[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF BRITISH COLUMBIA RESEARCH COUNCIL]

Nucleoside Polyphosphates. III.[1] Syntheses of Pyrimidine Nucleoside-2'(3'),5'-diphosphates

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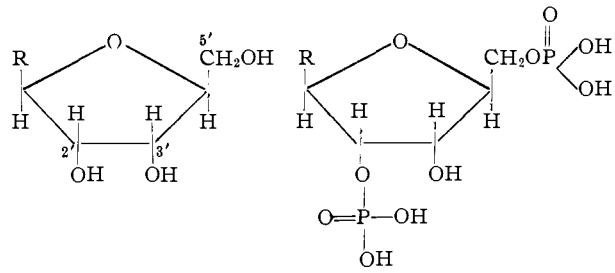
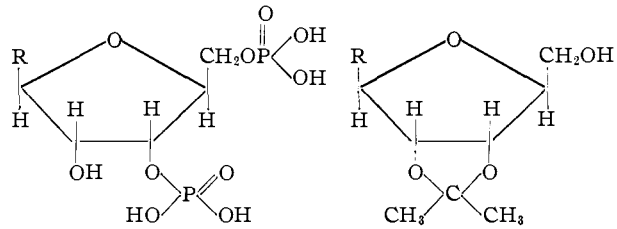
Simple and effective syntheses of uridine- and cytidine-2'(3'),5'-diphosphates have been developed. The reagent employed for the phosphorylation of the unprotected pyrimidine nucleosides was a mixture of phosphorus pentoxide and 85% orthophosphoric acid. By this method, uridine-5'-phosphate was prepared in excellent yield through the use of the readily accessible 2',3'-isopropylidene-uridine.

Of the three possible monophosphates which can be formed from each of the four naturally occurring ribonucleosides (I), only the one bearing the phosphate group on the 5'-position has been identified as a component of a large number of the nucleotide coenzymes.[2] In addition, nucleoside-5'-phosphates have been obtained as products of enzymic degradation of ribonucleic acids.[3] All the members of this group have been synthesized.[4] The second main group of ribomononucleotides comprises the four pairs of isomeric products obtained from the chemical hydrolysis of ribonucleic acids.[5] The isomerism in each of these four pairs is due to the location of the phosphate grouping on either the 2'- or 3'-position (I). Extensive investigations in recent years have established the absolute structures of these degradation products and the synthesis of members of this group also has been recorded.[6] More recently adenosine-2',5'-diphosphate[7] (IIa) and -3',5'-diphosphate[8] (IIIa) have been obtained by the enzymatic degradation, respectively, of triphosphorpyridine nucleotide (TPN) and coenzyme A and the presence of these fragments in the intact coenzymes thus proved. Mixtures of pyrimidine nucleoside-2',5'- and -3',5'-diphosphates (IIb and IIIb) have been encountered by Cohn and Volkin[3a] as products of enzymatic (snake venom diesterase) degradation of ribonucleic acid. These findings are of great interest in connection with the problem of the gross structure of ribonucleic acids. For certain enzymatic studies which will be reported[9] later, cytidine and uridine diphosphates were required and it is the purpose of this communication to describe a simple and effective method for the preparation of these substances. The method developed has also resulted in new and convenient procedures for the preparation in quantity of pyrimidine nucleoside-5'-monophosphates through the use of the readily accessible 2',3'-isopropylidene derivatives[10] (IV).



I, R = purine or pyrimidine

IIa, R = adenine
IIb, R = uracil or cytosine



IIIa, R = adenine
IIIb, R = uracil or cytosine

IV, R = uracil

The problem of the chemical phosphorylation of nucleosides has been the subject of extensive studies in recent years. Although the use of phosphorus oxychloride[10,11] and diphenyl phosphorochloridate[12,13] has been recorded, dibenzyl phosphorochloridate[12,14] has proved to be a very satisfactory phosphorylating agent in the nucleotide field. A

(1) Nucleoside Polyphosphates. II. Ross H. Hall and H. G. Khorana, THIS JOURNAL, **76**, 5056 (1954).

(2) All the known nucleoside triphosphates are derivatives of nucleoside-5'-phosphates. Adenosine-5'-phosphate is a component of certain well-known coenzymes, *e.g.*, flavin-adenine dinucleotide, diphosphopyridine nucleotide. More recent examples of biologically important pyrophosphates containing nucleoside-5'-phosphates are uridine diphosphate glucose (R. Caputto, L. F. Leloir, C. E. Cardini and A. C. Paladini, *J. Biol. Chem.*, **184**, 333 (1950)) and guanosine diphosphate mannose (E. Cabib and L. F. Leloir, *ibid.*, **206**, 779 (1954).

(3) (a) W. E. Cohn and E. Volkin, *ibid.*, **203**, 319 (1953); (b) *Nature*, **167**, 483 (1951); (c) *Arch. Biochem. Biophys.*, **35**, 465 (1952).

(4) (a) For the synthesis of adenosine-5'-phosphate see J. Baddiley and A. R. Todd, *J. Chem. Soc.*, 648 (1947), and the references cited therein. (b) Uridine-5'-phosphate was first synthesized by Levene and Tipson (*J. Biol. Chem.*, **106**, 113 (1934)), later by Gulland and Hobday (*J. Chem. Soc.*, 746 (1934)) and more recently by A. M. Michelson and A. R. Todd (*ibid.*, 2476 (1949)). An improved synthesis has been described by R. H. Hall and H. G. Khorana, THIS JOURNAL, **76**, 5056 (1954)). (c) For cytidine- and guanosine-5'-phsphates see A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2476 (1949).

(5) For a complete survey of pertinent literature on the subject see the excellent article by J. X. Khym and W. E. Cohn, THIS JOURNAL, **76**, 1818 (1954).

(6) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952); D. M. Brown, G. D. Fasman, D. I. Nagrath and A. R. Todd, *ibid.*, 1448 (1954).

(7) A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **186**, 557 (1950).

(8) T. P. Wang, L. Shuster and N. O. Kaplan, *ibid.*, **206**, 299 (1954) and references to earlier literature cited therein.

(9) This work is in progress in the laboratory of Dr. C. A. Dekker, Department of Biochemistry, University of California, Berkeley.

(10) (a) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934); (b) **121**, 131 (1937); (c) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2476 (1949).

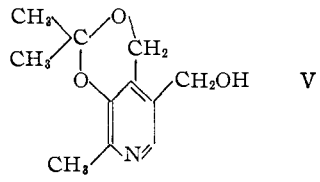
(11) J. M. Gulland and G. I. Hobday, *ibid.*, 746 (1940).

(12) New nomenclature of phosphorus compounds adopted by the International Union of Pure and Applied Chemistry; see *e.g.*, *J. Chem. Soc.*, 5122 (1952).

(13) H. Bredereck, E. Berger and J. Ehrenberg, *Ber.*, **73**, 269 (1940).

(14) (a) G. W. Kenner, "The Chemistry of Nucleotides," in L. Zechmeister, "Progress in the Chemistry of Organic Natural Products," Vol. VIII, 1951, p. 97; (b) F. R. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945), and subequent papers in the series.

straightforward extension of the general method developed by the Cambridge workers to the preparation of nucleoside-2′(3′),5′-diphosphates is exemplified by the phosphorylation of thymidine and desoxycytidine to give the corresponding 3′,5′-diphosphates.[15] In view of our interest, however, in developing alternative methods of simpler nature for the preparation of biologically interesting phosphates we have investigated the use of a mixture of phosphorus pentoxide and orthophosphoric acid[16] for the phosphorylation of nucleosides. Although numerous examples of the use of phosphorylating agents of "polyphosphoric acid"[17] type have been recorded, their possible application in the nucleotide field does not appear to have been examined. This is probably due to the well-known lability of the N-glycosidic linkage especially in the purine nucleosides. Our interest in the possible utility of phosphorus pentoxide–phosphoric acid mixtures[18] as phosphorylating agents was enhanced by the knowledge of the successful phosphorylation by Baddiley and Mathias[19] of isopropylidene-pyridoxine (V) with the retention of the highly acid labile isopropylidene group. It was clear that a similar method if applicable to the phosphorylation of the easily prepared 2′,3′-isopropylidine nucleo-



V

sides would furnish a convenient method for the preparation of the important nucleoside-5′-monophosphates.

2′,3′-Isopropylidene uridine (IV) suspended in a mixture of phosphorus pentoxide and phosphoric acid dissolved gradually at 60° and the nature and extent of the reaction was followed by paper chromatography after heating aqueous solutions of aliquots of the reaction mixture at 100° to hydrolyse inorganic polyphosphates and to remove the isopropylidene group. Table I (Experimental part) summarises the results thus obtained. After a two-hour reaction period uridine-5′-phosphate was the main product of the reaction, longer periods of time resulting in some "phosphorolytic" cleavage of the isopropylidene group to form the diphosphates. (The identification of these ultimate products of the reaction is discussed below.) In a large scale (5 g.) two hour experiment, after hydrolytic treatment, most of the resulting orthophosphoric acid was removed as lithium phosphate and uridine-5′-phosphate, the major product, was freed from the small amounts of the accompanying uridine and uridine diphosphates on a Dowex 2 ion-exchange column. After a water wash which removed uridine, uridine-5′-phosphate was eluted, along with the residual orthophosphoric acid, with 0.015 *N* hydrochloric acid. Pure uridine-5′-phosphate was finally isolated by the procedure described in the Experimental section as the barium salt in a yield of around 65%. The use of 2′,3′-isopropylidene uridine and the study of the time course of the reaction leaves little doubt concerning the identity of this product. The R_f values of this material on paper chromatograms in several solvent systems were identical with those of a sample of uridine-5′-phosphate prepared by the previously described methods. The elegant ion-exchange technique of Cohn and Volkin[3] which successfully differentiated uridine-5′-phosphate from the isomeric 2′- and 3′-phosphates was used as a further check and the product prepared by the present method was shown quantitatively to be the 5′-isomer. Addition of a small proportion of yeast uridylic acid (mixture of 2′- and 3′-phosphates) to the synthetic product gave rise to new peaks upon ion-exchange analysis. Finally rigid chemical evidence was also obtained concerning the identity of the above product. It has been established that nucleoside-5′-phosphates on treatment with dicyclohexylcarbodiimide in aqueous pyridine form exclusively the P^1,P^2-dinucleoside-5′-pyrophosphates[20] whereas nucleoside-2′-or-3′-phosphates under identical conditions produce initially the corresponding 2′,3′-cyclic phosphates and the latter react further to give N-phosphorylureas.[21] This reaction was carried out on the synthetic sample and P^1,P^2-diuridine-5′-pyrophosphate[20a] was shown to be the only product of reaction. The identification of the synthetic sample having been established, a highly practical method becomes available for the preparation of uridine-5′-phosphate. By a similar procedure we have also prepared cytidine-5′-phosphate in excellent yield and will report on it in another place. The phosphorylation of suitably protected purine nucleosides by the use of phosphorus pentoxide–phosphoric acid mixtures was not satisfactory, the nucleosides undergoing extensive decomposition.

The phosphorylation of unprotected uridine and cytidine was now examined. Treatment of these nucleosides with phosphorus pentoxide–phosphoric acid mixture for a period of as long as 20 hours did not cause any significant degradation of these substances and on working up and ion-exchange analysis the corresponding diphosphates were obtained in yields of the order of 80%. The identity of these products which were finally isolated as their barium salts was deduced first from their behavior on ion-exchange columns and paper chromatograms and more firmly from the elemental analyses of the barium salts. Electrometric titration of the

(15) C. A. Dekker, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 947 (1953).

(16) Use has been made of this mixture in the phosphorylation of hydroxyl groups in hydroxyamino acids and proteins; P. A. Levene and Schormueller, *J. Biol. Chem.*, **105**, 547 (1934); R. H. A. Plimmer, *Biochem. J.*, **35**, 461 (1941); R. E. Ferrell, H. S. Olcott and H. Fraenkel-Conrat, THIS JOURNAL, **70**, 2102 (1948). More recently, pyridoxal phosphate and related compounds have been prepared in good yield through this reagent; A. N. Wilson and S. A. Harris, THIS JOURNAL, **73**, 4693 (1951); E. A. Peterson and H. A. Sober, *ibid.*, **76**, 169 (1954). See also reference (19).

(17) G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley and Sons, Inc., New York, N. Y., 1950.

(18) Some information concerning the reaction of orthophosphoric acid with phosphorus pentoxide is given by E. Cherbuliez, J. P. Leber and M. Schwarz (*Helv. Chim. Acta*, **36**, 1189 (1953)).

(19) J. Baddiley and A. P. Mathias, *J. Chem. Soc.*, 2583 (1952).

(20) (a) S. M. H. Christie, D. T. Elmore, G. W. Kenner, A. R. Todd and F. J. Weymouth, *J. Chem. Soc.*, 2947 (1953); (b) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954).

(21) C. A. Dekker and H. G. Khorana, *ibid.*, **76**, 3522 (1954); C. A. Dekker, *Federation Proc.*, **13**, 197 (1954).

dibarium salt of uridine diphosphate showed that the ratio of the secondary phosphoryl groups (titrated between *p*H 4 and 8) to the enolic hydroxyl groups (located at the 6-position of the uracil ring and titrated between *p*H 8 and 10) was two to one, thus confirming that two monoesterified phosphoric acid residues were located on every uridine molecule. Because of the well-known high reactivity of the 5′-hydroxyl group, compared with that of 2′- or 3′-hydroxyl groups in substitution reactions, and the above described rapid formation of 5′-phosphate from 2′,3′-isopropylidene uridine it is certain that one of the two phosphate residues in the synthetic diphosphates is located at the 5′-position. (Further evidence in support of this conclusion is presented below.) Because of the acidic treatment involved during the isolation procedure and the known facile migration of the phosphate grouping between the 2′- and 3′-positions under acidic conditions, it is clear that the above products were an equilibrium mixture of 2′,5′- and 3′,5′-diphosphates. That the two isomers were present roughly in equal amounts was concluded from the study of the rate of degradation of the synthetic materials by the specific b(3′)-nucleotidase recently discovered by Shuster and Kaplan.[22] The degradation of uridine diphosphate followed an exponential curve levelling off when approximately 50% of the diphosphate had been degraded. The course of the action of the enzyme on cytidine diphosphate was less clear but appeared to follow the same general order. The corresponding monophosphates which were the main products of degradation were shown to be the 5′-phosphates. This work was essentially the repetition of Cohn and Volkin's experiments which enabled these authors to ascertain the location of the phosphate groups in diphosphates obtained from the enzymatic degradation of ribonucleic acid.

Exhaustive phosphorylation of a nucleoside, *e.g.*, uridine, should in principle lead to the formation of nucleoside-2′,3′,5′-triphosphate. In view of the importance of compounds of this type for the elucidation of the structure of ribonucleic acids we have devoted some effort to the preparation of uridine-2′,3′,5′-triphosphate by the prolonged treatment of uridine and yeast uridylic acid (mixture of uridine-2′-and-3′-phosphates) with phosphorus pentoxide and phosphoric acid mixtures and examination of polyphosphates eluted from ion-exchange columns after the removal of the above described diphosphates. The results of this work as well as the results of a general study of the properties of vicinal diphosphates will be reported in a subsequent communication.

Experimental

Phosphorylation of 2′,3′-Isopropylidene Uridine.—Two hundred mg. of 2′,3′-isopropylidene uridine (previously dried at 110° overnight in a vacuum) was stirred into a warm freshly prepared solution (1 cc.) of phosphorus pentoxide in 85% phosphoric acid (1:1.3, w./w.) and the mixture, which was stirred at intervals, was maintained at 60° with the exclusion of moisture. Isopropylidene uridine gradually dissolved to form a light yellow solution. 0.2-cc. aliquots were removed at intervals and diluted with 2 cc. of water. The clear solutions were heated at 100° for one-half hour and then neutralized with 4.5 *N* lithium hydroxide solution. The supernatants after centrifugation of lithium phosphate were examined by paper chromatography in the solvent system 1% ammonium sulfate–isopropyl alcohol (1:2, v./v.). Three spots located by their absorption of ultraviolet light had R_f values corresponding to uridine, uridine-5′-phosphate and uridine-2′(3′),5′-diphosphate. Their relative concentrations were determined by elution with 3 cc. of 0.01 *N* hydrochloric acid and measurement of the optical density of the resulting solutions at 260 mμ. Table I records the results thus obtained.

TABLE I

PHOSPHORYLATION OF 2′,3′ ISOPROPYLIDENE URIDINE

Time, hr.	Uridine, %	Uridine-5′-phosphate, %	Uridine-2′(3′),5′-diphosphate, %
1	37.4	62.6	..
2	11.7	80.4	7.9
3.5	6.3	70.6	23.1

Uridine-5′-phosphate.—Five grams of dry 2′,3′-isopropylidene uridine (prepared in 90% yield from uridine by the method of Levene and Tipson[10a]) was phosphorylated with 25 cc. of warm freshly prepared solution of phosphorus pentoxide in 85% phosphoric acid as described above. After a reaction period of two hours, 100 cc. of cold water was added and the clear solution heated at 100° for one-half hour when the solution attained a light pink color. It was neutralized with 4.5 *N* lithium hydroxide solution to *p*H 9 and the heavy precipitate of lithium phosphate removed by centrifugation, the precipitate being thoroughly washed with three 40-cc. portions of water. The combined filtrate and washings (*ca.* 500 cc.) were concentrated under reduced pressure to around 50 cc. when some more lithium phosphate precipitated. This was removed and the clear solution absorbed on the top of a Dowex 2 ion-exchange resin (200–325 mesh, chloride form) column (14 cm. long × 4 cm. diameter) and the column washed with water until the optical density of the effluent fell below 0.05. After removal of uridine in this way (*ca.* 1 liter of water being required), 0.015 *N* hydrochloric acid solution was passed through the column at a flow rate of 15 cc. per minute. The optical density (at 260 mμ) of the effluent began to rise after 800 cc. of the solution had passed through the column. Four liters of eluate containing uridine-5′-phosphate (orthophosphoric acid also comes off simultaneously) were collected before the optical density fell below 0.8 again. This solution was concentrated at 30–35° under reduced pressure to *ca.* 20 cc. and the concentrate sucked under a high vacuum for six hours and the final residue kept in an evacuated desiccator over potassium hydroxide and phosphorus pentoxide for two days. It was then washed twice with 50-cc. portions of dry ether, dissolved in 10 cc. of anhydrous ethyl alcohol and uridine-5′-phosphate precipitated by the addition of 100 cc. of anhydrous ether. The last operation was repeated twice, the ethereal layer being clarified through centrifugation. The residual gum which was almost completely free from orthophosphoric acid changed into a brittle resin on storage in an evacuated desiccator over phosphorus pentoxide. It was taken up in 40 cc. of water, neutralized to *p*H 9 with 4.5 *N* lithium hydroxide and to it was then added 10 cc. of 2 *M* barium acetate solution and the mixture set aside for several hours. The precipitate (0.340 g. dry weight) of barium phosphate (admixed with some barium uridine-5′-phosphate) was removed by centrifugation and barium uridine-5′-phosphate was now precipitated by the addition of an equal volume of ethyl alcohol. It was collected by centrifugation and washed twice with 25-cc. portions of 50% ethyl alcohol, then ethyl alcohol and finally ether. Yield of hydrated barium salt[22a] 5.42 g., found to contain 3.74 g. of free uridine-5′-phosphate (65%). Paper chromatography in a number of solvent systems[1] gave a single strong spot, having R_f values identical with those of a sample of uridine-5′-phosphate prepared by the method described earlier.[1]

(22) L. Shuster and N. O. Kaplan, *J. Biol. Chem.*, **201**, 535 (1953).

(22a) ADDED IN PROOF.—The barium salt has now been converted quantitatively to the highly crystalline disodium salt. An aqueous solution of the barium salt was passed through a Dowex 50 ion exchange resin (hydrogen form) column and the solution of the free acid was neutralized to *p*H 8 with sodium hydroxide and concentrated to a small volume. On the addition of a small proportion of acetone, the disodium salt crystallized spontaneously.

Reaction of Synthetic Uridine-5′-phosphate with Dicyclohexylcarbodiimide.—Samples of 10 mg. each of the free uridine-5′-phosphate (as prepared above in the form of a brittle resin, before conversion to the barium salt) and yeast uridylic acid (Schwarz Laboratories) were brought into reaction in aqueous pyridine (0.05 cc. water and 0.3 cc. pyridine) with 100 mg. of dicyclohexylcarbodiimide. The reaction mixtures were shaken for ten hours before dilution with water, removal of precipitated urea and removal of excess of pyridine through repeated extraction with ether. Paper chromatography in isopropyl alcohol–ammonia–water (70-5-25 by volume) showed the formation in the case of yeast uridylic acid of uridine-2′,3′-cyclic phosphate (R_f, 0.43) and N-uridyl urea (R_f, 0.90); a strong spot near the base line (R_f, 0.14) appeared as the only reaction product from synthetic uridine-5′-phosphate. Paper chromatography in 1% ammonium sulfate–isopropyl alcohol (1:2 v./v.) resolved the reaction product from uridine-5′-phosphate to two spots, one (R_f, 0.55) corresponding to unchanged material and the second heavy spot (R_f, 0.39) corresponding to P¹,P²-diuridine-5′-pyrophosphate.

Ion-exchange Analysis of Uridine Phosphates.—Four mg. of yeast uridylic acid was dissolved in 1 cc. of water and the solution after neutralization to *p*H 9 with ammonium hydroxide was applied to the top of a Dowex 2 ion-exchange resin (200–325 mesh, formate form) column (8 cm. long × 1 cm. diameter) and after a water wash elution was carried out with 0.01 *M* formic acid + 0.05 *M* sodium formate solution. Five-cc. fractions were collected and a flow rate of 1 cc./min. was maintained. Ultraviolet absorbing material was eluted between fractions 90 and 140, the elution diagram indicating lack of one sharp peak. On a fresh column of the same dimensions and under identical conditions 5 mg. of synthetic uridine-5-phosphate emerged as a single sharp peak between fractions 40 and 56, no further ultraviolet absorbing material appearing on prolonged elution with the above eluent or one containing higher concentration of sodium formate. In a third experiment, 1 mg. of yeast uridylic acid was added to 5 mg. of synthetic uridine-5′-phosphate and ion-exchange analysis repeated again. After the main peak between fractions 37 and 52 (uridine-5′-phosphate), the added yeast uridylic acid was eluted between fractions 110 and 142.

Uridine-2′(3′),5′-diphosphate.—To one gram (4.1 mmoles) of uridine (dried previously at 110° (0.1 mm.) over phosphorus pentoxide for 12 hours) was added 5 cc. of warm freshly prepared phosphorylating agent (see above) and the sealed reaction flask maintained at 60° in an oven. Uridine dissolved under frequent agitation during the first one-half hour to form a clear dark sirup. Direct examination by paper chromatography of a suitable amount of the fluid removed after a period of 2.5 hours and diluted with water showed only a small amount of unreacted uridine. After a total period of 20 hours the sirup was dissolved in 60 cc. of water and a small quantity of 6 *N* hydrochloric acid added to reduce the *p*H of the aqueous solution to 0. After being heated at 100° for 15 minutes, the solution was neutralized with 4.5 *N* lithium hydroxide solution to *p*H 9. The heavy precipitate of lithium phosphate was removed by centrifugation and washed thoroughly with small portions of water. The combined supernatants were allowed to pass slowly through a bed (12.5 sq. cm. × 4.2 cm.) of Dowex 2 ion-exchange resin (200–325 mesh,[23] chloride form). After a water wash which removed some uridine (1500 cc. of eluate with optical density at 260 mμ of 1.19, equivalent to 4.4% of the amount of uridine used), uridine monophosphates and orthophosphoric acid were eluted with 0.01 hydrochloric acid +0.015 *M* sodium chloride solution (total volume, 4 liters[24] of optical density at 260 mμ, 0.47, representing 4.7% of the amount of uridine used). The diphosphates were then eluted with 0.01 *N* hydrochloric acid +0.1 *M* sodium chloride solution [900 cc. of optical density at 260 mμ, 36.3 (measured by appropriate dilution) representing a conversion of 80.6% of the amount of uridine used to the diphosphates]. This eluate was neutralized with sodium hydroxide and then concentrated under partial pressure to *ca.* 18 cc. After filtration of the solution, to remove any suspended matter, through a sinter glass funnel, which was later washed with 2 cc. of water, the *p*H was adjusted to 9 with lithium hydroxide solution and the barium salts were precipitated by the addition of 7 cc. of 2 *M* barium acetate solution and collected through centrifugation. These were washed thrice with 50% ethyl alcohol, then ethyl alcohol and ether and allowed to equilibrate with air at room temperature; wt. 2.52 g., 75% yield based on the molecular formula $C_9H_{10}N_2O_{12}P_2Ba_2 \cdot 8H_2O$ (mol. wt., 819.04). *Anal.*[25] Calcd. for $C_9H_{10}N_2O_{12}P_2Ba_2 \cdot 8H_2O$: C, 13.2; H, 3.2; N, 3.4; P, 7.6. Found: C, 13.3; H, 3.25; N, 3.2; P, 7.4.

41.7 mg. of this sample was suspended in 0.15 *M* sodium chloride solution and brought into solution by the gradual addition of hydrochloric acid solution. The solution was then titrated with 1 *N* sodium hydroxide solution at 25° under nitrogen atmosphere using an automatic electrometric titration apparatus.[26] One hundred micromoles of the base was required to titrate the secondary P–OH groups between *p*H 4 and 8, 50 micromoles being further required to titrate the enolic hydroxyl group of the uracil ring between *p*H 8 and 10.3. From this the molecular weight of the barium salts was calculated to be 834.

Cytidine-2′(3′),5′-diphosphate.—A mixture of two hundred mg. (0.824 mmole) of cytidine (previously dried at 110° (0.1 mm.) over phosphorus pentoxide for 12 hours) and 1 cc. of a freshly prepared solution of phosphorus pentoxide in phosphoric acid (1:1.3, w./w.) was heated when cytidine slowly dissolved to give a clear homogeneous sirup. After a period of 20 hours the sirup was dissolved in 15 cc. of water and the solution treated as described above for uridine diphosphate. After removal of lithium phosphate the solution (*ca.* 50 cc.) was slowly passed through a Dowex 2 (200–325 mesh, formate form) ion-exchange bed (12.5 sq. cm. × 2.0 cm.). After a water wash (100 cc.) which removed only negligible amount of ultraviolet absorbing material (absence of cytidine), 1500 cc. of 0.01 *N* formic acid +0.05 *N* sodium formate[27] solution were passed, a flow rate of 15 cc. per minute being maintained during this and the following elutions. Cytidine monophosphates thus removed corresponded to 4.7% of the amount of cytidine employed. The diphosphates were next eluted with 4.0 *N* formic acid +0.01 *M* sodium formate[28] (volume, 450 cc. of optical density at 280 mμ, 17.2 corresponding to 0.61 mmole[29] or 74% of the amount of cytidine employed). Subsequent elution with 1.0 *N* formic + 1.0 *M* sodium formate removed a small amount of material (840 cc. of optical density at 280 mμ, 0.363; 3% of the amount of cytidine employed) corresponding presumably to 2′,3′,5′-triphosphate. The eluate containing the diphosphates now was concentrated to half its volume *in vacuo*, then diluted with an equal volume of water and re-evaporated. This process was repeated four times before the volume was reduced to 25 cc., this solution being freeze-dried. The residue was dissolved in 2.5 cc. of water, filtered through a fritted glass funnel, which was subsequently washed with 1 cc. of water. To the combined filtrate and washing, after neutralization with 4.5 *N* lithium hydroxide solution to *p*H 9, was added 1.6 cc. of 2 *M* barium acetate solution. Ten cc. of 95% ethyl alcohol was added and the precipitated barium salts were collected by centrifugation and washed thoroughly with three portions of 60% ethyl alcohol, then with ethyl alcohol and ether; yield 430 mg. *Anal.* Calcd. for $C_9H_{11}N_3O_{11}P_2Ba_2 \cdot 4H_2O$: C, 14.5; H, 2.6; N, 5.6. Found after drying at 110° (0.1 mm.) over phosphorus pentoxide for 12 hours, the sample being hygroscopic: C, 14.8; H, 2.6; N, 5.2.

Action of Barley b(3′)-Nucleotidase on Uridine and Cytidine Diphosphates.—The specific b(3)-nucleotidase was prepared from *Olli* strain of barley according to the directions of Shuster and Kaplan.[22] The preparation retained a slight amount of a- and 5-nucleotidase activity as was

(23) Prepared from the commercially available 200–400 mesh resin by removing fines with 325 mesh sieve.

(24) This large volume of eluate was collected to ensure complete removal from the column of orthophosphoric acid.

(25) Analyses were performed by Mr. V. Tashinian, Microchemical Laboratory, University of California, Berkeley 4.

(26) The unit employs a Model R Beckman *p*H meter, a Minneapolis–Honeywell Brown Electronic Recorder and a Gilmont ultramicroburet.

(27) *Cf.* reference 3b. The large volume of this eluant used ensured complete removal of inorganic phosphate.

(28) *Cf.* R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *J. Biol. Chem.*, **209**, 23 (1954). This eluant was used in order to elute the diphosphate rapidly, maintaining very low salt concentration.

(29) The figure of 12700 at *p*H 2 has been used for the molecular extinction coefficient of cytidine nucleotides at 280 mμ.

shown by tests on standard mononucleotides. A solution of 32 micromoles of the synthetic nucleoside diphosphates in 1 cc. of water was added to 16 cc. of tris-(hydroxymethyl)-aminomethane buffer (*p*H 7.6) containing 1 cc. of enzyme preparation and the mixture incubated at 37°. Ion-exchange analyses by the standard procedures were carried out on aliquots withdrawn at intervals. In the case of uridine diphosphate,[30] after 16 hours 43.6% and after 40 hours 51.5% of the diphosphate had been degraded to the monophosphate, no nucleoside being formed. In the case of cytidine diphosphate[30] after 16 hours 23.8%, after 40 hours 37% and after 64 hours 39% of the diphosphate had been converted to the monophosphate and 13.3% to cytidine. The identification of the monophosphates as the 5′-phosphates was established by ion-exchange procedure similar to the one described above under uridine-5′-phosphate.

Paper Chromatography of Uridine and Cytidine Diphosphates.—The solvent system isopropyl alcohol–1% ammonium sulfate (2:1, v./v.), using Whatman filter paper 1 previously soaked in 1% ammonium sulfate solution and dried, was found to be completely satisfactory in the present investigation; R_f values: uridine, 0.64; uridine-5′-phosphate, 0.45; uridine-2′(3′)-5′-diphosphate, 0.34; cytidine, 0.51; cytidine-5′-phosphate, 0.38; cytidine-2′(3′),5′-diphosphate, 0.22. A second solvent system also found useful, especially for the separation of inorganic phosphate from the nucleotides is that of Ebel[31] isopropyl alcohol (75 cc.)–water (25 cc.)–trichloroacetic acid (5 g.)–ammonia (sp. gr., 0.9); 25 cc. R_f values: inorganic phosphate, 0.68; uridine and cytidine diphosphates, around 0.32.

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The Oxidation of Glucose by Chlorine in Acid Aqueous Solution[1]

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Although the reaction of glucose with chlorine in aqueous solution buffered at *p*H 2.2 by H_3PO_4/NaH_2PO_4 proceeds beyond the gluconic acid stage, the kinetics of reaction to this stage has been investigated through utilization of glucose in great excess. In order to prevent errors arising from the volatility of chlorine in acid solution, a convenient syringe-reactor technique has been devised. Variation of specific rate, both during the course of a reaction and with change in initial concentration of oxidant, added chloride ion and hydrogen ion, leads to the conclusion that Cl_2 is much more reactive than HOCl. At *p*H 2.2 the reaction follows the rate law: $v = k_2(Cl_2)(\text{glucose})$. For anomerically equilibrated glucose at 35.7°, k_2 is approximately 15 liters mole^{-1} hr.$^{-1}$. At a *p*H of 3, the specific rate increases with increased buffer concentration. An analogous phenomenon is discernible in prior work with aqueous bromine.[9,32] A mechanism consistent with the data involves attack of a base on a complex of halogen and carbohydrate.

Introduction

Earlier studies[3] of the oxidation of D-glucose and other simple carbohydrates by aqueous solutions of halogens have been largely devoted to bromine water. These have demonstrated that initial reaction is to the D-gluconic acid stage in strongly acid,[4] alkaline,[5] or buffered feebly acidic[6] media. Under the latter conditions the primary identifiable product is D-glucono-δ-lactone,[7–9] Br_2 is the active component of bromine water[9] and β-D-aldoses react more rapidly than their respective α-anomers.[10] With excess unbuffered bromine water further oxidation occurs very slowly to yield 5-keto-D-gluconic acid[11] along with lesser amounts of D-glucaric acid.[12]

Meager available information suggests that reaction with aqueous chlorine follows a course similar to that with bromine. Thus D-glucose yields D-gluconic acid in both acidic[13] medium and alkaline[5] solution, but at a slower rate and in poorer yield than with bromine. Unlike bromine, unbuffered chlorine water attacks D-gluconic acid rapidly.[14]

The most significant studies involving aqueous chlorine have been devoted to its action in unbuffered solution on various glycosides. The reaction with methyl α- and β-D-glucoside[15] is slower than with glucose, yet it proceeds initially to the D-gluconic acid stage, with 5-keto-D-gluconic acid[15] and D-glucaric acid[16] being formed subsequently. Analogous primary reactions have been established for methyl α- and β-D-galactoside, mannoside, xyloside and β-D-cellobioside.[16,17] In all these cases oxidation is not preceded by hydrolysis[15–17] and the β-glycosides react more rapidly than the respective α-anomers.[15,17]

Since initial attack by chlorine takes place solely at the acetal carbon of the relatively unreactive gly-

(1) Based on a dissertation submitted by Melvin H. Saxe in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Kendall Company Fellow 1949–1952.

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