. The Synthesis of 5′-Deoxyuridine 5′-Phosphonic Acids and Derivatives*

L. Yengoyan† and D. H. Rammler

ABSTRACT: The chemical syntheses of the uridylic acid analogs, 5′-deoxyuridine 5′-phosphonic acid (I), 5′-deoxyuridine 5′-phosphonylphosphate (II), and 5′-deoxyuridine 5′-phosphonylpyrophosphate (III), are described. These substances are analogs corresponding to the mono-, di-, and triphosphates of uridine. 5′-Deoxyuridine 5′-phosphonic acid was prepared *via* a Michaelis–Arbuzov-type reaction on 5′-iodo-2′,3′-*O*-isopropylideneuridine with triallyl phosphite. The product of this reaction, diallyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (IV), was isolated by silicic acid chromatography. Removal of the isopropylidene group on IV was accomplished by treatment with hot 80% aqueous acetic acid, and essentially pure diallyl 5′-deoxyuridine 5′-phosphonate (V) (28%) was isolated after paper chromatography. Catalytic hydrogenolysis of V yielded a mixture of products, 5′-deoxyuridine 5′-phosphonic acid (I) (35%), *n*-propyl 5′-deoxyuridine 5′-phosphonate (50%), and di-*n*-propyl 5′-deoxyuridine 5′-phosphonate (15%), which were separated and isolated by ion-exchange chromatography. The uridine pyrophosphate analogs II and III were prepared by treating inorganic phosphate and pyrophosphate, respectively, with the imidazolidate of I which was prepared by treating I with 1,1′-carbonyldiimidazole. Preliminary attempts to polymerize 5′-deoxyuridine 5′-phosphonylphosphate using polynucleotide phosphorylase were unsuccessful.

A variety of nucleoside analogs have been prepared and their usefulness in biochemical and chemical experimentation is well documented (Bessman *et al.*, 1958; Kahan and Hurwitz, 1962). In general, these analogs have differed from the parent nucleoside or nucleotide by being altered either in the purine or pyrimidine nucleus and/or the carbohydrate portion of the nucleoside. Recently analogs of nucleotides have been described which have a phosphite (Hall *et al.*, 1957; Holy *et al.*, 1965) and phosphonate (Parikh *et al.*, 1957; Wolff and Burger, 1959; Bannister and Kagan, 1960) group in place of the naturally occurring phosphate group. The synthesis of a nucleoside triphosphate analog has been reported (Myers *et al.*, 1963) which contains a methylene group in place of the terminal pyrophosphate oxygen. This analog, 5′-adenyl methylenediphosphonate, is capable of being enzymatically polymerized by the deoxyribonucleic acid- (DNA) directed ribonucleic acid (RNA) polymerase of *Escherichia coli* (Simon *et al.*, 1965). Presently very little is known of the chemistry and biochemistry of nucleoside phosphonic acids, the analogs of nucleo-

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* From the Institute of Molecular Biology, Syntex Research, Palo Alto, California. *Received July 8, 1966.* Contribution No. 43.

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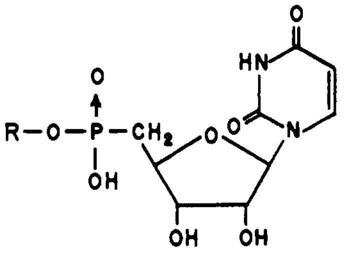
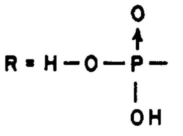
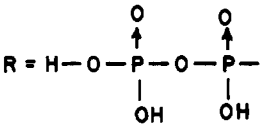
tides in which the phosphonyl group is joined to the ribose of the nucleoside by a carbon–phosphorus bond, primarily because these compounds have not been readily available. In this report the authors present the chemical synthesis of 5′-deoxyuridine 5′-phosphonic acid (I), 5′-deoxyuridine 5′-phosphonylphosphoric acid (II), and 5′-deoxyuridine 5′-phosphonylpyrophosphoric acid (III), these substances being analogs corresponding to the mono-, di-, and triphosphates of uridine.



I, R=H

II, 

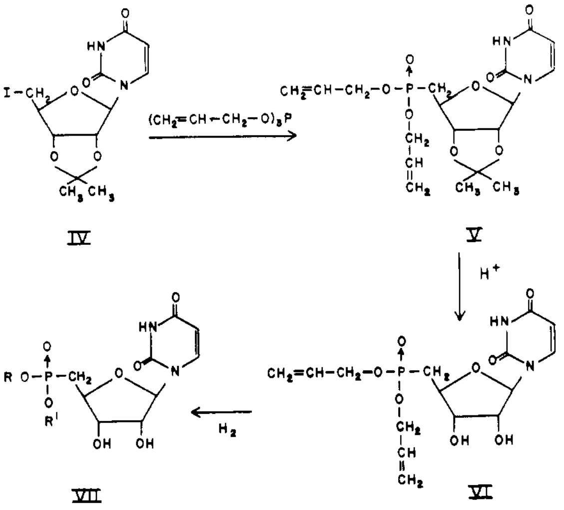
$$\mathrm{R = H{-}O{-}\underset{\mathrm{OH}}{\overset{\mathrm{O}}{\overset{\uparrow}{P}}}{-}}$$

III, 

$$\mathrm{R = H{-}O{-}\underset{\mathrm{OH}}{\overset{\mathrm{O}}{\overset{\uparrow}{P}}}{-}O{-}\underset{\mathrm{OH}}{\overset{\mathrm{O}}{\overset{\uparrow}{P}}}{-}}$$

5′-Deoxyuridine 5′-Phosphonic Acid. In this synthesis 2′,3′-*O*-isopropylideneuridine (Hampton, 1961) was converted into the 5′-*p*-toluenesulfonate. Displacement of the tosylate group with sodium iodide gave the expected 5′-deoxy-5′-iodo-2′,3′-*O*-isopropylideneuridine (IV) (Scheme I) (Levene and Tipson, 1934). Reaction (Michaelis–Arbuzov; see Kosolapoff (1950) for general references) of IV with excess triallyl phosphite at 130° gave as one of the products diallyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (V). The allyl iodide produced in the reaction was removed by entrainment in dry nitrogen gas saturated with triallyl phosphite. Use of this procedure prevented excessive formation of diallyl allylphosphonate formed by the reaction of catalytic amounts of allyl iodide and triallyl phosphite (Kosolapoff, 1950). Diallyl 5′-deoxyuridine 5′-phosphonate (VI) was isolated in 28% yield after the removal of the isopropylidene group by heating in aqueous acetic acid and by a combination of preparative silicic acid thin layer, column, and paper chromatography. The phosphonate V contained considerable amounts of triallyl phosphate presumably formed by air oxidation of the phosphite and lesser amounts of diallyl allylphosphonate in addition to other ultraviolet-absorbing contaminants. Effective separation of contaminating nonultraviolet-absorbing substances such as triallyl phosphate and diallyl allylphosphonate from the nucleoside phosphonate was accomplished by the chromatography of VI on silicic acid. Subsequent preparative paper chromatography separated the ultraviolet-absorbing side products, and the diallyl 5′-deoxyuridine 5′-phosphonate isolated in this manner was shown to be homogeneous by paper chromatography in two different chromatographic systems. The isolated nucleoside phosphonate derivative VI was obtained as an oil and attempts to further purify it by crystallization were unsuccessful.

SCHEME I: The Synthesis of 5′-Deoxyuridine 5′-Phosphonic Acid.



VII R = R′ = $CH_3CH_2CH_2$–
VIII R = $CH_3CH_2CH_2$– ; R′ = H
IX R = R′ = H

Structure VI was confirmed by a variety of techniques. Analysis of its ultraviolet spectrum in acid and base demonstrated the intactness of its uridine moiety and its molar extinction coefficient, based on phosphorus, was essentially the same as that of uridine 5′-phosphate. The presence of the phosphonate bond was demonstrated in the following manner. The nmr[1] spectrum of VI showed the presence of the $C_{5'}$-methylene protons (2.25 ppm) adjacent to the phosphorus atom. The nmr spectrum of diethyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (Bannister and Kagan, 1960) prepared and crystallized by an independent method also showed the same multiplet at 2.25 ppm. In addition, the nmr spectrum indicated the presence of the vinyl protons in the allyl groups of the ester. Further confirmation of the phosphonate linkage was afforded by treatment of VI with alkali. The monoanion similar to that expected from base hydrolysis of a nucleoside diester (Montgomery *et al.*, 1961) could not be detected in the alkaline hydrolysate under a variety of hydrolytic conditions, the principal ultraviolet-absorbing component present being uracil. The formation of uracil from VI is rationalized in Scheme II and most likely proceeds *via* base abstraction

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[1] Abbreviation used: nmr, nuclear magnetic resonance.

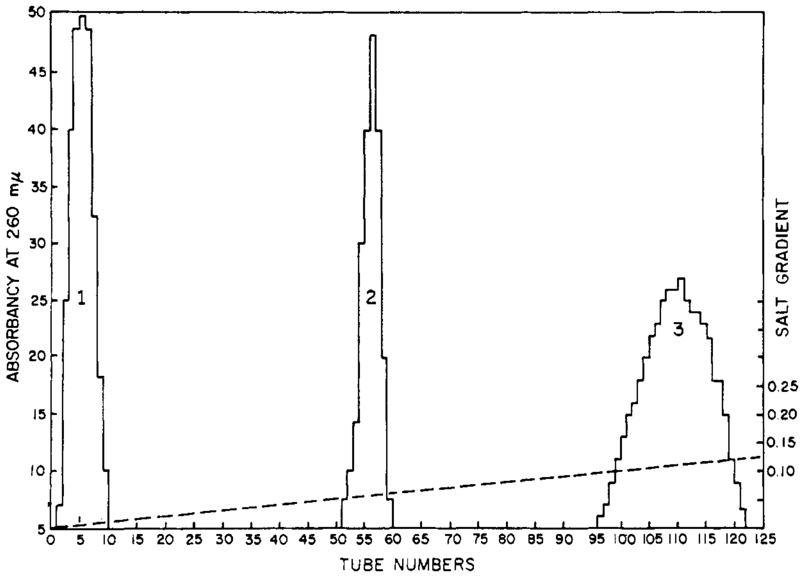
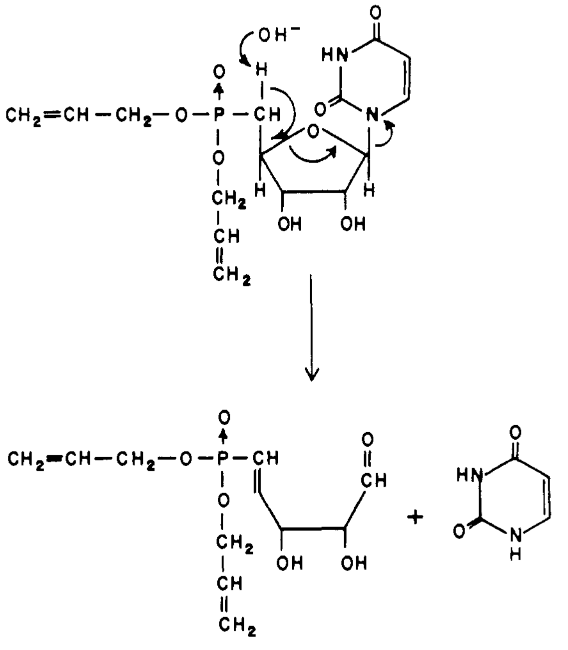


FIGURE 1: The elution pattern of di-*n*-propyl 5′-deoxyuridine 5′-phosphonate, *n*-propyl 5′-deoxyuridine 5′-phosphonate, and 5′-deoxyuridine 5′-phosphonic acid from DEAE-Sephadex (see Experimental Section for elution conditions).

SCHEME II: The Alkaline Hydrolysis of Diallyl 5′-Deoxyuridine 5′-Phosphonate.



of a $C_{5'}$ proton and β elimination to form uracil. A similar type of reaction has been proposed for the unusual base lability of the glycosidic linkage of 5′-adenosylmethionine (Schlenk and Dainko, 1962). This type of facile alkaline hydrolysis is consistent with phosphonate structure VI.

The $C_{5'}$ position of the phosphonate group in structure VI was confirmed by periodate oxidation. Periodate oxidation of VI led to the consumption of 1 mole of oxidant, indicating the presence of free 2′- and 3′-hydroxyl groups and thereby locating the phosphonate group at $C_{5'}$. The infrared spectrum (film) of VI indicated the presence of the semipolar phosphorus–oxygen bond of the phosphonate (1250 cm^{-1}).

Removal of the allyl groups on VI was achieved by catalytic hydrogenolysis.[2] The mixture of products obtained by catalytic hydrogenolysis was resolved by chromatography on DEAE-Sephadex. The chromatographic elution pattern is shown in Figure 1. The initial neutral peak 1 was shown to be di-*n*-propyl 5′-deoxyuridine 5′-phosphonate (VII) by the following criteria. It did not migrate on paper electrophoresis at pH 4 and 7.5; repeated catalytic hydrogenolysis failed to yield any anionic nucleoside phosphonate; and its nmr spectrum was consistent for the di-*n*-propyl 5′-deoxyuridine 5′-phosphonate structure.

Peak 2 was shown to be *n*-propyl 5′-deoxyuridine 5′-phosphonate (VIII) in the following manner. It was eluted from the DEAE-Sephadex at a salt concentration consistent with a singly charged uridine diester; it resisted change by further catalytic hydrogenation; and its migration on paper electrophoresis and chromatography was similar to *n*-propyl 5′-deoxyuridine 5′-phosphonate prepared by treating IX with *n*-propyl alcohol and dicyclohexylcarbodiimide (Rammler and Khorana, 1961). Its nmr spectrum showed the expected $C_{5'}$-methylene protons as a quartet centered

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[2] The silicic acid used for thin layer chromatography often contains sulfides which inhibit catalytic hydrogenation. These substances can be removed by either paper chromatography in solvent A or by chromatography on sulfide-free silicic acid.

at 2.45 ppm with a coupling constant of 20 cycles/sec. A well-characterized phosphonate, dimethyl methylphosphonate (Kosolapoff, 1950), had a methylene–phosphorus coupling constant of about 20 cycles/sec. The methyl protons of the propyl group of VIII showed the expected triplet at 1.05 ppm with a coupling constant of 7 cycles/sec. Attempts to hydrolyze the *n*-propyl ester with alkali were unsuccessful as were attempts using the phosphodiesterase of *Crotalus adamanteus.*

Peak 3 was shown to contain essentially pure 5′-deoxyuridine 5′-phosphonic acid (IX). When this substance was treated with alkaline phosphomonoesterase and the products were chromatographed on DEAE-Sephadex, a small amount of uridine (generally less than 2%) was obtained. The nucleoside phosphonates isolated from peaks 1 and 2 are most likely contaminated with di-*n*-propyl uridine 5′-phosphate and *n*-propyl uridine 5′-phosphate, respectively; however, the amounts of these contaminants do not significantly alter the physical constants reported for these substances. It is suggested that these phosphates arise by nucleophilic displacement of the iodo group on IV by diallyl phosphate. This latter substance can be generated *in situ* by oxidation of triallyl phosphite and its subsequent partial hydrolysis to yield the monoanion. This reaction is analogous to the published method (Anand *et al.*, 1952) for preparing uridine 5′-phosphate from IV and dibenzyl phosphate. 5′-Deoxyuridine 5′-phosphonic acid obtained after phosphomonoesterase hydrolysis and DEAE chromatography was homogeneous in two paper chromatographic solvent systems and migrated as a dianion on paper electrophoresis at pH 7.5. Its ultraviolet absorption spectrum in acid and base and its molar extinction coefficient based on phosphorus were consistent for a uridine derivative. It reduced 1 mole of periodate/mole of uracil demonstrating free vicinal hydroxyl groups at positions $C_{2'}$ and $C_{3'}$. The nmr datum was consistent with structure IX. 5′-Deoxyuridine 5′-phosphonic acid was, as expected, completely resistant to hydrolysis with *E. coli* alkaline phosphomonoesterase.

5′-Deoxy-2′,3′-O-isopropylideneuridine 5′-Phosphonic Acid. In an alternate method, diallyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (V) was isolated by chromatography on silicic acid. This substance was catalytically hydrogenolyzed in methanol containing 2 equiv of triethylamine for each equivalent of V. The reduction mixture was chromatographed on DEAE-Sephadex and again three principal fractions were obtained. Fraction 1 contained di-*n*-propyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (4.5%); fraction 2, *n*-propyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (70%); and fraction 3, 5′-deoxy-2′,3′-*O*-isopropylideneuridine-5′-phosphonic acid (23%). Treatment of each of these fractions with 80% acetic acid at 100° for 1 hr yielded the corresponding deacetonated phosphonates. When the reduction was carried out in the absence of triethylamine, partially deacetonated products were obtained because of acid-catalyzed hydrolysis by the free phosphonic acid formed during the reduction.

The acetonated phosphonate derivatives always contained a small amount of extraneous phosphorus-containing material as evidenced by high phosphorus/uracil ratios; however, this small amount of nonultraviolet-absorbing impurity should not preclude the use of these derivatives in studies where protected nucleoside phosphonates are required.[3]

Deoxyuridine 5′-Phosphonylphosphate (II). This substance was prepared using the method described by Hoard and Ott (1964). The nucleoside phosphonic acid derivative IX was treated with 1,1′-carbonyldiimidazole to form the phosphonoimidazolidate in essentially quantitative yield. No attempt was made to isolate this intermediate and it was treated with excess orthophosphate to yield 5′-deoxyuridine 5′-phosphonylphosphate which was isolated in 30% yield after chromatography on DEAE-Sephadex. 5′-Deoxyuridine 5′-phosphonylphosphate was identified by the following criteria. Its paper chromatographic, electrophoretic, and ion-exchange behavior were closely related to those of uridine 5′-diphosphate. Treatment with 1 N hydrochloric acid for 7 min at 100° gave only 5′-deoxyuridine 5′-phosphonic acid and treatment with *C. adamanteus* phosphodiesterase or with *E. coli* phosphomonoesterase removed the terminal phosphate and yielded 5′-deoxyuridine 5′-phosphonic acid as the only ultraviolet-absorbing substance. This substance is stable at pH 8.

Attempts to prepare 5′-deoxyuridine 5′-phosphonylphosphate *via* its morpholidate (Moffatt and Khorana, 1961) led to a somewhat complex mixture of products. Although 5′-deoxyuridine 5′-phosphonylmorpholidate could be isolated in good yields (70%), its conversion to 5′-deoxyuridine 5′-phosphonylphosphate was accomplished in poor yields (5%). Neither the reasons for this failure nor the identity of the reaction products was investigated further.

5′-Deoxyuridine 5′-Phosphonylpyrophosphate (III). The method described by Hoard and Ott (1965) was used to prepare this substance, which was isolated in 67% yield after chromatography on DEAE-Sephadex. The salt concentration required to elute III from DEAE-Sephadex at pH 7.5 and its electrophoretic mobility at pH 7.5 were those expected for a nucleoside derivative bearing four negative charges. Treatment with acid released 2 moles of inorganic phosphate/mole of uracil and hydrolysis with alkaline phosphomonoesterase and *C. adamanteus* diesterase yielded IX as the only ultraviolet-absorbing component. Furthermore, it could be oxidized by sodium periodate, indicating the presence of free vicinal hydroxyl groups. These lines of evidence are consistent with the structure of 5′-deoxyuridine 5′-phosphonylpyrophosphate.

Enzymatic Studies. In preliminary studies, 5′-deoxyuridine 5′-phosphonylphosphate could not be polym-

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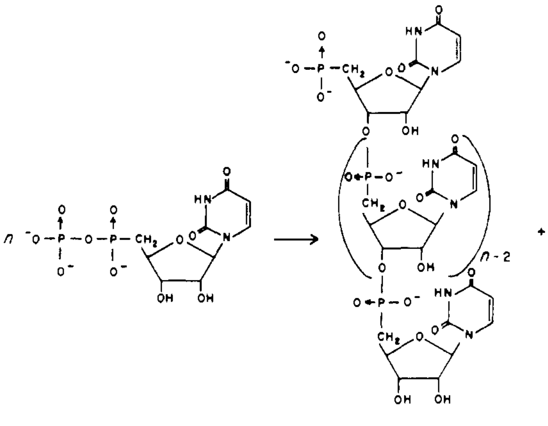
[3] Removal of extraneous nonultraviolet-absorbing material can be achieved by absorption and elution from carbon (Myers *et al.*, 1965).

erized by a purified preparation of polynucleotide phosphorylase (Singer and Guss, 1962), either in the presence or absence of uridylic oligonucleotide primers. In experiments with incubation periods of over 12 hr, virtually all of the 5′-deoxyuridine 5′-phosphonylphosphate in the reaction could be recovered intact. In control experiments using uridine 5′-pyrophosphate, maximum formation of polyuridylic acid was achieved within 90 min.

Discussion

Several aliphatic phosphonic acids have been found in nature (Horiguchi and Kandatsu, 1959; Kittredge and Hughes, 1964). Although their biological function and mode of biosynthesis are not known, the similarity of this type of phosphate analog to the ubiquitous phosphate esters of the cell is of interest because of the possibility that enzymes which require pyrophosphate activation for synthetic reactions, such as polymerizations, could utilize pyrophosphate derivatives of the phosphonic acids in place of the natural pyrophosphates. Polymeric phosphonates derived from these condensations would be extremely useful in studying reactions which require polymer integrity and which are subject to the hydrolytic action of phosphomono- and -diesterases. A particularly interesting reaction of this type is polyuridylic acid directed phenylalanine incorporation into polyphenylalanine by cell-free extracts (Nirenberg and Matthaei, 1961). The phosphomono- and -diesterase activities of this system have been characterized (Elson and Tal, 1959; Spahr, 1964; Neu and Heppel, 1964) and their effects on studies concerned with ribopolynucleotide-dependent polypeptide synthesis are described (Salas *et al.*, 1965). A ribopolynucleotide of the type shown in Scheme III would be expected to be stable to the action of phosphodiesterase II (a ribonuclease-type cleavage). Although it has one oxygen group less per nucleotide, this type of polymer would have the same number of negative charges that a similar natural polyuridylic acid would contain, and could thus possibly serve as a nuclease-stable messenger. With this view in mind, the authors sought a general procedure for the synthesis of nucleoside phosphonates.

SCHEME III: Poly-5′-deoxyuridine 5′-Phosphonate.



A procedure for the synthesis of aliphatic phosphonates has been described by Michaelis and Arbuzov (see Kosolapoff (1950) for summarizing references). Using this approach a crystalline uridine phosphonate, diethyl 5′-deoxyuridine 5′-phosphonate, was prepared (Bannister and Kagan, 1960); however, these authors were unable to convert this diester into its free acid. Our initial attempts to prepare 5′-deoxyuridine 5′-phosphonic acid employed a similar method except that we entrained the ethyl iodide generated in the reaction of IV with triethyl phosphite in a stream of nitrogen gas and lowered the reaction temperature to around 90°. In this way, it was possible to increase the yield of diethyl 5′-deoxyuridine 5′-phosphonate to around 90%, the product crystallizing directly from the reaction medium after removal of excess triethyl phosphite, thus obviating the need for chromatography. Attempts to hydrolyze the diester in dilute alkali were unsuccessful owing to the extreme alkaline lability of this substance (see Scheme II).

Although the yields of nucleoside phosphonate were less using triallyl phosphite, the use of this phosphite provided two mild routes for the removal of the aliphatic ester groups from the nucleoside phosphonate and, hopefully, a general route to the synthesis of these derivatives. For adenine-, guanine-, and uracil-containing nucleosides, the removal of the allyl groups can be readily achieved by hydrogenolysis; however, with cytosine-containing nucleosides the conditions of hydrogenolysis are sufficient to cause reduction of the cytosine nucleus. In this latter case, removal of the allyl groups could be achieved by mild oxidation of the vinyl ester to a *cis*-diol (Lemieux and von Rudloff, 1955) followed by periodate oxidation and by treatment with phenylhydrazine (Brown *et al.*, 1960). Work on this procedure is now in progress, as well as the synthesis of deoxyribonucleoside phosphonates and 5′-deoxyuridine 5′-phosphonic acid containing coenzymes (L. Yengoyan and D. H. Rammler, manuscript in preparation).

As indicated, our early efforts to enzymatically polymerize 5′-deoxyuridine 5′-phosphonylphosphate using a purified preparation of polynucleotide phosphorylase have been unsuccessful. Although the reasons for this nonreactivity are obscure, they most probably are not predominantly attributable to the phosphonylphosphate (pyrophosphate) linkage. This follows from the fact that the phosphonate, 5′-adenylylmethylene diphosphonate (Simon *et al.*, 1965), can serve as a substrate for a DNA-directed RNA polymerase. In general, phosphonates are weaker acids than their corresponding phosphates; for example, *n*-butylphosphonic acid has a pK_{a_1} of 2.5 and a pK_{a_2} of 8.1 (Freedman and Doak, 1957) while *n*-butyl phosphate has pK_a values of 1.8 and 6.8 (Kumler and Eiler, 1943). Although the pK_a values of 5′-deoxyuridine-5′-phosphonic acid were not determined, its electrophoretic mobility compared with uridine 5′-phosphate at pH 7.5 (see Experimental Section)

TABLE I: R_F Values of the Nucleoside Phosphonic Acids and Derivatives.

Compounds Chromatographed	Chromatographic Solvent Systems A	Chromatographic Solvent Systems B
5′-Deoxyuridine 5′-phosphonomorpholidate	0.31	0.50
Diallyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate	0.83	—
Di-*n*-propyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate	0.82	—
5′-Deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonic acid	0.28	0.49
Diallyl 5′-deoxyuridine 5′-phosphonate	0.69	0.80
Di-*n*-propyl 5′-deoxyuridine 5′-phosphonate	0.73	—
n-Propyl 5′-deoxyuridine 5′-phosphonate	0.44	0.66
5′-Deoxyuridine 5′-phosphonic acid	0.16	0.41
5′-Deoxyuridine 5′-phosphonylphosphate	0.12	0.24
5′-Deoxyuridine 5′-phosphonylpyrophosphate	0.09	0.15
Electrophoretic Mobilities Relative to Uridine 5′-Phosphate		
	pH 7.5	pH 4.0
Uridine 5′-phosphate	1.00	1.00
5′-Deoxyuridine 5′-phosphonic acid	0.83	1.03
5′-Deoxyuridine 5′-phosphonylphosphate	1.61	1.75
5′-Deoxyuridine 5′-phosphonylpyrophosphate	2.08	2.22
n-Propyl 5′-deoxyuridine 5′-phosphonate	0.42	
5′-Deoxyuridine 5′-phosphonomorpholidate	0.53	

indicated that it is a weaker acid than uridine 5′-phosphate. At the pH of the enzymatic polymerization, pH 9.0, it is probable that the difference in acid strengths of the phosphonate in a phosphonylphosphate linkage compared with the normal pyrophosphate bond will be reflected in different rates of polymerization rather than in the absence of polymerization (Todd, 1956). The principal difference between the nucleoside phosphonic acid and nucleotide is the absence of an oxygen atom at $C_{5'}$. This group can function as an enzyme binding site during polymerization or, because polynucleotide phosphorylase is specific for both the pyrophosphate bond and the ribose (Grunberg-Manago *et al.*, 1956) of the nucleotide, the absence of this group can be sufficient to prevent a good fit on the surface of the enzyme. Inhibitor studies with 5′-deoxyuridine 5′-phosphonic acid and the synthesis of an isosteric homonucleoside phosphonic acid are necessary in order to delineate these different effects. These studies are now in progress.

The phosphodiesterase found in the venom of *C. adamanteus* requires as a substrate a nucleoside 5′-phosphoryl residue bearing a free or at least a nonphosphorylated 3′-hydroxyl function (Razzell and Khorana, 1959). In the studies presented here, both 5′-deoxyuridine 5′-phosphonylphosphate and 5′-deoxyuridine 5′-phosphonylpyrophosphate serve as substrates for this enzyme despite the fact that they do not contain a 5′-phosphoryl residue. The phosphodiesterase contained little or no phosphomonoesterase activity as evidenced by the fact that no uridine could be detected in a control reaction with uridine 5′-diphosphate even after 12 hr and, hence, the hydrolytic action on the uridine phosphonic acid derivatives is ascribed to the phosphodiesterase. The reason why the phosphodiesterase did not hydrolyze *n*-propyl 5′-deoxyuridine 5′-phosphonate is not known; however, alkyl esters of 5′ nucleotides are not good substrates for this enzyme (Razzell, 1963). A clearer understanding of the specificity of this enzyme toward nucleoside phosphonic acid derivatives must await kinetic studies which are now in progress.

Experimental Section

Materials and Methods. Triallyl phosphite and 1,1′-carbonyldiimidazole were products of Aldrich Chemical Co., Milwaukee, Wis. *E. coli* alkaline phosphomonoesterase and *C. adamanteus* phosphodiesterase were obtained from Worthington Biochemicals, Freehold, N. J. Merck silicic acid for both thin layer and column chromatography was obtained from Brinkmann Instruments, Westbury, N. Y. Paper chromatography and electrophoresis were carried out using Schleicher and Schuell No. 589 orange ribbon paper. The paper chromatographic solvents were: solvent A, isopropyl alcohol–ammonia–water (7:1:2, v/v); solvent B, isobutyric acid–ammonia–water (57:4:39, v/v). Paper electrophoresis was carried out using the following buffer systems: ammonium bicarbonate (0.05 M, pH 7.5) and sodium acetate buffer (0.05 M, pH 4.0). Phosphorus analysis was performed using the method described by Ames and Dubin (1960). All phosphorus values are related to uridine 5′-phosphate as a standard.

Sodium periodate oxidations were carried out as described by Rammler and Rabinowitz (1962). The R_F values of the nucleoside phosphonates and derivatives and their electrophoretic mobilities are given in Table I.

2′,3′-O-Isopropylideneuridine. This substance was prepared as described by Hampton (1961), mp 159–60°, reported 160°.

2′,3′-O-Isopropylidene-5′-O-p-toluenesulfonyluridine. This substance was prepared as described by Levene and Tipson (1934) and was isolated as a glass.

5′-Deoxy-5′-iodo-2′,3′-O-isopropylideneuridine. This substance was prepared as described by Levene and Tipson (1934), mp 163°, reported 164°.

Diallyl 5′-Deoxyuridine 5′-Phosphonate. Triallyl phosphite (15 ml) was added to 5′-deoxy-5′-iodo-2′,3′-*O*-isopropylideneuridine (2.02 g, 5.14 mmoles). The nucleoside derivative dissolved while the mixture was being heated to 130°. Dry nitrogen gas, saturated with triallyl phosphite at 130°, was bubbled through the reaction solution to entrain the allyl iodide generated from the reaction. After 36 hr at 130°, about 90% of the starting material had reacted, as evidenced by thin layer chromatography (5% methanol in chloroform) of the reaction solution. At this time, the reaction solution was cooled and the excess triallyl phosphite was removed by evaporation under high vacuum. The residue, an oil (12.1 g), containing diallyl allylphosphonate and triallyl phosphate, was chromatographed on preparative silicic acid plates (10 plates, 1 m × 20 cm × 1.3 mm) using 5% methanol in chloroform as the solvent. Good resolution of the ultraviolet-absorbing reaction products was obtained when the eluting solvent was run about one-third of the plate distance initially, dried at room temperature, rechromatographed to two-thirds of the plate distance, dried again, and finally allowed to run to the top of the plate. By chromatographing in this fashion, five distinct ultraviolet-absorbing bands were obtained, in addition to a band remaining at the origin. The band with a mobility of 0.9 relative to 5′-deoxy-5′-iodo-2′,3′-*O*-isopropylideneuridine contained diallyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate. This band was removed and repeatedly extracted with hot methanol until the ultraviolet absorbancy of the extract was less than 1. The yellow oil, which contained diallyl allylphosphonate and triallyl phosphate in addition to the product (4.18 g) remaining after evaporation of the methanol, was dissolved in aqueous acetic acid (70%, 25 ml) and heated at 100° for 2 hr. After evaporation of the acetic acid, the residue in a small amount of chloroform was passed onto a column of silicic acid (250 g). All of the nonpolar material absorbed on the silicic acid was removed with chloroform, and subsequent elution with methanol removed most (90%) of the ultraviolet-absorbing material. The oil (0.69 g) remaining after removal of the methanol was next chromatographed on paper (Whatman 3MM) and five ultraviolet-absorbing bands were readily separable, diallyl 5′-deoxyuridine 5′-phosphonate (R_F 0.65, 28%) being the principal band. On periodate oxidation, it reduced 0.98 mole of periodate/mole of uracil. In addition to the proton resonance frequencies expected for uridine, its nmr spectrum in deuterium oxide revealed the following frequencies as multiplets: $C_{5'}$-methylene protons [$CH_2P(=O)$] centered at 2.2 ppm, allyl vinyl proton ($=CH$) centered at 6.1 ppm, and allyl vinyl protons ($CH_2=$) centered at 5.4 ppm. The infrared spectra (film) indicated the presence of the ester bond (POC) at 1075 cm^{-1} and the semipolar oxygen bond (P=O) at 1250 cm^{-1}. The ultraviolet spectrum in methanol had a λ_{max} at 261 mμ and a λ_{min} at 231 mμ; its molar extinction coefficient (ϵ) (weighed sample) based on a molecular weight of 388 was 10,000 and ϵ/phosphorus (ϵ/P) ratio was 9200.

Diallyl 5′-deoxyuridine 5′-phosphonate (0.49 g, 1.44 mmoles) in methanol (10 ml) was added to a suspension (25 mg in 50% aqueous acetic acid) of reduced palladium on barium sulfate (Kuhn and Hass, 1955). After 3 hr, the hydrogenolysis was judged complete, and the catalyst was removed by centrifugation, washed, and recentrifuged. The combined filtrates were evaporated to an oil which was dissolved into water adjusted to pH 8.0 and passed onto a column (2.5 × 25 cm) of DEAE-Sephadex (A-25) in the bicarbonate form. The elution was carried out using a linear gradient of triethylammonium bicarbonate at pH 7.5. The mixing vessel contained 500 ml of 0.005 M triethylammonium bicarbonate, pH 7.5, and the reservoir contained 500 ml of 0.2 M triethylammonium bicarbonate, pH 7.5. The flow rate was 1 ml/min and 15-ml fractions were collected. The elution pattern is shown in Figure 1. The fractions of each of the peaks were combined and evaporated to dryness at room temperature under high vacuum. Excess triethylamine was carefully removed by repeated evaporations with water, leaving each fraction as an oil.

Di-n-propyl 5′-Deoxyuridine 5′-Phosphonate. The first peak (15%) obtained by DEAE chromatography was di-*n*-propyl 5′-deoxyuridine 5′-phosphonate. It was characteristically labile to alkali; treatment with potassium hydroxide (0.1 M) at 37° for 1 hr yielded only uracil as evidenced by paper chromatography. It was completely stable to hydrolysis by *C. adamanteus* phosphodiesterase. Its ultraviolet spectrum was λ_{max}^{MeOH} 261 mμ, λ_{min}^{MeOH} 231 mμ ($\epsilon/P = 9800$); it reduced 0.98 equiv of sodium periodate/equiv of uracil.

n-Propyl 5′-Deoxyuridine 5′-Phosphonate. The second peak obtained by DEAE chromatography contained *n*-propyl 5′-deoxyuridine 5′-phosphonate (50%). It was homogeneous in the two paper chromatographic systems and migrated with a single negative charge at pH 7.5 and 4.0. Its mobility in both systems was similar to that of *n*-propyl 5′-deoxyuridine 5′-phosphonate prepared from 5′-deoxyuridine 5′-phosphonic acid, dicyclohexylcarbodiimide, and *n*-propyl alcohol (Rammler and Khorana, 1961). This substance was completely resistant to *C. adamanteus* phosphodiesterase after 12 hr of treatment (Razzell, 1963). Its ultraviolet spectrum was λ_{max}^{MeOH} 261 mμ, λ_{min}^{MeOH} 231 mμ ($\epsilon/P = 8900$). The nmr spectrum of the free acid in deuterium oxide contained $C_{5'}$-methylene protons [CH_2P-

(=O)] as a quartet at 2.4 ppm, 20 cycles/sec, propyl methylene proton (CH$_2$) as a multiplet centered at 1.4 ppm, and the methyl group (CH$_3$) as a triplet centered at 1.0 ppm, 7 cycles/sec.

5′-Deoxyuridine 5′-Phosphonic Acid. The third peak contained 5′-deoxyuridine 5′-phosphonic acid (35%). After removal of the eluent by evaporation, the residual oil was dissolved in ammonium bicarbonate solution (1 ml, 0.1 M, pH 8.5) and *E. coli* phosphomonoesterase (0.1 mg of the salt-fractionated enzyme) was added. The reaction solution was kept at 37° for 18 hr and then chromatographed on DEAE-Sephadex A-25 using the same conditions described above. Two ultraviolet-absorbing peaks were obtained: a neutral peak contained uridine (1.3%) and the second peak 5′-deoxyuridine 5′-phosphonic acid (98%). Uridine was identified by its paper chromatographic behavior in two solvent systems and by its ultraviolet spectral characteristics. The nmr spectrum of 5′-deoxyuridine 5′-phosphonic acid demonstrated the presence of the C$_{5'}$-methylene protons [CH$_2$P(=O)] as a quartet at 2.4 ppm, 19 cycles/sec, and was consistent with the over-all nucleoside phosphonate structure. It reduced 0.93 mole of periodate/uracil residue. Its ultraviolet absorption spectrum was $\lambda_{\max}^{\text{MeOH}}$ 261 mμ, $\lambda_{\min}^{\text{MeOH}}$ 231 mμ (ε 9800) (mol wt 298), and the ε/P was 9200. This material was homogenous in the two paper chromatographic systems and on electrophoresis in the two buffer systems.

5′-Deoxy-2′,3′-O-isopropylideneuridine-5′-phosphonic Acid. Crude diallyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (200 mg, 0.53 mmole) isolated by preparative silicic acid plates was hydrogenolyzed without the previously indicated acetic acid hydrolysis step. The reduction was carried out with prereduced Kuhn's catalyst (10 mg) in methanol (5 ml) containing 2 equiv of triethylamine/equiv of nucleoside derivative. After 3 hr, the catalyst was removed as described previously and the supernatant containing a drop of ammonia was passed onto a DEAE column and chromatographed as previously described. Three principal peaks were eluted at salt concentrations similar to those found for the deacetonated products. Peak I contained di-*n*-propyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (4.5%), peak II contained *n*-propyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (70%), and peak III contained 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonic acid (24%). Confirmation of the structure of each of these substances was obtained by relating them to their deacetonated derivatives. Each of the samples was contaminated with a small amount of nonultraviolet-absorbing phosphonate esters which could be removed by absorbing the nucleotide derivative onto charcoal, washing with water, and then eluting the nucleotide with 10% concentrated ammonium hydroxide in 50% aqueous ethanol (v/v).

5′-Deoxyuridine 5′-Phosphonomorpholidate. This substance was prepared essentially by the general procedure of Moffatt and Khorana (1961). The reaction was complete in 2 hr as evidenced by a single ultraviolet-absorbing component after paper chromatography and electrophoresis. The isolatable yield was 70% ($\lambda_{\max}^{\text{MeOH}}$ 260 mμ, $\lambda_{\min}^{\text{MeOH}}$ 231 mμ (ε/P = 8900)). Treating with hydrochloric acid (1 N) for 20 min at room temperature completely hydrolyzed the morpholidate to yield 5′-deoxyuridine 5′-phosphonic acid. An attempt to prepare 5′-deoxyuridine 5′-phosphonylphosphate from the morpholidate using the method of Moffatt and Khorana (1961) yielded a somewhat complex mixture of ultraviolet-absorbing substances, one component being the desired product.

5′-Deoxyuridine 5′-Phosphonylphosphate. 5′-Deoxyuridine 5′-phosphonic acid (10 μmoles) as the anhydrous mono(tri-*n*-butylammonium) salt was dissolved in dry dimethylformamide (0.5 ml). To this solution was added 1,1′-carbonyldiimidazole (15 mg) and the mixture was shaken until a solution was obtained. The tightly stoppered reaction mixture was kept at room temperature in a desiccator for 8 hr. Analysis of a portion of the reaction mixture by paper chromatography in solvent A and paper electrophoresis at pH 7.5 showed only one ultraviolet-absorbing component with behavior consistent to that expected for the nucleoside phosphonoimidazolidate. Anhydrous mono(tri-*n*-butylammonium) phosphate (100 μmoles) (Hoard and Ott, 1964) in dry dimethylformamide (0.5 ml) was added to the nucleoside phosphonoimidazolidate and the reaction solution was kept in a desiccator at room temperature for 12 hr. After this time, water (4 ml) was added and the solution was kept in the cold for 1 hr. Ammonium hydroxide (0.1 ml, 28%) was then added and the solution was passed onto a column of DEAE-Sephadex A-25 (2.5 × 25 cm) in the bicarbonate form. The elution was carried out at 0° using a linear gradient of triethylammonium bicarbonate as previously described except that the reservoir contained 0.45 M triethylammonium bicarbonate. Three main fractions were obtained from the column. Each fraction was combined and concentrated by lyophilization. The first fraction was identified as 5′-deoxyuridine 5′-phosphonic acid (60%) as previously described. The second fraction obtained at a salt concentration of 0.18 M was identified as 5′-deoxyuridine 5′-phosphonylphosphoric acid (30%) by the following criteria. On paper electrophoresis at pH 4.0 it migrated as a dianion, while at pH 7.5 its migration was that of a trianion; hydrolysis by *E. coli* phosphomonoesterase and *C. adamanteus* phosphodiesterase for 8 hr (1 μmole of phosphonylphosphate in 0.1 ml of 0.05 M Tris·HCl buffer, pH 8.9, containing 10 μg of either enzyme) yielded only 5′-deoxyuridine 5′-phosphonic acid and inorganic phosphate as evidenced by paper chromatography. 5′-Deoxyuridine 5′-phosphonylphosphate is stable at pH 8.9 for 18 hr at 37°; hydrolysis in 1 N HCl at 100° for 7 min yielded 1 mole of inorganic phosphate (Fiske and Subbarow, 1925) per mole of 5′-deoxyuridine 5′-phosphonic acid. Its ultraviolet spectrum at pH 7.0 (0.01 M Tris·HCl) showed a $\lambda_{\max}^{\text{pH 7.0}}$ at 262 mμ and a $\lambda_{\min}^{\text{pH 7.0}}$ at 233 mμ (ε/P = 5400).

The third fraction was eluted at a salt concentration (0.35 M) which was consistent for a tetraanion and was identified as 5′-deoxyuridine 5′-phosphonylpyrophosphate (8%) by comparing its chromatographic properties with that of the same derivative synthesized by an independent method (see below).

5′-Deoxyuridine 5′-Phosphonylpyrophosphate. To an anhydrous solution of dimethylformamide (1 ml) containing the mono(tri-*n*-butylammonium) salt of 5′-deoxyuridine 5′-phosphonic acid (10 μmoles) was added solid 1,1′-carbonyldiimidazole (15 mg). The solution was tightly stoppered and kept in a desiccator at room temperature for 9 hr. After this period, an anhydrous solution of mono(tri-*n*-butylammonium) pyrophosphate (50 μmoles) in dimethylformamide (1 ml) was added and the reaction was kept in a desiccator at room temperature for 18 hr. This solution was processed in a manner similar to that described for 5′-deoxyuridine 5′-phosphonylphosphate except that the eluting solvent in the reservoir contained 0.55 M triethylammonium bicarbonate. Three fractions were obtained by chromatography on DEAE-Sephadex. The first fraction was eluted at a salt concentration of 0.15 M and contained 5′-deoxyuridine 5′-phosphonylphosphate (14%). The second fraction was eluted at a salt concentration of 0.35 M and contained 5′-deoxyuridine 5′-phosphonylpyrophosphate (67%). On paper electrophoresis at pH 4.0 this substance migrated as a trianion and at pH 7.5 as a tetraanion. It was shown to be homogeneous in two paper chromatographic systems where its R_F values were consistent with those expected for a 5′-phosphonylpyrophosphate structure. Enzymatic hydrolysis using the phosphomonoesterase and phosphodiesterase, using the same conditions as described for 5′-deoxyuridine 5′-phosphonylphosphate, yielded 5′-deoxyuridine 5′-phosphonate as the only ultraviolet-absorbing component. Treatment with 1 N HCl for 7 min at 100° yielded 5′-deoxyuridine 5′-phosphonate and 2 moles of inorganic phosphate (Fiske and Subbarow, 1925). Its ultraviolet spectrum at pH 7.0 (0.01 M Tris·HCl) had a $\lambda_{\max}^{\text{pH 7.0}}$ at 261 mμ and a $\lambda_{\min}^{\text{pH 7.0}}$ at 231 mμ. The ϵ/P ratio was 3400. The third fraction was eluted at a salt concentration of 0.45 M and was tentatively identified as 5′-deoxyuridine 5′-phosphonyltriphosphate (14%) by its migration rate on paper electrophoresis at pH 4.0 and 7.5.

Attempts at Enzymatic Polymerization of 5′-Deoxyuridine 5′-Phosphonylphosphate. The polymerization solution contained 5′-deoxyuridine 5′-phosphonylphosphate (2 μmoles), $MgCl_2$ (0.01 M), Tris·HCl (0.5 M, pH 8.9), KCl (1 M), and purified (Singer and Guss, 1962) *Micrococcus lysodeikticus* polynucleotide phosphorylase (10 μg). The total volume was 0.15 ml. The reaction was followed by paper chromatography using *n*-propyl alcohol–ammonia–water (6:3:1, v/v) as the developing solvent. Aliquots (0.02 ml) of the reaction solution were removed at 30, 60, 90, and 240 min and 8 hr after addition of the enzyme. The only ultraviolet-absorbing component detected on paper was 5′-deoxyuridine 5′-phosphonylphosphate. In another experiment, the same polymerization solution was used except with 2 ODU (the absorbancy at 260 mμ, pH 7, of a 1-ml solution of the nucleotide in a cuvet with a 1-cm light path) of polyuridylic acid having a terminal 5′-phosphate group (a gift from Dr. Leon Heppel) and having average chain length of 18 nucleotides. Aliquots (0.02 ml) were removed as described and chromatographed after a pretreatment with pancreatic ribonuclease (1 μg) for 1 hr at 37°. The chromatograms demonstrated the presence of only 5′-deoxyuridine 5′-phosphonylphosphate and uridine 3′-phosphate. In control experiments using uridine 5′-diphosphate in place of the nucleoside phosphonate, maximum polymerization to polyuridylic acid was achieved in 90 min.

Acknowledgment

The authors are indebted to Dr. J. G. Moffatt of this institute for his helpful comments.

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Some Observations Relating to Acyl Mobility in Aminoacyl Soluble Ribonucleic Acids*

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ABSTRACT: A technique, based on nuclear magnetic resonance spectroscopy, has been developed for determining the rate of acyl migration in ribonucleoside derivatives. In buffered dimethyl sulfoxide solution at 20°, the rate of conversion of 3′-*O*-formyladenosine into an equilibrium mixture of 2′ and 3′ isomers has been found to be *ca.* 7000 times faster than the equilibration rates of 3′-*O*-acetyladenosine and -uridine. The rate of hydrolysis of 2′(3′)-*O*-formyladenosine in 0.1 M phosphate buffer (pH 7) at 20° is close to that reported for an average aminoacyl derivative of adenosine or soluble ribonucleic acid (s-RNA), but *ca.* 230 times faster than that of 2′(3′)-*O*-acetyladenosine. The equilibration and hydrolysis rates of 2′(3′)-*O*-acyl derivatives of adenosine are slightly (10–15%) faster than those of the corresponding uridine derivatives. The respective half-times of hydrolysis and equilibration of 2′(3′)-*O*-acetyluridine in 0.1 M phosphate buffer at 20° are *ca.* 30 days and 7.5 sec; their ratio is 350,000.

From these data and a suitable temperature correction, the equilibration half-time of an average aminoacyl-s-RNA derivative has been estimated to be *ca.* 2×10^{-4} sec. Thus such derivatives are likely to exist in living organisms as mixtures of 2′ and 3′ isomers.

In the process of *in vivo* protein synthesis, it has been established (Zachau *et al.*, 1958; Preiss *et al.*, 1959; Hecht *et al.*, 1959) that each amino acid is esterified to one of the hydroxyl groups of the *cis*-2′,3′-diol system of the terminal adenosine residue of a specific soluble ribonucleic acid (s-RNA) molecule before it is transferred in the reaction or reactions leading to the formation of a peptide bond (step b). However, it is not known whether the aminoacyl group is attached to the 2′- or the 3′-hydroxyl function of the adenosine residue when this acylation reaction occurs. Indeed, it is not even clear that this is a meaningful question to consider.

$$\text{s-RNA} + \text{aminoacyl-AMP}^{1} \rightleftharpoons \text{aminoacyl-s-RNA} + \text{AMP} \quad \text{(a)}$$

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* From the University Chemical Laboratory, Cambridge, England. *Received June 20, 1966.* This work was supported in part by Research Grant CA-06066-05 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

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[1] Adenosine 5′-monophosphate is abbreviated as AMP. Aminoacyl-AMP represents the mixed anhydride of an amino acid and AMP; aminoacyl-s-RNA represents an aminoacyl derivative of s-RNA in which the amino acid is esterified to the 2′- (or 3′-) hydroxyl group of the terminal adenosine residue.