A L E X A N D E R R . T O D D

Synthesis in the study of nucleotides

Nobel Lecture, December 11, 1957

I am deeply conscious of the very great honour which the Academy has done me by its recognition of my work in the field of nucleotides and nucleotide coenzymes, and it is in these circumstances a very special privilege to review this work before its members. And yet it is not an easy matter to review in the compass of one lecture a group of researches which have been my major interest over a period of some fifteen years, and to steer a safe course between the Scylla of generalities and the Charybdis of excessive detail. Nor is the content of these researches readily described in other than a highly technical way. For I am an organic chemist, albeit one who adheres to the definition of organic chemistry given by the great Swedish chemist Berzelius, namely, the chemistry of substances found in living matter, and my science is one of the more abstruse insofar as it rests on concepts and employs a jargon neither of which is a part of everyday experience. Nevertheless, organic chemistry deals with matters of truly vital importance and in some of its aspects with which I myself have been particularly concerned it may prove to hold the keys to Life itself.

In retrospect, the path which led me to the nucleotides and to the methods of study which I employ is relatively clear. After my undergraduate work in Glasgow, I had my first introduction to natural product chemistry in Frankfurt where I worked for two years with the late Professor Borsche on bile acids. Thereafter at Oxford I had the privilege of working on the anthocyanins and other natural colouring matters with Robert Robinson. It was from him that I learnt that synthesis is not merely a means of confirming a structure arrived at by degradative studies. Synthesis and degradation are complementary tools in structural work, and, properly used, synthesis can serve not only in the elucidation of structure, but can in many instances open up wider vistas regarding the significance and function of biologically important compounds. This view of synthesis in organic chemistry I have held ever since and it has played a major role in determining the pattern of all my subsequent researches.

In 1934, at the invitation of the late Professor Barger, I went to Edinburgh

to take up the structural investigation of the anti-beriberi factor, vitamin B_1. It was only natural that, following the synthesis of the vitamin with Bergel, I became interested in the specificity of vitamins and the reasons for their importance to living organisms. It was a fortunate circumstance that about this time biochemical studies had begun to reveal that the vitamins of the B group owe their importance to the fact that they are converted in the organism to more complex derivatives which function as coenzymes in metabolic systems. In these studies Swedish workers played a prominent part and some aspects of them were discussed by Theorell in his Nobel Lecture in 1955; coenzymes related to the B vitamins include *inter alia* flavin-adenine-dinucleotide (FAD) and cozymase or diphosphopyridine nucleotide (DPN). It seemed to me that, if the vitamins functioned in the form of nucleotide coenzymes, then it would be necessary to study the chemistry and mode of action of the latter if any progress were to be made in understanding vitamin specificity. But to do this meant in the first instance an attack on the chemistry of the nucleotides, a group of compounds which had been rather neglected by most chemists and about which we were consequently rather ill-informed.

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 hydrolysing nucleic acids. To-day 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 hydrolysing 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 4 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 glyco-
sidic linkage were unknown, while the point of attachment of the sugar
residue in the purine nucleosides was still in dispute, although the spectro-
scopic evidence of Gulland and Holiday 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-ribo-
sides 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 correspond-
ingly 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 im-
portant reason is to be found in the physical properties of compounds of the
nucleotide group. As water-soluble polar compounds with no proper melt-
ing 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 coun-
tercurrent distribution, peculiarly appropriate to compounds of this group.
Without them and 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 meth-
od 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 syn-
thetic 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 ribonucleo-
sides 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.
The deoxyribonucleosides were similarly shown to be 9-β-2-deoxy-D-ribo-
furanosides in the case of the purine, and 3-β-2-deoxy-D-ribofuranosides in
the pyrimidine members. The difficulty of obtaining and of handling deriva-

tives of 2-deoxy-D-ribose has hampered synthesis of the natural deoxyribo-nucleosides, but deoxyuridine has recently been. synthesized 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 the deoxyribonucleoside deoxycytidine (11) are given below.

OH OH   OH H

HOH₂C   HOH₂C

(I)   (II)
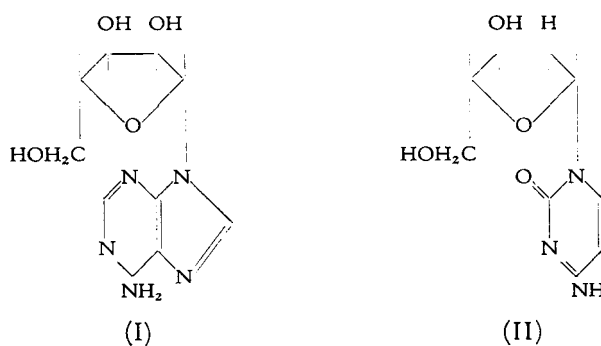
The simple nucleotides are phosphates of the nucleosides, the phosphate residue being attached to one or other of the hydroxyls in the sugar portion of the molecule. Phosphorylation of the nucleosides was thus a second essential phase in our studies. Although organic phosphates and polyphosphates are of widespread occurrence in living matter, relatively little attention had been paid in the past to their synthesis and still less to their chemical behaviour. 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 $(C_6H_5CH_2O)_2POCl$. This rather unstable substance is readily prepared in solution from dibenzyl phosphite by chlorination either with chlorine itself or with *N*-chlorosuccinimide, and it reacts smoothly with alcohols in 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, by anionic fission, or by partial hydrogenation; complete debenzylation can be brought about by e.g. 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 tetra-esters of pyrophosphoric acid, with mixed anhydrides of diesters of phosphoric acid with stronger acids (e.g. sulphonic acids), the reaction of dialkyl phosphites with polyhalogen compounds in presence of a base and the substance to be phosphorylated, 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 either via the phosphorochloridate or by direct oxidation. 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. 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 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 behaviour on hydrolytic breakdown, when they yield simple nucleotides which can be hydrolysed 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 showed, individual nucleotides are linked one to the other through phosphodiester groups - pyrophosphate and ether link-

ages are absent. Although the nucleic acid molecules are very large - molec-
cular 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 4 nucleosides are obtained - adenosine, gua-
nosine, 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 4 main nucleosides - deoxyadenosine, deoxy-
guanosine, deoxycytidine, and thymidine - but at least two others, 5-methyl-
and 5-hydroxymethyl-cytidine, are also of fairly frequent occurrence. Know-
ing from our initial studies the complete structure of the nucleosides the
outstanding problem in nucleic acid chemistry was the precise location of
the internucleotidic linkage.

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 chro-
matography to alkaline hydrolysates 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, i.e. there were
adenylic acid *a* and adenylic acid *b*, uridylic acid *a* and uridylic acid *b,* etc.
By a fortunate coincidence this work was carried 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'-deriva-
tive. 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 inabil-
ity to differentiate them immediately are at once interesting and of profound
significance for the understanding of nucleic acid structure and behaviour,
and have had wide repercussions in other areas of phosphate chemistry.
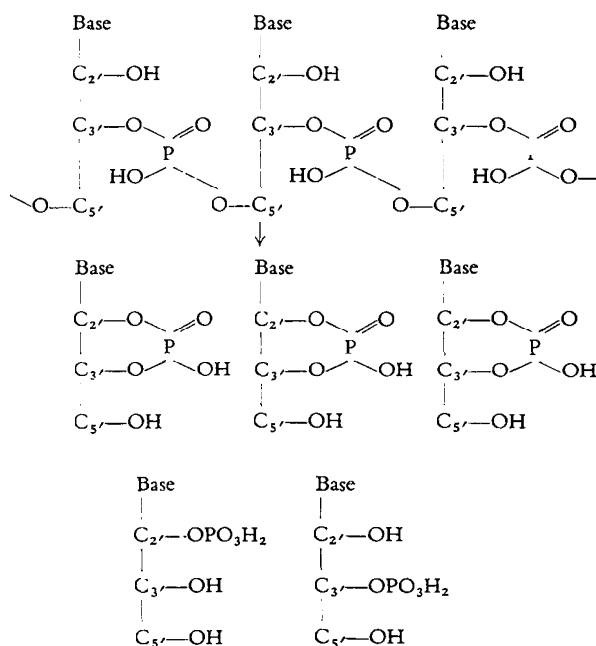
Our early attempts to prepare 2'- or 3'-nucleotides individually by phos-
phorylation of ribonucleoside derivatives failed partly because of difficulties
in group protection but more particularly because of phosphoryl migration
which always led to mixtures of the 2'- and 3'-isomers being obtained. Al-

though 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 behaviour of mono-esters of the 2'- and 3'-nucleotides. Normally, phospho-diesters are remarkably stable towards alkali, but these esters are not. They hydrolyse readily in dilute aqueous alkali yielding always a mixture of both isomeric nucleotides. The mechanism of this hydrolysis is, we believe, that pictured below (only the 2'- and 3'-positions of the sugar residue are here shown since the rest of the molecule is irrelevant to our argument).
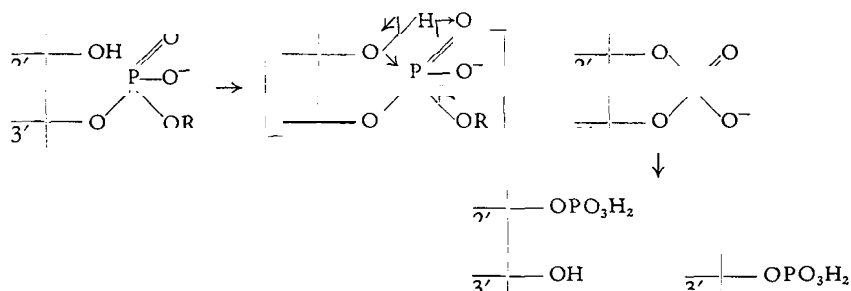
It should be noted that the overall reaction as indicated above is a two-stage process; the first is formation of the cyclic phosphate with simultaneous ex-pulsion of the group R, and the second the hydrolysis of the cyclic phosphate in either of two ways to give a mixture of nucleotides. It may also be men-tioned 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 behaviour 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 vicinal carbon atom. An analogous behaviour is shown, for example, by the mono-esters of the glycerophosphoric acids. But it is of particular rel-evance to an understanding of nucleic acid behaviour, for, if in the abbre-viated formula of the nucleotide ester used above, the group R represents a polynucleotide chain, then the analogy with ribonucleic acid itself becomes clear. The following scheme (in which the expression **Base**—$C_{2'}$,—$C_{3'}$,—$C_{5'}$, 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 hy-drolytic 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

Base
|
$C_{2'}$—OH

$C_{3'}$—O\ \ ⁄O
$\quad$ P
$\quad$ HO⁄ \
\O—$C_{5'}$

Base
|
$C_{2'}$—OH

$C_{3'}$—O\ \ ⁄O
$\quad$ P
$\quad$ HO⁄ \
O--$C_{5'}$

Base
|
$C_{2'}$—OH
|
$C_{3'}$—O\ \ ⁄O
$\quad$ HO⁄ \O—

O—$C_{5'}$

↓

Base
|
$C_{2'}$—O\ ⁄O
| $\quad$ P
$C_{3'}$—O⁄ \OH

$C_{5'}$—OH

Base
|
$C_{2'}$—O\ ⁄O
| $\quad$ P
$C_{3'}$—O⁄ \OH

$C_{5'}$—OH

Base
|
$C_{2'}$—O\ ⁄O
| $\quad$ P
$C_{3'}$—O⁄ \OH

$C_{5'}$—OH

Base
|
$C_{2'}$—OPO_3H_2
|
$C_{3'}$—OH
|
$C_{5'}$—OH

Base
|
$C_{2'}$—OH
|
$C_{3'}$—OPO_3H_2
|
$C_{5'}$—OH

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 di-esters of phosphoric acid. On these considerations, coupled with certain other evidence, Brown and I 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. 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 shows that only $C_{(3')}$ is involved in the internucleotidic linkage. This is supported also by the behaviour of synthetic dinucleoside phosphates (5':5', 2':5' and 3':5) towards chemical and enzymic hydrolysis.

Deoxyribonucleic acids. 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.

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 determinations 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 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.

Time 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 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.
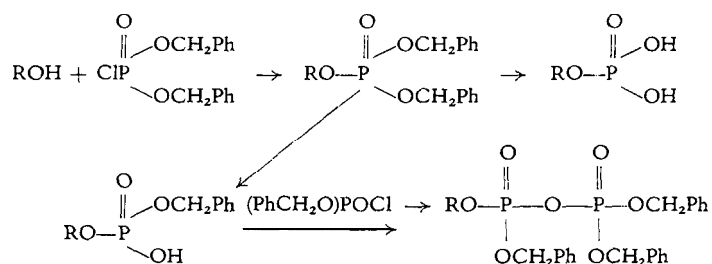
Nucleotide coenzymes

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. They function in association with specific proteins or apoenzymes, the complete enzyme system being made up of the combination apoenzyme + coenzyme (v. 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 (v. Euler and Schlenk); it functions as coenzyme in a group of oxidation-reduction enzymes belonging to the pyridinoprotein group. Other examples are flavin-adenine-dinucleotide (FAD) found in many flavoproteins, adenosine triphosphate (ATP) acting as a cophosphorylase and also as a provider of the energy used in muscular conkaction and many others. All known members of the group belong to one or other of two types: (a) monoesters of polyphosphoric acids in which the esterifying group is a nucleoside derivative, or (b) unsymmetrical P^1P^2-diesters of pyrophosphoric acid in which at least one of the esterifying groups is a nucleoside derivative. Adenosine triphosphate (III) is an example of type (a) and cozymase or DPN (IV) of type (b).

(III)

(IV)
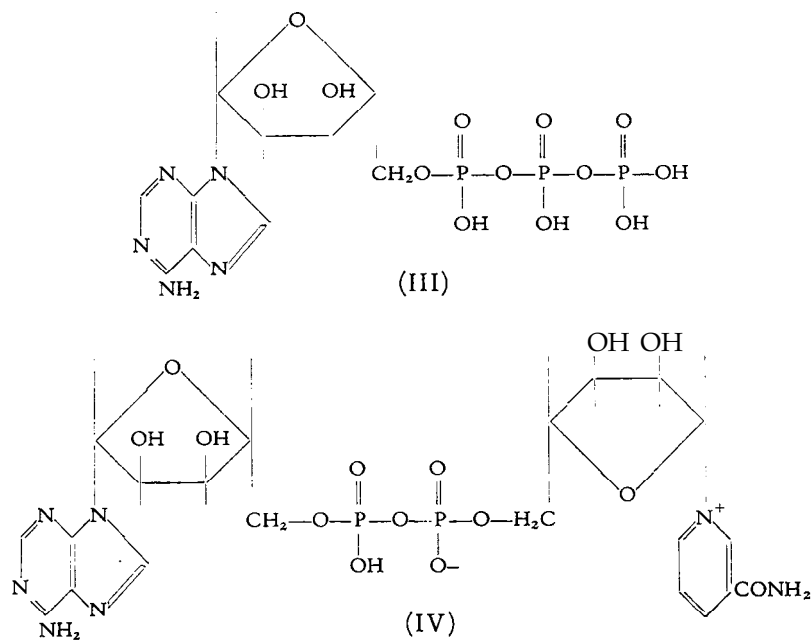
An examination of structures (III) and (IV) at once reveals the three basic problems of nucleotide coenzyme synthesis: (1) the synthesis of nucleosides, (2) the phosphorylation and polyphosphorylation of nucleosides, and (3) the linkage of dissimilar molecules one to another by pyrophosphate residues. Of these problems, solutions to (1) and to the simple phosphorylation part of (2) 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.

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 below can give not only monoesters of phosphoric and pyrophosphoric acids but also, by simple extension, either mono- or di-esters of polyphosphoric acids in general.

$$
\begin{array}{cccc}
 & \overset{O}{\underset{\displaystyle \|}{}} & \overset{O}{\underset{\displaystyle \|}{}} & \overset{O}{\underset{\displaystyle \|}{}} \\
\text{ROH} + \text{ClP}\big\langle{}^{\text{OCH}_2\text{Ph}}_{\text{OCH}_2\text{Ph}} & \rightarrow\ \text{RO—P}\big\langle{}^{\text{OCH}_2\text{Ph}}_{\text{OCH}_2\text{Ph}} & \rightarrow\ \text{RO—P}\big\langle{}^{\text{OH}}_{\text{OH}}
\end{array}
$$

$$
\overset{O}{\underset{\displaystyle \|}{\text{RO—P}}}\big\langle{}^{\text{OCH}_2\text{Ph}}_{\text{OH}}\ \ \xrightarrow[\ \ \ \ \ \ \ \ \ \ \ \ \ \ ]{(\text{PhCH}_2\text{O})\text{POCl}}\ \ \text{RO—}\overset{O}{\overset{\|}{\underset{\text{OCH}_2\text{Ph}}{\underset{|}{\text{P}}}}}\text{—O—}\overset{O}{\overset{\|}{\underset{\text{OCH}_2\text{Ph}}{\underset{|}{\text{P}}}}}\text{—OCH}_2\text{Ph}
$$

This simple method of polyphosphate synthesis was used by us in the first total syntheses of adenosine-5'-pyrophosphate (ADP) and adenosine-5'-triphosphate (ATP). 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 $0°$ to yield tetrabenzyl pyrophosphate and diphenyl phosphoric acid; this reaction is clearly a two-stage nucleophilic substitution, the first stage being formation. of the unsymmetrical ester P[1]-diphenyl P²-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 (i.e.
has a more stable anion) than B, and C is weaker than A we will have the
reaction AB + C→ 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 polyphosphate synthesis can be consider-
ably improved if partially esterified phosphates are employed as starting ma-
terials. By this type of procedure we were able to effect total syntheses of the
coenzymes flavin-adeninedinucleotide (FAD), uridine-diphosphate-glucose
(UDPG) and uridine-diphosphate-galactose (UDPGal), as well as to pro-
vide 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 ex-
amples could be given but one will suffice. The enzymic synthesis of FAD
from riboflavin phosphate and ATP described by Kornberg is clearly a reac-
tion of this type. It is interesting to note that in such cases Nature appears to
use mono-esterified pyrophosphates whereas in the laboratory analogous
reactions seem to occur readily only with fully esterified compounds. Pre-
sumably the protein component of the enzyme involved in the biological
reaction produces an effect on the polyphosphates akin to that brought about
by ester&cation. 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 com-
pound with β–cyclodextrin, and if so it suggests a new approach to some
of the problems of enzyme action and specificity. In the laboratory the ex-
change reaction, using fully esterified pyrophosphates, has not proved very
useful for coenzyme synthesis since the extreme lability of the initially form-
ed products makes the reaction too difficult to control.

Quite apart from difficulties caused by the occurrence of exchange reac-
tions, the phosphorochloridate route has also the disadvantage that, where
nucleoside phosphorochloridates are employed, protection of the sugar hy-

droxyl groups by acylation or alkylation is necessary and, moreover, non-hydroxyhc 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). These substances - the one most frequently employed has been dicyclohexyl carbodi-imide - react smoothly with mono- and di-esters of phosphoric acid to yield respectively di- and tetra-esters of pyrophosphoric acid and they do so even in the presence of moderate amounts of water provided that excess of the carbodi-in-ride is employed. The reaction goes well both in polar and non-polar 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), which is then attacked by phosphate anion to give a pyrophosphate and the dialkyl urea. The overall 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 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 ofnucleoside polyphosphates, uridine-diphosphate-glucose (UDP) and most recently to effect the total synthesis of cozymase (DPN) and of triphospho-pyridine nucleotide (TPN). It is of some interest to note that in the cozymase synthesis from nicotinamide nucleotide and adenosine-5′ phosphate using dicyclohexyl carbodi-imide 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 favourable 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 in his preparation of cytidine-diphosphatecholine.

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 keten-imines, cyanamide and the dialkyl cyanamides. All these share with the carbodi-imides the disadvantage 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.
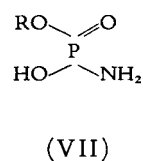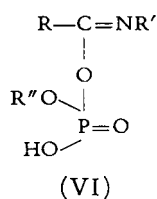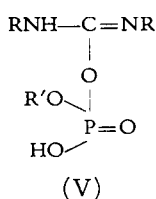
We have devoted much effort to devising other reagents and methods for pyrophosphate synthesis specifically designed to produce unsymmetrical esters and so to avoid the waste of materials and the inconvenience of the carbodi-imide method. Of these I shall mention only briefly two interesting methods which have emerged - the imidoyl phosphate method and the phosphoramidic acid method. The first of these employs as reagents imidoyl phosphates (e.g. VI) for which several 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 mono-esters of phosphoramidic acid (e.g. VII), when protonated, react readily with phosphoric acids to yield pyrophosphates; they can be used to excellent effect in the synthesis of mono-esters of polyphosphoric acids (e.g. of 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 to-day

lies very much in the methods of pyrophosphate formation and in the behaviour 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.

To-day 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 specially 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.