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Synthesis in the Study of Nucleotides

Basic work on phosphorylation opens the way to an attack on nucleic acids and nucleotide coenzymes.

Alexander Todd

The term *nucleotide* requires definition, for, like many other terms, it is now used in a much broader sense than when it was first introduced. Originally it was applied only to the phosphate esters of certain N-glycosides of purine and pyrimidine bases (the nucleosides) obtained on hydrolyzing nucleic acids. Today it is applied generally and rather loosely to phosphates of N-glycosides of heterocyclic bases, and it includes not merely the simple nucleotides of the original definition but also the nucleic acids (polynucleotides) and such substances as nicotinamide nucleotide (5′-phosphate of the quaternary N-ribofuranosylnicotinamide), and adenosine triphosphate (ATP). The nucleotide coenzymes are, in general, characterized by the presence in them of at least one simple nucleotide residue and, although derivatives of riboflavin phosphate (FMN) are not glycosidic in nature, they are commonly listed among the nucleotides because of their close similarity to, and association with, true nucleotides.

In 1939 when I first began experiments in this field the fundamental substances of the group—the nucleosides obtained by hydrolyzing nucleic acids—had long been known and had been the subject of study by various workers. The early studies of Fischer had been followed by those of a few other investigators, among whom one thinks particularly of Levene. As a result it was established that the four nucleosides derived from ribonucleic acids were N-D-ribosides of adenine, guanine, uracil, and cytosine, respectively, but the size of the lactol ring in the sugar residue and the configuration at the glycosidic linkage were unknown while the point of attachment of the sugar residue in the purine nucleosides was still in dispute, although the spectroscopic evidence of Gulland and Holiday (*1*) indicated with a high degree of probability that it was N_9. Of the nucleosides from deoxyribonucleic acids, all that was known with any certainty was that they were 2-deoxy-D-ribosides of the bases adenine, guanine, thymine, and cytosine, and it was assumed that they were structurally analogous to the ribonucleosides.

The chemistry of the nucleotides—the phosphates of the nucleosides—was in a correspondingly primitive state. It may well be asked why the chemistry of these groups of compounds was not further advanced, particularly since we recognize today that they occupy a central place in the chemistry of the living cell. True, their full significance was for long unrecognized and emerged only slowly as biochemical research got into its stride, but I think a more important reason is to be found in the physical properties of compounds of the nucleotide group. As water-soluble polar compounds with no proper melting points, they were extremely difficult to handle by the classical techniques of organic chemistry and were accordingly very discouraging substances to early workers. It is surely no accident that the major advances in the field have coincided with the appearance of new experimental techniques such as paper and ion-exchange chromatography, paper electrophoresis and countercurrent distribution peculiarly appropriate to compounds of this group. Without them and without the availability of convenient and precise spectroscopic methods, I doubt whether our work would have been possible.

I decided that we should seek to clarify the nucleotide field beginning with the simplest units—the nucleosides. To do so we applied primarily the method of synthesis since the amount of preliminary information available from earlier work had at least given sufficient indication of the nature of the nucleosides to make such an attack appropriate. This phase of our work, although providing, I believe, an interesting example of the power of synthetic methods in structural work, would take an entire lecture to describe in itself, and I shall not, therefore, discuss it here. Suffice to say that this work led to the rigid establishment of the structure of the individual ribonucleosides as the 9-β-D-ribofuranosides of adenine and guanine and the 3-β-D-ribofuranosides of uracil and cytosine and to the total synthesis of all of them (*2*). The deoxyribonucleosides were similarly shown to be 9-β-2-deoxy-D-ribofuranosides in the case of the purine, and 3- β-2-deoxy-D-ribofuranosides in the pyrimidine members (*3*).

The difficulty of obtaining and of handling derivatives of 2-deoxy-D-ribose has hampered synthesis of the natural deoxyribonucleosides, but deoxyuridine has recently been synthesized (*4*) and it is likely that synthesis of the others will shortly follow. For reference the structural formulae of two typical nucleosides, the ribonucleoside adenosine (I) and

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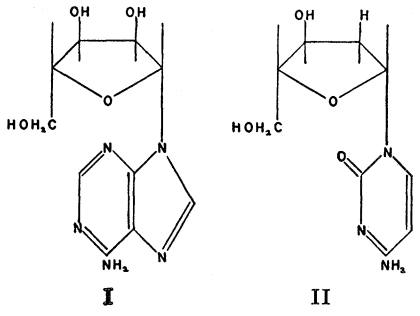
the deoxyribonucleoside deoxycytidine (II), are given in Fig. 1.

The simple nucleotides are phosphates of the nucleosides, the phosphate residue being attached to one or other of the hydroxyls in the sugar portion of the molecule. Phosphorylation of the nucleosides was thus a second essential phase in our studies. Although organic phosphates and polyphosphates are of widespread occurrence in living matter, relatively little attention had been paid in the past to their synthesis and still less to their chemical behavior. True, a number of organic phosphates had been prepared, usually by rather crude procedures not well suited to use with sensitive molecules, and we found it necessary to undertake a general study of phosphorylation in all its aspects so as to make available methods which might be satisfactory in dealing with the rather wide range of delicate structural features to be encountered in the nucleotide and nucleotide coenzyme field.

The most widely employed phosphorylating agent which emerged from these studies is dibenzyl phosphorochloridate $(C_6H_5CH_2O)_2POCl$ (*5*). This rather unstable substance may readily be prepared in solution from dibenzyl phosphite by chlorination either with chlorine itself or with N-chlorosuccinimide, and it reacts smoothly with alcohols in the presence of tertiary bases to yield alkyl dibenzyl phosphates from which the protecting groups may be removed by a variety of methods. Monodebenzylation may be effected by treatment with strong tertiary bases (*6*), by anionic fission (*7*), or by partial hydrogenation; complete debenzylation can be brought about by, for example, hydrolysis, catalytic hydrogenolysis, or ammonolysis.

Although dibenzyl phosphorochloridate has been the most generally used reagent for the phosphorylation of alcohols, other methods have been developed by us which are also effective and find uses in particular cases. These include phosphorylation with tetraesters of pyrophosphoric acid (*8*), with mixed anhydrides of diesters of phosphoric acid with stronger acids (for example, sulfonic acids) (*9*), the reaction of dialkyl phosphites with polyhalogen compounds in presence of a base and the substance to be phosphorylated (*10*), and the important phosphite route in which an alcohol is converted first to an alkyl benzyl phosphite by treatment with the mixed anhydride of benzyl phosphorous acid and diphenyl phosphoric acid, the phosphite then being converted to phosphate



I II

Fig. 1. Structural formulae of two typical nucleosides, ribonucleoside adenosine (I) and the deoxyribonucleoside deoxycytidine (II).

either via the phosphorochloridate or by direct oxidation (*11*).

As a result of these basic studies on phosphorylation we were able not only to synthesize all the theoretically possible simple nucleotides derived from the natural ribonucleosides and deoxyribonucleosides but to open the way to an attack on the more complex problems presented by the nucleic acids and the nucleotide coenzymes (*2, 12*). Since these represent two distinct though interrelated aspects of nucleotide chemistry, it will be convenient to treat them separately in this discussion.

Nucleic Acids

It is now well over eighty years since the first nucleic acid was isolated by Miescher from pus cells. Since that time it has become clear that they are essential constituents of all living cells, commonly occurring in association with proteins in the so-called nucleoproteins among which are numbered many viruses and enzymes, and that they are among the most complex of all the substances occurring in living matter. Over the years many early misconceptions have been removed, and it is now recognized that the number of individual nucleic acids is large and that all so far discovered belong to one or the other of two types—the ribonucleic acids and the deoxyribonucleic acids, the former yielding on hydrolysis D-ribose and the latter 2-deoxy-D-ribose.

The general nature of the nucleic acids is indicated by their behavior on hydrolytic breakdown, when they yield simple nucleotides which can be hydrolyzed further to give nucleosides and phosphoric acid in equimolecular proportions. The nucleic acids are thus to be regarded as polynucleotides in which, as Levene and Simms (*13*) showed, individual nucleotides are linked one to the other through phosphodiester groups; pyrophosphate and ether linkages are absent. Although the nucleic acid molecules are very large—molecular weights may be as high as several million in some cases—the number of individual nucleosides involved in them is surprisingly small. Indeed, from the ribonucleic acids in general four nucleosides are obtained—adenosine, guanosine, uridine, and cytidine—although recently there have been reports of trace amounts of one or two other nucleosides in certain individual acids. Deoxyribonucleic acids yield four main nucleosides—deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine—but at least two others, 5-methyl- and 5-hydroxymethyldeoxycytidine, are also of fairly frequent occurrence. Knowing from our intial studies the complete structure of the nucleosides, the outstanding problem in nucleic acid chemistry was the precise location of the internucleotidic linkage (*14*).

Until 1949 it was believed that alkaline hydrolysis of ribonucleic acids yielded only four simple nucleotides, which, mainly on evidence adduced by Levene, some of which we now know to have been of doubtful validity, were regarded as the 3′-phosphates of the respective nucleosides. On this basis it was not at all easy to provide a rational polynucleotide structure. In 1949, however, Cohn began to apply the technique of ion-exchange chromatography to alkaline hydrolyzates of ribonucleic acids, and he showed that they contained not four, but eight, nucleotides made up of four pairs of isomeric nucleotides, each pair derived from one of the four nucleosides; these pairs he described originally as the *a* and *b* nucleotides—that is, there were adenylic acid *a* and adenylic acid *b*, uridylic acid *a* and uridylic acid *b*, and so forth. By a fortunate coincidence this work was car-

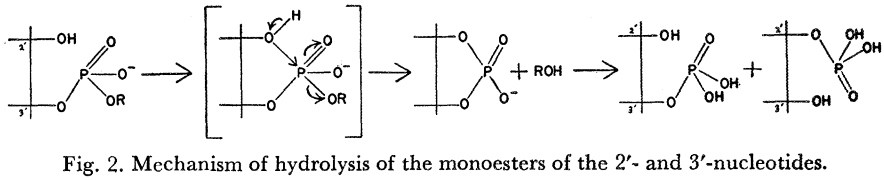


Fig. 2. Mechanism of hydrolysis of the monoesters of the 2′- and 3′-nucleotides.

ried out at much the same time as we were carrying out the synthesis of the individual ribonucleotides, and it soon became clear that the *a* and *b* nucleotides were, in fact, the 2′- and 3′-phosphates of the respective nucleosides, although at the time we were unable to say with any certainty which was the 2′- and which the 3′-derivative. It will simplify our story if I say here that we were able later to establish firmly that Cohn's *a* nucleotides are the 2′-phosphates and his *b* nucleotides the 3′-phosphates of the respective nucleosides, but the reasons for our inability to differentiate them immediately are at once interesting and of profound significance for the understanding of nucleic acid structure and behavior, and have had wide repercussions in other areas of phosphate chemistry.

Our early attempts to prepare 2′- or 3′-nucleotides individually by phosphorylation of ribonucleoside derivatives failed partly because of difficulties in group protection but more particularly because of phosphoryl migration which always led to mixtures of the 2′- and 3-′isomers being obtained. Although stable in alkaline solution, either pure isomer is converted in acid solution by phosphoryl migration into an equilibrium mixture of the two, probably by way of a cyclic intermediate. Even more interesting is the behavior of monoesters of the 2′- and 3′-nucleotides. Normally phosphodiesters are remarkably stable toward alkali, but these esters are not. They hydrolyze readily in dilute aqueous alkali, yielding always a mixture of both isomeric nucleotides. The mechanism of this hydrolysis is, we believe, that pictured in Fig. 2 (only the 2′- and 3′-positions of the sugar residue are shown since the rest of the molecule is irrelevant to our argument).

It should be noted that the over-all reaction as indicated in Fig. 2 is a two-stage process; the first is formation of the cyclic phosphate with simultaneous expulsion of the group *R*, and the second the hydrolysis of the cyclic phosphate in either of two ways to give a mixture of nucleotides. It may also be mentioned that the cyclic phosphates have indeed been isolated as first products in the above hydrolysis and have also been prepared synthetically and shown to have the expected properties. This interesting behavior is, of course, not confined to the esters of the 2′- and 3′-nucleotides; it is indeed observed in all phosphodiesters where a hydroxyl occurs *cis* to the phosphate group on the

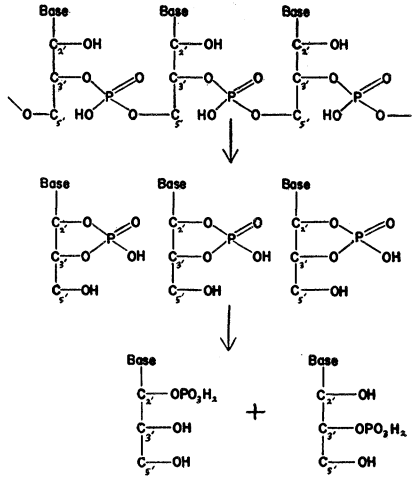


Fig. 3. Hydrolytic breakdown of a ribonucleic acid with alkali.

vicinal carbon atom. An analogous behavior is shown, for example, by the monoesters of the glycerophosphoric acids. But it is of particular relevance to an understanding of nucleic acid behavior, for, if in the abbreviated formula of the nucleotide ester used in Fig. 2 the group *R* represents a polynucleotide chain, then the analogy with ribonucleic acid itself becomes clear. The scheme shown in Fig. 3 (in which the expression "Base—$C_{2'}$—$C_{3'}$—$C_{6'}$" is used to represent a ribonucleoside residue, 2′, 3′, and 5′ being the only possible points of attachment of phosphate) represents our view of the hydrolytic breakdown of a ribonucleic acid with alkali to give exclusively a mixture of the 2′- and 3′-phosphates of the ribonucleosides.

This theory of hydrolytic breakdown provides a simple explanation for the fact that ribonucleic acids yield simple nucleotides with alkali, no larger fragments being obtained, and also explains why, in contradistinction to ribonucleic acids, the deoxyribonucleic acids are alkali stable; lacking the vicinal hydroxyl group on C_2, the latter compounds show the normal stability of simple diesters of phosphoric acid. On these considerations, coupled with certain other evidence, Brown and I (*15*) postulated both types of nucleic acids as 3′:5′-linked polynucleotides. All subsequent work has confirmed this view of their structure and it is today generally accepted. The main evidence as now available may be briefly summarized for the two types of nucleic acid as follows.

Ribonucleic acids. Ribonucleic acids are unstable to alkali, yielding simple nucleotides; the cyclic phosphates postulated as intermediates have been in fact isolated. Enzymic hydrolysis (snake venom) shows the participation of $C_{5'}$ in the internucleotidic linkage. Studies of the action of ribonuclease and spleen nuclease on the cyclic 2′:3′-phosphates of nucleosides and on esters of 2′- and 3′-nucleotides show that only $C_{3'}$ is involved in the internucleotidic linkage. This is supported also by the behavior of synthetic dinucleoside phosphates (5′:5′, 2′:5′ and 3′:5′) toward chemical and enzymic hydrolysis.

Deoxyribonucleic acids. Deoxyribonucleic acids are stable to alkali; hydrolysis by appropriate enzymes can yield 3′- or 5′-nucleotides. Acid hydrolysis yields *inter alia* pyrimidine nucleoside 3′:5′-diphosphates. Moreover, dithymidine-3′:5′-dinucleotide occurs in digests of deoxyribonucleic acids and has been identified with a synthetic specimen (*16*).

It seems probable that the nucleic acids are linear rather than branched polyesters. This is certainly true of isolated deoxyribonucleic acids, since only branching on phosphorus is theoretically possible, and such branching points involving phosphotriester linkages would not have the necessary alkali stability. In the case of ribonucleic acids we have shown that branching on phosphorus is incompatible with stability requirements, but chain branching on $C_{2'}$ of the sugar residue of the nucleoside remains a theoretical possibility although no experimental evidence for its occurrence in any ribonucleic acid has yet been adduced.

Since the essential difference between individual nucleic acids must reside in the different sequence of nucleoside residues in them, methods for sequence determination are clearly of importance for further work. Studies on this problem have not been encouraged by the fact that, as yet, no truly homogeneous nucleic acid preparations have been made, but we have devised one method for stepwise degradation which has been shown to be effective for oligonucleotides (*17*) and may be capable of application to ribonucleic acids themselves.

As yet chemical synthesis in the polynucleotide field is in its infancy. Unambiguous syntheses of dinucleoside phosphates and of at least one dinucleotide have been realized, and a start has been made using, essentially, mixed anhydride methods to develop polycondensation methods suitable for rapid polynucleotide synthesis. The increased activity now evident in this field, both in my own laboratories and in those of my

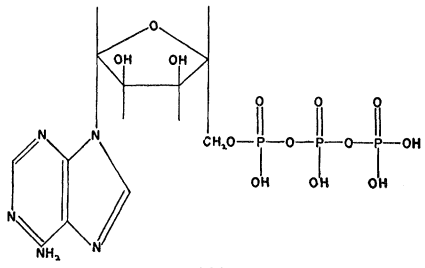
former students and colleagues, encourages me to predict rapid advances in this important aspect of nucleic acid research.

Space does not permit me to pursue this topic further and to trace how the chemical information discussed above has been combined with the results of x-ray and other studies to build up current views on the macromolecular structure of the deoxyribonucleic acids. Suffice to say that the double helical structure of the DNA molecule adumbrated first by Watson and Crick (*18*) on these foundations bids fair to open a new era in molecular biology. For it offers clues to the significance of nucleic acids in the transmission of hereditary characteristics and, taken in conjunction with our greater understanding of the properties and reactions of organic phosphates, it permits an approach to a closer understanding of the role of nucleic acid in cellular processes.

Nucleotide Coenzymes

The term *nucleotide coenzyme* is applied to a large and growing group of substances which are vital components of many enzyme systems involved in metabolic processes (*19*). These substances function in association with specific proteins or apoenzymes, the complete enzyme system being made up of the combination apoenzyme + coenzyme (von Euler). Historically, the first member of the group is cozymase or diphosphopyridine nucleotide (DPN) whose existence was recognized in 1906 by Harden and Young, although it was not in fact isolated in a pure state until 1936 (von Euler and Schlenk); it functions as coenzyme in a group of oxidation-reduction enzymes belonging to the pyridinoprotein group. Other examples are flavinadenine dinucleotide (FAD), found in many flavoproteins, adenosine triphosphate (ATP), acting as a cophosphorylase and also as a provider of the energy used in muscular contraction, and many others. All known members of the group belong to one or other of two types: (i) monoesters of polyphosphoric acids in which the esterifying group is a nucleoside derivative or (ii) unsymmetrical P^1P^2-diesters of pyrophosphoric acid in which at least one of the esterifying groups is a nucleoside derivative. Adenosine triphosphate (III) (Fig. 4) is an example of type i and cozymase or DPN (IV) (Fig. 5) of type ii.

An examination of structures III (Fig. 4) and IV (Fig. 5) at once reveals the three basic problems of nucleotide coenzyme synthesis: (i) the synthesis of nucleosides, (ii) the phosphorylation and polyphosphorylation of nucleosides, and (iii) the linkage of dissimilar molecules one to another by pyrophosphate residues. Of these problems, solutions to i and to the simple phosphorylation part of ii were available to us from the work I have already described. Here I shall discuss only the synthesis of polyphosphates and of unsymmetrical diesters of pyrophosphoric acid leading to actual coenzyme synthesis.



III

Fig. 4. Adenosine triphosphate.

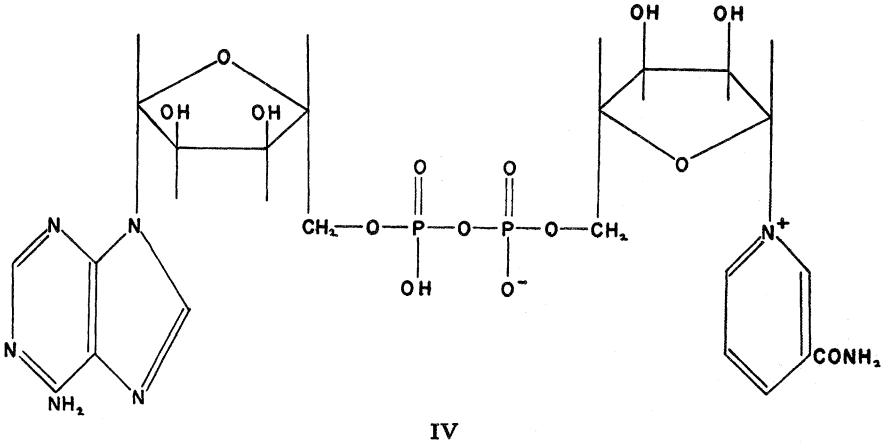
Starting from our most frequently used phosphorylating agent, the simplest and most direct route to pyrophosphates is the reaction between dibenzyl phosphorochloridate and the salt of a phosphodiester. With the variety of procedures open to us for partial or complete removal of benzyl groups, the scheme shown schematically in Fig. 6 can give not only monoesters of phosphoric and pyrophosphoric acids but also, by simple extension, either mono- or diesters of polyphosphoric acids in general.

This simple method of polyphosphate synthesis was used by us in the first total synthesis of adenosine-5′-pyrophosphate (ADP) and adenosine-5′-triphosphate (ATP) (*20*). The yields obtained in these early syntheses were usually poor for reasons which shortly became apparent to us as efforts to discover other and better procedures increased our knowledge of the properties and reactions of esters of pyrophosphoric acid.

Fully esterified pyrophosphates are very labile substances. Not only do they phosphorylate amines and alcohols but they readily undergo exchange reactions with other anions. For example, in presence of a base, tetraphenyl pyrophosphate reacts with dibenzyl phosphoric acid very rapidly even at 0°C to yield tetrabenzyl pyrophosphate and diphenyl phosphoric acid (*21*); this reaction is clearly a two-stage nucleophilic substitution, the first stage being formation of the unsymmetrical ester P^1-diphenyl P^2-dibenzyl pyrophosphate. The reaction appears to be a general one for pyrophosphates and for mixed anhydrides of phosphoric with other acids. In its most simplified form we may say that if two acids *A* and *B* form an anhydride *AB*, then if *AB* is brought into contact with the anion of an acid *C*, then if *A* is stronger (that is, has a more stable anion) than *B*, and *C* is weaker than *A*, we will have the reaction

$$AB + C \rightarrow BC + A.$$

It is this reaction that is primarily responsible for the low yields obtained in the ADP and ATP syntheses just mentioned, for the initial products of reaction are fully esterified polyphosphates and as such they rapidly undergo exchange reactions leading to reduced yields and to complex and difficultly separable mixtures. This tendency to undergo exchange reactions is less evident in the partially esterified polyphosphates (which are correspondingly less effective phosphorylating agents) and the phosphorochloridate method of poly-



IV

Fig. 5. Cozymase or diphosphopyridine nucleotide.

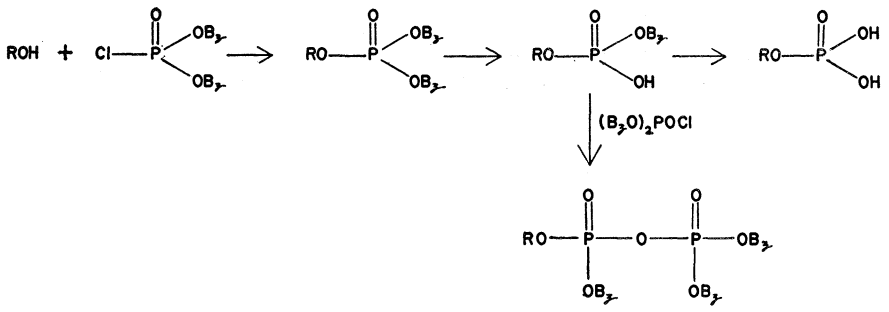


Fig. 6. Method of polyphosphate synthesis.

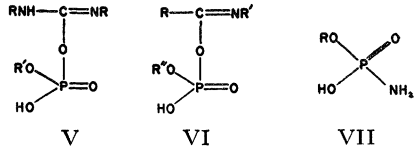
phosphate synthesis can be considerably improved if partially esterified phosphates are employed as starting materials. By this type of procedure we were able to effect total syntheses of the coenzymes flavinadenine dinucleotide (FAD) (*22*), uridine-diphosphate-glucose (UDPG) (*23*), and uridine-diphosphate-galactose (UDPGal) (*22*) as well as to provide much improved syntheses of the nucleoside-5′-polyphosphates.

The exchange reaction of polyphosphates which I have mentioned above is of particular interest because I have no doubt that it represents the main reaction employed in nature for polyphosphate synthesis. Numerous examples could be given but one will suffice. The enzymic synthesis of FAD from riboflavin phosphate and ATP described by Kornberg (*24*) is clearly a reaction of this type. It is interesting to note that in such cases nature appears to use monoesterified polyphosphates whereas in the laboratory analogous reactions seem to occur readily only with fully esterified compounds. Presumably the protein component of the enzyme involved in the biological reaction produces an effect on the polyphosphates akin to that brought about by esterification. This labilizing effect of the enzyme protein may well be analogous to the labilization of the terminal phosphate residue of ATP which we have found can be achieved by allowing it to form an inclusion compound with β-cyclodextrin (*25*) and if so it suggests a new approach to some of the problems of enzyme action and specificity. In the laboratory the exchange reaction, in which fully esterified pyrophosphates are used, has not proved very useful for coenzyme synthesis since the extreme lability of the initially formed products makes the reaction too difficult to control.

Quite apart from difficulties caused by the occurrence of exchange reactions, the phosphorochloridate route has also the disadvantage that, where nucleoside phosphorochloridates are employed, protection of the sugar hydroxyl groups by acylation or alkylation is necessary and, moreover, nonhydroxylic solvents must be used since phosphorochloridates react very readily with alcohols and with water. We have therefore devoted a good deal of effort to developing reagents for synthesizing pyrophosphates from phosphates without the need for protecting groups and which might be used even in presence of water since nucleotides are most easily handled in polar and particularly aqueous solvents. This has meant, in fact, the use of reagents which react with acids to form anhydrides more readily than they will react with water.

The first successful reagents of this type which we used were the dialkyl and diaryl carbodi-imides (RN=C=NR) (*26*). These substances—the one most frequently employed has been dicyclohexyl carbodi-imide—react smoothly with mono- and diesters of phosphoric acid to yield, respectively, di- and tetraesters of pyrophosphoric acid, and they do so even in the presence of moderate amounts of water provided that excess of the carbodi-imide is employed. The reaction goes well both in polar and nonpolar media and, although the mechanism has not been studied in detail, the first step is almost certainly an addition of phosphate to the carbodi-imide to give an adduct of type V (Fig. 7) which is then attacked by phosphate anion to give a pyrophosphate and the dialkyl urea.

The over-all reaction is very rapid and it has not been possible to halt it at the ψ-urea phosphate (V) stage; as a result, although the reaction is well nigh



V VI VII

Fig. 7. Structures V, VI, and VII.

ideal for the production of symmetrical pyrophosphates, it is less satisfactory for unsymmetrical pyrophosphates of the nucleotide coenzyme type, since treatment of a mixture of two different phosphates with a carbodi-imide normally gives a mixture of all possible symmetrical and unsymmetrical pyrophosphates, the resolution of which into its components is often a matter of considerable difficulty. Despite this defect we have used the carbodi-imide reaction to synthesize a variety of nucleoside polyphosphates, uridine-diphosphate-glucose (UDPG) (*27*) and most recently to effect the total synthesis of cozymase (DPN) and of triphosphopyridine nucleotide (TPN) (*28*). It is of some interest to note that in the cozymase synthesis from nicotinamide nucleotide and adenosine-5′-phosphate in which di-*cyclo*hexyl carbodi-imide is used, very little di-(nicotinamide nucleoside-5′)-pyrophosphate was produced with a corresponding increase in the yield of the desired unsymmetrical product (DPN). Although the reason for this unexpectedly favorable result is not yet fully understood, it may be a characteristic of carbodi-imide reactions involving dipolar-ionic molecules, since a similar result has been obtained by Kennedy (*29*) in his preparation of cytidine-diphosphate-choline.

In addition to the carbodi-imides, several other reagents have been studied, which, although in general somewhat less reactive, function in the same way and are of practical value in certain cases. These reagents include the ketenimines, cyanamide, and the dialkylcyanamides. All these share with the carbodi-imides the disadvantages of producing mixtures of products when applied to unsymmetrical pyrophosphate synthesis; the dialkylcyanamides, however, are of particular value when it is desired to operate in aqueous solvents since they are considerably more resistant to hydrolysis than the carbodi-imides (*30*).

We have devoted much effort to devising other reagents and methods for pyrophosphate synthesis specifically designed to produce unsymmetrical esters and so to avoid the waste of materials and the inconvenience of the carbodi-imide method. Of these I shall mention only briefly two interesting methods which have emerged—the imidoyl phosphate method and the phosphoramidic acid method. The first of these employs as reagents imidoyl phosphates (for example, VI, Fig. 7) (*31*) for which sev-

eral preparative methods have been devised. These substances, which bear some formal resemblance to the enol-phosphates occurring in nature, react readily enough with phosphate anions to give pyrophosphates; unfortunately, however, in polar media some exchange between phosphate and imidoyl phosphate occurs and mixed products are again obtained. Similarly monoesters of phosphoramidic acid (for example, VII, Fig. 7), when protonated, react readily with phosphoric acids to yield pyrophosphates (*31*); they can be used to excellent effect in the synthesis of monesters of polyphosphoric acids (for example, ADP and ATP) but once again they yield mixtures of symmetrical and unsymmetrical esters when applied to the synthesis of diesters of pyrophosphoric acid.

In this field of nucleotide coenzyme synthesis, we are thus still seeking an ideal method for unsymmetrical pyrophosphate synthesis, although, having already developed five distinct types of synthetic method, we have reached a point at which the synthesis of any coenzyme molecule can be undertaken with reasonable certainty of success. Our practical interest in the field today lies very much in the methods of pyrophosphate formation and in the behavior of our pyrophosphates and mixed anhydride intermediates. For in the properties of such anhydrides lies the secret of many biological processes, and it is noteworthy that in our search for new methods we are in many ways coming closer and closer to the methods of nature—not only by working in aqueous media but also by using in some of our synthetic routes intermediates which bear a striking resemblance to some of the reactive phosphate derivatives such as enol-phosphates which are widespread in living organisms.

Today the nucleotides occupy a prominent place in chemical, biochemical, and biological research, and new vistas are opening before us which may in a relatively short time lead to a far deeper understanding of the mechanisms of the living cell than seemed possible only a few years ago. And this is surely a matter of profound importance to humanity in its ceaseless struggle against disease. We still have far to go, but if, in a small way, our chemical researches contribute to such an understanding then my colleagues and I are more than satisfied. I specifically include my colleagues in this statement, for the work I have attempted to review would not have been possible without the untiring efforts of a large number of brilliant and enthusiastic students and co-workers, among whom I would mention particularly Lythgoe, Baddiley, Atherton, Kenner, Michelson, Brown, Clark and Webb. It has been a privilege to be associated with them and to all of them I owe a deep debt of gratitude.

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