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NEW METHODS FOR THE SYNTHESIS OF NUCLEOTIDE COENZYMES

V.M.Berezovskii and R.V.Artemkina

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I. INTRODUCTION

Consideration of synthetic methods for obtaining nucleotide coenzymes is of considerable interest in view of their important role in metabolic processes and especially in view of the fact that most nucleotide coenzymes have been obtained artificially only during recent years by new methods of organic synthesis.

Nucleotide coenzymes combined with a specific protein form numerous enzyme systems, in which they catalyse various chemical reactions occurring in the living organism. Nucleotide coenzymes are the monomers of nucleotides (phosphoric esters of nucleosides) and are structurally similar or analogous to units in ribonucleic acids (RNA) or deoxyribonucleic acids (DNA), which are polymers of nucleotides.

The molecules of nucleotide coenzymes are based on those of nucleosides, *N*-glycosides of heterocyclic bases, linked *via* the ring nitrogen atom with the primary carbon of D-ribose, and in this respect all natural nucleotide coezymes have the β-configuration.

Complex nucleotide coenzymes can be regarded as made up of two simpler compounds joined by a pyrophosphate link: flavin-adenine dinucleotide (FAD) consists of adenosine-5′ phosphate and riboflavin-5′ phosphate, uridine diphosphate glucose (UDPG) of uridine-5′ phosphate and α-D-glucose-1-phosphate, nicotinamide-adenine dinucleotide (NAD)† of adenosine-5′ phosphate and nicotinamide mononucleotide, nicotinamide-adenine dinucleotide phosphate (NADP)† of adenosine-2′,5′ diphosphate and nicotinamide mononucleotide, and coenzyme A (CoA) of adenosine-3′,5′ diphosphate and the 4′ phosphate of D-pantetheine, etc.

Among the various methods for obtaining nucleotide coenzymes from nucleosides or simple nucleotides, the very complex specific methods for producing a pyrophosphate link, which we shall examine in the present paper, are of considerable interest.

In a simple nucleotide coenzyme molecule, which is a monoester of a polyphosphoric acid, the pyrophosphate link is produced either by direct phosphorylation of a nucleoside or by forming di- and tri-phosphates from the nucleoside monophosphate. In the molecule of a complex nucleotide coenzyme which is an unsymmetrical P^1P^2-diester of pyrophosphoric acid, the pyrophosphate link is produced mainly in two ways: (*a*) condensation of two different

† According to the nomenclature recommended by the Commission on Enzymes of the International Biochemical Congress and by the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry–Chem.Abs., 55, 6558 (1961); Biokhimiya, 26, 563 (1961).

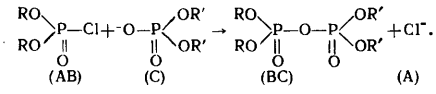
nucleoside monophosphates, for example adenosine-5′ phosphate and riboflavin-5′ phosphate, nicotinamide nucleoside monophosphate and adenosine-5′ phosphate, etc.; (*b*) condensation of a nucleoside diphosphate with an unphosphorylated nucleoside, such as adenosine-5′ diphosphate and riboflavin, or riboflavin-5′ diphosphate and adenosine, etc.

Up to the present time attention has been paid mainly to the study of methods for condensing two molecules of nucleoside monophosphates. The second method for producing a pyrophosphate link appears very infrequently in the literature.

The formation of a pyrophosphate link by the condensation of two nucleoside monophosphates can be accomplished by three main methods: (1) the phosphorochloridate method, consisting in the condensation of halogenated phosphates or halogenated nucleotides, containing protected hydroxyl groups, with salts of phosphates or of nucleotides, followed by removal of the protective groups; (2) the carbodiimide method, consisting in the direct condensation, under the influence of carbodiimides (dicyclohexylcarbodiimide, di-*p*-tolylcarbodiimide, etc.), of two nucleoside monophosphates; and (3) the phosphoramidate method, consisting in the condensation of a phosphoramidate derivative of a nucleoside containing unprotected hydroxyl groups with another mononucleotide, which is likewise unprotected.

II. SYNTHESIS OF NUCLEOTIDE COENZYMES BY THE PHOSPHOROCHLORIDATE METHOD

The phosphorochloridate method is based on exchange between pyrophosphates, or mixed anhydrides of phosphoric and other acids, and phosphate anions. Two different acids A and B form an anhydride AB, which comes into contact with the anion of acid C, and if A is stronger than B and C, exchange takes place with the formation of the free acid A and the anhydride BC:



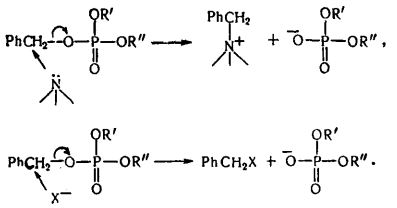
In this example B and C are phosphate anions, A is hydrogen chloride, AB is a phosphorochloridate, derived from the anhydride of phosphoric acid and hydrogen chloride, and BC is the pyrophosphate to be synthesised.

Completely or partly esterified phosphates are most often used in phosphorylation by the phosphorochloridate method. Completely esterified pyrophosphates are extremely labile, readily undergoing exchange with other anions, as a result of which they are more active phosphorylating agents than the partly esterified phosphates. However, owing to this ready reaction with other anions, very complex mixtures, which are difficult to separate, are formed. This may explain the low yields obtained in many syntheses.

The subsequent removal of the protective groups, which is necessary at the final stages of the synthesis, results in some breakdown in the pyrophosphate link, which also decreases the yield. Since partly esterified phosphates have far less tendency to undergo exchange reactions, their use enables the formation of side-products to be avoided, and thereby an increased yield of polyphosphates can be achieved.

The choice of protective groups and their removal constitute a separate problem. Benzyl groups are most commonly employed as protective groups, since debenzylation is possible under relatively mild conditions. Dibenzyl phosphorochloridate is usually employed as phosphorylating agent, even though it is less stable and has a weaker phosphorylating action than, for example, diphenyl phosphorochloridate.

The traditional method of debenzylation is catalytic reduction over Pt, Pd, or Ni catalysts in ethanolic or dioxane solution. Reduction with sodium in liquid ammonia is also used, but partial breakdown of the pyrophosphate link then takes place. A very successful method for the removal of benzyl groups from phosphate compounds has been solvolysis in phenol. Selective monodebenzylation can be successfully achieved by nucleophilic substitution in the presence of quaternary bases[1] or of anions (chlorides, thiocyanates, iodides):



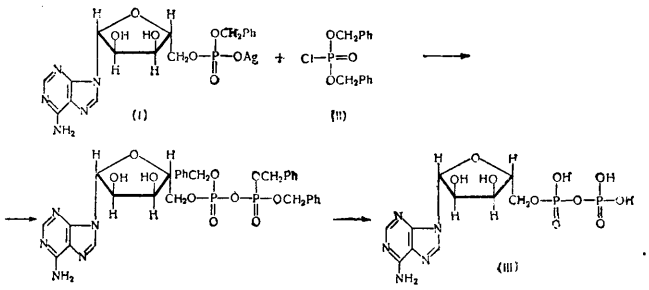
Removal of all the benzyl groups from completely esterified pyrophosphates cannot be achieved by means of lithium chloride, for example, but P^1P^2-dibenzyl pyrophosphate can be obtained from tetrabenzyl pyrophosphate. Such incomplete debenzylation can be conveniently employed because partly esterified pyrophosphates are more stable during isolation than are the completely esterified pyrophosphates. Undesirable debenzylation during formation of the pyrophosphate link can be suppressed by the use of 2,6-lutidine. The initial phosphates are normally employed in the form of their metal or amine salts, the latter being the more advantageous, since they are much more soluble. Anhydrous benzene, phenol, acetonitrile, or their mixtures are used as solvents.

1. Adenosine-5′ Di- and Tri-phosphates

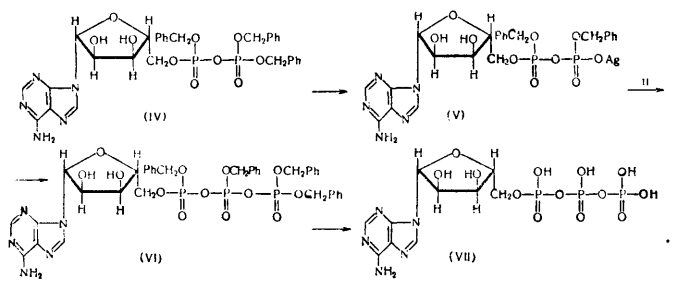
Nucleotide coenzymes such as FAD and UDPG were first obtained by the phosphorochloridate method of synthesis. This method had first been used to synthesise several simple coenzymes, such as adenosine-5′ diphosphate (ADP), adenosine-5′ triphosphate (ATP), uridine-5′ diphosphate (UDP), uridine-5′ triphosphate (UTP), and also a series of symmetrical pyrophosphates (P^1P^2-diadenosine-5′ and P^1P^2-diuridine-5′ pyrophosphates).

ADP (III) was obtained by the reaction of silver adenosine-5′ benzyl phosphate (I) with dibenzyl phosphorochloridate (II) in warm glacial acetic acid followed by catalytic hydrogenation of the resulting adenosine-5′ tribenzyl diphosphate in alcoholic solution[2]. ADP was isolated in low

yield as its acridinium salt. However, its yield can be increased to 55% by using a mixture of phenol and acetonitrile in the place of glacial acetic acid[3].



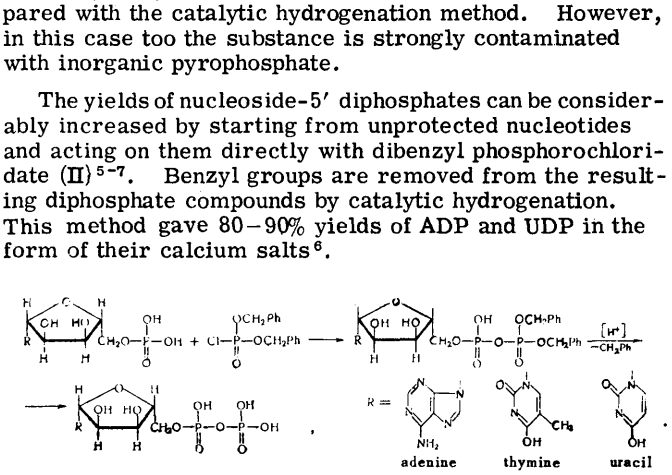
The starting material used for the synthesis of ATP (VII) was silver adenosine-5′ dibenzyl diphosphate (V), obtained by the monodebenzylation of adenosine-5′ tribenzyl diphosphate (IV)[4]. Compound (V) was condensed with dibenzyl phosphorochloridate (II) to yield the tetrabenzyl ester of ATP (VI):



If reduction of compound (VI) is carried out in glacial acetic acid, the product of the reaction contains 7–15% of ATP.[3] When the medium is a mixture of phenol and acetonitrile, the ATP content increases to 40%. The chief impurities were ADP and inorganic polyphosphates. The triacridinium salt of ATP which was isolated was identical with the same salt of natural adenosine-5′ triphosphate.

The principal difficulties in the synthesis of ADP and ATP by the phosphorochloridate method are encountered in the choice of conditions for the selective monodebenzylation of compound (IV). If 4-methylmorpholine is used for this purpose, the yield of ATP can be almost doubled[3] compared with the catalytic hydrogenation method. However, in this case too the substance is strongly contaminated with inorganic pyrophosphate.

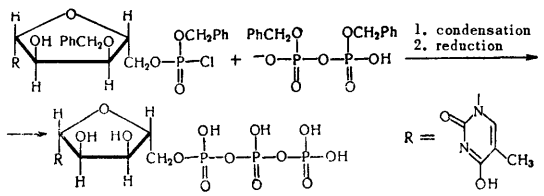
The yields of nucleoside-5′ diphosphates can be considerably increased by starting from unprotected nucleotides and acting on them directly with dibenzyl phosphorochloridate (II)[5-7]. Benzyl groups are removed from the resulting diphosphate compounds by catalytic hydrogenation. This method gave 80–90% yields of ADP and UDP in the form of their calcium salts[6].



2. Thymidine-5′ Di- and Tri-phosphates

Thymidine-5′ diphosphate (TDP) was prepared by the condensation of the tri-n-octylammonium salt of thymidine-5′ phosphate with dibenzyl phosphorochloridate in anhydrous dioxane at room temperature, the yield being 85% and the purity 92% (as the calcium salt)[6]. Thymidine-5′ phosphate (TMP) and inorganic phosphate were detected among the small amount of impurity. The benzyl groups were removed by reduction.

A 43% yield of thymidine-5′ triphosphate (TTP) was obtained by the interaction of 3′-*O*-benzylthymidine-5′ benzyl phosphorochloridate with the di(phenyltrimethylammonium)salt of P^1P^2-dibenzyl pyrophosphate in a benzene–acetonitrile mixture at room temperature, followed by debenzylation. It was shown by means of paper chromatography that the main product was TTP with small amounts of TMP and inorganic pyrophosphates and traces of TDP. After neutralisation of the solution with ammonia to pH 6, the reaction products were separated on Dowex-2 ion-exchange resin (in the chloride form). The TTP was finally purified *via* the barium salt.



3. Flavin-Adenine Dinucleotide

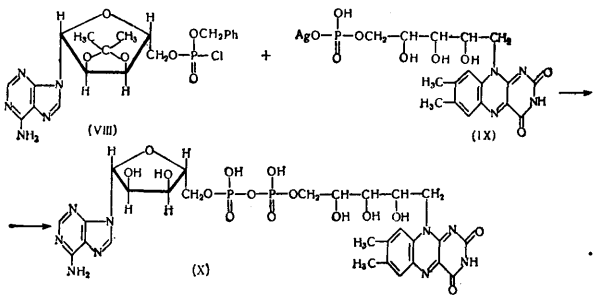
In the search for methods of synthesising natural unsymmetrical pyrophosphates (FAD, UDPG, NAD, CoA), symmetrical pyrophosphates were obtained, in particular such compounds as P^1P^2-diadenosine-5′ and P^1P^2-diuridine-5′ pyrophosphates[8]. The results of these investigations showed that synthesis of unsymmetrical nucleotide coenzymes occurring in Nature is possible. In subsequent work an effort was made to avoid reaction conditions which would result in symmetrical pyrophosphates.

The first complex coenzyme to be obtained synthetically was FAD. One of the components of the FAD molecule – adenosine-5′ monophosphate (muscle adenylic acid, AMP) – was prepared by the direct phosphorylation of adenosine with phosphoryl chloride in pyridine[9,10], and also by the treatment of 2′,3′-*O*-isopropylideneadenosine with phosphoryl chloride[11], by the interaction of 2′,3′-diacetyladenosine with diphenyl phosphorochloridate followed by removal of the protective groups[12], by the phosphorylation of 2′,3′-*O*-isopropylideneadenosine with phosphoric acid in the presence of trichloroacetonitrile[13], and by phosphorylation with a phosphorodichloridate[14].

The second component of the FAD molecule, riboflavin-5′ phosphate, usually termed "flavin mononucleotide" (FMN)‡, has been synthesised by several methods: by the phosphorylation of riboflavin with phosphoryl chloride after preliminary protection of the primary and secondary hydroxyl groups[15-17], by phosphorylation with phosphorochloridates without preliminary protection of the hydroxyls[18-20], and by phosphorylation with metaphorphoric acid[21,22] and with alkyl phosphorochloridates also without protection of the hydroxyl groups[23-26].

‡ Riboflavin-5′ phosphate is a hydrogenated nucleotide.

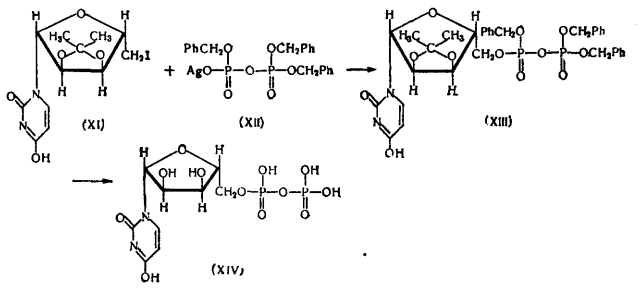
An attempt to use 4',5'-anhydroriboflavin as an intermediate in the synthesis of FAD proved unsuccessful[27]. The first successful synthesis of FAD (X), by condensation of the silver salt of riboflavin-5' phosphate (IX) with 2',3'-*O*-isopropylideneadenosine-5' benzyl phosphorochloridate (VIII) in the presence of triethylamine, was accomplished by Christie *et al.*[28,29] in 1954. Phenol was chosen as solvent, since FMN is readily soluble in it, and formation of a pyrophosphate link takes place quite readily in this medium. Use of this solvent offered the further advantage that, as noted above, phenols are able to monodebenzylate the benzyl pyrophosphates which are formed. The protective isopropylidene group was subsequently removed by treatment with acid. However, the yield of FAD was only 6%, partly owing to cleavage of the pyrophosphate link on elimination of the protective groups.



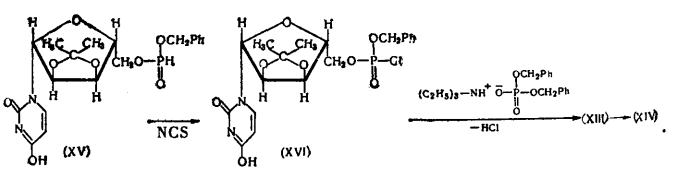
The isolation of coenzymes was always a complex and laborious task, both from natural sources and from synthetic products. With the development of chromatography, which in recent years has found wide application in organic chemistry, more effective isolation and separation of nucleotide coenzymes has become possible. Various methods were applied, for example, to the isolation and purification of FAD: partial separation of flavin nucleotides can be achieved on IRC-50 ion-exchange resin, whereas anion-exchange resins in general adsorb them very strongly. Better results were obtained by the use of adsorption chromatography on Florisil[30], partition chromatography in the phenol–butanol–water system[30], paper chromatography[31], column electrophoresis[32,33], and chromatography on powdered cellulose[34-36], the last method proving the most effective. Paper chromatography in various solvent systems has been used for the identification of flavin compounds[37,38].

4. Uridine Diphosphate Glucose

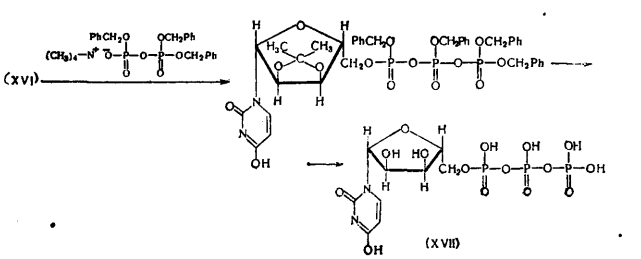
One of the products of the cleavage of UDPG is UDP which was synthesised by condensing 5'-deoxy-5'-iodo-2',3'-*O*-isopropylideneuridine (XI) with silver tribenzyl pyrophosphate (XII) in benzene solution[39]. The resulting good yield of 2',3'-*O*-isopropylideneuridine-5' tribenzyl diphosphate (XIII) was debenzylated with lithium chloride in 2-ethoxyethanol to uridine-5' dibenzyl diphosphate. The use of lithium chloride for the debenzylation proved to be very convenient, since it enabled the reaction to be carried out under mild conditions[40]. Although all the protective groups could not be removed in this way, this reaction was nevertheless of great importance, since the partly debenzylated compound was more stable in acid medium than the completely esterified compound. This facilitated the removal of the remaining benzyl groups by catalytic hydrogenation with subsequent elimination of the isopropylidene groups by mild acid hydrolysis:



UDP (XIV) can be synthesised also by the reaction of 2',3'-*O*-isopropylideneuridine-5' benzyl phosphorochloridate (XVI) with triethylammonium dibenzyl phosphate[41] and subsequent removal of benzyl and isopropylidene groups from the intermediate product in a manner similar to that described above. Compound (XVI) is obtained from 2',3'-*O*-isopropylideneuridine-5' monobenzyl phosphite (XV) by treatment with *N*-chlorosuccinimide (NCS). UDP (XIV) was isolated as the barium salt in 25% yield; the use of phenols for partial debenzylation considerably increases the yield[42].

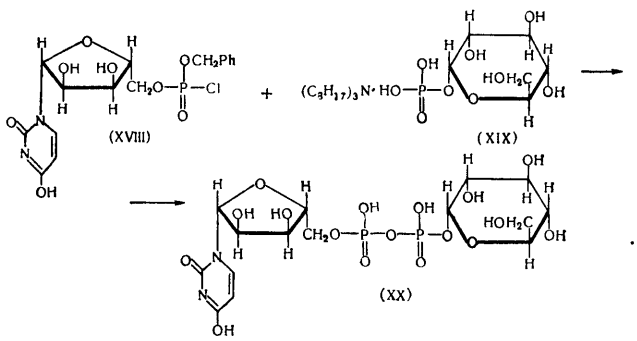


UTP (XVII), which probably takes part in the enzymic synthesis of uridine derivatives[43], was prepared by condensation of compound (XVI) with tetramethylammonium tribenzyl pyrophosphate[42]. Partial debenzylation of the intermediate product was effected by means of *m*-cresol in the presence of small amounts of hydrochloric acid, with subsequent removal of the remaining benzyl groups by catalytic hydrogenation and removal of the isopropylide group by the usual method:



After treatment with lithium hydroxide, the reaction mixture contained ~ 37% of UTP as the lithium salt. The

pure barium salt of UTP was isolated in 29% yield after chromatographic purification on carbon or on Dowex-2 ion-exchange resin.



(XVIII) (XIX) (XX)

UDPG (XX) was obtained by condensing 2',3'-dibenzyluridine-5' benzyl phosphorochloridate (XVIII) with tri-n-octylammonium α-D-glucose-1-phosphate (XIX) in the presence of tri-n-butylamine in benzene[44]. Uridine diphosphate galactose also was obtained by the same method.

The choice of the benzyl group instead of isopropylidene for protecting the hydroxyls is explained by the considerably greater lability in acid medium of UDPG than, for example, FAD, where isopropylidene protection is employed, since the isopropylidene group in the condensation product is removed in acid medium. After the pyrophosphate link had been formed, the benzyl groups were removed by catalytic hydrogenation.

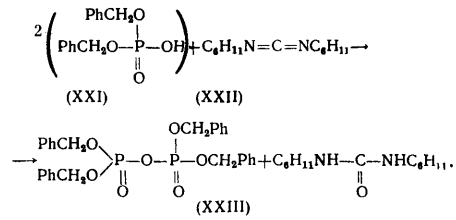
UDPG was isolated as its calcium salt (30% UDPG) with an overall yield of 15%. The chief impurities were UMP and α-D-glucose-1-phosphate. Further purification resulted in the calcium salt of UDPG of 92% purity (according to enzymic assay).

The above phosphorochloridate method for producing a pyrophosphate link possesses, in spite of its apparent specificity, several substantial defects: (1) the necessity for protecting the hydroxyl groups of the nucleoside makes it a multistage method; (2) removal of the protective groups after the condensation is always accompanied by partial cleavage of the pyrophosphate link, resulting in low yields of the nucleotide coenzymes; (3) the laborious synthesis of the initial phosphorochloridates gives rise to additional complications.

The low yields are also due to the fact that completely esterified pyrophosphates readily undergo exchange reactions, leading to complex mixtures[45,46], which are difficult to separate.

III. SYNTHESIS OF NUCLEOTIDE COENZYMES BY THE CARBODIIMIDE METHOD

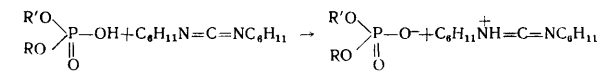
In the search for a more specific and simple method of synthesising nucleotide coenzymes, the condensation of two nucleotides in the presence of such condensing agents as dicyclohexylcarbodiimide, di-*p*-tolylcarbodiimide, etc. was investigated. This method was first tried with the synthesis of benzyl esters of pyrophosphoric acid[47]. In anhydrous pyridine dibenzyl phosphate (XXI) reacts instantaneously with dicyclohexylcarbodiimide (DCC) (XXII) to form tetrabenzyl pyrophosphate (XXIII) and *NN'*-dicyclohexylurea. It was found that even a considerable excess of water does not interfere with the formation of the anhydride, the yield of tetrabenzyl pyrophosphate being in all cases ~ 50%.



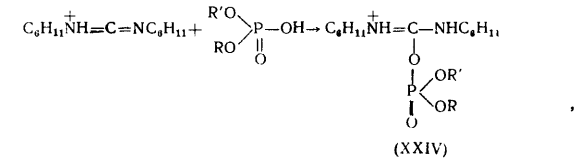
(XXI) (XXII) (XXIII)

This constitutes the great value of the carbodiimide method, since even a small amount of water greatly increases the solubility of nucleotides and orthophosphoric acid, which are insoluble in the anhydrous pyridine usually employed as reaction medium.

Formation of the pyrophosphate link probably consists in the initial addition of a proton to the dicyclohexylcarbodiimide[48,49]:

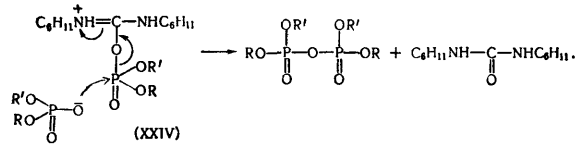


with subsequent addition to the protonated carbodiimide of a phosphoric acid or ester molecule to yield an adduct such as the intermediate compound (XXIV)[49-52]:



(XXIV)

which is then attacked by the phosphate anion to form a pyrophosphate and a substituted urea. The intermediate compound was not isolated from the reaction mixture



(XXIV)

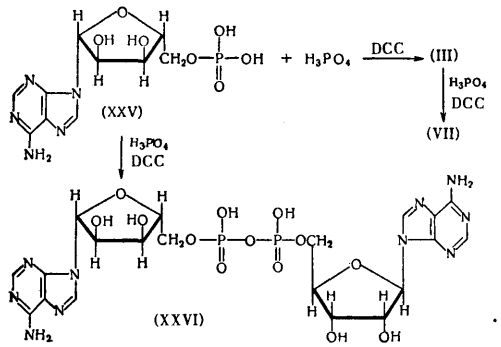
A pyrophosphate link can be formed only with the protonated form of carbodiimides, when, under the influence of the proton, an O–P instead of a C–O bond is ruptured in the intermediate adduct (XXIV) on attack by the phosphate anion, and a new O–P bond is established in the resulting pyrophosphate.

In the absence of bases, carbodiimides react quite readily with acids[49]. In the presence of bases, especially tri-n-butylamine, the reaction velocity is considerably diminished owing to the decrease in concentration of the protonated carbodiimide and the intermediate compound (XXIV). In pyridine the reactions take place at a great rate, obviously because the carbodiimide is sufficiently basic compared with the pyridine to complete with the latter.

The starting materials for condensations in the presence of carbodiimides are taken in the form of the free acids with unprotected hydroxyl groups, or as their pyridinium or trialkylammonium salts. Apart from pyridine containing moderate amounts of water, dimethylformamide is used as solvent. The presence of considerable amounts of water is undesirable, since it is then necessary to use a large excess of the carbodiimide.

1. Adenosine and Uridine Di- and Tri-phosphates

The one-stage synthesis of ADP (III) and ATP (VII) from AMP (XXV) and 85% orthophosphoric acid in the presence of an excess of dicyclohexylcarbodiimide (DCC) in aqueous pyridine was undertaken by Khorana[53]:



ADP and ATP were isolated from the reaction mixture by precipitation as the mercuric salts with Lohmann's reagent [100 g of $Hg(NO_3)_2.8H_2O$ in 25 ml of water + 25 ml of concentrated nitric acid] at a low pH, with further separation on Dowex-2 ion-exchange resin. The ADP and ATP were isolated as their barium salts with an overall yield of 33%. Subsequent purification was effected *via* their mercuric salts by the method of LePage[54]. They were identified by paper chromatography in different solvent systems. It was found by means of ion-exchange chromatography that the ADP obtained was contaminated with ATP (up to 2%), while the ATP was in turn contaminated with ADP (up to 5%).

Use of a slight excess of 85% orthophosphoric acid results in the formation of a considerable amount (up to 55%) of the symmetrical P^1P^2-diadenosine-5′ pyrophosphate (XXVI). Since the presence of symmetrical pyrophosphates in the reaction mixture is undesirable in the preparation of natural unsymmetrical nucleotide coenzymes, conditions were studied under which the formation of by-products would be reduced to a minimum. It was found that use of a fivefold molar excess of 85% orthophosphoric acid yields only ~ 10% of P^1P^2-diadenosine-5′ pyrophosphate; a tenfold excess of the acid almost completely suppresses the formation of this compound, but it is then difficult to obtain a homogeneous mixture of the reactants.

UDP and UTP were obtained in an analogous fashion from UMP and 85% orthophosphoric acid in the presence of dicyclohexylcarbodiimide in aqueous pyridine at room temperature[55], the reaction occurring at a great rate. Increase in the duration of the reaction is undesirable, since this can lead to the formation of a large amount of organic and inorganic polyphosphates. A considerable excess of orthophosphoric acid is also undesirable, since it involves the formation of higher inorganic polyphosphates. Isolation of uridine phosphates by selective precipitation at different pH values as the barium, magnesium, and mercuric salts was unsuccessful. Direct purification on ion-exchange resins did not give yields of UTP exceeding 50%. The most satisfactory method was to isolate a mixture of UDP and UTP from the reaction mixture by passing the latter through Dowex-50 ion-exchange; further separation of the nucleotides was effected on Dowex-2. UDP and UTP were isolated as their barium salts. In spite of the considerable losses, the yield of UDP was 20–25% (purity 98%), and that of UTP 20% (purity 96%).

Reaction of FMN with 85% orthophosphoric acid in the presence of dicyclohexylcarbodiimide yielded riboflavin-5′ diphosphate[56], while uridine-5′ phosphate gave the symmetrical P^1P^2-diuridine-5′ pyrophosphate[8].

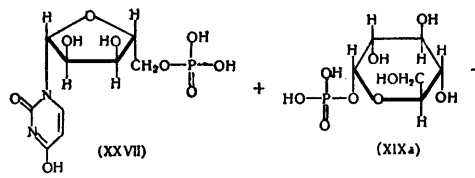
As mentioned above, the value of the carbodiimide method for producing a pyrophosphate link consists in the possibility of carrying out the reaction in aqueous pyridine. In many cases, however, a homogeneous solution of the nucleotide and the dicyclohexylcarbodiimide cannot be achieved, which probably explains the relatively low yields of coenzymes obtained. Attempts were therefore made to use the more polar, water-soluble carbodiimide[57] $(C_2H_5)_2\overset{+}{N}(CH_3)-[CH_2]_3-N=C=N-C_6H_{11}$, which was employed in the condensation of AMP to P^1P^2-diadenosine-5′ pyrophosphate[57]. The reaction velocity was found to be greater in homogeneous solutions, but the presence of an aqueous medium makes it necessary to use a very large excess of the carbodiimide.

Trialkylammonium salts tend to increase the solubility of phosphoric esters in anhydrous solvents, so that both carbodiimides and orthophosphoric acid can be used in this form for the preparation of nucleoside polyphosphates. Thus condensation of uridine-5′ phosphate in a mixture of pyridine, tri-n-butylamine, and dicyclohexylcarbodiimide gives a quantitative yield of P^1P^2-diuridine-5′ pyrophosphate[57]. The same carbodiimide and 85% orthophosphoric acid were used in the presence of two equivalents of tri-n-butylamine in anhydrous pyridine for the synthesis of nucleoside-5′ triphosphates, the yield of UTP being ~ 65%, and of ATP ~ 40%.[57]

This last variant of the carbodiimide method can be recommended in many cases for the preparation of nucleoside-5′ triphosphates.

2. Uridine Diphosphate Glucose

Direct condensation of the unprotected initial phosphates was of some interest for the preparation of UDPG, which is extremely unstable towards acids and alkalis, since this method eliminated the necessity for employing the acid or alkaline media used in the removal of protective groups. UDPG (XX) was obtained by the condensation of UMP (XXVII) with α-D-glucose-1-phosphate (XIXa) under the influence of dicyclohexylcarbodiimide[58]. The proportions of the reactants were as follows: 1 mole of the pyridinium salt of UMP + 1.9 moles of the pyridinium salt of α-D-glucose-1-phosphate + 1.7 moles of the carbodiimide in dimethylformamide. The reaction resulted in a complex mixture of substances containing only ~ 3.5% of UDPG.

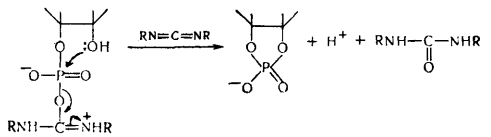


Anion-exchange chromatography proved to be unsuitable for separating the substances in the reaction mixture, since the UDPG apparently decomposed on the ion-exchange resin. Chromatography on activated charocal was more effective. One of the fractions contained ~ 42% of the ammonium salt of UDPG, which was identical with the

natural coenzyme. However, the overall yield of the substance was low. The reaction was accompanied by the formation of considerable quantities of a cyclic α-D-glucose-1,2-phosphate[58].

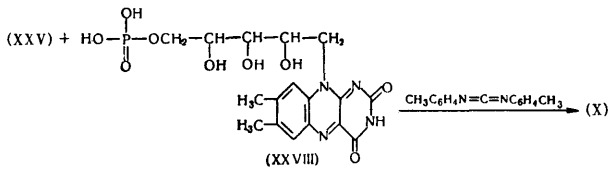
A similar result occurred in the reaction of uridine-2′ or uridine-3′ phosphate with dicyclohexylcarbodiimide in aqueous pyridine, when cyclic uridine-2′,3′ phosphate was obtained, while yeast adenylic acid (a mixture of adenosine-2′ and adenosine-3′ phosphates) under the same conditions yielded cyclic adenosine-2′,3′ phosphate[54]. However, no cyclic phosphate is formed even by the prolonged action of this carbodiimide on AMP, the symmetrical P^1P^2-diadenosine-5′ pyrophosphate being obtained in good yield instead[53].

The tendency of cyclic phosphates to be formed is probably associated with the *cis*-configuration of the hydroxyl and phosphate groups in such compounds as adenosine- and uridine-2′ and -3′ phosphates, which creates conditions favourable to intramolecular cyclisation[49]

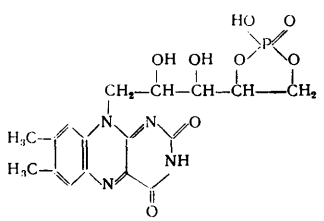


3. Flavin-Adenine Dinucleotide

FAD (X) was prepared from FMN (XXVIII) and AMP (XXV) in the presence of di-*p*-tolylcarbodiimide in aqueous pyridine[31]. Reaction was continued in the dark for 24 h, after which the resulting di-*p*-tolylurea was filtered off, and the coenzyme isolated from the reaction mixture by paper chromatography with subsequent purification *via* the uranyl salt[59]. The yield was ~ 4% and the purity 90%. The solvent systems employed were n-butanol – acetic acid – water (4 : 1 : 5), in which FAD has R_f = 0.12, for the isolation, and t-butanol – water (60 : 40), in which R_f = 0.40, for the purification.



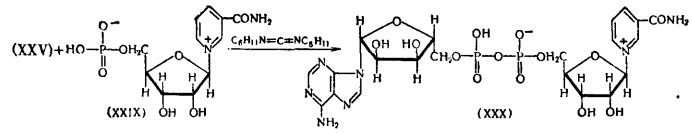
As was to be expected, the reaction product contained large quantities of the cyclic riboflavin-4′,5′ phosphate. This is consistent with the tendency of reactions with dicyclohexylcarbodiimide to form cyclic nucleotides[60,61], *e.g.* the sole product of the reaction between FMN and AMP (XXV) in the presence of this carbodiimide was the cyclic riboflavin-4′,5′ phosphate[17]:



The low yield of FAD synthesised by the carbodiimide method is associated with the fact that the reaction is directed mainly towards formation of a cyclic monophosphate owing to the *cis*-configuration of the hydroxyl groups relative to the phosphate group.

4. Nicotinamide-Adenine Dinucleotide

Little work has been done on the synthesis of this coenzyme. Nicotinamide mononucleotide (XXIX) is insoluble in anhydrous pyridine and in dimethylformamide, the solvents which are most widely used in syntheses of nucleotide coenzymes by the carbodiimide method, and therefore aqueous pyridine in the presence of excess dicyclohexylcarbodiimide was used to obtain NAD (XXX) from AMP (XXV) and nicotinamide mononucleotide (XXIX)[62], the reaction being carried out at 0°. After separation of the dicyclohexylurea which had been formed, more of the carbodiimide was added, and the reaction continued at the same temperature; this was repeated three times



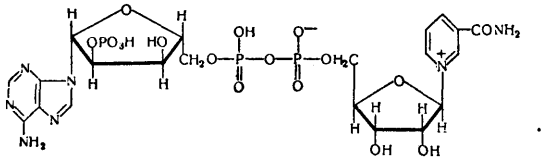
Paper chromatography enabled NAD, two symmetrical pyrophosphates (P^1P^2-dinicotinamide-nucleoside-5′ and P^1P^2-diadenosine-5′ pyrophosphates), and small amounts of the starting materials to be detected in the reaction mixture. About 50% of the nicotinamide nucleotide was converted into NAD, and only about 10% into the symmetrical P^1P^2-dinicotinamide-nucleoside-5′ pyrophosphate.

The substances in the reaction mixture were separated on Dowex-2 ion-exchange resin. The aqueous fractions contained nicotinamide nucleotide and symmetrical P^1P^2-dinicotinamide-nucleoside-5′ pyrophosphate, while the NAD was retained on the resin together with the AMP and P^1P^2-diadenosine-5′ pyrophosphate. These last three substances were eluted from the resin with 0.01 *N*, 0.1 *N*, and 1 *N* formic acid respectively. The NAD was isolated as an amorphous powder containing ~ 70% of the coenzyme.

The α- and β-anomers of nicotinamide nucleotide (approximately in the proportions 80 : 20) were used in the reaction, and therefore condensation resulted in a mixture of α- and β-anomeric forms of the coenzyme, which were difficult to separate on ion-exchange resins. An elegant method was therefore used in which the NAD was reduced with alcohol dehydrogenase to dihydro-NAD with subsequent alkali treatment to cleave the α-anomer. The NAD obtained in this way was identical with the natural product, as shown by paper chromatography and electrophoresis. The optical data ($[\alpha]_{0D}$ = 31.5 ± 0.5°) and the enzymic activity indicated that the sample was 90% pure.

In a similar way nicotinamide-adenine dinucleotide phosphate (NADP) was synthesised from adenosine-2′(3′),5′ diphosphate and nicotinamide mononucleotide in the presence of dicyclohexylcarbodiimide in aqueous pyridine. The NADP was isolated on a column containing Dowex-50, and paper electrophoresis showed it to contain small

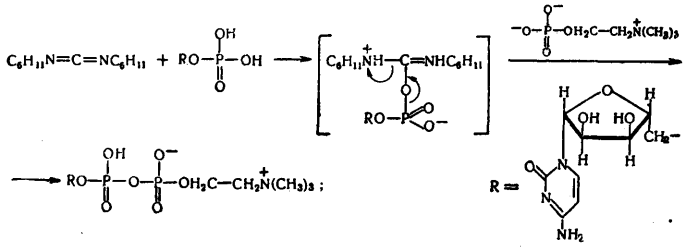
amounts of nicotinamide mononucleotide and adenosine-2′,5′ and -3′,5′ diphosphates as impurities



The considerably higher yields of NAD (XXX) and of cytidine diphosphate choline (see below) compared with FAD and UDPG can be explained by the fact that both nicotinamide mononucleotide and choline phosphate are zwitterions, and are therefore weaker nucleophilic agents than the anions of adenosine-5′ phosphate and cytidine-5′ phosphate. An adduct like the intermediate compound (XXIV) will therefore be formed mainly by attack on the protonated carbodiimide by the adenosine-5′ or cytidine-5′ phosphate, not by that of the nicotinamide mononucleotide or choline phosphate respectively. Subsequent attack on the electrophilic phosphorus of the phosphate–carbodiimide adduct of the (XXIV) type is possible both from the nicotinamide mononucleotide or choline phosphate anion and from the adenosine-5′ or cytidine-5′ phosphate anion. This leads to the formation of the required unsymmetrical pyrophosphate together with some of the symmetrical pyrophosphates P^1P^2-diadenosine-5′ and P^1P^2-dicytidine-5′ pyrophosphates, and traces of P^1P^2-dinicotinamide-nucleoside-5′ pyrophosphate, but no P^1P^2-dicholine pyrophosphate.

5. Cytidine Diphosphate Choline

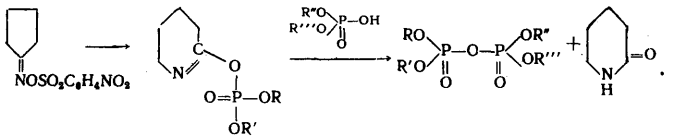
Cytidine diphosphate choline was obtained by the interaction of cytidine-5′ phosphate and choline phosphate in the presence of excess dicyclohexylcarbodiimide in aqueous pyridine at room temperature[63]. The condensation products were separated on Dowex-1 (in the formate form). The yield was 46%.



The carbodiimide method has also been used to synthesise adenosine diphosphate choline (yield 30–40%), uridine diphosphate choline (30–40%), and guanosine diphosphate choline (30–40%)[63], as well as cytidine diphosphate glycerol[64], cytidine diphosphate ribitol[65], and cytidine diphosphate ethanolamine in moderate yields.

Such reagents as derivatives of imidoyl phosphates[66–68], trihalogenoacetonitriles[13,49,51], isocyanates[69], and cyanamides[70] were also used in the formation of a pyrophosphate link, for they react with orthophosphoric acid or its esters by a mechanism similar to that characteristic of the carbodiimides, with the formation of analogous intermediate compounds.

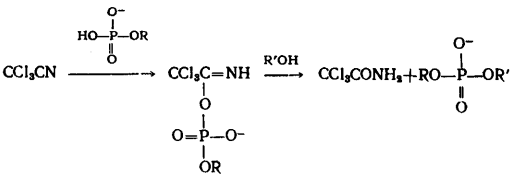
For example, derivatives of the imidoyl phosphate (XXXII),[66–68] which are structurally analogous to the intermediate compound (XXIV), are obtained by Beckmann rearrangement of the *p*-nitrobenzenesulphonate of cyclopentanone oxime (XXXI), and interact with diesters of orthophosphoric acid to form a tetraester of pyrophosphoric acid (XXXIII):



(XXXI) (XXXII) (XXXIII)

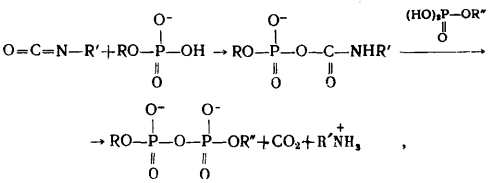
In this way UDP was obtained by the interaction of tetra-n-butylammonium 2′,3′-isopropylideneuridine-5′ benzyl phosphate and compound (XXXI), with subsequent treatment of the intermediate substance (XXXII) with dibenzyl phosphate, the yield being 40%. However, this method too is not strictly specific in syntheses of nucleotide coenzymes.

Imidoyl phosphate derivatives, formed from trichloroacetonitrile by the action of monoesters of orthophosphoric acid in the presence of pyridine[13], are able to give unsymmetrical diesters of orthophosphoric acid with alcohols, and pyrophosphates with monoesters of phosphoric acid:



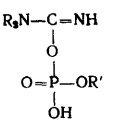
The preparation of monobenzyl phosphate and of di-*p*-chlorophenyl pyrophosphate by means of this reagent has been described.

Isocyanates[69] react with orthophosphoric acid and its monoesters to give carbamoyl phosphates[71,72], which react further with phosphoric monoesters to yield pyrophosphates:



where R = C_6H_5 and R′ = C_3H_7. P^1-Adenosine-5′ P^2-uridine-5′ pyrophosphate has been synthesised by this method using dimethylcyanamide[70].

Reactive adducts of the type

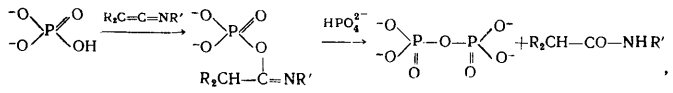


structurally similar to the intermediate (XXIV) which probably occurs in reactions with carbodiimides, are formed from dialkylcyanamides and orthophosphoric mono- and di-esters[70]. The tautomeric form of substituted cyanamides is of the carbodiimide type:

$$R_2N{-}C{\equiv}N \rightleftarrows RN{=}C{=}NR.$$

A 40% yield of cytidine-5′ diphosphate was obtained by this method from the monophosphate, by reaction with an aqueous solution of dimethylcyanamide.

Diphenylketen *p*-tolylimide and dimethylsulphonylketen methylimide are able to give symmetrical and unsymmetrical pyrophosphates with dibenzyl and diphenyl phosphates[73]:

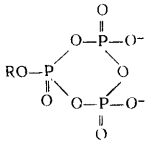


where R = C_6H_5 and R′ = $C_6H_4CH_3$, or R = CH_3SO_2 and R′ = = CH_3. Diphenylketen *p*-tolylimide with dibenzyl phosphate in benzene gives a 44% yield of tetrabenzyl pyrophosphate[73]. On treatment with an equimolar mixture of dibenzyl and diphenyl phosphates, dimethylsulphonylketen methylimide yields mainly the unsymmetrical P^1-dibenzyl P^2 diphenyl pyrophosphate[73].

The above reagents, which can be used to obtain pyrophosphates, have not yet found extensive application in the synthesis of nucleotide coenzymes.

Thus the advantages of the carbodiimide method of condensation over the phosphorochloridate method are firstly that it can be carried out in one stage without preliminary protection of the hydroxyl groups of the nucleoside, and secondly that the reaction can conveniently be carried out in a medium containing water. Nevertheless, it is not always possible to obtain homogeneous solutions of the reactants with the carbodiimide method. The formation of cyclic phosphates of the origina' nucleotides (for example in the synthesis of FAD and UDPG) also imposes considerable limitations on this method. Furthermore, in several cases symmetrical pyrophosphates are obtained as by-products with carbodiimides. All this results in yields of the synthetic nucleotide coenzymes which are not very high.

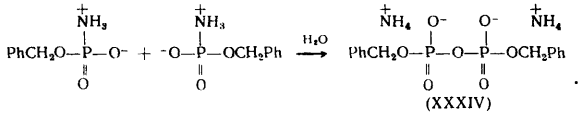
In many cases, however, the carbodiimide method can be recommended for the preparation of nucleoside-5′ triphosphates. The preferential formation of triphosphates perhaps results from hydrolysis of a cyclic metaphosphate, which is stable during the reaction but is converted into a linear phosphate in the process of isolation



IV. SYNTHESIS OF NUCLEOTIDE COENZYMES BY THE PHOSPHORAMIDATE METHOD

The efforts of research workers have been directed towards finding derivatives of phosphoric acid and its esters which would be readily available, sufficiently reactive, stable under the experimental conditions, and specific with respect to the synthesis of nucleotide coenzymes.

For example, the phosphorylation of alcohols with monobenzyl phosphoramidate yielded, not the intended phosphoric esters of the alcohol, but the diammonium salt of P^1P^2-dibenzyl pyrophosphate (XXXIV).[74] The same compound was obtained on prolonged storage (for about 5 years) of monobenzyl phosphoramidate:

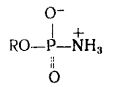


(XXXIV)

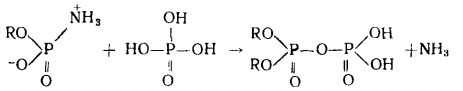
These observations suggested that monoesters of the amide of orthophosphoric acid are able to react with phosphate anions to form a pyrophosphate link. No phosphorylation of alcoholic hydroxyl groups in the molecule takes place, so that phosphoramidates can be used for the synthesis of unsymmetrical dinucleotides.

The insignificant reactivity of phosphoramidates towards alcoholic hydroxyl groups allows unprotected nucleotides to be used, which avoids the exchange reactions possible in the presence of completely esterified pyrophosphates, as was observed with the phosphorochloridate method.

Crystallographic analysis showed[75] that phosphoramidic monoesters are zwitterions, with the nitrogen atom carrying a positive charge:



Such a compound can react with nucleophilic phosphates to form pyrophosphates and polyphosphates:



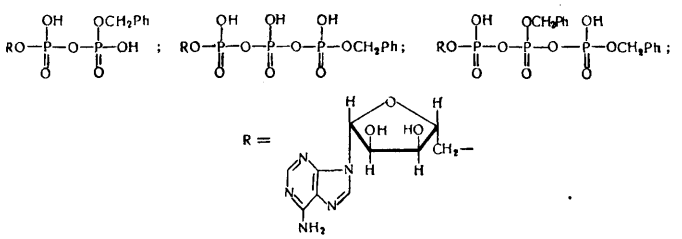
Phosphorylation takes place smoothly in the presence of pyridine or a trialkylamine. The starting materials are most often used in the form of their salts with metals (potassium, sodium) and organic bases (pyridine, trialkylamines). These salts are readily soluble in formamide, dimethylformamide, pyridine, *o*-chlorophenol, and a mixture of pyridine and *o*-chlorophenol, which are the solvents most frequently employed in this method.

1. Adenosine, Uridine, and Cytidine Di- and Tri-phosphates

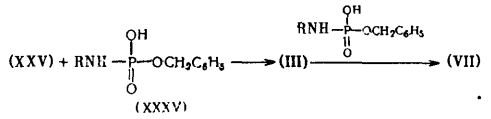
Reaction of the dipyridine salt of AMP (XXV) with benzyl phosphoramidate (XXXV)[74] in dimethylformamide containing 5% of water at 100° yielded the monobenzyl ester of adenosine-5′ diphosphate, from which a 48% yield of ADP (III), isolated as the lithium salt, was obtained after catalytic elimination of the benzyl group.

A side-reaction, resulting in the formation of a minute amount of P^1P^2-diadenosine-5′ pyrophosphate, occurs in an anhydrous medium. The presence of a small quantity of water completely suppresses this reaction, though the yield of ADP is then not very high, and a large proportion of the AMP remains unchanged.

Subsequent reaction of the ADP with compound (XXXV) or with *N*-benzyl- or *N*-cyclohexyl-phosphoramidate gave a 71% yield of ATP (VII) contaminated with a minute quantity of higher polyphosphates of adenosine. The presence of only small amounts of polyphosphates is explained by the lability of benzyl polyphosphates compared with the free polyphosphoric acids and monoesterified phosphates. Thus, for example, the monobenzyl esters of ADP and ATP proved to be quite stable during isolation, whereas the dibenzyl ester of ATP was broken down

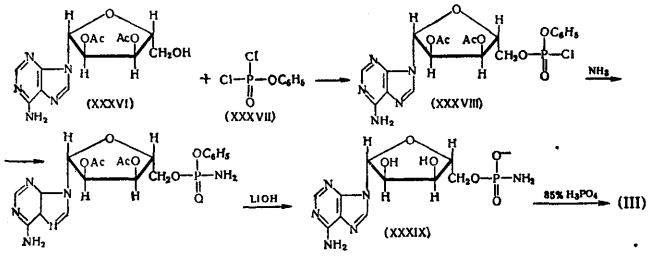


It is interesting that the phosphorylation of unprotected nucleotides with monoesterified phosphoramidates takes place in stages, ATP (VII) not being obtained directly from AMP (XXV). In order to ascertain whether it was possible to use the phosphoramidate method to synthesise natural nucleotide coenzymes, an investigation was made of the reaction between AMP and an excess of phosphoramidate in diethylformamide[76]. Ion-exchange chromatography showed the presence in the product of 34% AMP + 27% ADP + 21% ATP + 18% of higher nucleotide polyphosphates. Hence it follows that a pyrophosphate link can be produced by the phosphoramidate method, but, like the carbodiimide method, this method is not absolutely specific.



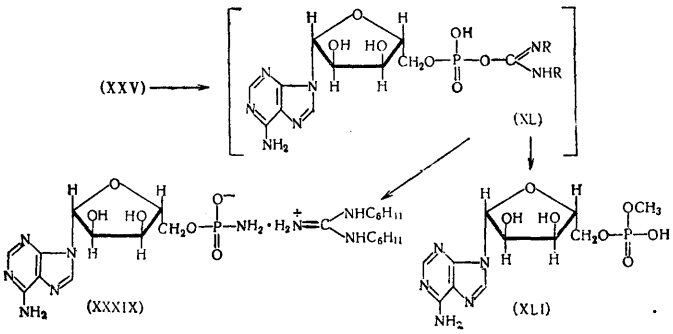
(XXXV)

Another variant of the phosphoramidate method was suggested, based on the use of a phosphoramidate derivative of a nucleotide instead of an amide of phosphoric acid. Thus 2',3'-di-*O*-acetyladenosine (XXXVI) and phenyl phosphorodichloridate (XXXVII) in the presence of quinoline were used to synthesise 2',3'-di-*O*-acetyladenosine-5' phenyl phosphorochloridate (XXXVIII), treatment of which with dry ammonia in dioxane followed by lithium hydroxide removed the acetyl and phenyl groups, giving a 25% yield of adenosine-5' phosphoramidate (XXXIX):[76]



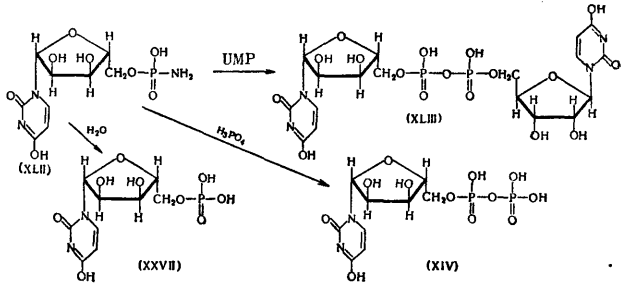
A 50% yield of ADP (III) was obtained from compound (XXXIX) by the action of a fivefold excess of 85% orthophosphoric acid in *o*-chlorophenol at 0°. No side-reactions were observed during this process, apart, perhaps, from some hydrolysis of the adenosine-5' phosphoramidate to AMP. However, such a multistage route for the synthesis of phosphoramidates is unsuitable for preparative purposes.

A simpler and more convenient method for preparing nucleoside-5' phosphoramidates from nucleotides and an excess of dicyclohexylcarbodiimide in the presence of ammonia was proposed by Khorana[77]. It was found that, in the preparation of adenosine-5' phosphoramidate from AMP (XXV) by the action of an excess of this carbodiimide, the treatment with ammonia was best carried out in t-butanol and formamide at 80° for 3 h.[78] By this method, adenosine-5' phosphoramidate (XXXIX) was isolated as the crystalline dicyclohexylguanidinium salt with a yield of 85–92%.



When the reaction was carried out in aqueous pyridine, the main product was unchanged AMP. It is difficult to believe that the AMP is obtained as a result of hydrolysis of the adenosine-5' phosphoramidate in aqueous pyridine, since phosphoramidates are known to be stable in basic and neutral media. This is most probably explained by the lability of the hypothetical intermediate compound (XL) in this medium. Methyl alcohol also proved to be unsuitable as a solvent, since the methyl ester of AMP (XLI) was then formed instead of the expected phosphoramidate.

A 75% yield of the crystalline dicyclohexylguanidinium salt of uridine-5' phosphoramidate (XLII) was obtained[79] in a similar manner, and from it UDP (XIV) was synthesised in 50% yield by the action of 85% orthophosphoric acid. The presence of UMP (XXVII) in the reaction product can be explained by the possible hydrolysis of uridine-5' phosphoramidate in acid medium, and the presence of traces of the symmetrical P^1P^2-diuridine-5' pyrophosphate (XLIII) by reaction of UMP with uridine-5' phosphoramidate. An effective method for separating the components of the reaction mixture was to absorb them on Dowex-1 (in the chloride form) and then to elute them with lithium chloride solution.



The phosphoramidate method for producing a pyrophosphate link was extended to the synthesis of cytidine-5' diphosphate (CDP) and guanosine-5' diphosphate (GDP).[30] Cytidine-5' phosphoramidate was obtained in the usual way, but difficulties were encountered in obtaining the phosphoramidate derivative of guanosine-5' phosphate, especially in isolating it, since the substance decomposed at room temperature into guanosine-5' monophosphate (GMP) and ammonia.

GDP was obtained from the ammonium salt of guanosine-5' phosphoramidate and 85% orthophosphoric acid in *o*-chlorophenol at 0°. Apart from the GDP (58%), the reaction product was found to contain GMP (34%) and P^1P^2-diguanosine-5' pyrophosphate (~ 1%).[80]

Similar results were obtained by the reaction of dicyclohexylguanidinium cytidine-5' phosphoramidate with 85% orthophosphoric acid in *o*-chlorophenol[80], the products

being 50% CDP + 31% cytidine-5′ phosphate + 3% P^1P^2-dicytidine-5′ pyrophosphate.

If phosphorylation is carried out with anhydrous orthophosphoric acid in dioxane solution, the yield of the diphosphate can be raised to 83%.[80]

Condensation of 1,3-dicyclohexylguanidinium adenosine-5′ phosphoramidate (XXXIX) with di(triethylammonium) pyrophosphate in a mixture of "tricresol" and acetonitrile yields a mixture of products containing 78% ATP + 10% ADP + 12% AMP (in molar proportions). After purification on the resin Amberlite CG-400 (in the chloride form), the ATP was isolated as its barium salt with an overall yield of 43%.[81] The 5′-triphosphates of uridine, cytidine, and deoxyadenosine were obtained in a similar manner[81].

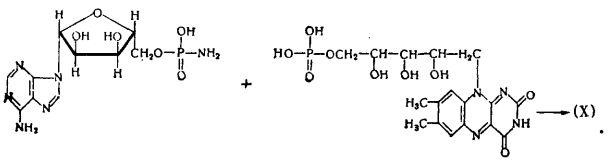
Reaction of the dicyclohexylguanidinium salts of adenosine-5′ and uridine-5′ phosphoramidates with phenyl phosphate in anhydrous pyridine gave P^1-adenosine-5′ P^2-phenyl pyrophosphate and P^1-uridine-5′ P^2-phenyl pyrophosphate in yields of 70% and 87% respectively[77].

The application of derivatives of nucleoside phosphoramidates (morpholidates, imidazolidates) to the synthesis of di- and tri-phosphates is described below.

The method described above for the synthesis of ADP (III)[75] from adenosine-5′ phosphoramidate and an excess of orthophosphoric acid was utilised by Moffatt and Khorana to prepare complex nucleotide coenzymes such as UDPG and FAD.

2. Flavin-Adenine Dinucleotide

An important problem in the synthesis of FAD (X) by the phosphoramidate method was to find a solvent system which would dissolve riboflavin-5′ phosphate (XXVIII) and adenosine-5′ phosphoramidate (XXXIX) equally well. Anhydrous pyridine is unsuitable for this purpose, but a mixture of it with *o*-chlorophenol proved satisfactory. Condensation was carried out in the presence of a 2.5 molar excess of the pyridine salt of FMN in a mixture of these solvents at room temperature for 4 days[82].

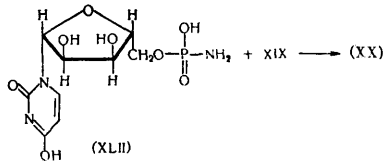


(XXXIX) (XXVIII)

A diethylaminoethylcellulose column (in the chloride form)[82] was successfully used to separate the reaction products, the FAD being isolated as the lithium salt (90%) with a yield of 40%.

3. Uridine Diphosphate Glucose

The starting materials for the synthesis of UDPG (XX)[82] were the dicyclohexylguanidinium salt of uridine-5′ phosphoramidate (XLII) and the tri-n-octylammonium salt of α-D-glucose-1-phosphate (XIX). Since these salts are readily soluble in anhydrous pyridine, the reaction can be carried out in a homogeneous medium, a fourfold excess of the sugar phosphate being used:



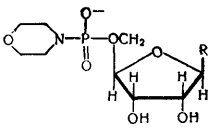
(XLII)

Apart from UDPG the reaction mixture contained UMP and small amounts of the symmetrical P^1P^2-diuridine-5′ pyrophosphate. A very effective method for isolating the coenzyme was based on the use of Dowex-2 anion-exchange resin in the chloride form. The yield of UDPG (as the amorphous lithium salt) was 59%, and the material was quite pure.

Uridine-5′ phosphoramidate is stable in aqueous pyridine, which could therefore be employed in the reaction. It was found, however, that increasing quantities of water lead to a diminution in the yield of UDPG, with a simultaneous increase in the uridine-5′ phosphate content and the formation of small quantities of P^1P^2-diuridine-5′ pyrophosphate. Phosphate anions may catalyse hydrolysis of the phosphoramidate, and the appearance of the symmetrical P^1P^2-diuridine-5′ pyrophosphate can be explained by interaction between the uridine-5′ phosphate and the uridine-5′ phosphoramidate. For the preparation of unsymmetrical pyrophosphates, therefore, it is better to employ an anhydrous medium in order to avoid the formation of undesirable by-products.

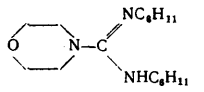
The phosphoramidate method, while possessing certain advantages over the methods previously described, is not sufficiently general or satisfactory for the synthesis of nucleotide coenzymes. Further research was therefore directed towards reactive substituted nucleoside phosphoramidates derived from various aromatic amines and nitrogen heterocycles. Nucleoside phosphoropiperidates, phosphoranisidates, and phosphoromorpholidates were therefore prepared and investigated[83]. With strong bases, for example piperidine, the extent of reaction was only 20%, whereas with weak bases (*p*-anisidine and morpholine) the yield is almost quantitative. The nucleoside phosphoropiperidates were found to be more reactive than the anisidates, the morpholidates occupying an intermediate position. Subsequent syntheses of nucleotides and nucleotide coezymes were based on the use of nucleoside phosphoromorpholidates because they were quite reactive and readily obtainable.

Nucleoside phosphoromorpholidates are obtained as their crystalline morpholino-*NN*′-dicyclohexylamidinium salts by slowly running a solution of dicyclohexylcarbodiimide in t-butanol into a boiling solution of the nucleoside-5′ phosphate and morpholine in aqueous t-butanol:



where R = adenine, uracil, cytosine, guanine.

The formation of nucleoside phosphoromorpholidates is accompanied by the side-reaction of morpholine with the carbodiimide to form guanidines of the type



The stronger the base, the more rapidly the guanidine is formed.

The possibility of using substituted nucleoside phosphoramidates in the formation of a pyrophosphate link was investigated with the synthesis of P^1-adenosine-5′ P^2-phenyl pyrophosphate as a model. In the case of the phosphoranisidate this was obtained in 90% yield, although a long time was required. The phosphoropiperidate was the most reactive, but its application was restricted by the difficulty of preparing it[83].

The phosphoramidate method using nucleoside phosphoromorpholidate derivatives was extended further to the preparation of complex nucleotide coenzymes.

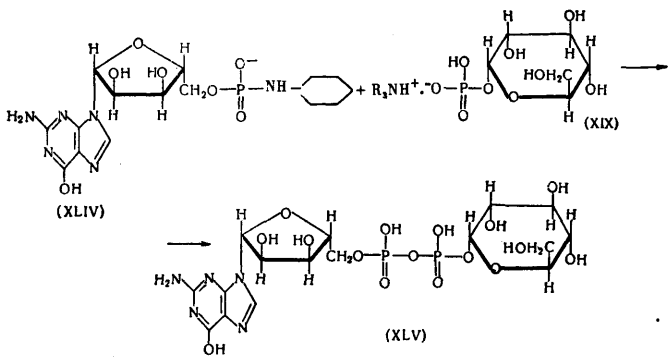
UDPG (XX) was obtained by condensing morpholino-*NN*′--dicyclohexylamidinium uridine-5′ phosphoromorpholidate with three equivalents of tri-n-octylammonium α-D-glucose-1-phosphate (XIX) in anhydrous pyridine at room temperature for 3 days[84,85]. The reaction product was chromatographed on Dowex-1 (in the chloride form), and the UDPG was isolated as its lithium salt in 70% yield. UDPG is obtained in better yield and by a far shorter route by the phosphoramidate than by other methods.

Uridine diphosphate galactose, cytidine diphosphate glycerol, and guanosine diphosphate mannose were prepared in a similar manner, the overall yields being 63–70%.[84] The same method was utilised to obtain nucleoside diphosphates. Nucleoside-5′ phosphoromorpholidates were condensed with trialkylammonium orthophosphates in anhydrous pyridine at room temperature for 2–3 days. Cytidine-5′ diphosphate and uridine-5′ diphosphate were obtained with yields of 78% and 67% respectively[83].

An overall yield of 43% of ATP could be obtained[83] by the reaction of adenosine-5′ phosphoromorpholidate with the inorganic di(tri-n-butylammonium) pyrophosphate in anhydrous pyridine for 5 h. It is interesting that a considerable quantity of ATP is formed right at the start of the reaction, but then its concentration falls rapidly, most probably owing to the effect of the excess of inorganic pyrophosphate present in the reaction mixture.

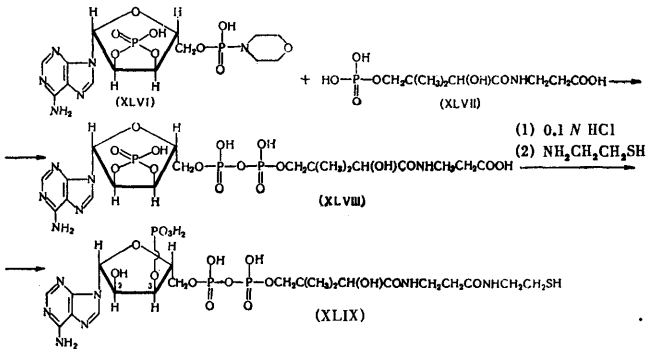
4. Guanosine Diphosphate Glucose

The phosphoramidate method has been used in the synthesis of guanosine diphosphate glucose (XLV) (isolated from *Eremothecium ashbyii*). The starting material was guanosine-5′ *N*-cyclohexylphosphoramidate (XLIV). The phosphoramidate itself and the phosphoromorpholidate did not react owing to their poor solubility in pyridine[86]. Compound (XLIV) was condensed with tri-n-octylammonium α-D-glucose-1-phosphate (XIX) in anhydrous pyridine. The synthetic product was purified by ion-exchange chromatography on Dowex-1 in the chloride form and isolated as the lithium salt.



5. Coenzyme A

Moffatt and Khorana[87,88] based their synthesis of CoA (XLIX) on the principle of the phosphoramidate method, using a phosphoromorpholidate derivative for the purpose. A 98% yield of adenosine-2′(3′)phosphate-5′ phosphoromorpholidate (XLVI) was first obtained from adenosine--2′(3′),5′ diphosphate and morpholine in the presence of dicyclohexylcarbodiimide. Compound (XLVI) was then condensed with the 4′-phosphate of DL-pantothenic acid (XLVII) in anhydrous pyridine at room temperature. The condensation product (XLVIII), after isolation and purification by ion-exchange chromatography, was treated with 0.1 *N* hydrochloric acid to open the cyclic phosphate link, after which it was brought into reaction with 2-aminoethanethiol:

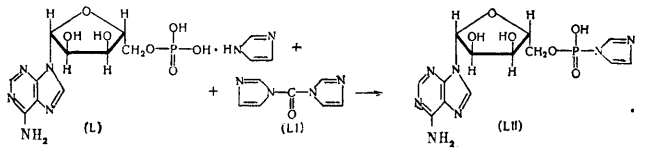


A mixture of CoA and iso-CoA (the 2′-phosphate isomer) was isolated, with an overall yield of 50%, by chromatography on an adsorption column; it possessed only 33% of the biological activity of natural coenzyme A, since the isomeric form is practically inactive.

Compound (XLVI) was also condensed with the 4′-phosphate of D-pantetheine, which also yielded the isomeric forms CoA and iso-CoA[87]. The enzymic activity of the synthetic coenzyme A was greater (86%) than that of the isocoenzyme (4%).

The possibilities of using nucleoside phosphoramidates derived from various aromatic amines and nitrogen heterocycles to produce a pyrophosphate link were further investigated by preparing adenosine-5′ phosphorimidazolidate (AMP-I) (LII) and studying its reactions with nucleophilic reagents[89–92]. The imidazolium salt of AMP-I is readily

obtained by the action of 1,1′-carbonyldiimidazole (LI) on imidazolium adenosine monophosphate (L) in anhydrous dimethylformamide, and also, in almost quantitative yield, by the reaction of 1 mole of AMP with 2–4 moles of 1,1′-carbonyldiimidazole:



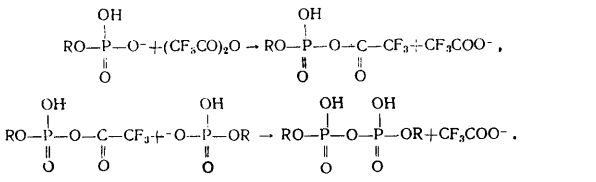
P^1P^2-Diadenosine-5′ pyrophosphate was prepared from the imidazolium salt of AMP-I and AMP, and a 57% yield of the crystalline form was isolated by chromatography on Dowex-1 (in the formate form). Condensation of AMP-I with uridine-5′ phosphate produces P^1-adenosine-5′ P^2-uridine-5′ pyrophosphate.

Reaction of the imidazolium salt of AMP-I with an excess of 85% H_3PO_4 at -10° to -20° gives a 25% yield of ADP. Treatment of the salt with aqueous ammonia in dimethylformamide–t-butanol results in cleavage of compound (LII) and the formation of adenosine-5′ phosphoramidate[89].

The phosphoramidate method of producing a pyrophosphate link is more satisfactory and simpler than other methods. Firstly, the condensation of two nucleotide molecules is carried out without preliminary protection of the hydroxyl groups, which greatly shortens and simplifies the synthesis; the carbodiimide method also requires no preliminary protection of the hydroxyl groups, but condensation often produces a complex mixture of substances, which makes it difficult to isolate the synthetic coenzyme. Secondly, the value of the method consists in the possibility of using an aqueous medium, which is very important in those cases in which the presence of even a small amount of water increases the solubility of the reactants. Furthermore, higher yields of nucleotide coenzymes can be obtained by this method.

Apart from the above three most important methods of forming the pyrophosphate link in nucleotide coenzymes, other methods, employing various condensing agents, have been used[93].

Trifluoroacetic anhydride has been used as condensing agent for the synthesis of FAD from FMN (as the sodium salt) and AMP[94]. Preliminary work on these lines was carried out by Shuster and his collaborators[95]. The phosphate anion attacks the trifluoroacetic anhydride to form an intermediate anhydride, which reacts with a second phosphate anion to form the P^1P^2-dinucleoside-5′ pyrophosphate:



Although considerable amounts of FAD can be obtained by this method, the yield is not very high (~ 10%). The FAD was isolated and purified by chromatography on powered cellulose[35].

A low yield of nicotinamide-adenine dinucleotide was obtained by a similar method from adenosine-5′ phosphate and nicotinamide mononucleotide[95].

It is interesting that condensation in the presence of acetic anhydride[96] leads to the exclusive formation of symmetrical compounds. A yield of ~ 55% of P^1P^2-dithymidine-5′ pyrophosphate has been obtained by this method from 3′-*O*-acetylthymidine-5′ phosphate by treatment with 0.275 millimole of acetic anhydride; it was shown that a twentyfold molar excess of acetic anhydride results in complete cleavage of the pyrophosphate link.

Use of a mixture of phosphoryl chloride and phosphorus pentoxide for the condensation of the appropriate two unprotected mononucleotides makes it possible to prepare FAD[97]. The reaction was carried out in phenol, in which the reactants are readily soluble[98,99]. Apart from FMN and AMP, riboflavin-5′ diphosphate and adenosine, riboflavin and ADP, and also other starting materials have been used in this synthesis.

When phosphoryl chloride was used as condensing agent, 2′,3′-*O*-isopropylideneadenosine gave 2′,3′-*O*-isopropylideneadenosine-5′ triphosphate, the yield being 45% and the purity 60–65%.[100]

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THE CHEMICAL EFFECTS OF NUCLEAR REACTIONS. THE ISOTOPIC EFFECT IN SECONDARY REACTIONS OF RECOIL ATOMS, ACCOMPANYING (n,γ) REACTIONS

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