Preparation of Adenosine-5′ Methyl Phosphate (Ammonium Salt).[27]—Adenosine-5′ phosphate (105 mg., 0.3 mmole) was dissolved in 0.35 ml. of 1 *N* ammonium hydroxide, 0.6 ml. of water and 6.3 ml. of methanol. Dicyclohexylcarbodiimide (309 mg., 1.5 mmoles) was added and the mixture was stirred until it was homogeneous. After 4.5 hours the mixture was worked up using a procedure similar to that described for the amidates (A). The aqueous solution which was obtained after the ether extractions was concentrated to a small volume (*ca.* 1 ml.) streaked on 4 sheets of Whatman 3 MM paper (7 inches wide) and chromatographed in solvent I. The fastest band, corresponding to AMP-OMe was eluted with water and the effluent was evaporated to a dry powder under reduced pressure. This slightly yellow powder was dissolved in aqueous methanol and precipitated by the addition of acetone. The precipitate was collected by centrifugation, washed with acetone and ether, and dried, while still wet with ether, *in vacuo* over phosphorus pentoxide and paraffin at room temperature. The yield was 60 mg. (53%). This material gave a positive Nessler test which is consistent with its formulation as the ammonium salt; R_f solvent I, 0.43; solvent III, 0.60.

Anal. Calcd. for $C_{11}H_{19}N_6O_7P\cdot H_2O$ (396.36): P, 7.82; Ad/P, 1.00. Found: P, 7.49; Ad/P, 0.96. Equilibration of the sample with air gave a weight increase corresponding to 2 additional molecules of water. Calcd. for trihydrate: P, 7.17. Found: P, 6.96.

Characterization of Adenosine-5′ Methyl Phosphate.—A small amount of the above solid (*ca.* 5 mg.) was dissolved in 1 drop of water and 1 drop of 0.1 *M* sodium periodate was added. After 1.5 hours the solution was streaked on a piece of Whatman 1 filter paper (7″ wide) and chromatographed in solvent I for 16 hours. Three bands were located by ultraviolet light: R_f 0.16 (iodate?), 0.26 (dialdehyde, see text) and 0.41 (unreacted starting material?). The 0.26 band was cut out, trimmed to a point at one end and placed flat end down in a small beaker of water. The ultraviolet material was allowed to collect at the pointed tip of the paper. The tip was cut off and placed in 0.5 *M* glycine buffer, *p*H 10. After 24 hours, the solution was concentrated to a small volume (less than 0.1 ml.) and a portion examined by chromatography in solvent I. Ultraviolet absorbing spots were found at R_f 0.18 and 0.47 in addition to a fluorescent spot at 0.29. None of these contained phosphorus, since when the paper was tested for the presence of phosphate the only blue spot which appeared was at R_f 0.12 corresponding exactly to an authentic sample of methyl phosphate which was run simultaneously.

Preparation of a Compound Having the Properties of Adenosine-5′ phosphoroimidazole.—Adenosine-5′ phosphate (35 mg., 0.1 mmole) and imidazole (24 mg., 0.35 mmole) were dissolved in 0.5 ml. of formamide, and 1 ml. of *t*-butyl alcohol was added to give a slightly cloudy solution. Dicyclohexylcarbodiimide (103 mg., 0.5 mmole) was added giving a much more turbid solution. Addition of 0.15 ml. of formamide gave an almost clear reaction mixture. After 24 hours at room temperature, an aliquot was removed for paper chromatography in solvent I. Another small aliquot of the reaction mixture was treated with 1 *N* sodium hydroxide for 1 hour. No change in the spot distribution could be detected. The chromatograms were inspected for ultraviolet absorption, imidazole and phosphorus according to the procedures given under "Chromatography." Besides spots corresponding to AMP and imidazole, a new major component (R_f 0.45) was present. This material absorbed ultraviolet light[20] and gave a positive test for esterified phosphate.[19] When the paper was sprayed with diazotized sulfanilic acid the only spot which appeared corresponded to free imidazole, but the R_f 0.45 material gave a rose-colored spot after the sodium carbonate spray, indicating the presence of an imidazole group.[22]

The *t*-butyl alcohol was removed from the reaction mixture under reduced pressure, and 10 ml. of acetone was added to the remaining solution. After the mixture had stood overnight at −10°, the solid which had precipitated was removed by centrifugation, washed with acetone and dried over phosphorus pentoxide at room temperature *in vacuo* giving 17 mg. of material which was chromatographically shown to be a mixture of AMP and AMP-imidazole. The solid was unstable at room temperature and slowly broke down to AMP and imidazole. The reaction appeared to be somewhat over 50% complete under these conditions. A few other preliminary experiments under different conditions failed to improve the yield. On heating a small sample in boiling water for 15 minutes it was almost completely hydrolyzed to AMP. Similarly, addition of 0.1 *N* hydrochloric acid to an acetone solution caused complete hydrolysis after 0.5 hour at room temperature.

(27) For a more efficient preparative route to this compound see M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, in preparation.

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

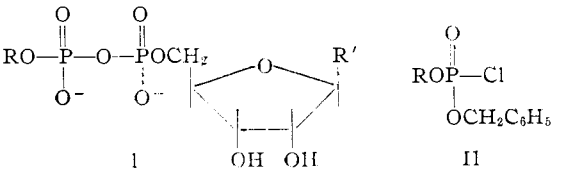
Nucleoside Polyphosphates. VIII.[1] New and Improved Syntheses of Uridine Diphosphate Glucose and Flavin Adenine Dinucleotide Using Nucleoside-5′ Phosphoramidates[2]

BY J. G. MOFFATT AND H. G. KHORANA

RECEIVED JANUARY 27, 1958

The reaction of uridine-5′ phosphoramidate with a fourfold excess of monophenyl phosphate in anhydrous pyridine gave after three days at room temperature P^1-uridine-5′,P^2-phenyl pyrophosphate in 78% yield. By a similar procedure the coenzyme uridine diphosphate glucose was synthesized in an isolated yield of 59% from uridine-5′ phosphoramidate and α-glucose-1 phosphate. Similarly, the reaction of adenosine-5′ phosphoramidate with monophenyl phosphate gave P^1-adenosine-5′,P^2-phenyl pyrophosphate in a high yield. The coenzyme flavin adenine dinucleotide was synthesized in a final yield of 40% from adenosine-5′ phosphoramidate and riboflavin-5′ phosphate using a mixture of pyridine and *o*-chlorophenol as the solvent. A new chromatographic procedure for the preparative separation of flavin nucleotides using cellulose anion exchangers is described. Finally, general observations on the present specific method for the synthesis of nucleotide coenzymes are presented.

Chemical syntheses of a number of nucleotide coenzymes (general formula, I) have been reported in recent years. The procedures employed have broadly been of two types. The first type involves the condensation of a protected nucleoside



I

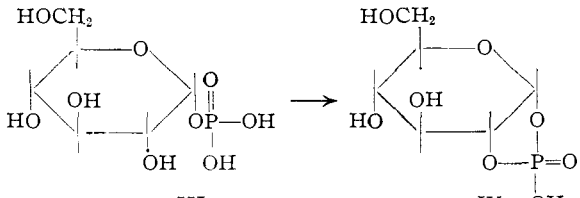
II

Uridine diphosphate glucose; I, R = glucose; R′ = uracil
Flavin adenine dinucleotide; I, R = riboflavin; R′ = adenine

(1) Paper VII, R. W. Chambers and H. G. Khorana, THIS JOURNAL, **80**, 3749 (1957).

(2) This work has been supported by a grant (G 56 29) from the Life Insurance Medical Research Fund, New York.

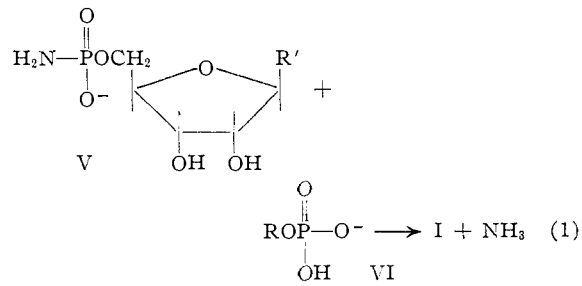
benzyl phosphorochloridate (II, R = protected nucleoside) with the salt of a second phosphate es-



III IV

ter, and then removal of the protecting groups.[3,4] The second method, which has been used more widely recently, utilizes the direct condensation of two unprotected phosphomonoesters in the presence of dehydrating agents such as carbodiimides[5-8] or trifluoroacetic anhydride.[9] Neither method is, however, very satisfactory. Thus, the first method requires the tedious preparation of the necessary intermediates, and the final removal of the protecting groups takes place under conditions which cause considerable breakdown of the pyrophosphate linkage. The second method suffers from the lack of specificity in effecting condensation and a further limitation is encountered in the synthesis of those nucleotide coenzymes where one of the reacting components (*e.g.*, α-glucose-1 phosphate (III) necessary in the synthesis of uridine diphosphate glucose (I)) bears a hydroxyl function suitably placed for an intramolecular cyclic phosphate formation (III → IV).[10] Consequently, the yields of synthetic products of the type I have in general been exceedingly low. Exceptions are the satisfactory syntheses of cytidine diphosphate choline[6] and of diphosphopyridine nucleotide[8] by the carbodiimide method. It is to be noted that in both cases the possibility of cyclic phosphate formation is absent and also that the formation of one of the symmetrical pyrophosphates apparently does not occur, due to an as yet unknown reason.

With a view to devising a more generally satisfactory procedure for the synthesis of the unsymmetrical nucleoside pyrophosphates, Chambers and Khorana[11] investigated the use of phosphoramidic acid derivatives and recorded a satisfactory synthesis of adenosine-5′ diphosphate by treating adenosine-5′ phosphoramidate (V, R′ = adenine; hereafter abbreviated to AMP-NH_2) with an excess of orthophosphoric acid. The results were particularly promising for the problem of the synthesis of true nucleotide coenzymes (I) according to eq. 1, since by providing specific activation of the nucleotide component, only the anions of the second reactant (VI) would be required and, therefore, side reactions such as self-condensation of the latter or its intramolecular cyclic phosphate formation would be precluded. The present communication records the successful application of this approach to highly improved syntheses of the two well-known nucleotide coenzymes, uridine diphosphate glucose (UDPG) and flavin adenine dinucleotide



V VI

(FAD). These two examples, which have been the focus of much previous synthetic work,[3-5,7,9] were considered to provide stringent tests of the superiority of the new method over the existing ones. A brief report of a part of this work has already appeared.[12] The use of monobenzylphosphoramidate in the synthesis of adenosine-5′ di- and triphosphates has been reported recently by Clark, Kirby and Todd.[13]

The problem of the preparation of the nucleoside-5′ phosphoramidates themselves having been solved in a highly satisfactory manner,[14] the outstanding questions in pyrophosphate synthesis pertain to the selection of the media and the reaction conditions. In the earlier work[11] free acids were employed with the view that the phosphorolytic decomposition of the phosphoramidic acids would be enhanced by protonation of these substances, in analogy with their greater lability in aqueous solutions at low *p*H. It has now been found that pyrophosphate synthesis proceeds well in the presence of bases such as pyridine and trialkylamines. The conditions are clearly preferable to those used earlier and all the present experiments were therefore carried out using basic systems.

1,3-Dicyclohexylguanidinium uridine-5′ phosphoramidate[14] (UMP-NH_2) was found to be soluble in pyridine and in a model experiment on its "phosphorolysis" by phosphomonoesters (eq. 1) we employed the readily available, crystalline and anhydrous monophenylphosphoric acid. Paper chromatographic examination of the homogeneous pyridine solution of the reactants showed that UMP-NH_2 had disappeared completely after three days at room temperature and that the major uracil-containing product was the desired P^1-uridine-5′,P^2-phenyl pyrophosphate (UDP-phenol, I, R = phenyl, R′ = uracil). Other substances present, in addition to the excess of phenyl phosphate used, were some uridine-5′ phosphate and a trace of P^1,P^2-diuridine-5′ pyrophosphate. (The formation of this last product is discussed later.) UDP-phenol was separated readily from the contaminants on an ion exchange column and ac-

(3) S. M. H. Christie, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 46 (1954).

(4) A. M. Michelson and A. R. Todd, *ibid.*, 3459 (1956).

(5) G. W. Kenner, A. R. Todd and R. F. Webb, *ibid.*, 2843 (1954).

(6) E. P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956).

(7) F. M. Huennekens and G. L. Kilgour, THIS JOURNAL, **77**, 6716 (1955).

(8) N. A. Hughes, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 3733 (1957).

(9) C. Deluca and N. O. Kaplan, *J. Biol. Chem.*, **223**, 569 (1956).

(10) H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, THIS JOURNAL, **79**, 430 (1957).

(11) R. W. Chambers and H. G. Khorana, *Chemistry & Industry*, 1022 (1956); THIS JOURNAL, **80**, 3749 (1958).

(12) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 4240 (1957).

(13) V. M. Clark, G. W. Kirby and A. R. Todd, *J. Chem. Soc.*, 1497 (1957).

(14) R. W. Chambers and J. G. Moffatt, THIS JOURNAL, **80**, 3752 (1958).

counted for 78% of the total nucleotidic material, as estimated spectrophotometrically. Even though some loss occurred during the isolation, the final yield (66%) of the pure material was very satisfactory. Full characterization of this product by chemical and enzymatic means is described in the Experimental section.

The success of the above experiment encouraged us to undertake the synthesis of UDPG (I) by a similar procedure. The mono-(trioctylammonium) salt[4] of α-glucose-1 phosphate (III) was freely soluble in anhydrous pyridine and, consequently, no difficulty was encountered in obtaining a homogeneous solution in this solvent using UMP-NH_2 and a fourfold excess of the sugar phosphate (III) salt. After a total period of four days at room temperature, the desired UDPG was indeed the major product and, again, only very small amounts of uridine-5′ phosphate and of the symmetrical diuridine pyrophosphate were present. The coenzyme was purified by ion exchange chromatography using mildly acidic eluents (0.003 *N* hydrochloric acid containing increasing amounts of lithium chloride) and finally isolated as the amorphous lithium salt in an over-all yield of 59%, as estimated spectrophotometrically. The completely satisfactory ion exchange chromatography using the chloride form[15] of an anion exchanger may be noted, in view of the earlier report of decomposition of the coenzyme on formate columns.[5]

Synthetic UDPG was completely homogeneous by the criteria of paper chromatography, paper electrophoresis and ion exchange analysis. The ratio of uridine to phosphorus to glucose was ascertained to be 1.0:1.98:0.98 (theoretical 1:2:1). Spectrophotometric examination of a weighed sample showed that UDPG comprised 85% of the total weight, the rest apparently being water. On the basis of its ultraviolet absorption, the sample was 100% active in the UDPG pyrophosphorylase system.[16,17] Its behavior toward acid and alkali[18] is described in the Experimental section.

The above results should be compared with those obtained in the previous syntheses of UDPG. The products of reaction of a mixture of uridine-5′ phosphate and α-glucose-1 phosphate with dicyclohexylcarbodiimide gave some enzymatic activity expected of UDPG but, apparently, the total mixture was so complex that isolation of pure UDPG was not attempted.[5] An alternative method involving a much lengthier route[4] gave about 15% yield of UDPG, about 30% pure on a weight basis and admixed with other ultraviolet absorbing material and glucose derivatives.

The procedure just described for the synthesis of UDPG should be directly applicable to the synthesis of a number of related compounds, *e.g.*, uridine diphosphate galactose and uridine diphosphate acetylglucosamine.

Attention was next turned to the synthesis of unsymmetrical pyrophosphates from AMP-NH_2. Its crystalline 1,3-dicyclohexylguanidinium salt proved to be rather insoluble in anhydrous pyridine. However, in the model experiment with an excess of phenylphosphoric acid it was drawn largely into solution in this solvent. After a reaction period of three days, the results were as satisfactory as those in the parallel experiment with UMP-NH_2. Thus, P^1-adenosine-5′,P^2-phenyl pyrophosphate (ADP-phenol) was the major product, with some adenosine-5′ phosphate and a trace of the symmetrical diadenosine-5′ pyrophosphate being present.

Since the solubilizing effect of phenylphosphoric acid in the above experiment was apparently a special case, attempts were made to find alternative solvent systems for the AMP-NH_2 salt. Dimethyl sulfoxide and formamide, or mixtures of either[19] with pyridine, readily gave clear solutions of the amidate but, while some ADP-phenol was formed in experiments using these solvents, hydrolysis to AMP was extensive. More satisfactory results were obtained using a mixture of *o*-chlorophenol and pyridine, which also gave a homogeneous solution of the reactants. The formation of a side product, apparently the *o*-chlorophenyl ester of AMP which was encountered in this experiment, is peculiar to the use of phenylphosphoric acid.[20] It has not been observed in the experiments on adenosine-5′ diphosphate[1] and coenzyme syntheses (see below) in which solvent systems containing *o*-chlorophenol have been satisfactorily used.

In extending the above experiments to the synthesis of FAD, both dicyclohexylguanidinium AMP-NH_2 and riboflavin-5′ phosphate (FMN) were found to be soluble in a mixture of *o*-chlorophenol and anhydrous pyridine. Using a 2.5 molar excess of FMN the reaction appeared to be complete after a period of four days as judged by paper chromatography in suitable solvent systems (see Experimental). The total mixture of reaction products was separated satisfactorily on a diethylaminoethyl (DEAE)[21] cellulose (chloride form) column. FAD, which was eluted as a discrete peak with dilute hydrochloric acid (0.003 *N*) containing lithium chloride (0.035 *N*), was finally isolated as its lithium salt in 40% yield, based on AMP-NH_2. The product was pure paper chromatographically and electrophoretically and possessed spectral properties identical to those established by the careful work of Whitby.[22] After drying in a vacuum the material was still apparently somewhat hydrated, FAD comprising 90% of the weight of the sample. An enzymatic assay[17,23] by the D-amino acid oxidase system

(15) E. Cabib, L. F. Leloir and C. E. Cardini, *J. Biol. Chem.*, **203**, 1055 (1953).

(16) H. M. Kalckar in "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 676.

(17) We are grateful to Dr. W. E. Razzell of this Laboratory for the enzymatic assays reported in this paper.

(18) A. C. Paladini and L. F. Leloir, *Biochem. J.*, **51**, 426 (1952).

(19) Some observations on alternative solvent systems also were made during experiments on UDP-phenol synthesis. The use of a 1:1 mixture of dimethylformamide and pyridine was satisfactory. However, dimethylformamide alone, which gave a clear solution of the reactants, was unsatisfactory. It therefore appears that solvents such as formamide and dimethylformamide inhibit in some way the pyrophosphate synthesis.

(20) Phenylphosphoric acid is a stronger acid than orthophosphoric acid or the other phosphomonoesters used, and, accordingly, its anion is a weaker nucleophilic agent. This results in effective competition by the solvent molecules during attack on the amidate.

(21) Purchased from the Brown Co., Berlin, N. H.

(22) L. G. Whitby, *Biochem. J.*, **54**, 437 (1953).

(23) The procedure used was that of C. DeLuca, M. M. Weber and N. O. Kaplan, *J. Biol. Chem.*, **223**, 559 (1956).

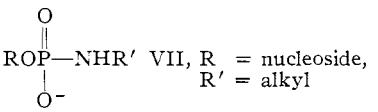
showed it to be slightly more active than a commercial preparation stated to be not less than 90% pure.

The new procedure used above for the purification of FAD on a preparative scale deserves further comment. In the past the isolation of pure FAD from either natural sources or from synthetic reaction mixtures has been a difficult and tedious problem. Since Warburg and Christian's[24] original isolation and purification of FAD, there have been numerous attempts to apply chromatographic techniques to the separation of the flavin compounds. Of these, partition chromatography on cellulose columns[9,22] probably has been the most useful on a preparative scale, but even on the relatively simple mixtures resulting from attempted syntheses repeated chromatography is necessary in order to obtain a pure product. Chromatography on columns of alumina,[25] Florisil, Celite and calcium phosphate[26] has been used with moderate success. Very small samples of FAD have been purified by repeated paper chromatography[7] or paper electrophoresis[27] and by column electrophoresis.[28,29] Counter-current distribution has recently been suggested as a useful method.[30] While a partial separation of naturally occurring flavins has been achieved by elution with water from a column of IRC-50 resin,[28] anion exchange resins are generally of little use in the flavin field since the flavins are very strongly held by the resins. The chromatography using cellulose anion exchangers now described would appear to be the method of choice for the separation of the flavin nucleotides on both analytical and preparative scales.

The results described above illustrate the general usefulness of the phosphoramidate approach to the specific synthesis of nucleotide coenzymes and clearly represent an advance on the methods that have hitherto been available in this field. While these studies are being extended further some general observations may be made on the new method at the present time. The first concerns the possible use of partly aqueous media, a feature which would obviously add to the value of the method. This possibility appeared to be feasible since the nucleoside-5′ phosphoramidates themselves were found to be completely stable both in aqueous and anhydrous pyridine over extended periods of time.[31] However, a series of experiments on UDPG synthesis in pyridine containing 0, 5 and 15% water showed a sharp decrease in the yield of the desired product with increase in the water content. In fact the major reaction in aqueous pyridine was the hydrolysis of the UMP-NH_2 to the parent nucleotide. The use of an excessive amount of water therefore appears to be precluded unless an excess of the amidate is used to offset loss through simple hydrolysis. Such a variant of the present technique may find application in certain cases where small amounts of water must be incorporated to enhance solubility.

The formation of some of the mononucleotides corresponding to the amidates and of small amounts of the symmetrical dinucleoside pyrophosphates was observed in the present experiments. The following observations may be made on the origin of these by-products in view of the complete stability of the amidates themselves in both anhydrous and aqueous pyridine. Firstly, hydrolysis of the amidate to the parent nucleotide is in some way governed by the presence of the added phosphomonoester ions. Secondly, the dinucleoside pyrophosphates apparently arise through the attack of this free mononucleotide on the amidate rather than by self-condensation of two molecules of the amidate followed by hydrolysis of the remaining amide linkage. From the practical standpoint, the formation of the mononucleotide and, consequently, that of the symmetrical pyrophosphate, necessitates the use of an excess of the second phosphomonoester, *e.g.*, glucose-1 phosphate in the synthesis of UDPG. This is not a serious disadvantage since the non-nucleotide components of the nucleotide coenzymes are usually readily available. When this is not the case, an adaptation of the method using an excess of the amidate readily can be foreseen.

Because of the usually high insolubility of certain nucleotides and their derivatives, solubilization of the reactants is often a problem in this field. In the present work satisfactory results were obtained using either pyridine alone or a mixture of this solvent and *o*-chlorophenol. It is clear that the use of N-substituted nucleoside-5′ phosphoramidates (VII) may offer distinct advantages in the matter of solubility as well as reactivity.



VII, R = nucleoside, R′ = alkyl

While studies along this line will be reported later, adenosine-5′ N-cyclohexylphosphoramidate (VII, R = adenosine, R′ = cyclohexyl) already has been shown to possess very convenient solubility properties.

Experimental

Chromatography.—Descending paper chromatography was carried out in the following solvent systems: solvent I, isopropyl alcohol–ammonia (sp. gr. 0.9)–water (7–1–2, v./v.)[32]; solvent II, isopropyl alcohol–1% aqueous ammonium sulfate (2–1)[33]; solvent III, ethyl alcohol–0.5 *M* ammonium acetate buffer (*p*H 3.8) (5:2).[18] Analytical chromatography in solvent II was done on Whatman No. 1 paper which had previously been soaked in 1% aqueous ammonium sulfate and then dried.[33] For the chromatography of flavins it was necessary to use Whatman No. 1

(24) O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150 (1938).

(25) K. Burton, *Biochem. J.*, **48**, 458 (1951).

(26) E. Dimant, D. R. Sanadi and F. M. Huennekens, THIS JOURNAL, **74**, 5440 (1952).

(27) O. Walaas and E. Walaas, *Acta Chem. Scand.*, **10**, 118 (1956).

(28) N. Siliprandi and P. Bianchi, *Biochim. et Biophys. Acta*, **16**, 424 (1955).

(29) K. Shimazu, *Ann. Rept. Shionogi Research Lab.*, **6**, 50 (1956) [*C. A.*, **51**, 4485 (1957)].

(30) F. Bergel, R. C. Bray and K. R. Harrap, *Ann.*, **607**, 219 (1957).

(31) The stability of the nucleoside-5′ phosphoramidates in aqueous pyridine should be contrasted with the pyridine-catalyzed hydrolysis of unsubstituted phosphoramidic acid; T. Rathlev and T. Rosenberg, *Arch. Biochem. and Biophys.*, **65**, 319 (1956).

(32) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

(33) N. Anand, V. M. Clark, R. H. Hall and A. R. Todd, *ibid.*, 3665 (1950).

paper which had been thoroughly washed with 2 N acetic acid, then with water and air-dried.[34]

Paper electrophoresis was carried out in an apparatus similar to that described by Markham and Smith[35] using either 0.1 M ammonium acetate buffer (pH 4.5) or 0.05 M phosphate buffer (pH 7.5).

Ultraviolet absorbing compounds on chromatograms were detected by visual observation under ultraviolet light[36] and phosphorus-containing compounds by the perchloric acid-molybdate spray of Hanes and Isherwood[34] followed by irradiation with ultraviolet light.[37] Total phosphorus determinations were done by King's method.[38]

P¹-Uridine-5′,P²-Phenyl Pyrophosphate (UDP-Phenol).—1,3-Dicyclohexylguanidinium uridine-5′ phosphoramidate[39] (0.4 mmole) was dried under vacuum at 95° overnight, and dissolved together with monophenylphosphoric acid (2.0 mmoles) in anhydrous pyridine (5 ml.). The sealed mixture was stored at room temperature for three days and then examined by paper chromatography. In solvent I, UDP-phenol (R_f 0.40) was the major product, UMP-NH_2 (R_f 0.23) was absent and a spot corresponding to UMP (R_f 0.11) was present. Chromatography in solvent II, which also separated UDP phenol (R_f 0.54) from UMP-NH_2 (R_f 0.38), UMP (R_f 0.42) and phenyl phosphate (R_f 0.67), showed in addition the presence of a small amount of diuridine pyrophosphate (R_f 0.28). The pyridine was evaporated under reduced pressure and the residual gum dissolved in water (25 ml.). After thorough stirring some insoluble guanidinium salts were removed by filtration and the aqueous filtrate was adjusted to pH 8 and applied to the top of a column (2.3 cm. diameter × 6 cm. long) of Dowex 2 (Cl^-) resin. After thorough washing with water the column was developed with 0.003 N hydrochloric acid containing increasing amounts of lithium chloride. Uridine-5′ phosphate (480 optical density (O.D.) units at 262 mμ) was eluted using 0.03 N lithium chloride; monophenyl phosphate and a little diuridine-5′ pyrophosphate (total 1164 O.D. units,[40] of which the pyrophosphate comprised 400 units) with 0.12 N lithium chloride; and UDP-phenol (3120 O.D. units, 78% based on UMP-NH_2) with 0.30 N lithium chloride. The UDP-phenol peak was directly stirred with acid-washed Norite[41] (6 g.) which adsorbed all ultraviolet absorbing materials from solution, and the charcoal was well washed with water. Repeated washing with 50% aqueous ethyl alcohol containing 2% ammonium hydroxide[15] eluted 2850 O.D. units (92% recovery) of UDP-phenol. The effluent was evaporated to dryness under reduced pressure giving a gum which was dissolved in water and passed through a column (1 × 5 cm.) of Dowex 50 (H^+) resin. The effluent was concentrated (below 30°) to a small volume and aqueous barium hydroxide added to pH 3.5. After removal of a trace of insoluble material, acetone (50 ml.) was added and the resulting white precipitate collected by centrifugation. After washing with acetone and drying under vacuum at 100° overnight barium UDP-phenol (189 mg., 2640 O.D. units, 66% based on UMP-NH_2) was isolated as an amorphous, hydrated white solid which was both chromatographically and electrophoretically homogeneous under a variety of conditions.

Anal. Calcd. for Ba·UDP-phenol·4H_2O: P, 9.02; phosphorus:uridine, 2.00. Found: P, 9.27; phosphorus:uridine,[40] 2.07.

Incubation[17] of this product with a snake venom fraction[42] free from mononucleotidase activity and rich in pyrophosphatase and diesterase activity led to rapid and quantitative cleavage to give uridine-5′ phosphate and monophenyl phosphate.

Uridine Diphosphate Glucose (UDPG).—Mono-(trioctylammonium)-α-glucose-1 phosphate[4] (1.5 mmoles) was dried by repeated azeotropic distillation with anhydrous benzene and then evaporated to dryness. The addition of anhydrous pyridine (20 ml.) and 1,3-dicyclohexylguanidinium UMP-NH_2 (0.35 mmole, dried at 100° under vacuum for four hours) gave a clear solution which was sealed and stored at room temperature for four days. Paper chromatography in solvent III showed that UDPG (R_f 0.21) was the major product and that only a trace of UMP-NH_2 (R_f 0.32) was present. The pyridine was evaporated under reduced pressure and water (25 ml. containing 1.0 g. of sodium acetate) was added to the residue. The aqueous solution was extracted five times with ether, adjusted to pH 7 and applied to the top of a column (2.3 × 8 cm. long) of Dowex 2 (Cl^-) resin. After thorough washing with water the column was eluted with 0.003 N hydrochloric acid containing lithium chloride. UMP-NH_2 (292 O.D. units at 262 mμ, 8.3% based on starting UMP-NH_2) followed by UMP (592 O.D. units, 16.9%) was eluted with 0.01 N lithium chloride. UDPG (2300 O.D. units, 66.0%) followed by diuridine-5′ pyrophosphate (312 O.D. units, 8.9%) was eluted with 0.06 N lithium chloride.

The UDPG peak was adjusted to pH 6.5 with lithium hydroxide and evaporated to dryness under reduced pressure and below room temperature. The resulting gum was dissolved in methyl alcohol (25 ml.) and concentrated to about 10 ml. The addition of acetone (200 ml.) precipitated a white solid which was collected by centrifugation and reprecipitated twice in the same manner. The final precipitate was dried under vacuum giving a hydrated lithium salt of UDPG (150 mg., 2050 O.D. units) in an over-all yield of 59%. This material was homogeneous by analytical ion exchange chromatography, paper chromatography and paper electrophoresis both before and after drying at 100° under vacuum. It was 85% pure on a weight basis as estimated spectrophotometrically. The product was completely active in the UDPG pyrophosphorylase system.[16,17]

Anal. Calcd. for Li_2·UDPG·6 H_2O: P, 9.03; uridine:phosphorus:glucose, 1:2:1. Found[43]: P, 9.06; uridine:phosphorus:glucose, 1.0:1.98:0.98; $[\alpha]^{22}D$ +43.6°, (c 0.34, H_2O).

As reported by Paladini and Leloir,[18] UDPG was completely hydrolyzed in 10 minutes at 100° in 0.01 N hydrochloric acid, the products being uridine-5′ pyrophosphate, glucose and a small amount of uridine-5′ phosphate, presumably arising from subsequent degradation of uridine-5′ pyrophosphate. In alkaline media, while the mode of hydrolysis was as discovered by the above workers,[18] UDPG was found to be considerably more stable. Thus, at 0° in concd. ammonia (sp. gr. 0.9) only a trace of uridine-5′ phosphate was detected in two hours. Heating at 100° in tris-(hydroxymethyl)-aminomethane buffer (pH 8.5) caused only about 40% hydrolysis, as judged by the visual observation of uridine-5′ phosphate and UDPG spots, in one hour.

P¹-Adenosine-5′,P²-phenyl Pyrophosphate (ADP-Phenol).

(a) **Using Pyridine as Solvent.**—1,3-Dicyclohexylguanidinium adenosine-5′ phosphoramidate monohydrate[14] (118 mg., 0.2 mmole) was dried under vacuum at 100° for 5 hours and then dissolved in anhydrous pyridine (10 ml.) by gentle warming with monophenylphosphoric acid (350 mg., 2.0 mmoles). The solution, which was almost completely homogeneous, was sealed and stored at room temperature for three days. Paper chromatography in solvent I then showed the amidate (R_f 0.23) to have disappeared, and ADP-phenol (R_f 0.36) to be the major product. After evaporation of the pyridine, water (10 ml.) was added and some insoluble guanidinium salts removed by filtration.

(34) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(35) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(36) E. R. Holiday and E. A. Johnson, *Nature*, **163**, 216 (1949).

(37) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

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

(39) The dicyclohexylguanidinium UMP-NH_2 used throughout this work was amorphous and as such readily soluble in pyridine. More recently this substance has been obtained crystalline and in this form it appears to have very little solubility in pyridine. The procedure recommended for handling the crystalline material is to lyophilize its aqueous solution and use the resulting fluffy material after drying as mentioned in the Experimental section.

(40) E_{max} of phenyl phosphate at 261 mμ is 455 at pH 2 whereas at 280 mμ the substance has negligible absorption. The diuridine pyrophosphate content was determined by measuring the absorption at the latter wave length and then calculating that at 262 mμ.

(41) Treated as described by D. Lipkin, P. T. Talbert and M. Cohn, THIS JOURNAL, **76**, 2871 (1954).

(42) J. F. Koerner and R. L. Sinsheimer, *J. Biol. Chem.*, **228**, 1049 (1957).

(43) Glucose was determined by the sensitive method of J. T. Park and M. J. Johnson, *ibid.*, **181**, 149 (1949), after hydrolysis of the UDPG by 0.1 N hydrochloric acid at 100° for 10 minutes followed by neutralization with sodium hydroxide. Uridine and UMP were shown not to interfere under these conditions. The uridine content was determined spectrophotometrically at 262 mμ.

The aqueous filtrate was concentrated to a small volume and applied as a streak to four 20 cm. wide strips of Whatman 3 MM paper.[44] The chromatograms were developed for 20 hours in solvent II and the bands corresponding to ADP-phenol (R_f 0.46) were eluted with water giving 2458 O.D. units at 257 mμ. This solvent separated ADP-phenol from monophenyl phosphate (R_f 0.70), AMP (R_f 0.36) and a trace of diadenosine-5′ pyrophosphate (R_f 0.23). A trace of contaminating AMP was removed by concentrating the effluent to a small volume and rechromatographing as a streak in solvent I. The ADP-phenol band (R_f 0.36) was eluted with water giving a recovery of 2000 O.D. units at 257 mμ. The effluent was evaporated to dryness and dissolved in methanol (5 ml.). The addition of ether (30 ml.) resulted in the precipitation of the hydrated ammonium salt of ADP-phenol (75 mg., 60% yield on an optical density basis after drying at room temperature under vacuum).

This material was homogeneous by paper chromatography, paper electrophoresis and ion exchange chromatography. It was rapidly and quantitatively cleaved by the snake venom fraction mentioned above to AMP and monophenyl phosphate.

Anal. Calcd. for $(NH_4)_2$ADP-phenol·5H_2O: P, 9.89; ratio of phosphorus:adenine, 2.00. Found: P, 9.90; phosphorus:adenine, 2.02.

(b) **Using *o*-Chlorophenol as Solvent.**—Freshly dried 1,3-dicyclohexylguanidinium adenosine-5′ phosphoramidate (0.2 mmole) and monophenyl phosphate (0.6 mmole) were mixed and dissolved in distilled *o*-chlorophenol (3 ml.). Anhydrous pyridine (2 ml.) was added, and the solution was stored at room temperature for three days. The pyridine was evaporated under reduced pressure and water (25 ml.) was added. The resulting mixture was extracted five times with ether and then the aqueous phase was concentrated to a small volume and chromatographed, as described above, in solvents II and then I. The products were ADP-phenol (1550 O.D. units at 257 μ, 52% based on AMP-NH_2), AMP (125 O.D. units, 4%), diadenosine-5′ pyrophosphate (47 O.D. units, 1.6%) and *o*-chlorophenyl AMP (680 O.D. units, 23%). The ADP-phenol fractions was isolated as above to give a homogeneous, white, amorphous product with a phosphorus to adenine ratio of 2.01.

Purification of Riboflavin-5′ Phosphate.[45]—Diethylaminoethyl cellulose[21] (DEAE cellulose, type 20) was washed with 5% sodium hydroxide and then converted to the chloride form by washing with water and then 5% hydrochloric acid. After washing until neutral, fine particles were removed by sedimentation and a 4.5 × 30 cm. long column was carefully packed under a slight pressure until the bed volume was constant. Commercial sodium riboflavin-5′ phosphate[46] (1.5 g.) was dissolved in water and carefully applied to the column. Riboflavin was removed with water and then riboflavin-5′ phosphate was eluted with aqueous 0.03 *N* lithium chloride which left flavin polyphosphates on the column as discrete bands. The riboflavin-5′ phosphate peak (*ca.* 2 l.) was evaporated to dryness under reduced pressure and thoroughly stirred with methanol (20 ml.). The addition of acetone (200 ml.) gave the lithium salt of riboflavin-5′ phosphate as an orange precipitate (1.0 g.) which was shown to be homogeneous by analytical ion exchange chromatography on DEAE cellulose, paper chromatography and paper electrophoresis.

This material was converted to the pyridinium salt by passage through a column of IR-120 (H^+) resin into an excess of pyridine and lyophilization. After drying at 100° under vacuum for four hours this material was shown to be the monohydrate of monopyridinium riboflavin-5′ phosphate.

Flavin Adenine Dinucleotide (FAD).—[45]Purified pyridinium riboflavin-5′ phosphate (297 mg., 0.52 mmole) and 1,3-dicyclohexylguanidinium adenosine-5′ phosphoramidate (90 mg., 0.15 mmole) were separately dried under vacuum at 100° for three hours. They were then mixed and dissolved in distilled *o*-chlorophenol (10 ml.) by gentle warming. Dry pyridine (10 ml.) then was added and the resulting solution was sealed and stored in the dark for four days.

The pyridine was then largely removed under reduced pressure, and water (20 ml.) was added to the residue. The *o*-chlorophenol was removed by three extractions with chloroform, the first extract being back-extracted with water. The resulting orange aqueous solution was adjusted to *p*H 7 with dilute ammonia and carefully applied to the top of a 21 cm. long × 4.5 cm. diameter column of DEAE cellulose (type 20) in the chloride form. The column was then washed with water until the optical density of the effluent at 260 mμ fell to zero (total of 222 O.D. units at 450 mμ). Elution was then continued using 0.003 *N* hydrochloric acid containing 0.015 *N* lithium chloride which removed adenosine-5′ phosphate (1291 O.D. units at 260 mμ) and then riboflavin-5′ phosphate (11730 O.D. units at 260 mμ, O.D. ratio 260 mμ/450 mμ = 2.26). In some runs a trace of diadenosine-5′ pyrophosphate was eluted with the tail of this peak. Hydrochloric acid (0.003 *N*) containing 0.035 *N* lithium chloride eluted FAD (2302 O.D. units at 260 mμ, ratio 260 mμ/450 mμ = 3.26 at *p*H 7) as a discrete peak. The yield at this stage was 41.5% based on AMP-NH_2.

The FAD peak was adjusted carefully to *p*H 5.8 with dilute lithium hydroxide and evaporated to a small volume under reduced pressure using a bath temperature below 30°. The *p*H of the evaporating solution was checked periodically and adjusted to 6.0 if necessary. After filtration from some traces of water-insoluble material the aqueous solution was evaporated to a gum. Methanol (20 ml.) was added and the mixture well swirled. The addition of acetone (200 ml.) and ether (20 ml.) resulted in the precipitation of the orange lithium salt of FAD which was collected by centrifugation and washed three times by stirring in methanol (4 ml.) and adding acetone (40 ml.) and ether (4 ml.). The final precipitate was dissolved in water and lyophilized giving Li_2-FAD as a fluffy yellow solid (63 mg.) which was completely homogeneous as judged by paper chromatography and paper electrophoresis under a variety of conditions. The material showed spectral properties identical to those described by Whitby,[22] having optical density ratios at 260 mμ/450 mμ of 3.28 and at 375 mμ/450 mμ of 0.82 at *p*H 7. However, it apparently was hydrated, being 75% FAD on a weight basis. The final yield was 2190 O.D. units at 260 mμ (40% over-all based on AMP-NH_2). Assayed enzymatically by the D-amino acid oxidase system[17,23] this product was found on an optical density basis to be 105% pure relative to FAD from the California Foundation. The ratio of phosphorus to riboflavin[47] was found to be 2.02.

After drying of a sample under vacuum at 100° for four hours the FAD content was raised to 89% with no change in chromatographic or spectral behavior. A sample of the lithium salt was converted to the free acid by passage through a small column of Dowex 50 (H^+) resin followed by lyophilization. After drying at 100° for two hours the free acid retained its biological activity and contained 90% FAD.

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(44) Ion exchange chromatography was not suitable for isolation since ADP-phenol and monophenyl phosphate failed to separate cleanly.

(45) The following experiments were conducted in subdued light.

(46) California Foundation, purified grade.

(47) Riboflavin was estimated spectrophotometrically at 450 mμ.