Activated Esters in the Field of Polynucleotides and Coenzymes

by

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I should like to thank Professor H. D. Springall who made facilities available for this work.

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All the work reported in this thesis was carried out by the author, under the supervision of Dr. D. Cohen.

ABSTRACT

The 1-ethoxyvinyl esters of a variety of acids have been prepared, and isolated in some cases. In other cases their presence was demonstrated by their spectra and chemical properties. Thus 1-ethoxyvinyl thiolates and sulphonates were isolated, characterised and were found to be readily attacked by nucleophiles. Monoesters of sulphuric acid reacted with ethoxyacetylene to give 1-ethoxyvinyl sulphates, attempts to purify these compounds being unsuccessful. They were, however, successfully used to prepare the coenzyme analogue adenosine-5'-sulphatophosphate in up to 45% yield.

The 1-ethoxyvinyl esters of pyruvic and oxalic acids were prepared and found to undergo rearrangement on heating to 70°. Carbon monoxide and β-keto esters were the products. A five centre rearrangement in the first case, and a five centre followed by a four centre rearrangement in the second case has been suggested for the mechanisms of these rearrangements.

Although the diethoxyvinyl esters of phthalic acid could not be isolated (although this was possible when terephthalic acid was used) phthaloyl protected amino acids and esters were prepared by activation of phthalic acid using ethoxyacetylene in the presence of a free amino acid or its ester. The reaction was conducted successfully in aqueous and non-aqueous solvents, and when an optically active amino-acid was used, no racemisation was detected. This method holds

potentialities both for protection and for end group labelling of peptide hydrolysis fragments.

Polynucleotides containing thymidylate and deoxyadenylate residues were synthesised using DCC as the condensing reagent. One of the mononucleotides was labelled with ^{32}P so that nearest-neighbour base sequence analyses could be conducted on the polymeric products. The results of these analyses suggest that the base sequence is essentially random, but that thymidylate residues predominate over deoxyadenylate residues in the ratio of ~~5.4 ± 2.6~~ 27·5 :1 in the polymers. Reasons for this predominance have been suggested.

The chemistry of alkoxyalkynes and of enol esters has been reviewed and a survey has been made of recent techniques of polynucleotide synthesis. The structure, biosynthesis and properties of nucleic acids and coenzymes have also been reviewed.

CONTENTS

PART I

Introduction

(A) Nucleic Acids and Coenzymes - Structure and Function.

The quantity of information about nucleic acids would probably show an exponential relationship with time if it were plotted from 1869 to the present day. The increase since 1953 is positively alarming to the layman.

These dates are not altogether arbitrary - in 1869 Friedrich Miescher discovered the nucleic acids whilst in 1953 Watson and Crick proposed a theory of DNA structure. These landmarks in biochemical history, have provided the seeds for the germination of a new outlook on cellular mechanisms, enlarging the horizons of biochemistry and biophysics, evolutionary theory, information theory, physiology and all of the other sub-divisions that now constitute the science of biology. Indeed, they have triggered off a reawakening of this science.

Some of the reasons for this feverish interest lie in the dual function of DNA which are fundamental properties of life, no matter how primitive. Thus DNA is the carrier of 'information' which is handed on from one generation to the next, and also directs the synthesis of new organisms by the construction of enzymes, some of which direct its own synthesis. Although the importance of fats, proteins, etc. in the life of the cell must be recognised, the 'fundamental' entity does appear

to be the nucleic acid, and some of the simplest examples of 'life' consist of no more than genetic material (DNA) surrounded by a protective protein coat.

Work on coenzyme chemistry and biology has proceeded in a similar fashion over the past decade, and their importance in cellular metabolism is now recognised.

1. Nucleic acids

The 'nucleic acid era' is generally acknowledged to have been initiated in 1869 when Miescher, then at Tübingen, digested pus cells from discarded surgical bandages with pepsin in the presence of hydrochloric acid, and extracted the digest with ether. The cell nuclei remained in the aqueous phase and were concentrated by gravity and filtered off. The material was appropriately named nuclein, shown to be acidic, insoluble in dilute acids but soluble in dilute alkali. The most interesting fact about nuclein was its high phosphorus content, since at that time the only known phosphorus compound in animal tissues was lecithin. Indeed, Hoppe Seyler, in whose laboratories Miescher was working, appears to have been sceptical of these results, and repeated the work himself before publishing it.

(a) The structure of nucleic acids

Many procedures for the extraction of nucleoprotein from cells from various sources have been developed. There also exists the problem of separating the two components of the nucleoprotein complex. Much of the early information about nucleic acids led to faulty conclusions

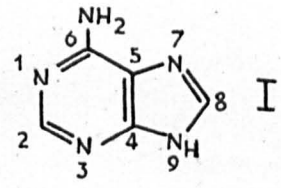
regarding their structure and size because of the drastic methods used for their extraction and purification and the inadequate procedures employed for determining their molecular weights. The procedures originally developed usually included alkaline extraction from the tissues, resulting in extensive depolymerisation of the ribonucleic acids, and denaturing of the deoxyribonucleic acids. Hence on this faulty basis the tetranucleotide theory for nucleic acid structure held sway for such a considerable period of time.[1] Thus molecular weights of about 1.3×10^3 for ribonucleic acids were quoted. The alkaline extraction procedure has now been completely abandoned in favour of milder procedures utilising extraction with salt solutions, anionic detergents, phenol and other reagents.[2,3,7] It is now well established that both ribonucleic acids and deoxyribonucleic acids are generally highly polymerised molecules in the native state, with molecular weights ranging up to 2×10^6 for tobacco mosaic virus ribonucleic acid and 8×10^6 for thymus gland deoxyribonucleic acid.

The basic characteristics of the two types of nucleic acid are summarised in Table 1 below.

TABLE 1

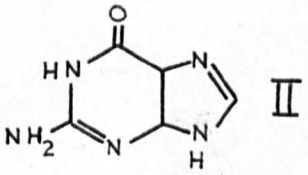
	DNA	RNA
Purines	Adenine and guanine	Adenine and guanine
Pyrimidines	Cytosine and thymine	Cytosine and uracil
Sugar	2-Deoxy-D-ribose	D-ribose
Phosphate	One per sugar	One per sugar
Source	Plant and animal nuclei	Plant and animal cytoplasm and nuclei
Former Identification	Thymus, or animal nucleic acid	Yeast, or plant nucleic acid

The structures of the purines and pyrimidines are shown in (I, II, III, IV and V), the carbon numbering system being shown.



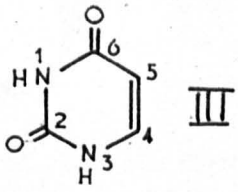
I

Adenine = 6-amino-purine



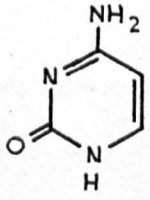
II

Guanine = 2-amino-1,6-di-hydro-6-oxopurine



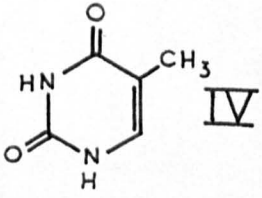
III

Uracil



V

Cytosine

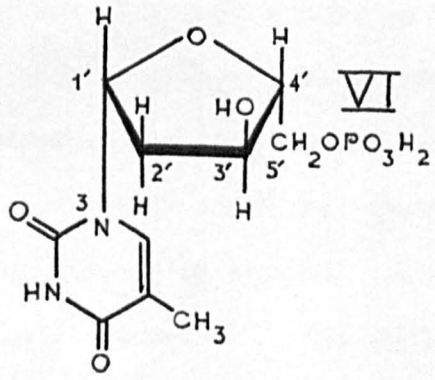


IV

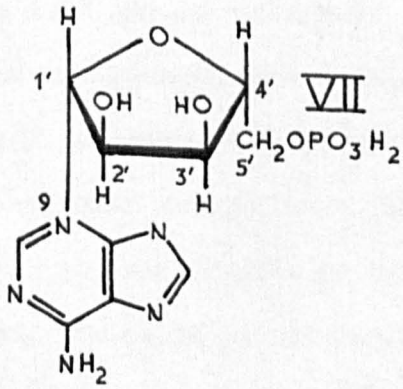
Thymine

Other bases are also found in trace quantities in nucleic acids, and 5-hydroxymethylcytosine replaces cytosine completely in the T-even bacteriophages.

The sugars in nucleic acids have the furanose configuration, and are linked from the 1-carbon atom of the pentose to the 3-N-atom of pyrimidines, or the 9-N-atom of purines, these glycosidic linkages having the β-configuration. Such a combination of a heterocyclic base is termed a nucleoside (ribonucleoside) or deoxynucleoside (deoxyribonucleoside). A nucleotide is a monophosphate ester of a nucleotide although the term has often been extended to include, for example, riboflavin, which has no glycosidic linkage, but is a derivative of ribitol. (VI and VII) show the structures of two typical nucleotides.



Thymidylic Acid = 3-β-D-2'-deoxyribofuranosylthymine-5'-phosphate

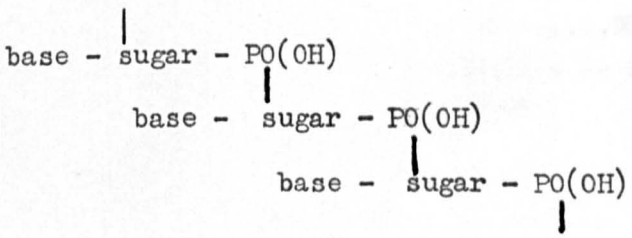


Adenylic Acid = 9-β-D-ribofuranosyladenine-5'-phosphate

Infrared and ultraviolet data of derivatives as well as X-ray crystallography indicate that the hydroxyl groups exist predominantly

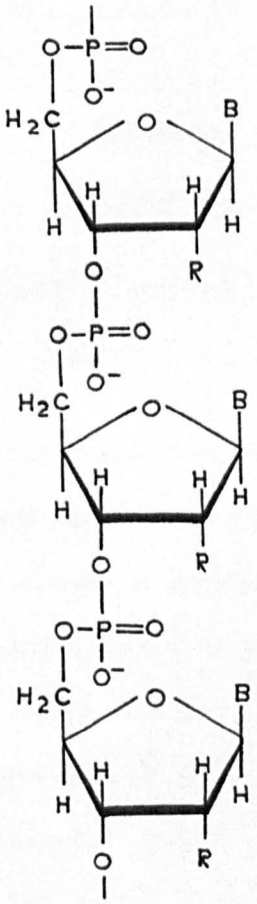
in the keto-form (as shown) and the amines exist predominantly in the amino form, as opposed to the imino form.

Electrometric titration of monoribonucleotides of RNA and of DNA led Levene and Simms[4] to the concept of nucleic acids as polynucleotides in which nucleoside residues were linked by phosphodiesters bridges, as shown in(VIII)



VIII

The now classical studies by Brown, Todd and co-workers at Cambridge, coupled with enzymatic studies on the specific hydrolysis of phosphodiester bonds in nucleic acids, and the subsequent polynucleotide syntheses, have established that the phosphodiester moiety links the 3'- and 5'-positions of adjacent nucleosides, and the details of this work have been well reviewed.[5,6] The nucleic acid structure is illustrated in (IX).



R = -OH .....RNA

R = -H .....DNA

B = Purine or Pyrimidine base

IX

IX

No sound evidence has been produced yet for chain-branching in DNA (formation of a tertiary phosphate ester) or RNA (tertiary phosphate or utilising a 2'-hydroxyl group of the D-ribose).

During the early 1950's, Chargaff and co-workers carefully

analysed the ratios of total pyrimidine to total purine bases in a variety of DNA species and found the following regularities:

$$[\text{adenine}] = [\text{thymine}]$$
$$[\text{guanine}] = [\text{cytosine}]$$

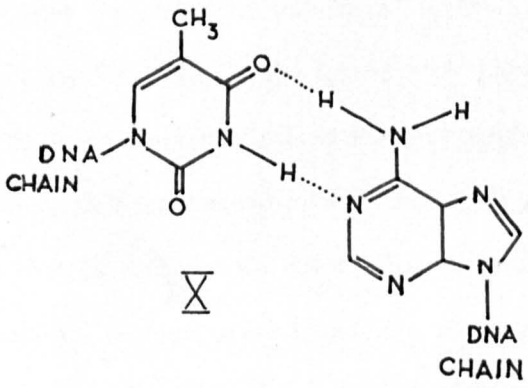
i.e. $[\text{adenine}] + [\text{guanine}] = [\text{thymine}] + [\text{cytosine}]$

$$[\text{adenine}] + [\text{cytosine}] = [\text{guanine}] + [\text{thymine}] \qquad (7)$$

These results plus those from the work of Astbury and Bell who reported that X-ray diffraction measurements on paracrystalline DNA fibres showed a regular spacing of c. 3.434 Å along the fiber axis, which was interpreted as a succession of flat nucleotides standing perpendicular to the axis,[8] led Watson and Crick to propose their theory for the structure of DNA, a theory which with one or two modifications, is still accepted today.[9] Before this, Furberg had proposed that the planes of the sugar rings were parallel to the long axis of the DNA molecule whilst the bases were at right angles to the sugars and to the long axis, with the latter oriented into a spiral.[10] Watson and Crick combined all of the available data into a double helix theory in which the two helical chains are each coiled around the same axis, with their components arranged linearly but in opposite directions. The pyrimidines and purines were located on the inside of the helix, and the phosphates on the outside. A pair of nucleotides occurs in the direction of the long axis every 3.4 Å. The structure repeats every 10 nucleotides or 34 Å,

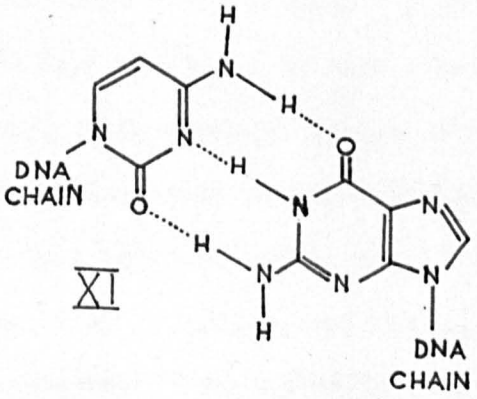
the angle between adjacent nucleotides in the same chain being assumed to be 36°.

One of the most attractive features of the postulated structure is the hydrogen bonding, which is in complete agreement with the analytical data of Chargaff. It is assumed that for every thymine nucleus in one chain, there is an adenine nucleus hydrogen bonded to it in the adjacent antiparallel chain, and similarly for every guanine in one chain there is a cytosine in the opposite chain. Thus each purine is opposite a pyrimidine in every pairing and two pyrimidines will not bridge the gap while two purines would be unable to enter the available space. (X, XI).



X

Adenine-Thymine
base pairing



XI

Guanine-Cytosine
base pairing

The DNA from the bacteriophage Φx 174 has been discovered to be single stranded in the natural state, and so the regularities in purine:pyrimidine ratios do not obtain. Thus ratios for adenine/thymine and guanine/cytosine of 0.75 and 1.3 were found respectively.[11]

The chemical analysis of RNA has not led to the clear generalisations concerning its base ratios that have been found with DNA. The X-ray diffraction patterns from RNA fibres have a broad similarity to those obtained from DNA fibres, but are too diffuse to be interpreted unequivocally.[12] Four varieties of ribonucleic acids have been distinguished - viral RNA, soluble RNA (sRNA, also termed transfer or adaptor RNA), ribosomal RNA and messenger RNA (mRNA). Viral RNA seems to replace DNA in some viruses, e.g. tobacco mosaic virus. sRNA consists of one polynucleotide chain made up of about 80 nucleotide residues with one half of the polynucleotide folded back on itself to make a helix. The sequences of nucleotides therefore, run in opposite directions (as in DNA), making possible the pairing of complementary bases, although base analysis indicates that such pairing does not take place to a large extent, a fact supported by the recent work of Holley, who has determined the exact base sequence of an sRNA molecule.[13] The molecule is about 100 Å long and contains roughly $3\frac{1}{2}$ turns of the helix.

Ribosomes are small particles, not resolved in the microscope, that contain about 60% RNA and 40% protein, and appear to consist of two subunits. Ribosomal RNA differs in size and base content from sRNA and the two classes (termed 16s and 23s according to the sedimentation

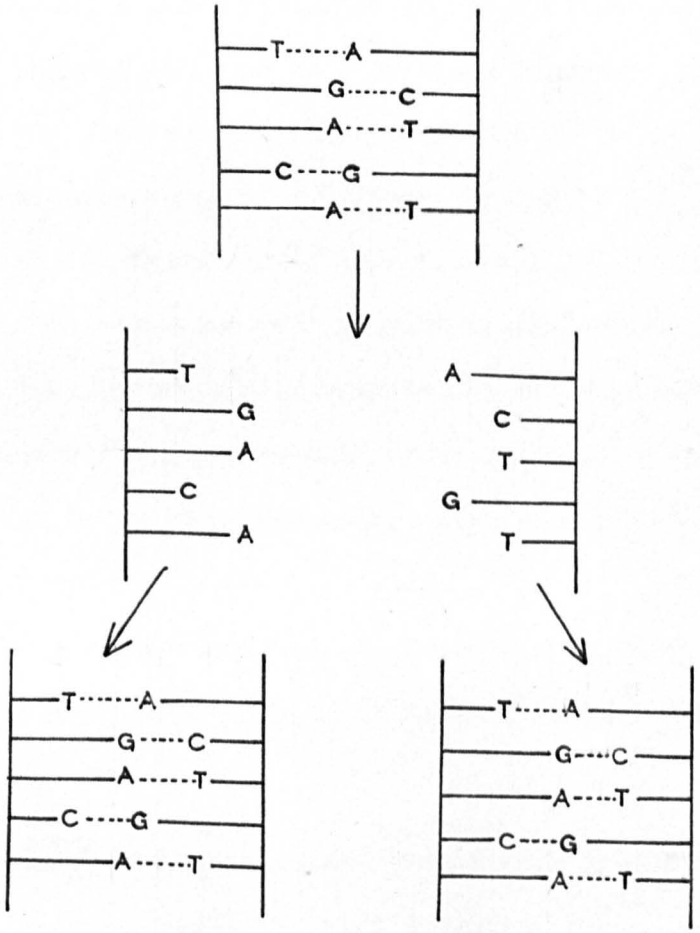
coefficients of their respective subunits) have base sequences that appear to be complementary to regions of the DNA, along which they are presumably synthesised. In E. coli, the rate of ribosomal RNA formation is about one molecule per second per gene (and each RNA molecule is over 1,000 nucleotides long!). The ribosomal RNA then somehow combines with proteins to form a 30s or 50s subunit. Once protected by combination with protein, ribosomal RNA is stable and is not easily degraded in vivo. In mammalian cells, synthesis of ribosomes appears to be a more complex process in which high molecular weight (45s) RNA is a precursor of ribosomal RNA. The reason for the complex structure and RNA content of ribosomes is not yet clear.

Current evidence indicates that mRNA molecules are polyribonucleotide complementary copies of one strand of the double-stranded DNA molecules. After its synthesis in the nucleus, mRNA becomes a component of the cytoplasm, and is found associated with the ribosomes. mRNA is synthesised very rapidly, but that of bacteria and some mammalian cells is unstable - its half-life is only a few minutes. About four-fifths of the RNA synthesised in bacteria is mRNA, but because it is degraded so rapidly, it constitutes only a few per cent of the total RNA. mRNA of other cells, e.g. reticulocytes, is much more stable.

(b) The function of nucleic acids

One of the main lines of evidence which implicated DNA as the genetic determinant and carrier of hereditary factors originated in the work of Griffith in the 1920's, and of Avery and co-workers in 1944.

This subject is discussed in detail by Stahl[14]. Thus during mitosis or meiosis of the cell, the DNA would have to undergo replication to produce copies of itself, and the Watson and Crick theory included a suggestion for such a mechanism. If a double-stranded DNA molecule separated into halves, then each half could provide a template on which a complementary DNA chain could be synthesised. This is represented diagramatically in Scheme 1.



SCHEME 1

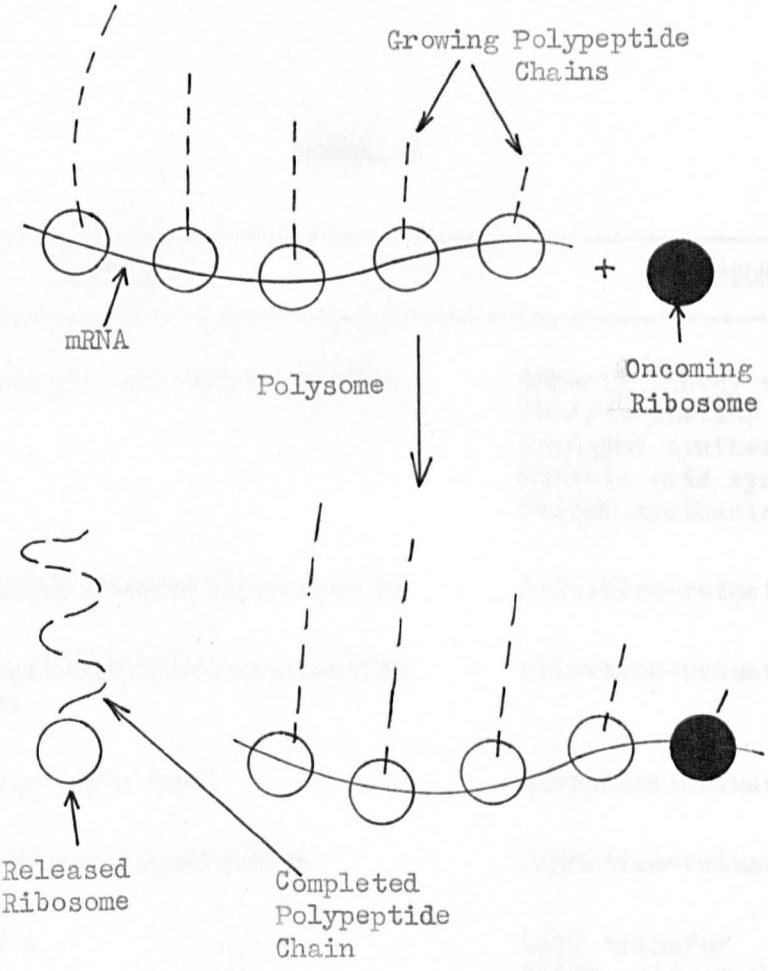
This semi-conservative mechanism was confirmed by the work of Meselson and Stahl, who used ^{15}N labelled bacterial DNA to follow the reproduction of E. coli.[15]

Work in recent years has indicated the importance of the base sequence along the DNA chain, this providing a triplet code for the synthesis of proteins. Thus a sequence of three bases is thought to code for one amino acid in structural genes.[16] A mRNA is synthesised on one or both of the DNA strands of the duplex thus forming a complementary copy, which becomes attached to a ribosome particle. It has been shown that each amino-acid possesses a specific sRNA, to which it becomes attached after activation of the former by ATP. It is postulated that three bases at the "bend" of an sRNA molecule represents a unit of the triplet code and which can pair up with a complementary triplet on the mRNA on the ribosome, thus orientating the amino-acids in the correct sequence for peptide-bond formation to occur from the N-terminal end, resulting in protein synthesis. This is represented diagramatically in the photograph.

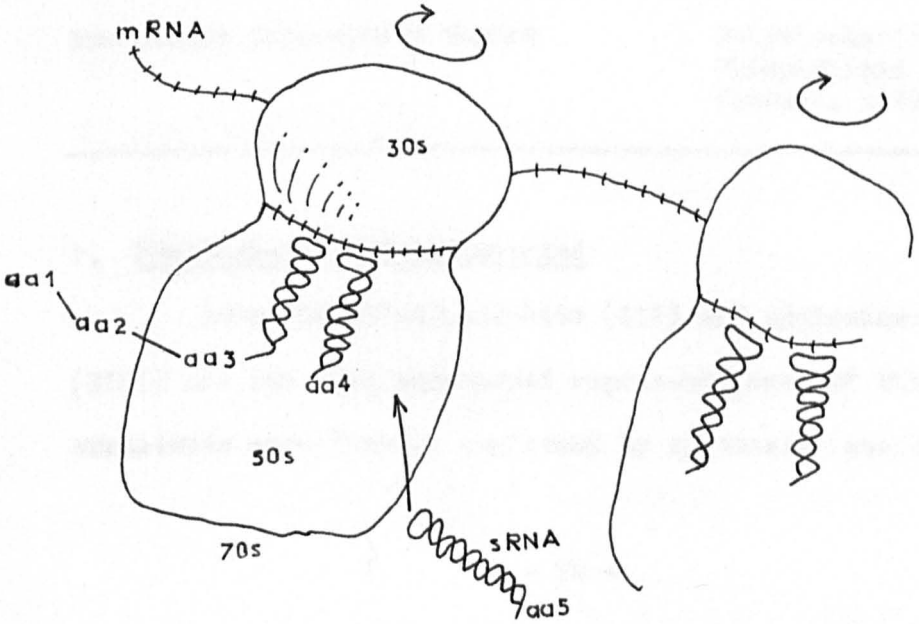
For a detailed description of protein synthesis, the reader is referred to the review by Hartman and Suskind.[17]

2. Coenzymes

Some of the most important coenzymes, together with their function, are listed in Table 2 below.



Schematic model of Polysome Function



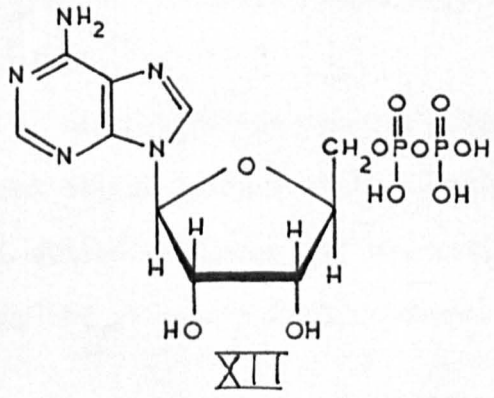
One portion of Polysome active in Protein Biosynthesis

TABLE 2

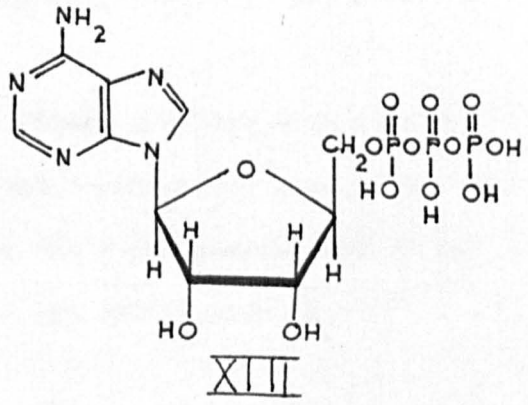
COENZYME	FUNCTION
Nucleoside di- and triphosphates	General energy source (ATP) Phosphorylation reactions Coenzyme synthesis Nucleic acid synthesis Protein synthesis
Nicotinamide-adenine-dinucleotide	Oxidation-reduction
Nicotinamide-adenine-dinucleotide phosphate	Oxidation-reduction
Flavin mononucleotide	Oxidation-reduction
Flavin-adenine dinucleotide	Oxidation-reduction
Coenzyme A	Acyl transfer Fatty acid synthesis Glyceride synthesis
Nucleoside diphosphate sugars	Polysaccharide synthesis Phospholipid synthesis Teichoic acid synthesis

1. Nucleoside-5'-polyphosphates

Adenosine-5'-diphosphate (XII) and adenosine-5'-triphosphate (XIII) are the most widespread representatives of this class. Their structures were finally confirmed by synthesis (see later section).



XII



XIII

Adenosine triphosphate is produced biosynthetically as the end product of three main metabolic processes - substrate level oxidative phosphorylation, respiratory chain oxidative phosphorylation and photosynthetic phosphorylation.[18] In every case the final stage in the synthesis of ATP is the phosphorylation of ADP by an "energy rich" phosphorylating agent such as phosphoenol pyruvate or acetyl phosphate (Scheme 2).

$$ADP + CH_3COOPO_3H_2 \rightleftharpoons ATP + CH_3CO_2H \qquad \text{- Scheme 2}$$

ADP can be formed by the myokinase catalysed phosphorylation

of AMP by ATP, again a reversible reaction. Thus ATP has been designated an "energy-rich" compound, or described as containing an "energy-rich" P-O bond. This approach has, however, been severely criticised.[19]

The α and γ-phosphorus atoms of ATP are more susceptible towards attack by nucleophiles than the β-phosphorus atom. Thus a nucleophilic substrate (S) can attack the γ-phosphorus atom of ATP giving the phosphorylated nucleophile and ADP (Scheme 3).

$$ATP + S \rightleftharpoons SH_2PO_4 + ADP \qquad \text{- Scheme 3}$$

This is a very common enzymatic route to phosphomonoesters, e.g. sugar phosphates, and is of importance in the biosynthesis of other nucleoside triphosphates.

Attack by a nucleophile can also occur on the α-phosphorus atom of ATP to produce an adenosine-5' pyrophosphate ester and inorganic pyrophosphate. This is the biosynthetic route to most nucleotide coenzymes, which are diesters of pyrophosphoric acid (Scheme 4).

$$ATP + FMN \rightleftharpoons FAD + H_4P_2O_7 \qquad \text{- Scheme 4}$$

(See (XVI) and (XVII) for the structures of FMN and FAD).
Other nucleoside triphosphates can react in a similar way, this being the biosynthetic route to most nucleoside diphosphate sugars.

Deoxyribonucleoside-5'-triphosphates are the precursors of deoxy-ribonucleic acids and are incorporated onto the ends of the chain,

resulting in the formation of phosphodiester bonds and expulsion of pyrophosphate. Nucleoside-5'-diphosphates are the precursors of ribonucleic acids (see later discussion).

An example of attack by a nucleophile on the β-phosphorus atom is the synthesis of ribose-1-pyrophosphate-5-phosphate from ribose-5-phosphate.[20]

An example of interest in the present context is the activation and transfer of sulphate, since one of the intermediates in this process, adenosine-5'-sulphatophosphate, has been chemically synthesised. The following equations (1 - 4) summarise this process of activation and transfer.[21,22]

$$HSO_4^- + ATP \rightleftharpoons \text{Adenosine-5'-sulphatophosphate (APS)} + H_4P_2O_7 \quad (1)$$

$$APS + ATP \rightleftharpoons \text{Adenosine-3'-phosphate-5'-sulphatophosphate (PAPS)} + ADP \quad (2)$$

$$PAPS + \text{acceptor} \rightleftharpoons \text{Adenosine-3,5'-diphosphate (PAP)} + \text{acceptor} - HSO_4 \quad (3)$$

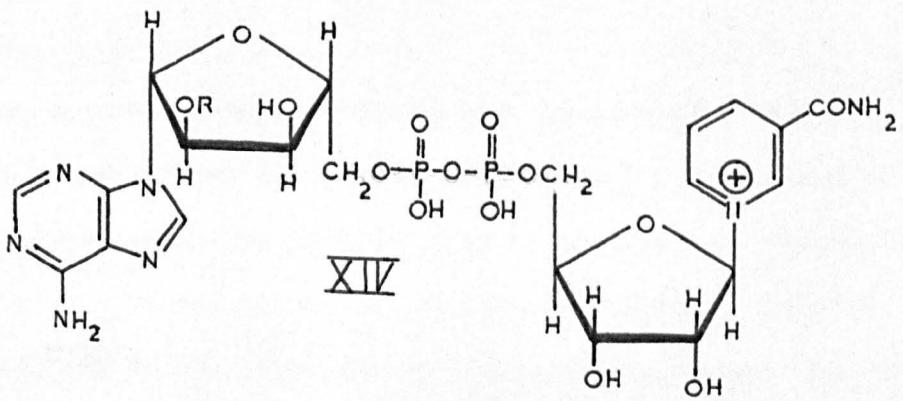
$$PAP \longrightarrow AMP + H_2PO_4 \qquad (?) \quad (4)$$

Reaction (4) is questioned since the fate of PAP has not been established in relation to the net uptake of sulphate in equation (1), and it is conceivable that PAP may be converted to ATP without the intermediate

formation of AMP and inorganic phosphate.

2. The pyridine coenzymes

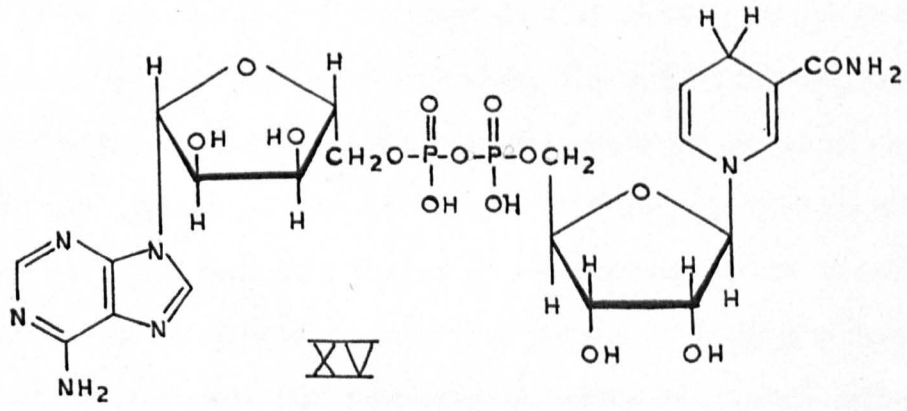
Nicotinamide adenine dinucleotide (NAD^+) (XIV; R = H), the stable cofactor of alcoholic fermentation, was the first coenzyme to be discovered, but had to wait thirty years for its isolation in the pure state.[23,24] The heat stable coenzyme of D-glucose-6-phosphate dehydrogenase, with properties similar to NAD^+ had been isolated five years earlier by Warburg and Christian (who also isolated NAD^+), and this coenzyme is now known to be nicotinamide-adenine dinucleotide phosphate ($NADP^+$) (XIV; R = H_2PO_3). Both are cofactors of a number of dehydrogenases.



XIV

The site of hydrogen transfer has been established as the 4-position of the pyridine ring, and thus reduced nicotinamide-adenine

dinucleotide (NADH) may be formulated as XV (see further discussion in [25]).



XV

Two classes of enzyme require NAD^+ as cofactor, and the difference between these two classes depends on the difference in the direction of attack on the pyridine ring by hydride ions during the reduction step. In one class, the hydride ion attacked from the "top" of the ring, and in the other the hydride ion attacks from the "underside" of the ring, with respect to the plane of the paper.[26]

These coenzymes, in association with appropriate protein apoenzymes, are cofactors for a wide variety of dehydrogenation reactions.[27] One common reaction, which is catalysed by NAD^+ is the oxidation of

alcohols to aldehydes or ketones; the alcohol dehydrogenases are found in yeast and liver (Scheme 5).

$$R_1CHOHR_2 + NAD^+ \rightleftharpoons R_1COR_2 + NADH \quad \text{- Scheme 5}$$

When a substrate is oxidised in a mitochondrion, electrons are transferred from the substrate along the cytochrome sequence to molecular oxygen. The first electron-transferring component in the chain is NAD^+, which then transfers the electrons to flavoprotein. This in turn transfers them to a series of cytochromes before the final transference of electrons to molecular oxygen. During the transference of one electron pair from the substrate to molecular oxygen, three molecules of ATP are synthesised from ADP - this process is known as oxidative phosphorylation.

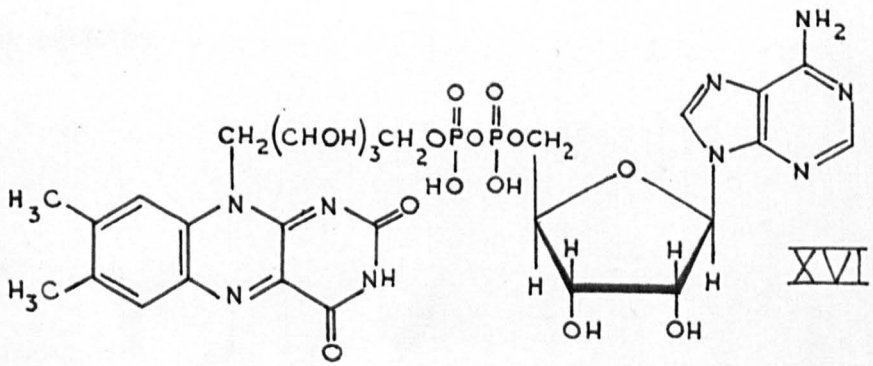
The biosynthesis of NAD^+ is thought to involve the preliminary formation of nicotinic acid - adenine dinucleotide and subsequent conversion of the carboxylic acid group to an amide function.[28]

3. The flavin coenzymes

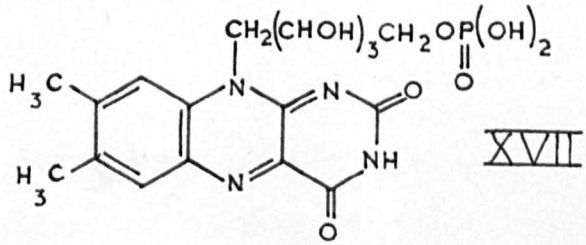
The flavin coenzymes are oxidation-reduction coenzymes by virtue of the reversible oxidation and reduction of the flavin moiety. In this respect they are similar to the nicotinamide coenzymes, and like the latter the flavin coenzymes can catalyse enzymatic dehydrogenation reactions and are electron carriers in the cytochrome chain of mitochondria.

Riboflavin (vitamin B_2) was one of the earliest vitamins to be

recognised, isolated and studied. Riboflavin itself, however, has a very low activity as a coenzyme for enzymatic dehydrogenation reactions, although phosphate esters of riboflavin are very active catalysts in such reactions, for example Flavin-adenine dinucleotide (XVI) and riboflavin-5'-phosphate (XVII) which, whilst not a derivative of D-ribose and hence not a nucleotide, has acquired the trivial name of flavin mononucleotide (FMN).



XVI



XVII

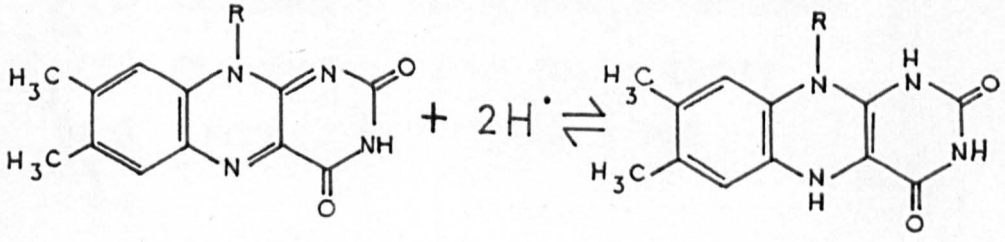
Methods for the chemical synthesis of FMN and FAD will be described in a later section. The biosynthesis involves phosphorylation of riboflavin by ATP in the presence of flavokinase[29] (Scheme 6).

$$\text{Riboflavin} + \text{ATP} \rightleftharpoons \text{FMN} + \text{ADP} \qquad \text{- Scheme 6}$$

The riboflavin-5'-phosphate is then converted into FAD by a further phosphorylation with ATP[30] (Scheme 7).

$$\text{FMN} + \text{ATP} \rightleftharpoons \text{FAD} + H_4P_2O_7 \qquad \text{- Scheme 7}$$

The oxidation-reduction process in vivo is a free radical reaction, as shown in Scheme 8.



Scheme 8

Both FMN and FAD are only active when bound to proteins, and can then oxidise, for example, the reduced pyridine coenzymes, α-amino acids, aldehydes and purines, and can also function as electron carriers in the cytochrome chain. When a substrate is oxidised by NAD^+ (or $NADP^+$), the reduced pyridine coenzyme (NADH or NADPH) results, and these can be

reoxidised by flavoprotein, which then transfers its electrons to another electron carrier such as a cytochrome (Scheme 9).

$$\text{Substrate H} + \text{NAD}^{+} \longrightarrow \text{Substrate} + \text{NADH}$$

$$2\ \text{NADH} + \text{FMN} \longrightarrow 2\ \text{NAD}^{+} + \text{FMNH}_2$$

$$\text{FMNH}_2 + 2\ \text{Fe}^{III} \longrightarrow \text{FMN} + 2\ \text{Fe}^{II} + 2\ \text{H}^{\oplus}$$

Scheme 9

The oxidation of both D- and L-α-amino acids is thought to be a three-stage process - (i) the amino acid is oxidised to an imine, (ii) the imine is hydrolysed and (iii) FAD regenerated from $FADH_2$ by electron transfer to molecular oxygen[31] (Scheme 10).

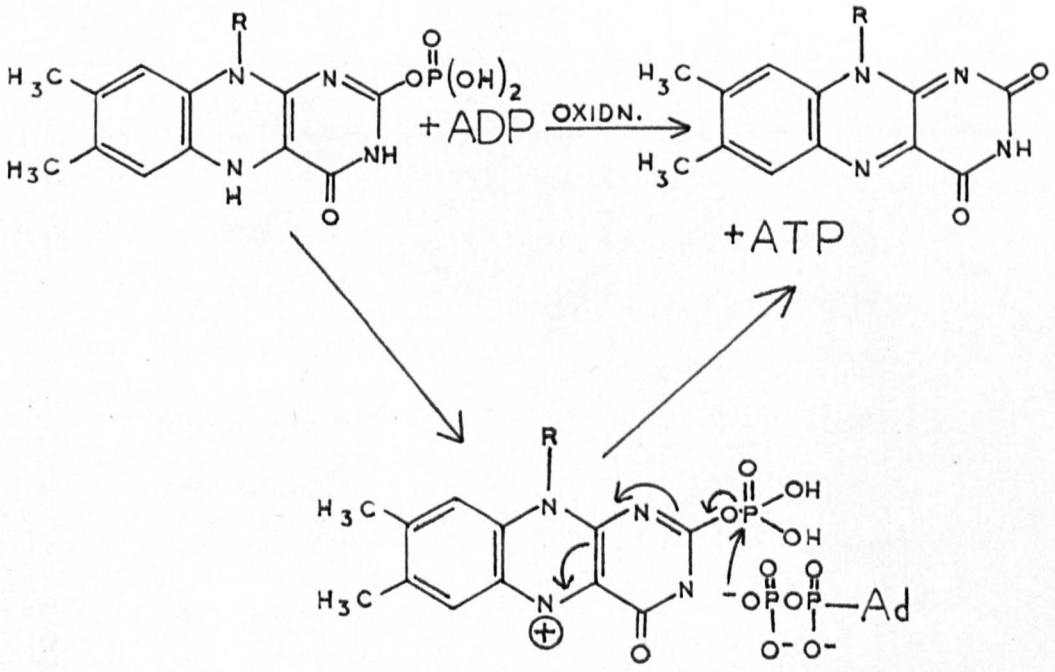
$$\text{(i)}\quad \text{NH}_2\text{CHRCOOH} + \text{FAD} \longrightarrow \text{FADH}_2 + \text{NH=CRCOOH}$$

$$\text{(ii)}\quad \text{NH=CHRCOOH} + \text{H}_2\text{O} \longrightarrow \text{NH}_3 + \text{RCOCOOH}$$

$$\text{(iii)}\quad 2\ \text{FADH}_2 + \text{O}_2 \longrightarrow 2\ \text{FAD} + \text{H}_2\text{O}$$

Scheme 10

One proposal for the phosphorylation process in the NAD^{+}-flavoprotein step in the respiratory chain is that one of the oxygen atoms of the flavin moiety is phosphorylated, which, under oxidative conditions, could then transfer the phosphate group to ADP[32] (Scheme 11).



SCHEME II

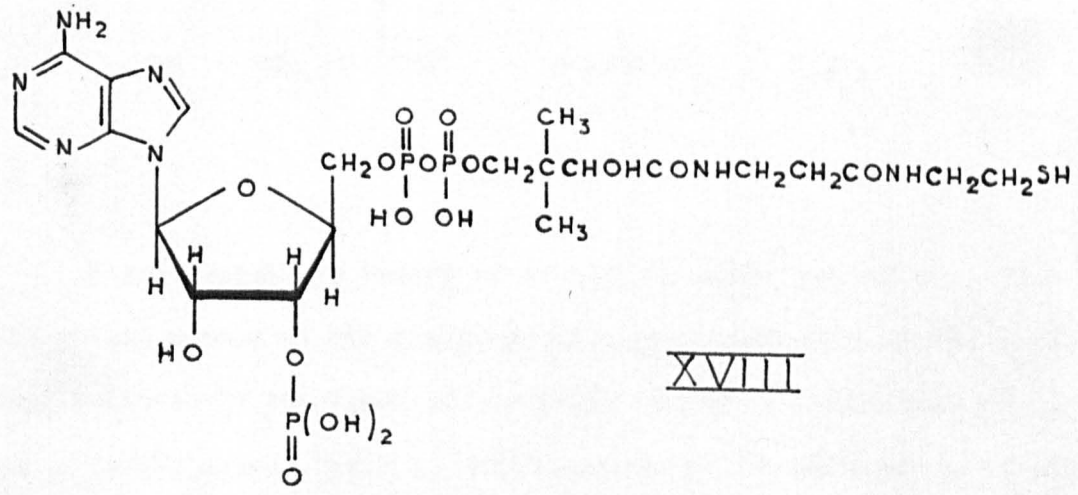
The principal function of this chain of coenzymes is to transport electrons between the $\geq$C - H bond of a substrate and molecular oxygen with a concomitant synthesis of "high-energy" phosphate. The efficiency of the over-all system demands that several molecules of ATP be formed from the oxidation of a single $\geq$C - H bond, and for this to take place it is necessary that the electron transport be broken into a series of steps, each of which can be coupled to a process of phosphorylation. Thus each coenzyme is capable of reducing the succeeding coenzymes as one moves down the chain to the reduction of molecular oxygen, and this is reflected in the increase in the redox potentials, some of which are reproduced in the table below.

Coenzyme	E_o' at pH7 and 25°C
NAD^+	-0.32
$NADP^+$	-0.32
FAD	-0.123
Cytochrome b	+0.04
Cytochrome c	+0.26
Cytochrome a	+0.29
Coenzyme Q (in ethanol)	+0.542
Oxygen	+0.82

4. Coenzyme A

This coenzyme was shown to be a constituent of most living systems by Lipmann,[33] but, in contrast to the last two types of coenzymes discussed, CoA is an acyl-transferring coenzyme, and is the cofactor for a wide variety of biological acylations, for example, the synthesis of acetoacetate from acetate and ATP in pigeon-liver. The acetoacetate is formed from two activated carbon fragments (both derived from acetyl phosphate) and not by the acetylation of the methyl group of an acetate molecule.[34]

The structure of CoA (XVIII) was elucidated by Baddiley,[35] Lipmann[36] and Snell, and confirmed by synthesis by Khorana,[37] Michelson[38] and Lynen.[39]

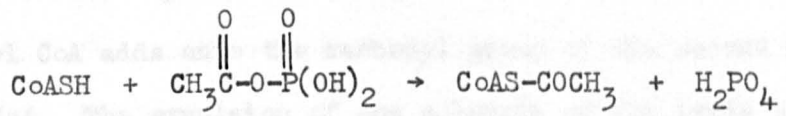


XVIII

The main biosynthetic route to CoA in most animals appears to involve phosphorylation of pantothenic acid by ATP to give pantothenic acid-4 phosphate. The latter then reacts with β-alanine in the presence of either ATP or CTP to produce the adenosine-pantetheine pyrophosphate - 'dephospho CoA', plus inorganic phosphate. This 'dephospho CoA' is then phosphorylated on the 3'-hydroxyl group of the adenosine moiety by ATP.[40]

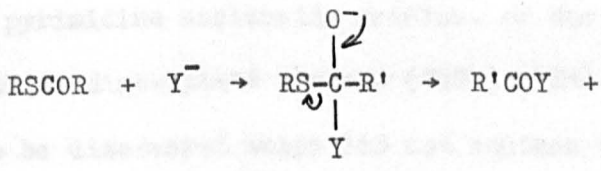
CoA can be acylated in vitro without difficulty to form S-acyl derivatives, which are the active species in acylating reactions in vivo.[41] S-acetyl CoA can be formed by the reaction between CoA and acetyl phosphate. The latter is a mixed anhydride of acetic and phosphoric acids and

consequently a good acetylating agent (Scheme 12).



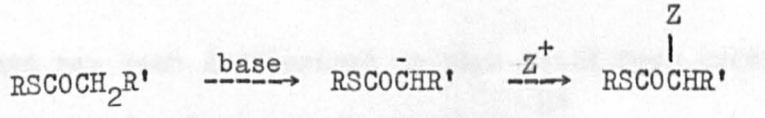
Scheme 12

S-Acyl thiols are esters of carboxylic acids and thiols. Thus nucleophilic attack on the electron-deficient carbon atom of the carbonyl group, followed by expulsion of the thiol, which is a weak nucleophile and forms a stable anion, leads to the formation of the acylated nucleophile (Scheme 13).



Scheme 13

S-Acyl thiols can also react in a second way - by formation of an anion on the carbon atom α to the carbonyl group, the resulting anion then being able to react with electrophiles such as carbonyl groups (Scheme 14).



Scheme 14

In the synthesis of S-acetoacetyl CoA from two molecules of S-acetyl CoA, both these reaction paths are used.[34] The carbanion of one molecule of S-acetyl CoA adds onto the carbonyl group of the second molecule of S-acetyl CoA. The expulsion of one molecule of CoA leads to the formation of S-acetoacetyl CoA, (Scheme 15).

$$CoASCOCH_3 + CoASCO\bar{C}H_2 \rightarrow CoASCOCH_2COCH_3 + CoAS^-$$

Scheme 15

5. Nucleoside diphosphate sugars

The most common members of this class are those which contain either the pyrimidine nucleoside uridine, or the purine nucleoside guanosine.

Uridine diphosphate glucose (UDPG) (XIX) was the first nucleotide coenzyme to be discovered which did not contain adenosine in the nucleoside moiety.

In vivo UDPG is synthesised from UTP and α-D-glucose-1-phosphate, this being the typical route to nucleoside pyrophosphate esters[42] (Scheme 16).

α-D-glucose-1-phosphate + UTP → UDPG + $H_4P_2O_7$

Scheme 16

In vitro UDPG has been synthesised in high yield from uridine-5'-phosphoramidate and α-D-glucose-1-phosphate.[43]

UDPG was discovered by Leloir during his studies on the metabolism

of galactose in yeast. D-Galactose is first converted by ATP into D-galactose-1-phosphate, which then reacts with UDPG to give D-glucose-1-phosphate and UDP-galactose, (Scheme 17).

Galactose + ATP → galactose-1-phosphate + ADP

Galactose-1-phosphate + UDPG → glucose-1-phosphate + UDPGal

Scheme 17

Both starch and glycogen are synthesised <u>in vivo</u> from UDPG, that of glycogen being represented by:

$$x(\text{UDPG}) + (\alpha\text{-}1,4\text{-D-glucose})_n \rightarrow (\alpha\text{-}1,4\text{-D-glucose})_{n+x} + x\text{UDP}$$

The reaction resembles the biosynthesis of polynucleotides, which is catalysed by polynucleotide phosphorylase, as a similar linkage is formed and a similar primer is required before the reaction will take place. This type of biosynthesis, however, proceeds by elongation of the primer, whilst in polynucleotide biosynthesis, the primer acts as a template. (See later section).

(B) General Methods for the Formation of Ester and Anhydride Linkages

In many syntheses of phosphate esters and anhydrides, as with their carboxylic analogues, activation of one or other of the reactants is generally required. Thus the two types of approach are exemplified by Schemes 18 and 19 below:

$$(RO)_2PO.O^- + R'X \rightarrow (RO)_2PO.OR' + X^- \qquad \text{Scheme 18}$$

$$(RO)_2PO.X + R'OH \rightarrow (RO)_2PO.OR' + HX \qquad \text{Scheme 19}$$

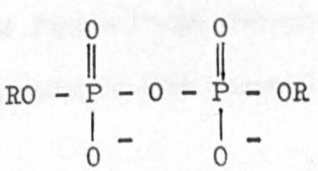
The methods employing activation of the alcohol have familiar counterparts in carboxylic acid chemistry - reaction of the acid or a salt with alkyl halides, epoxides, imino-ethers and diazoalkanes, resulting in the formation of esters.

The majority of chemical phosphorylations have been realised by activations of the phosphoryl group (Scheme 19), in which X is normally a group which undergoes nucleophilic displacement very readily to yield a stable anion, or, in some cases, by protonation, to release a neutral moiety. Thus in general, phosphorylation is effected if the acid HX is stronger than the phosphoric acid $(RO)_2PO.OH$, and so phosphorylating agents have been of two types - phosphorochloridates or anhydrides of pyrophosphate or "metaphosphate" type, although in broad terms, the phosphorochloridates can be considered as mixed acid anhydrides. There are obvious advantages in phosphorylation methods which involve activation of existing alkyl phosphates so that, for example, a naturally occurring

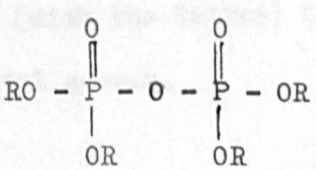
phosphoric acid ester may be used directly for the synthesis of more complex derivatives. This may be executed by conversion to a mixed anhydride with a strong acid, and subsequent reaction with an alcohol or a second phosphate anion in situ.

Activation, and the concomitant cleavage of the P-X bond can be accomplished if X is an efficient electron sink. This can be achieved by protonation of X or removal of electrons from X. Thus phosphoramidates are stable as anions, but will act as mild phosphorylating agents on protonation, whilst during oxidative phosphorylation, electrons are removed from a moiety X (see subsequent discussion).

One of the important factors in chemical phosphorylation, which does not obtain in carboxylic ester and anhydride chemistry, is the polyfunctional nature of phosphoric acid, and hence the chief problem in activating a phosphoric acid molecule to a sufficiently powerful phosphorylating agent is to "overcome" the negative changes on the oxygen atoms. Thus the simple anhydride of phosphoric acid, pyrophosphoric acid, is completely devoid of phosphorylating capacity under mild conditions. Its further conversion to a meta - or polyphosphoric acid is necessary to confer on it phosphorylating ability. Similarly, even P^1, P^2-dialkyl pyrophosphates (XX) are not phosphorylating agents, but the corresponding tetralkyl pyrophosphates (XXI) acquire the character of phosphorylating agents.



XX



XXI

As stated, the phosphoric acid molecule, is usually surrounded by electronegative oxygen atoms, and one of the major problems in the design of efficient phosphorylating agents has therefore been to confer a more electropositive character on the phosphorus atom.

The following section will attempt to summarise the developments in phosphorylation methods, drawing comparisons with carboxylic acid analogues, as excellent detailed reviews of this field have appeared recently by Brown,[44] Khorana[45] and Clark.[46]

1. Alcohol activation

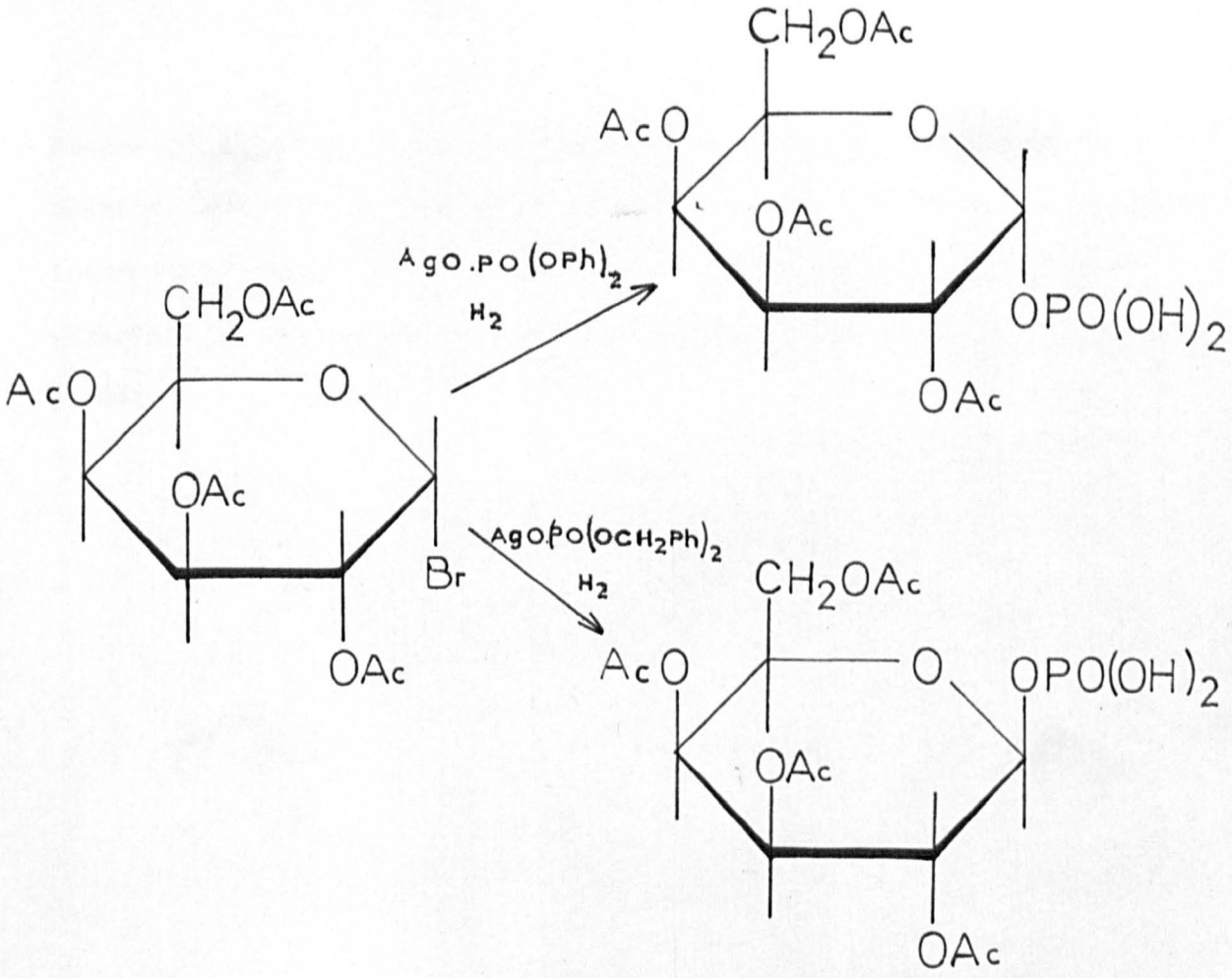
(a) Halide derivatives

The reactions of salts of carboxylic acids with alkyl halide or acyl chlorides are standard routes to the preparation of esters and anhydrides (Schemes 20 and 21).

$$RCOOAg + R'X \rightarrow RCOOR' + AgX \qquad \text{Scheme 20}$$

$$RCH_3COONa + CH_3COCl \rightarrow CH_3CO\text{-}O\text{-}CO CH_3 + NaCl \qquad \text{Scheme 21}$$

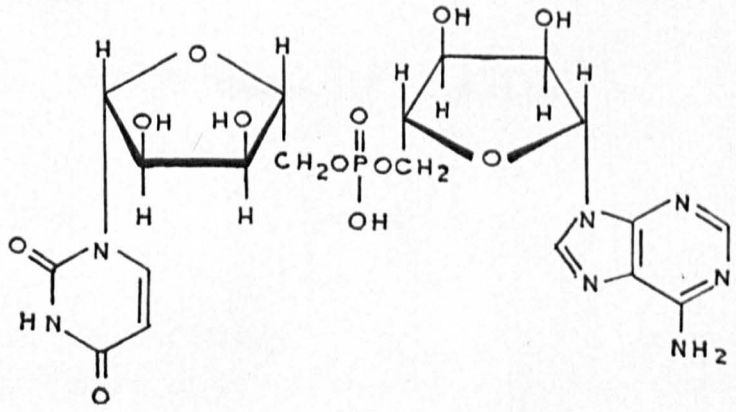
In the case of phosphates, the synthesis of glycosyl-1-phosphates have provided particularly favourable examples because of the high reactivity of the 1-halogeno derivatives. Thus reaction of the silver salts of diphenyl and dibenzyl phosphates with tetra-acetyl-α-D-galactosyl bromide gives the α - (with the former) and β-1-phosphate (with the latter) (Scheme 22)[47,48] after hydrogenolysis and removal of acetyl groups.



Scheme 22

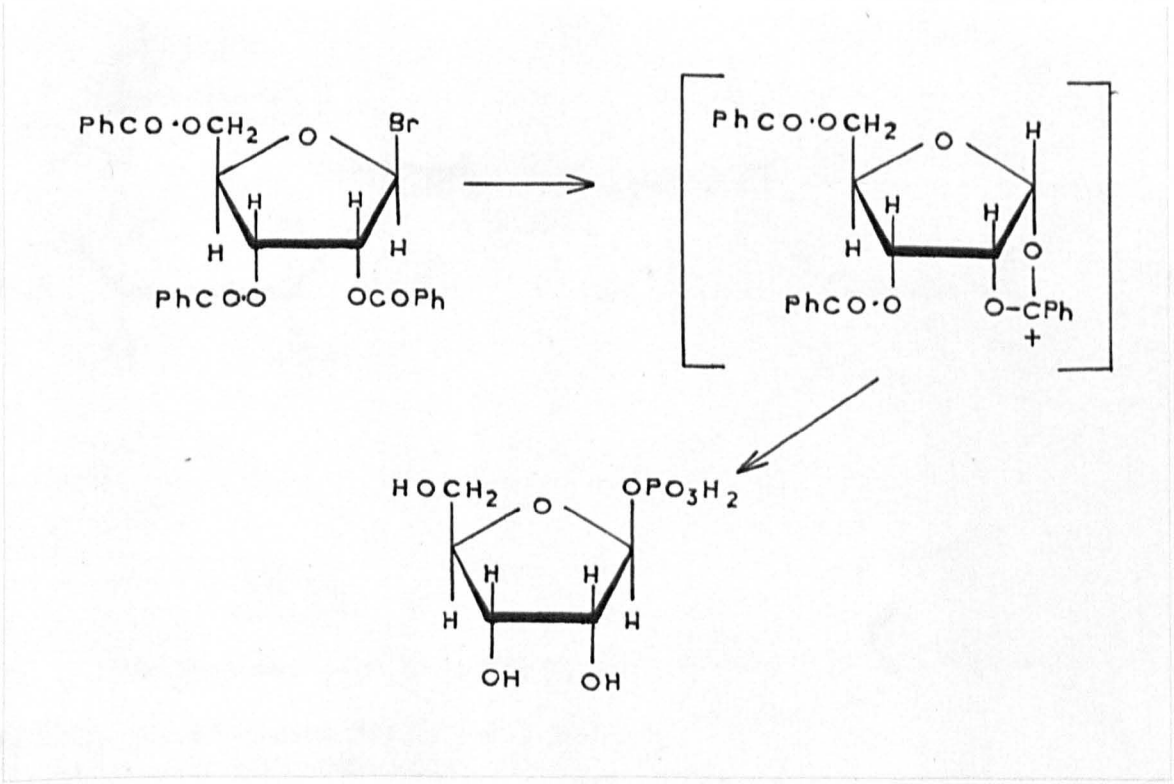
No satisfactory explanation has been found for the inversion during the synthesis of the phosphate using silver dibenzyl phosphate, but it is thought that displacements with this reagent proceed with participation from the 2-O-acyl group, whilst those involving silver diphenyl phosphate do not.[44] Application to nucleotide syntheses has been confined largely to derivatives of uridine. Thus 2',3'-O-isopropylidene-5'-iodo-5-deoxyuridine with silver dibenzyl phosphate gave the 5'-dibenzyl phosphate, from which the protecting groups were removed to yield uridine-5'-phosphoric acid.[49]

Elmore and Todd were able to synthesise the unsymmetrical phosphate diester, adenosine-5'-uridine-5'-phosphate (XXII) by condensing the above iododeoxyuridine with silver 2',3'-isopropylidene-adenosine-5'-benzyl phosphate in boiling toluene, with conventional removal of the protecting groups.[50]



XXII

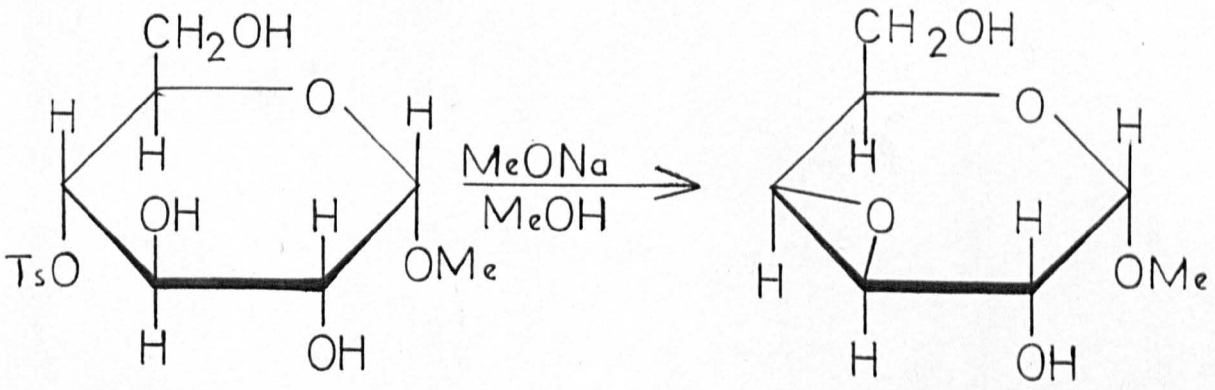
There has been a tendency recently in this field to use salts with organic cations instead of the silver salts, the former being soluble in non polar solvents. Thus Wright and Khorana reacted 2,3,5-tri-O-benzoyl-β-D-ribofuranosyl bromide (XXIII) with triethylammonium dibenzyl phosphate in benzene at 5°C. The extremely labile 1-(dibenzyl phosphate) was hydrogenolysed and benzoyl groups removed with alkali, giving β-D-ribofuranosyl-1 phosphate (XXIV). It was believed that the cation (XXV) was a transient intermediate in the condensation,[51] (Scheme 23).



Scheme 23

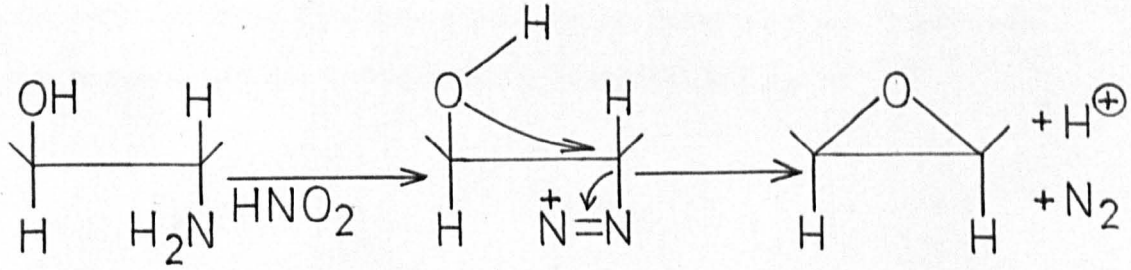
(b) Epoxides

Reaction of perbenzoic acid or monoperphthalic acid with an alkene yields an epoxide. These compounds can also be prepared by reaction of a chlorohydrin with alkali. Moreover, if a sulphonyl group is attached to a secondary carbon atom which is trans with respect to a vicinal hydroxyl group, or an ester group which is removed under the reaction conditions, mild alkaline hydrolysis leads to production of an epoxide again. This is illustrated by the conversion of methyl α-D-glucoside-4-toluene-p-sulphonate to methyl 3,4-anhydro-α-D-galactoside (Scheme 24).

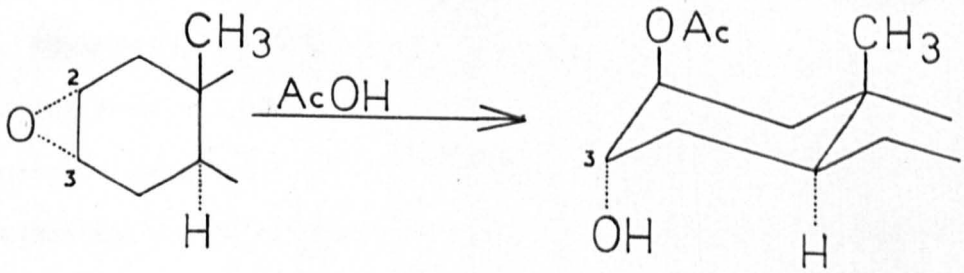


Scheme 24

Epoxides may also be prepared by deamination with nitrous acid of suitably blocked trans diaxial α-hydroxyamino compounds.

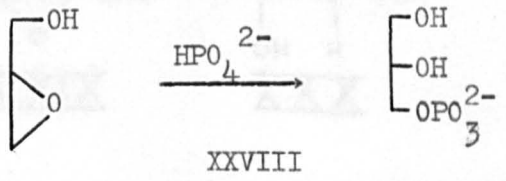


Acetolysis of the epoxide then yields a monoacetate ester, illustrated below in which an α-oxide (XXVI) of the cholestane series is attacked by acetic acid to cleave the C_2-bond, with inversion at C_2 (attack from the back) and formation of the trans-diaxial 2-monoacetate (XXVII) (Scheme 25).



Scheme 25

Similarly, in the phosphate field, Bailly showed that glycidol reacted with disodium hydrogen phosphate in water at room temperature to give disodium glycerol-1-phosphate (XXVIII) (Scheme 26).[52]



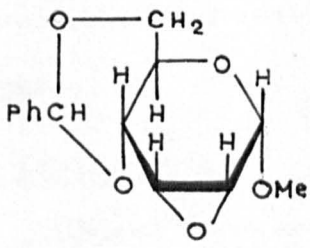
XXVIII

Scheme 26

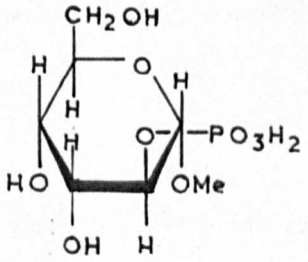
The ring opening may result from two main processes: (i) the attack on the netural epoxide or (ii) on its conjugate acid. As for other bimolecular nucleophilic displacements on carbon, a rate sequence primary > secondary > tertiary is general, and this seems to hold for reactions

with phosphate ions, although in practice deviations from this rule are observed in acid-catalysed openings.

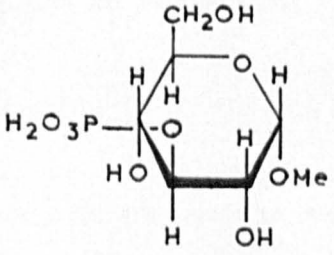
The reactions of epoxides with phosphates have, however, severe limitations when applied to the more complex phosphates. Stereochemically, trans opening is the rule, and moreover, the availability of complex epoxide derivatives may be limiting, as is also the fact that the phosphate anion can attack either carbon atom of the epoxide ring. Thus the acid-catalysed reaction of 2,3-anhydro-alloside (XXIX) with phosphate anion was extremely slow, and also, substitution at $C_{(2)}$ and $C_{(3)}$ occurred giving finally the altroside (XXX) and the glucoside[53] (XXXI).



XXIX



XXX



XXXI

(c) Diazoalkanes

Methylation of acids, phenols, enols, primary and unhindered secondary alcohols can be brought about very efficiently with diazomethane (Scheme 27).

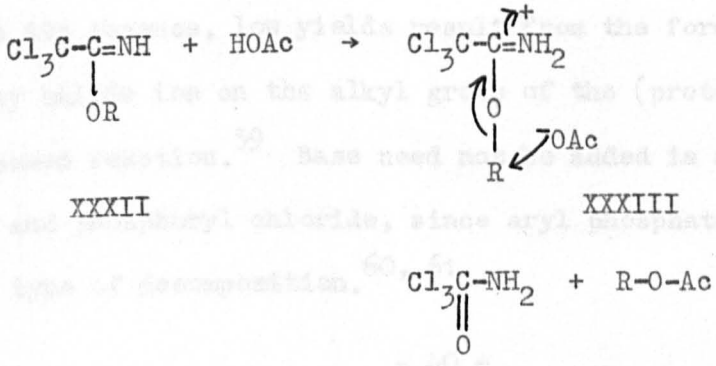
$$(RO)_2PO.OH + R'CHN_2 \rightarrow (RO)_2PO.OR' + N_2$$ Scheme 28

Thus uridine-3' phosphate was converted quantitatively to the neutral, and extremely unstable dimethyl and dibenzyl esters by titration in methanol with the corresponding diazoalkane.[54] With other nucleotides, solubility problems become important, but adenosine-2' (and -3')-phosphate and cytidine-2' (and -3')-phosphate were converted to their monobenzyl esters by phenyldiazomethane in dimethylformamide. Monoesterification of monoalkyl phosphates can be carried out by a diazoalkane reaction on an acid salt. Thus benzyl glycerol-1-phosphate was prepared, without formation of the dibenzyl ester, from phenyldiazomethane and cyclohexylammonium glycerol -1-hydrogen phosphate.[55]

The method is however, on the whole, severely limited by the inaccessability of many substituted diazomethanes and on solubility grounds.

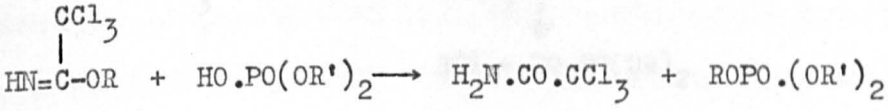
(d) Imino ethers

Cramer[56] has described a method of synthesis of carboxylic esters by reaction of imino-ethers derived from trichloroacetonitrile (XXXII) with acids (Scheme 29).



Scheme 29

Cramer then extended this reaction to prepare phosphate esters using the reaction of imino ethers with phosphoric acids (Scheme 30).[57]



Scheme 30

Thus the benzyl ether (XXXIII) with dibenzyl phosphoric acid gave tribenzyl phosphate in 90% yield. Once again, the difficulty of the preparation of more complex imino-ethers must impose limitations on this method.

2. Acid Activation

(a) Use of phosphorohalidates (i) Phosphoryl halides

One of the earliest phosphorylating agents to be used was phosphoryl chloride in the presence of base. Although it is not widely used nowadays, in nucleotide chemistry, it is still used extensively for the preparation of symmetrical trialkyl phosphates[58] (Scheme 31). The reaction may be carried out in pyridine as the solvent, or in an organic solvent with the calculated quantity of tertiary base. The presence of the base is necessary since in its absence, low yields result from the formation of alkyl halides. Attack by halide ion on the alkyl group of the (protonated) alkyl phosphate is the presumed reaction.[59] Base need not be added in reactions involving phenols and phosphoryl chloride, since aryl phosphates will not be subject to this type of decomposition.[60, 61]

$$POCl_3 + 3\ ROH \xrightarrow{3B} (RO)_3PO + 3B.HCl$$
$$\downarrow$$
$$RCl + HO.PO(OR)_2$$

Scheme 31

This reagent is obviously less satisfactory for the synthesis of primary and secondary phosphates, where mixtures of the three types of esters can result, although it is often possible to separate the products by distillation.

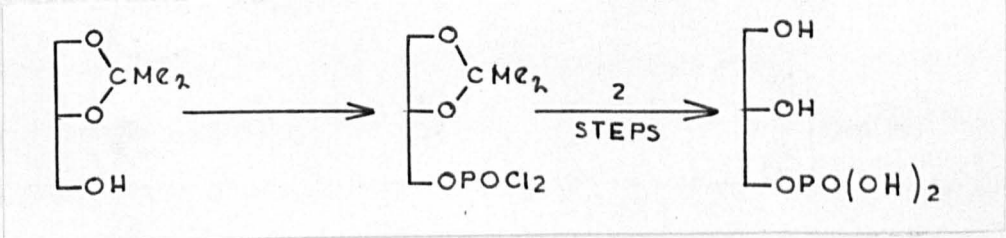
Phosphorylation using phosphoryl chloride has also been carried out with aqueous sodium or barium hydroxide as base,[62] but invariably low yields resulted. A probable reason for this is that polyphosphate formation occurs through further phosphorylation of the partially hydrolysed phosphorochloridate (Scheme 32).

$$RO.POCl_2 \longrightarrow RO.PO_3^{2-} \xrightarrow[2.\ OH^-]{1.\ POCl_3} RO.P_2O_6^{3-}$$

Scheme 32

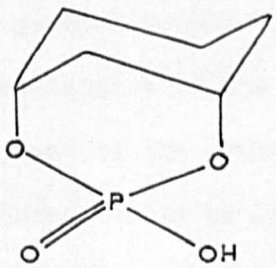
A very useful synthesis of pyrophosphate esters utilises this reaction.[63]

An example of a phosphorylation using phosphoryl chloride is provided by the preparation of (±)-glycerol-1-phosphate in quinoline by Fischer and Pfähler[64] (Scheme 33).



Scheme 33

If the reactant is a 1:2-diol, an intermediate cyclic phosphate is evidently formed, and has in certain cases been isolated, but mild hydrolysis during the work-up is sufficient to form the diol monophosphate. In the phosphorylation of 1:3-diols, the cyclic phosphate is the normal product,[67] (e.g. XXXIV).



XXXIV

The reasons for the instability of five-membered cyclic phosphate esters has been the subject of much discussion. The ratio of the rate of hydrolysis of ethylene phosphate to that of dimethyl phosphate is around $10^6 - 10^7$, and so the difference in free energy of activation is probably of the order of 10 kcal./mole^{-1}.[74] Conventional ring strain may contribute (c. 3 kcal./mole^{-1}) to the total difference in free energy of activation, but cannot alone account for the observed experimental results. The possibility that the eclipsing of the hydrogen atoms or unpaired electrons on the ester oxygen atoms creates

strain (or that it creates all the strain) is also improbable. Westheimer and co-workers have, however, suggested that greater emphasis should be placed on the stabilisation of non-cyclic esters, and less on conventional ring strain.[75] In the non-cyclic phosphate esters, stabilisation is possible by p-d overlap of the electron systems of the ester oxygen and phosphorus atoms, resulting in the P-O bond acquiring double-bond character. The stiffening and flattening of the ring in cyclic phosphate esters caused by this partial double-bonding might then produce strain. At the same time the resistance of the ring could result in less double-bond character, and to the extent that this is diminished, the five-membered cyclic esters would be less stable than their open-chain analogues. In support of this, Todd and co-workers, in a ^{31}P nuclear magnetic resonance study of cyclic phosphates, have shown that the five-membered cyclic esters show less electron-shielding of the phosphorus nucleus, than is observed for open-chain or six-membered cyclic esters. This observation is consistent with a diminution in the $p\pi$-$d\pi$ double bond character of the cyclic P-O bonds in the five-membered ring.[76]

Unsymmetrical diesters have been synthesised using phosphoryl chloride (Scheme 34), examples from the lipid field being reported.

$$ROH \rightarrow RO.PO.Cl_2 \rightarrow (RO)(R'O)PO.Cl \rightarrow (RO)(R'O)PO.OH$$

Scheme 34

(ii) Diphenylphosphorochloridate

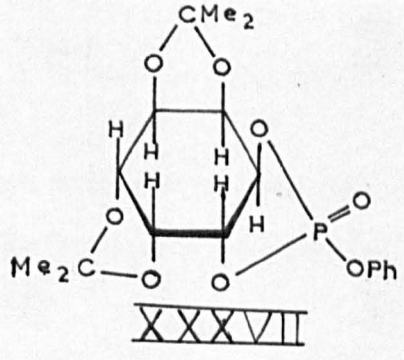
Phosphoryl chloride has been largely superseded as a

phosphorylating agent by ones carrying protecting groups, such as diphenyl phosphorochloridate (XXXV). The latter is readily prepared from phenol and phosphoryl chloride, and can be separated from the other two adducts by fractional distillation.[60] It reacts rapidly in pyridine with alcohols, often at low temperatures, and both phenyl groups may be removed from the intermediate triesters (XXXVI - Scheme 35), which are often highly crystalline, by hydrogenation over platinum.

$$(PhO)_2PO.Cl \xrightarrow{ROH} (PhO)_2PO.OR \xrightarrow{H_2/Pt} RO.PO(OH)_2 \qquad \text{Scheme 35)}$$

XXXV XXXVI

This reagent has found wide use in the sugar phosphate series. Thus Lardy and Fischer phosphorylated 1,2,3,4-tetra-O-acetyl glucose and after removal of the phenyl residues followed by deacetylation, obtained D-glucose-6-phosphate in high overall yield.[68] A hydroxyl group neighbouring the diphenyl phosphate residue leads to instability, and the probability of cyclic phosphate formation and/or phosphate migration. Thus the product isolated from the phosphorylation of di-isopropylidene-(-)-inositol was the cyclic phenyl phosphate (XXXVII).[69]



XXXVII

(iii) Phenylphosphorodichloridate

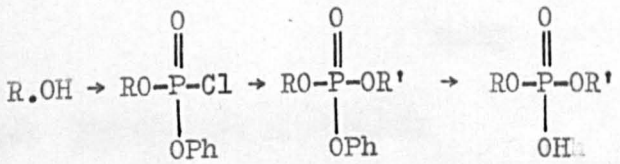
This reagent is very useful for the preparation of symmetrical dialkyl phosphates. Thus reaction of phenylphosphorodichloridate (XXXVIII - Scheme 36) with two moles of alcohol proceeds readily in the presence of a tertiary base, but the reaction with alkoxides can also be used.[70]

$$\underset{\text{XXXVIII}}{PhOPO.Cl_2} \rightarrow PhO.PO(OR)_2 \rightarrow \underset{\text{XXXIX}}{(RO)_2PO.OH}$$

Scheme 36

Thus Gulland and Smith synthesised di-uridine-5'-phosphate (XXXIX; R = uridine) from phenylphosphorodichloridate and two moles of 2',3'-O-isopropylideneuridine, followed by removal of the phenyl group from the intermediate triester by base, and the isopropylidene group by acid hydrolysis.[71]

The reagent has also been used occasionally for the synthesis of unsymmetrical diesters (XI - Scheme 37).

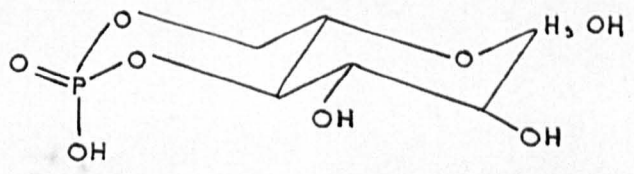


Scheme 37

XLI XLII XL

This method has been utilised by Baer and co-workers in the phospholipid field.[72,73] The intermediate phenylphosphorochloridate ester (XLI) was

not isolated, but was immediately put into reaction with the second alcohol. Invariably some of the symmetrical dialkyl phenyl phosphate (XLII; R = R') is formed, but it is generally easily removed.[77] The first halogen atom in phenylphosphorodichloridate appears to be more easily displaced than the second. This effect seems to be accentuated if quinoline rather than pyridine is used as the base.[78,64] Phenylphosphorodichloridate has also been used in the synthesis of six membered cyclic phosphates, such as glucose-9,6-phosphate (XLIII) from phenyl glucoside.[79]



XLIII

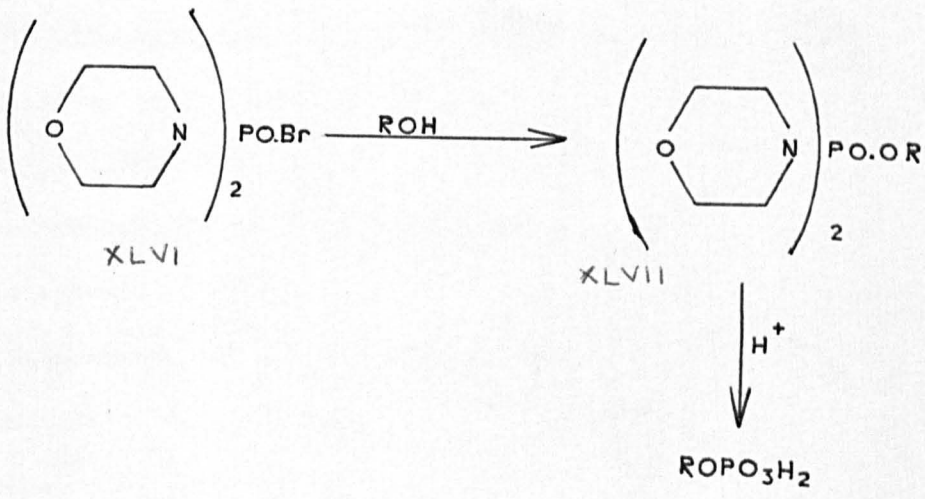
(iv) Phosphoramidic halides

Preliminary studies on compounds of the general type (XLIV) and (XLV) have been made with a view to their potentialities as phosphorylating agents.[80,81]

$(>N)_2PO.Hal$ $>NPOHal_2$

XLIV XLV

Probably the most successful reagent of this type is the unstable but crystalline phosphorodimorpholidic bromide (XLVI) which reacts with alcohols at room temperature in the presence of a tertiary base, (Scheme 38).[82]



Scheme 38

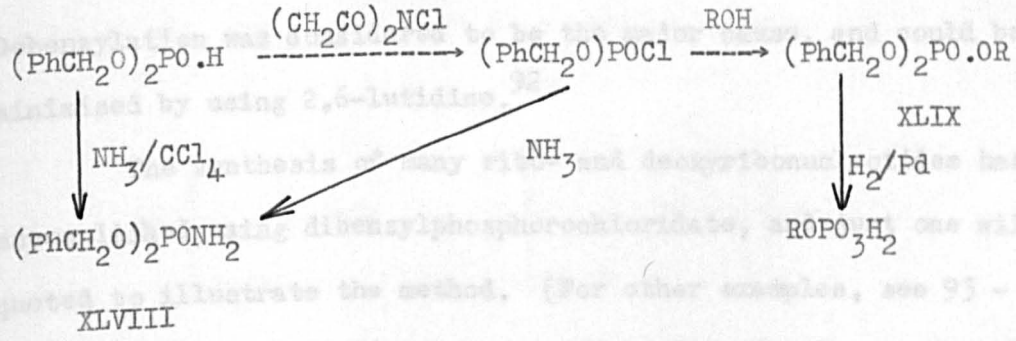
The ester (XLVII) on heating with acid (or an acidic ion-exchange resin) gives the primary phosphate. The reagent may find applications with compounds in which sensitivity to catalytic reduction, but not acid is the limiting factor. It is unlikely that powerful reagents of this type will be found, since the lone pair electrons on nitrogen are able to bond with phosphorus d-orbitals, thus depressing the overall reactivity.

(v) Benzyl esters of phosphorochloridic acid

The majority of studies to be discussed in this section relate

to the developments in phosphorylation methods first applied in the synthesis of nucleotides, and in which the Cambridge School has been pre-eminent. Their objective has been the rational synthesis of nucleotide coenzymes and of compounds related to the nucleic acids which have also provided evidence for the chemical structure of the latter.[83-88]

Dibenzylphosphorochloridate - the choice of the benzyl group as a protecting group was conditioned by the versatility of its reactions, and in particular its ease of controlled removal under mild conditions.[89] Dibenzylphosphorochloridate cannot be prepared from benzyl alcohol and phosphoryl chloride since it is relatively unstable and decomposes on distillation. It can, however, be prepared by reaction at room temperature of dibenzyl phosphite with N-chloro-succinimide in an inert solvent.[90] Another valuable synthesis of phosphorochloridates involves reaction between dialkyl phosphites, carbon tetrachloride and 10 -15 mole-% of a tertiary amine.[91] If an alcohol is present, phosphorylation occurs (Scheme 39) or if gaseous ammonia is present, dibenzyl



Scheme 39

phosphoramidate is deposited (XLVIII). The mechanism of the reaction is considered to involve attack of the dialkyl phosphite anion on the polyhalogenoalkane (Scheme 40) or on an intermediate N-haloamine[91] (Scheme 41).

$$(RO)_2PO^- + CCl_4 \rightarrow (RO)_2PO.Cl + CCl_3^- \qquad \text{Scheme 40}$$

$$(RO)_2PO^- + R_3\overset{+}{N}Cl \rightarrow (RO)_2PO.Cl + NR_3 \qquad \text{Scheme 41}$$

I. Monoesters: Dibenzylphosphorochloridate reacted with amines, and alcohols to yield phosphoramidates (Scheme 39) and tertiary phosphates (XLIX) respectively, and the benzyl groups were removed by hydrogenation over palladium catalysts.[89] Phenols do not appear to react, but their sodium salts do so with great ease, affording a route to aryl dihydrogen phosphates.[89] It was soon noticed that relatively low yields were sometimes encountered during phosphorylations, and this was traced to decomposition of the reagent by pyridine, the base usually employed. Debenzylation was considered to be the major cause, and could be minimised by using 2,6-lutidine.[92]

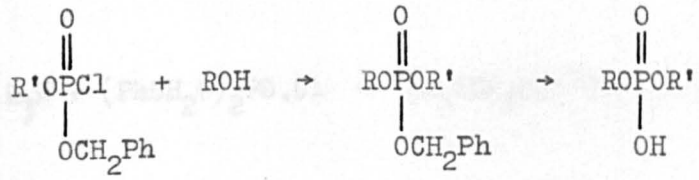
The synthesis of many ribo- and deoxyribonucleotides has been accomplished using dibenzylphosphorochloridate, and just one will be quoted to illustrate the method. (For other examples, see 93 - 100). Phosphorylation of 2',3'-O-isopropylideneadenosine in pyridine by dibenzylphosphorochloridate, followed by removal of benzyl groups by

hydrogenation and the isopropylidene group by mild acid hydrolysis gave the nucleotide adenosine-5'-phosphoric acid in excellent yield.[101]

In the early work with dibenzylphosphorochloridate, hydrogenolysis of the benzyl groups was, at times, slow and incomplete, and this was attributed to the presence of catalyst poisons, possibly traces of phosphites.[92] Cohen has, however, shown that hydrogenolysis of benzyl groups on phosphites is possible.[102] Thus one general procedure, which has been used extensively in nucleotide syntheses is to carry out partial debenzylation noncatalytically, and then complete the removal of protecting groups after purification of a salt of the intermediate.

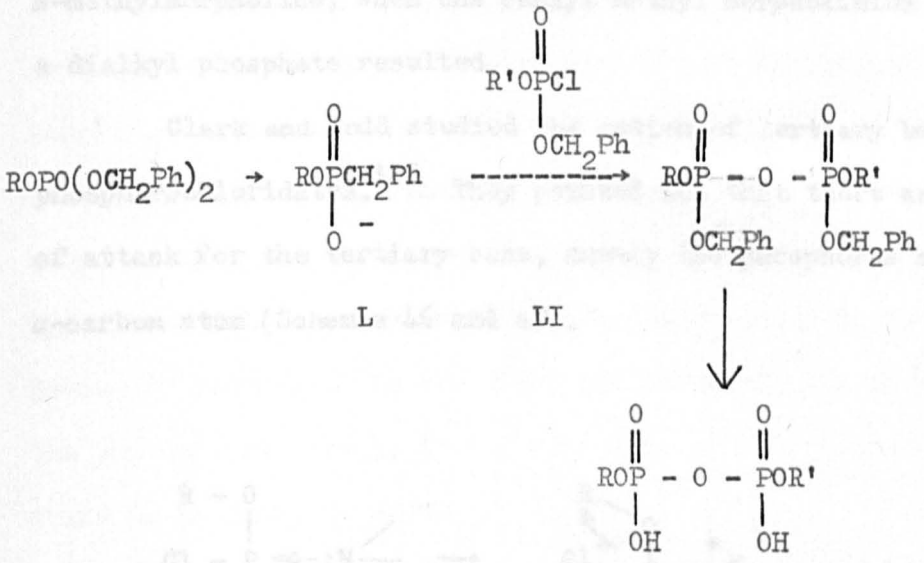
II. Unsymmetrical diesters and polyphosphates

The general scheme for synthesis of an unsymmetrical diester of phosphoric acid and an unsymmetrical pyrophosphate is presented below (Schemes 42 and 43 respectively) and raised two problems, namely the selective removal of one protecting group to yield the monoanion (L) and the elaboration of unsymmetrical dialkylphosphorochloridates (LI) in which one group (R') might be a complex residue.



LI

Scheme 42



Scheme 43

III. Selective Debenzylation

Todd and co-workers observed that dibenzylphosphorochloridate underwent rapid decomposition in the presence of a tertiary base to yield a quaternary chloride, and it was presumed, benzylmetaphosphate,[92] (Scheme 44).

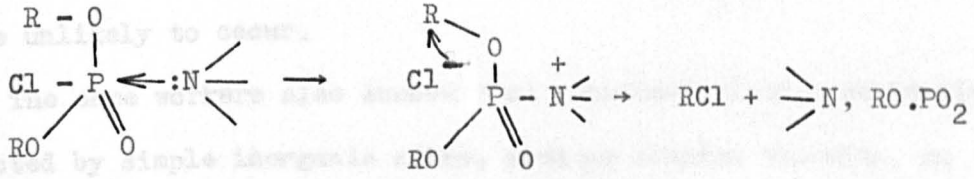
$$R_3N + (PhCH_2O)_2PO.Cl \rightarrow [R_3NCH_2Ph]^+Cl^- + [PhCH_2.OPO_2]$$

Scheme 44

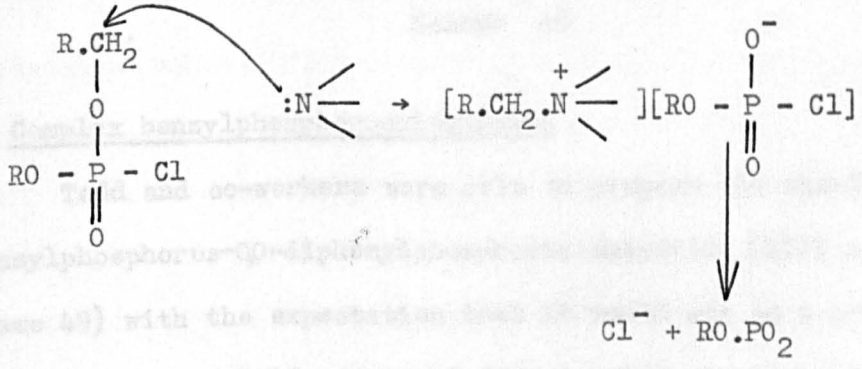
The method was applied to a variety of benzyl phosphates, in

N-methylmorpholine, when the benzyl methyl morpholinium salt of a dialkyl phosphate resulted.

Clark and Todd studied the action of tertiary bases on phosphorochloridates.[103] They pointed out that there are two centres of attack for the tertiary base, namely the phosphorus atom and the α-carbon atom (Schemes 46 and 47).



Scheme 46



Scheme 47

Scheme 46 results in the formation of alkyl chloride and

a base-metaphosphate complex, wherease Scheme 47 results in formation of a quaternary halide, the base becoming alkylated. Clark and Todd concluded that the centre of nucleophilic attack by a tertiary base, was influenced by the nature of the alkyl groups on the phosphorus atom, so that, for instance, for a triphosphate of type $(PhCH_2O)_2PO.OR$, by virtue of the susceptibility of a benzyl group to nucleophilic attack, Scheme 47 operates. In all cases the reaction went no further than the monodebenzyl stage, as was expected, since attack on the monoanion would be unlikely to occur.

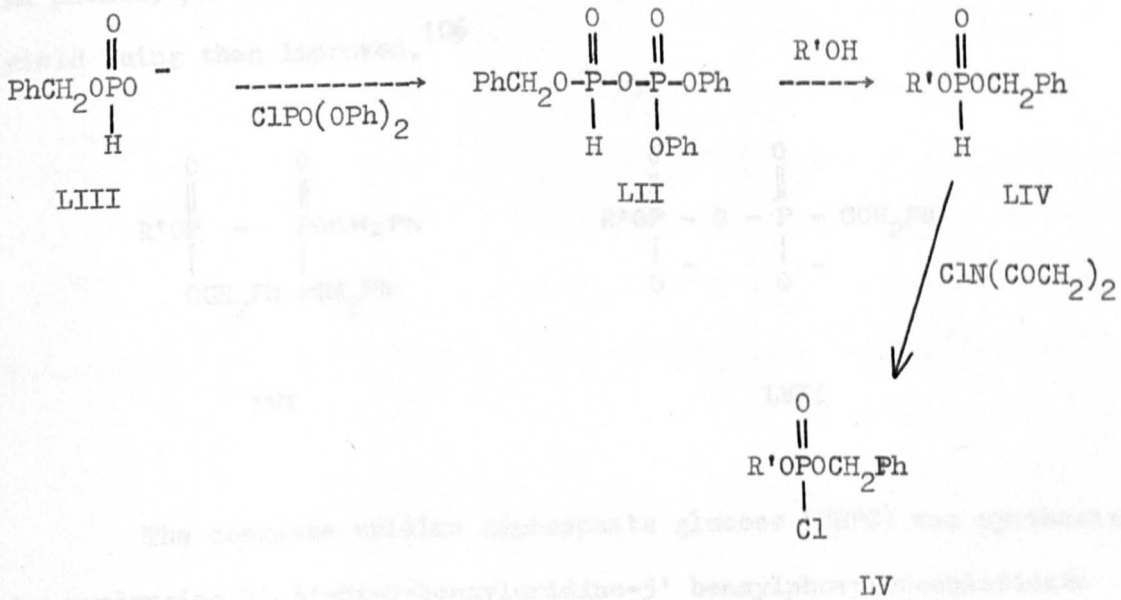
The same workers also showed that monodebenzylation could also be effected by simple inorganic salts, such as lithium chloride, in hot ethoxyethanol[104] (Scheme 48).

$$PhCH_2 - OPO(OR)_2 \xrightarrow{Cl^-} PhCH_2Cl + {}^-OPO(OR)_2$$

Scheme 48

IV. Complex benzylphosphorochloridates

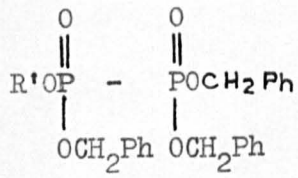
Todd and co-workers were able to prepare the mixed anhydride O-benzylphosphorus-O,O-diphenylphosphoric anhydride (LII) as shown (Scheme 49) with the expectation that it would act as a phosphitylating agent, and the anhydride prepared from monobenzyl phosphite (LIII) and diphenylphosphorochloridate, did, in fact, react with alcohols to give mixed secondary phosphites (LIV).[105] These were then converted, in the cold, to the phosphorochloridates by N-chlorosuccinimide.[90]



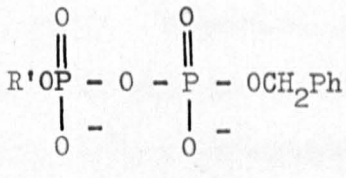
Scheme 49

Of the many syntheses involving a combination of the above techniques, the following examples may be considered as representative. The benzyl phosphite of 2',3'-O-isopropylideneuridine was prepared, and from this the phosphorochloridate (LV; R' = isopropylidene-5'). The latter with triethylammonium dibenzyl phosphate in methyl cyanide gave the pyrophosphate (LVI), from which two benzyl groups were removed by debenzylation using lithium chloride. The product (LVII), now stabilised, was converted to uridine-5'-pyrophosphate by hydrogenation and mild acid hydrolysis to remove the isopropylidene group.[90] The lithium chloride step could be avoided if the condensation was carried out

in phenol, partial debenzylation being affected by the solvent, and the yield being then improved.[106]



LVI



LVII

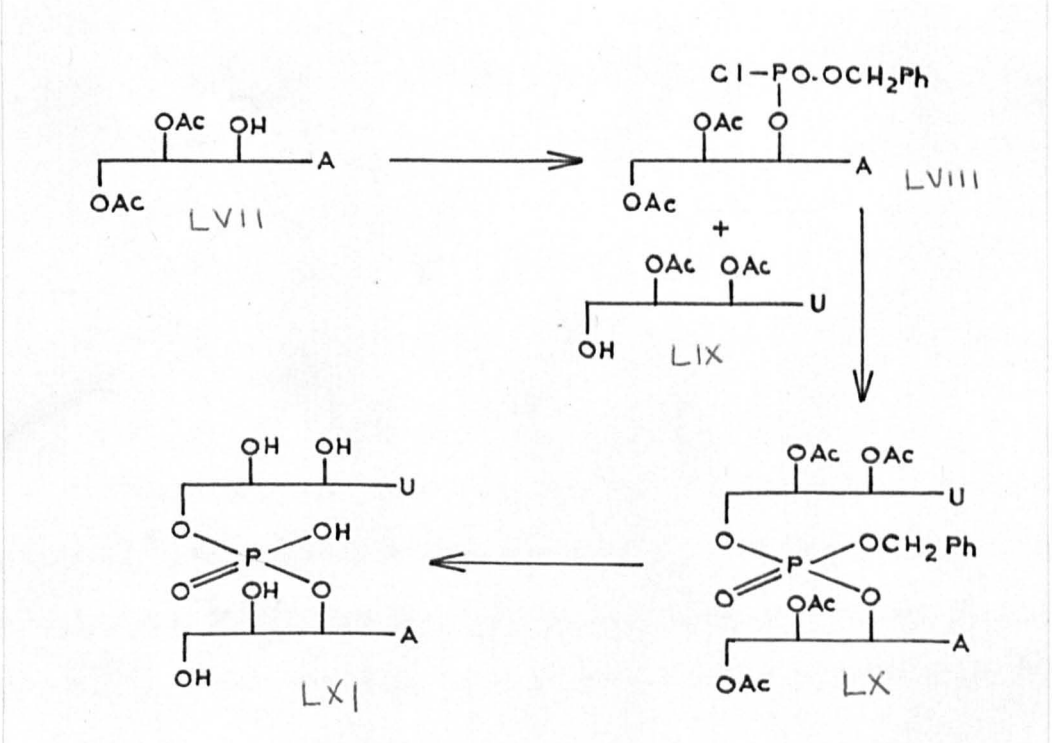
The coenzyme uridine diphosphate glucose (UDPG) was synthesised by condensing 2',3'-di-O-benzyluridine-5' benzylphosphorochloridate (LV; R = 2',3'-di-O-benzyluridine-5') with α-D-glucose-1-phosphate as the mono tri-n-octylammonium salt which was soluble in the solvent of the reaction-benzene. The intermediate so formed was converted directly to UDPG (XIX; R = H) by hydrogenolysis.[107]

An important demonstration of the versatility of these syntheses was that of flavin adenine dinucleotide (FAD) (XVI). Thus 2',3'-O-isopropylidene adenosine-5'-benzylphosphorochloridate (LV; R' = 2',3'-O-isopropylidene adenosine-5') with monothallous riboflavin-5'-phosphate in methyl cyanide-phenol solution. The benzyl group was solvolysed by the phenol, and mild acid hydrolysis removed the isopropylidene group. The product was finally purified by chromatography and through its silver salt, to give FAD in reasonable yield.[108,105]

Two factors do, however, reduce yields in these syntheses. The

first is base catalysed attack by a vicinal hydroxyl group, with fission of the pyrophosphate linkage to give the cyclic phosphate, and the second is the series of exchange reactions by which unsymmetrically substituted pyrophosphates revert to the two symmetrical pyrophosphates.

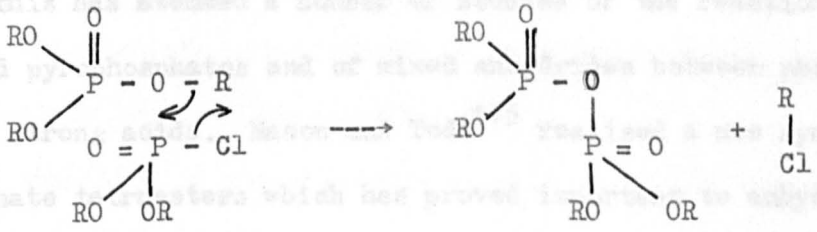
This method has also had limited success in the polynucleotide field, nucleic acids being essentially polyphosphodiesters. Thus in the polyribonucleotide field, 3',5'-di-O-acetyladenosine (LVII) was converted through the 2'-benzyl phosphite to the phosphorochloridate (LVIII),[109] which was then condensed with 2',3'-di-O-acetyl uridine (LIX), and the benzyl and acetyl groups removed from the product (LX) to yield adenosine-2'-uridine-5'-phosphate (LXI; A = adenine, U = uracil residues)[110] (Scheme 50).



Scheme 50

Similar syntheses in the polydeoxyribonucleotide field have also been accomplished. Low yields have been attributed to trace amounts of water present during the condensation (leading to pyrophosphates) and also the relatively low reactivity of alcoholic hydroxyl groups, by comparison with phosphate anions towards phosphorylations.

The products of such reactions were always contaminated by quantities of pyrophosphate. Hall[124] suggested that the latter arose by attack of the phosphorochloriate on the newly formed triester, as shown below:



$R = C_2H_5$

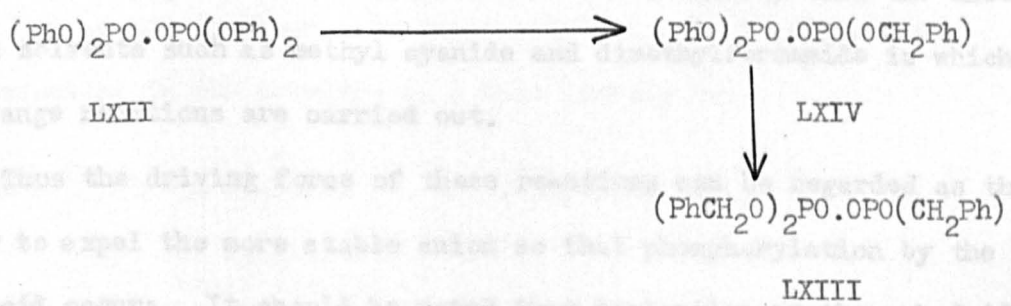
The use of the mixed anhydride (LII; benzyl moiety replaced by a suitably protected nucleoside residue) to synthesise dinucleotides by reaction with a suitably protected nucleoside also resulted in low yields.[102] It appears that in this case in the formation of (LII) the

nucleoside phosphite anions which were always present in excess, immediately attacked any mixed anhydride formed to give pyrophosphate, which would be hydrolysed during the work-up to appear as "unreacted" phosphite. These findings were in accord with the results of Corby and co-workers.[105]

(b) Use of anhydrides

There are a number of phosphorylating agents in this category, which, although useful in some specialised cases, do not seem to have general application. Thus phosphorus pentoxide,[58] polyphosphoric acid[111,112] and triethyl trimetaphosphate[113] have been used.

Turning to more definable reagents, it was recognised that tetraesters of pyrophosphoric acid could act as phosphorylating agents,[114] and from this has stemmed a number of studies of the reactions of esterified pyrophosphates and of mixed anhydrides between phosphates and other strong acids. Mason and Todd[115] realised a new synthesis of pyrophosphate tetraesters which has proved important to anhydride chemistry in general (Scheme 51).



Scheme 51

Tetraphenyl pyrophosphate (LXII) was shown to react rapidly with dibenzylphosphate in the presence of a tertiary base yielding tetrabenzyl pyrophosphate (LXIII). This exchange reaction was thought to involve two successive nucleophilic displacements by the dibenzyl phosphate anion, and to proceed via the unsymmetrical intermediate (LXIV). Corby and co-workers showed that two moles of base were required, and that lithium dibenzyl phosphate underwent reaction without added base, adding proof to the above mechanism.[116] The unsymmetrical ester (LXIV) was formed, although not isolated, when one mole of base was added, and compared with that prepared from diphenylphosphorochloridate and dibenzylphosphate with one mole of base. They also concluded that

(i) The exchange reaction proceeds so as to convert a tetraester of pyrophosphoric acid into a less reactive anhydride;

(ii) the intermediate mixed anhydride is correspondingly intermediate in its stability, e.g. to hydrolysis; and

(iii) the stability of the anhydride is related to the strength of the acid from which it is derived - diphenyl hydrogen phosphate is a considerably stronger acid (i.e. has a more stable anion) than the dibenzyl ester in solvents such as methyl cyanide and dimethylformamide in which the exchange reactions are carried out.

Thus the driving force of these reactions can be regarded as the tendency to expel the more stable anion so that phosphorylation by the weaker acid occurs. It should be noted that ionisation of the anhydride before reaction would lead to the same result, since the positive

phosphorylium ion (the phosphorylating entity) would derive from the weaker acid. The precise mechanism is in doubt, but it would seem likely that when pyrophosphates are used the intact anhydride molecule reacts, whereas ionisation might occur when the reagent is a dialkyl phosphorochloridate or a mixed anhydride with a strong acid.[86]

This reaction has been shown to be a general one. Thus p-toluenesulphonyl chloride with dibenzyl phosphate gave tetrabenzylpyrophosphate (LXV; Scheme 52), and trifluoroacetic anhydride with uridine-5'-phosphate gave P^1, P^2-di-uridine-5'-pyrophosphate.[115]

$$2(RO)_2PO_2^- + ArSO_2Cl \rightarrow (RO)_2PO.OPO(OR)_2 + Cl^- + RSO_3^-$$

LXV

Scheme 52

The nucleotide coenzymes are generally formed in nature by exchange reactions, but only recently has this process been utilised for their chemical synthesis. The difficulty of halting the reaction at the first stage has been overcome by allowing a pyrophosphate triester (generated in absence of basic catalysts) to react with a phosphomonoester in the presence of a base (Scheme 53).

$$ROPO_3^{2-} + (R'O)_2PO.Cl \rightarrow ROPO.OPO(OR')_2 + Cl^-$$

(or an active vinyl dialkyl phosphate - see next section)

$$ROPO.OPO(OR')_2 + R''OPO_3^{2-} \xrightarrow[\text{pyridine}]{} ROPO.OPO.OR'' + (R'O)_2PO_2^-$$

Scheme 53

Michelson has synthesised a number of nucleotide coenzymes by this method.[117,118,119] Thus in the synthesis of UDPG,[120] uridine-5'-phosphate is condensed with diphenylphosphorochloridate, and the intermediate treated with α-D-glucose-1-phosphate.

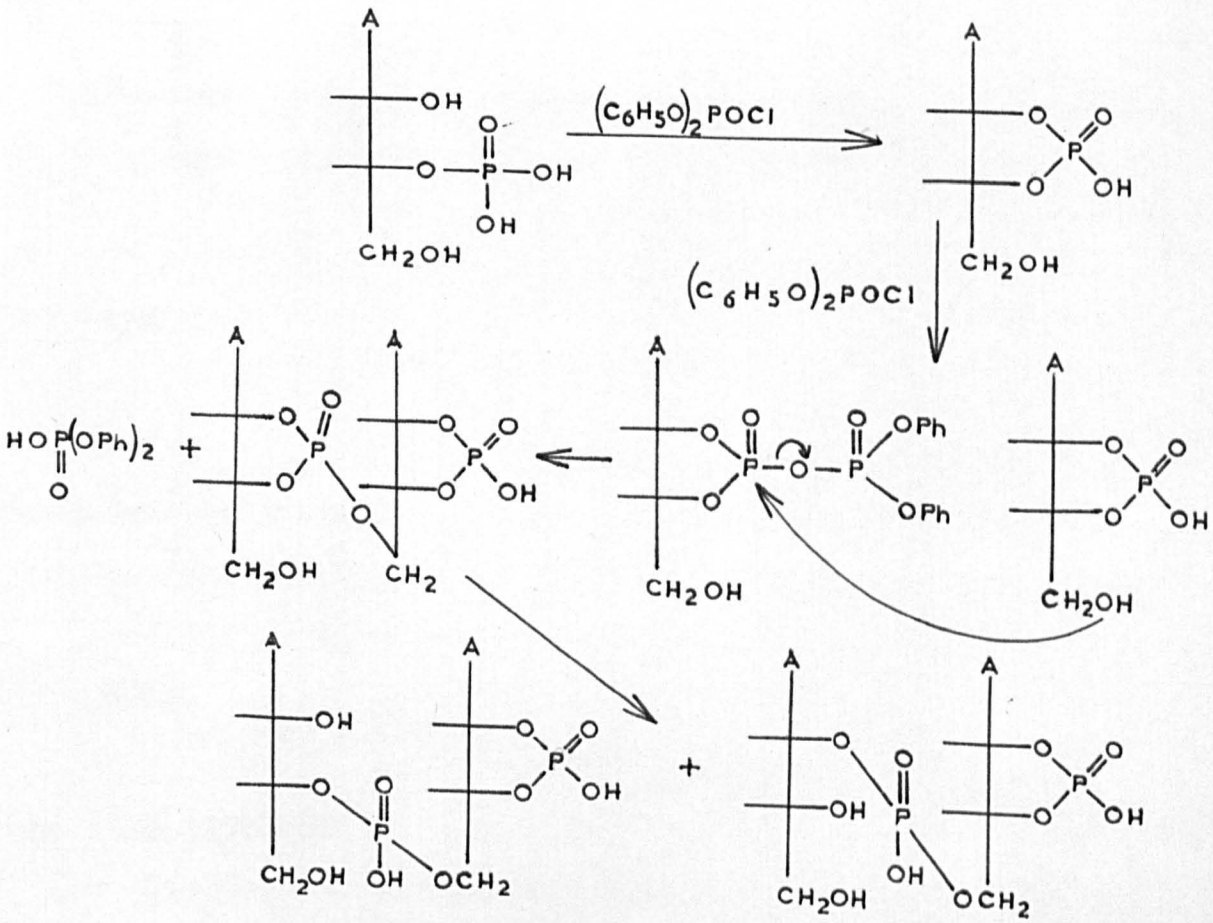
He has also synthesised adenosine-5'-sulphatophosphate by the method of anhydride exchange.[119] (For an alternative synthesis, see Experimental Section).

If phosphorylation of an alcohol was required, the phosphorochloridates rather than the corresponding pyrophosphates or mixed anhydrides have been used, although tetra-p-nitrophenyl pyrophosphate, an extremely powerful reagent, phosphorylated 2',3'-O-isopropylidene guanosine even in the absence of base, to give a convenient synthesis of guanosine-5'-phosphate.[116] These reagents have also been used to prepare cyclic phosphates. Thus Forrest and co-workers treated riboflavin-5'-phosphate either with tetraphenyl pyrophosphate, or with trifluoroacetic anhydrides and obtained in each case riboflavin-4',5'-cyclic phosphate.[120] The intermediate anhydride, which was presumed to form, effected internal phosphorylation, emulating the decomposition of FAD under mildly basic conditions.[121]

Khorana and co-workers have also used this principle to condense thymidine-5'-phosphate and 5'-tritylthymidine by means of p-toluene sulphonyl chloride in pyridine to effect a synthesis of thymidine-5' thymidine-3'-phosphate in good yield.[122,123] With thymidine-5'-phosphate

itself, polymerisation occurred via the intermediate dithymidine pyrophosphate which, itself, could be converted to poly (thymidine phosphate) by further reaction with p-toluene-sulphonyl chloride.[125]

Michelson was able to polymerise ribonucleoside-2'- and -3'-phosphates, by their conversion to the cyclic phosphate with one mole of diphenylphosphorochloridate, and using a second mole to bring about polymerisation,[126] (Scheme 54).

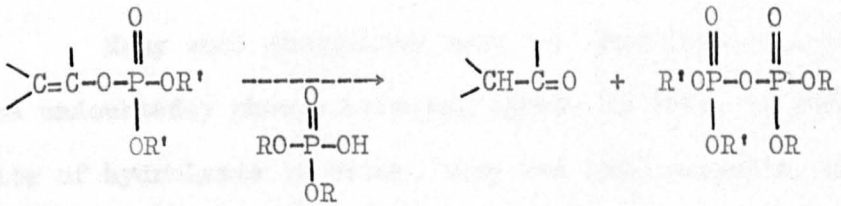


Scheme 54

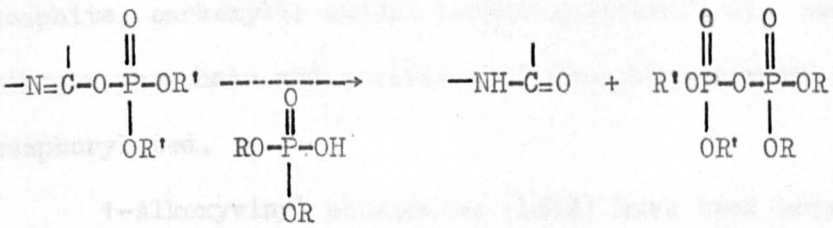
The products were linear polymers containing 3' to 5' and 2' to 5' phosphodiester linkages, with an average chain length of 10 - 12 nucleotides.

(c) Use of vinyl phosphates and related compounds

These reagents are represented by (LXVI) and (LXVII), which are either isolatable, or more frequently, hypothetical intermediates in phosphorylation reactions.



LXVI



LXVII

(i) Vinyl phosphates

This class of compounds will be discussed in detail in the section devoted to enol esters and their properties, hence only brief reference to them will be made here. Perkow discovered that reaction

between α-halo carbonyl compounds and trialkyl phosphites yielded enol phosphates; for example, trichloroacetaldehyde and triethyl phosphite rapidly formed 2,2-dichlorovinyl diethyl phosphate (LXVIII; Scheme 55).[127,128]

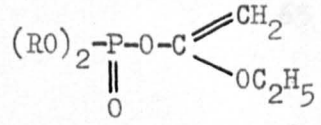
$$CCl_3.CHO + P(OEt)_3 \rightarrow CCl_2{=}CHOP(OEt)_2$$

LXVIII

Scheme 55

Many enol phosphates have now been prepared, but although they are undoubtedly phosphorylating agents in vivo, as judged from their rate of hydrolysis in water, they are weak reagents, and in general unlikely to have much value in the phosphorylation of alcohols. Acid anions, however, are phosphorylated, and using one of the more reactive enol phosphates, derived from diethyl bromomalonate and triethyl phosphite, carboxylic acids, benzyloxycarbonylamino acids, biphenyl hydrogen phosphate and a variety of phosphomonoesters have all been phosphorylated.

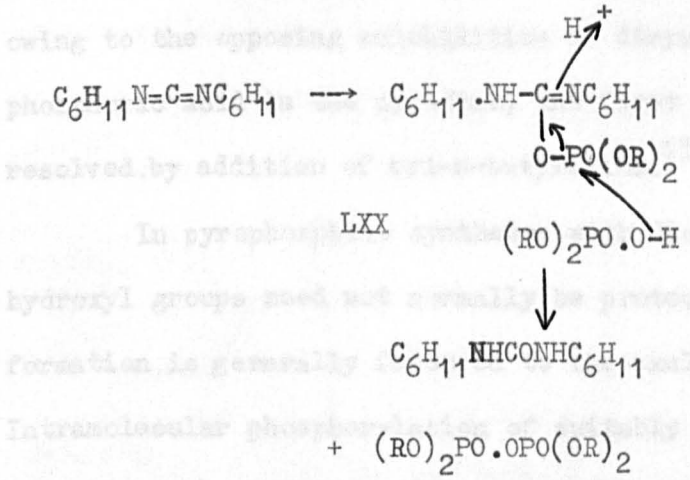
1-Alkoxyvinyl phosphates (LXIX) have been prepared by reaction of ethoxyacetylene with a phosphodiester, and have been shown to be successful phosphorylating agents for both acid anions and in some cases, alcohols.[129,130] This has been demonstrated to be a general method for the activation of acids, carboxylic, thiolic, sulphonic and sulphuric acids also baving been activated (see later section).



LXIX

(ii) Carbodiimides

This class of compounds will again be discussed in greater detail in a later section, and the general chemistry of the group has been reviewed in detail.[45,131] Khorana and Todd[132] found that under anhydrous conditions dicyclohexylcarbodiimide (DCC) with dibenzyl-, diphenyl- and di-p-nitrophenylphosphates gave the corresponding pyrophosphate quantitatively, whilst monophenyl phosphate gave P^1P^2-diphenyl pyrophosphate. They put forward the hypothesis that the initial step of the reaction is the formation of the adduct (LXX), and that the protonated form of this is attacked by another phosphate anion to give the pyrophosphate and the urea (Scheme 56). It has been found impossible to isolate a ψ-urea phosphate adduct (LXX).



Scheme 56

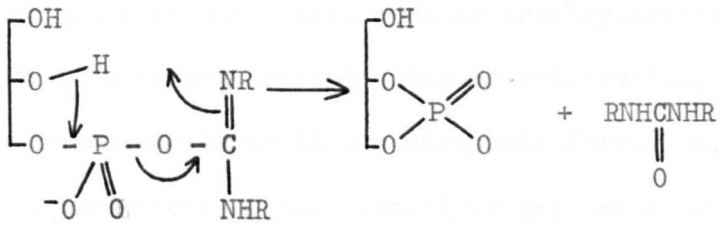
Dicyclohexylcarbodiimide can be used in inert solvents or in anhydrous

pyridine, and in some cases in organic solvents containing water.[132] Salts of dialkyl phosphates with stronger bases (e.g.triethylamine) do not react, possibly by preventing protonation of the intermediate LX, but those of monoalkyl phosphates do.[133,134] (See later discussion).

Many examples of syntheses utilising dicyclohexylcarbodiimide are known, a culmination of these being a synthesis of Nicotinamide Adenine Dinucleotide (NAD^+) (XIV; R = H) in relatively good yield.[135,136] In the majority of cases, random condensation occurs, and so ion-exchange and related fractionation methods have proved invaluable. Good yields of unsymmetrical pyrophosphates can be obtained by use of a large excess of one of the components. Thus Khorana and co-workers condensed nucleoside-5'-phosphates with an 85% excess of phosphoric acid to synthesise the 5'-pyrophosphates and the 5'-triphosphates. Difficulties owing to the opposing solubilities of dicyclohexylcarbodiimide and phosphoric acid in the pyridine, the first solvent to be used, were resolved by addition of tri-n-butylamine.[133]

In pyrophosphate syntheses with dicyclohexyl carbodiimide, hydroxyl groups need not normally be protected since pyrophosphate formation is generally favoured to the exclusion of esterification. Intramolecular phosphorylation of suitably placed hydroxyl groups does occur, however, providing a useful synthesis of cyclic phosphates. It appears likely that production of the latter depends on a displacement of dicyclohexylurea from the Ψ-urea phosphate adduct (LXXI) by the neighbouring group which, although weakly nucleophilic, is suitably

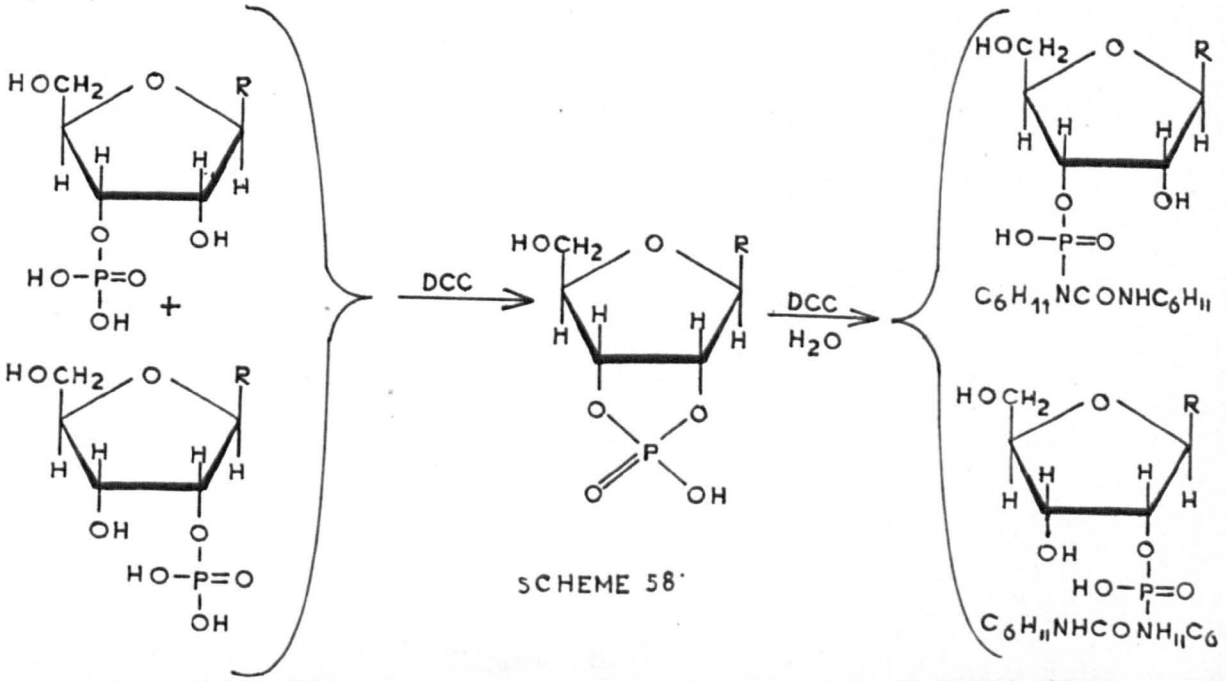
placed to compete favourably with attack by other phosphate anions (Scheme 57).



LXXI

Scheme 57

Nucleoside-2',3'-cyclic phosphates (LXXII; R = purine or pyrimidine base) are rapidly formed from the corresponding 2'- and 3'-nucleotides, but it was found that these labile five membered cyclic phosphates underwent subsequent reaction to form N-phosphoryl ureas (LXXIII), Scheme 58).[137]

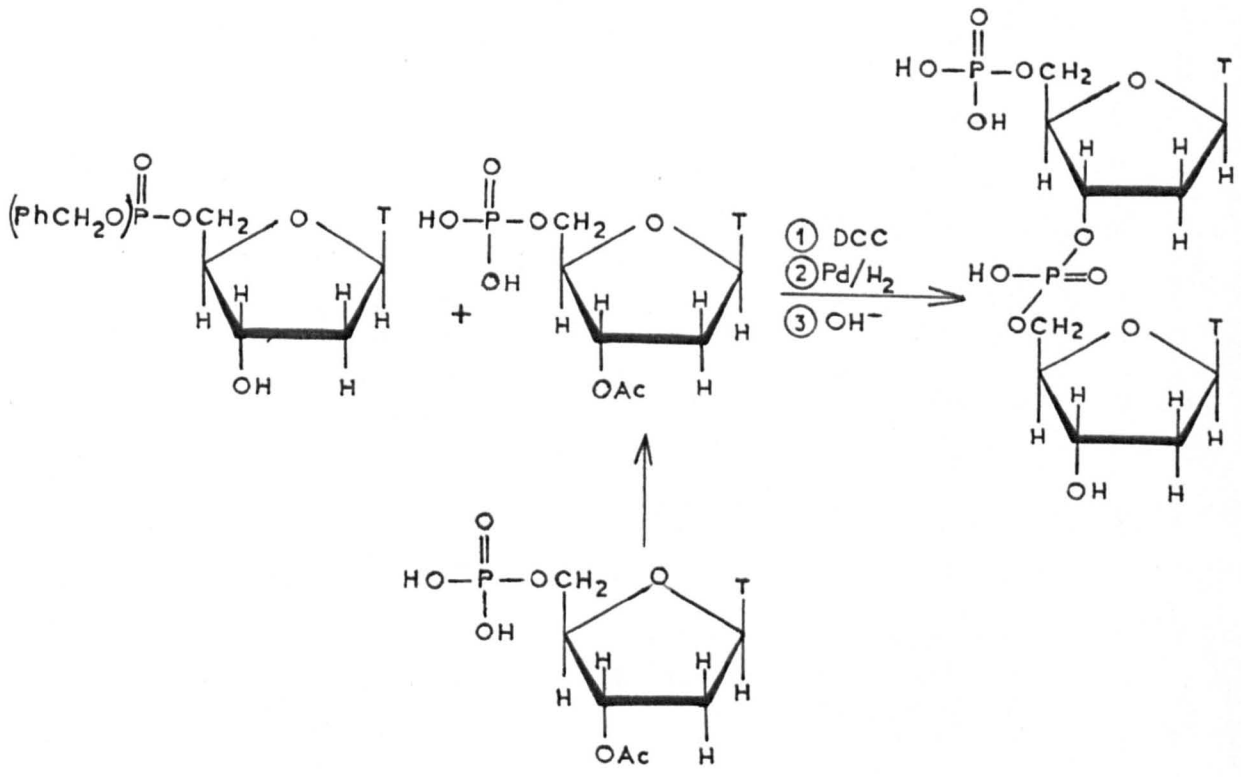


SCHEME 58

More recent work[134] has shown that these five-membered cyclic phosphates are obtained quantitatively as the ultimate products when the reaction with dicyclohexylcarbodiimide is carried out in the presence of ammonia or other strong bases, such as trialkylamines.

To accomplish intermolecular esterification, two approaches have been developed to avoid pyrophosphate formation.[134] In the first, a large excess of the alcohol is used with or without base, and pyrophosphate formation is presumably avoided by dilution. Thus anhydrous phosphoric acid in methanol with dicyclohexylcarbodiimide yields dimethyl phosphate. In the second, a monophosphate ester is condensed with more nearly equimolar quantities of alcohols[123] in the absence of base. (If base is added, pyrophosphate is the only product - see later discussion). This reaction has found use in the synthesis of nucleoside phosphates where a suitably protected nucleoside is condensed with 2-cyanoethyl phosphate followed by elimination of the cyanoethyl group.[138] Nucleoside protection is not always necessary (see Experimental Discussion) providing ion-exchange fractionation of the products is employed.

This method has been applied extensively in the polynucleotide field. Thus, as a simple example, thymidine-5'-phosphate (LXXIV) has been converted to dithymidylic acid (LXXV) as shown in Scheme 59.[123]

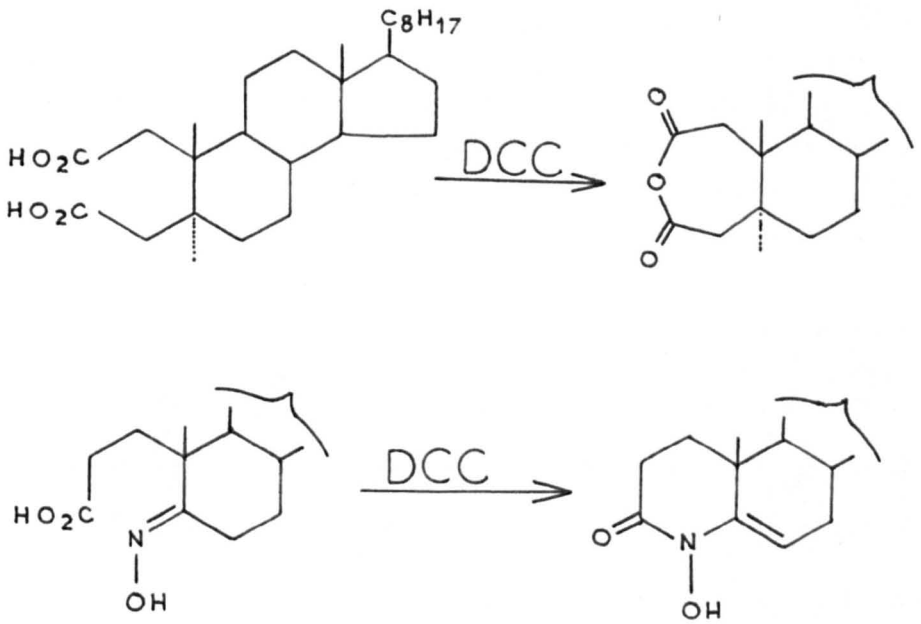


Scheme 59

An interesting extension of this, to be discussed in detail in a future section, is the polymerisation of pyridinium deoxynucleoside--5'-phosphates by dicyclohexycarbodiimide.

Carbodiimides can be used in a similar way to synthesise carboxylic and sulphonic anhydrides from their respective acids.[131,139]

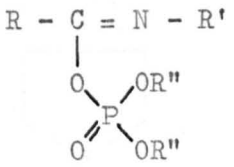
Applications in the heterocyclic steroid field illustrate a further extension of this, (Scheme 60).[140]



Scheme 60

(iii) Imidoyl phosphates

The postulated reactive intermediate (LXX) in the carbodiimide reaction, is an enol derivative of a urea (or an imidoyl phosphate). Consequently, synthesis of other imidoyl phosphates (LXXVI) has been attempted for use in pyrophosphate synthesis (Scheme 61).

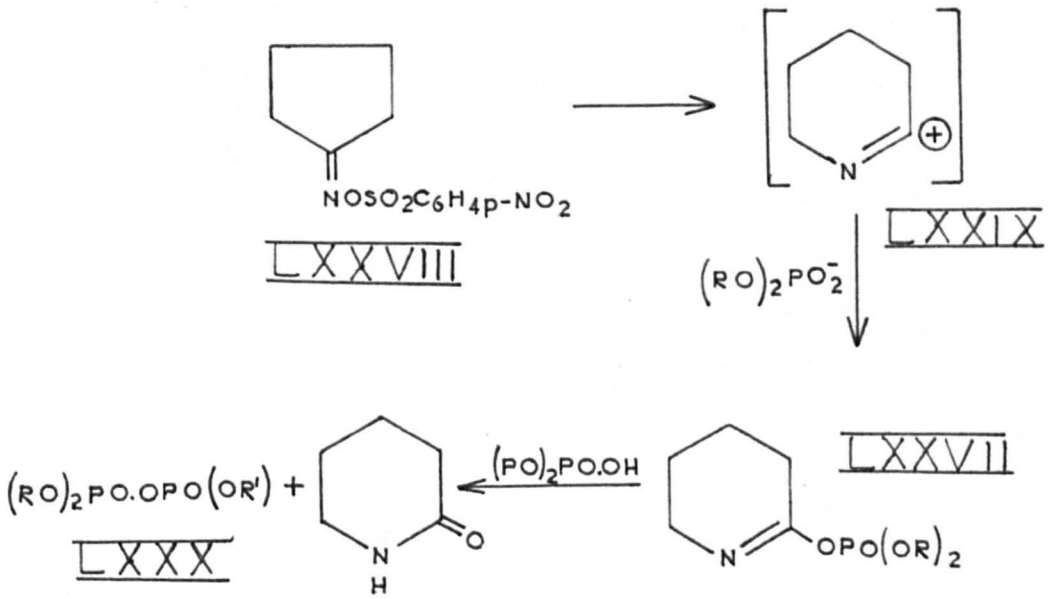


LXXVI

$$(R''O)_2PO.OCR = NR' + HOPO(OR''')_2 \rightarrow (R''O)_2PO.OPO(OR''')_2 + R'CO.NHR'$$

Scheme 61

Imidochlorides of carboxylic acids react with the silver salts of phosphoric acids to give imidoyl phosphates (LXXVI). An interesting variant involves the imidoyl phosphate (e.g. LXXVII) generated during the Beckmann rearrangement of an oxime ester.[101,102] Cyclopentanone oxime p-nitrobenzene sulphonate (LXXVIII) was chosen, since it could be isolated in a pure state; it rearranged rapidly, and, in addition, it was presumed that the arenesulphonate ion would not compete effectively with the added phosphate ion for the intermediate cation (LXXIX) (Scheme 62).

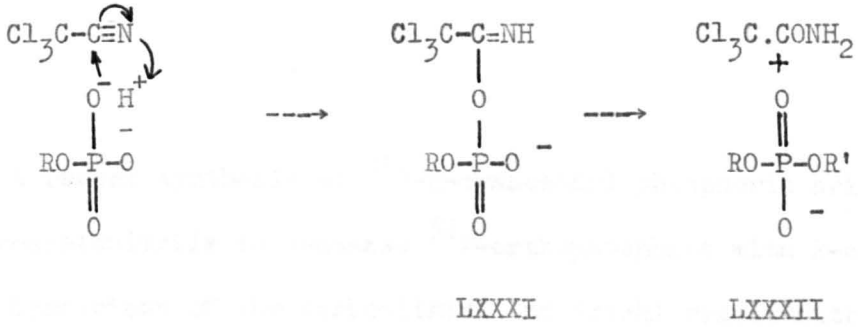


Scheme 62

Thus, using this method, pyrophosphates and nucleotide coenzymes were synthesised.[141,142] When (LXXVIII) and tetramethylammonium diphenyl phosphate were allowed to react in benzene, and then dibenzyl phosphate added, P'-dibenzyl P^2-diphenyl pyrophosphate (LXXX; R = Ph, R' = Ch_2Ph) was formed. Uridine-5'-pyrophosphate was synthesised by the same principle. However, when more polar solvents were used, mixtures of the unsymmetrical and symmetrical pyrophosphates resulted. Either imidoyl phosphates can undergo exchange reactions with other phosphate anions in polar solvents, or exchange occurred after the unsymmetrical

pyrophosphate had formed. Thus this reagent may have some advantage over carbodiimides with non polar solvents, but not if polar solvents have to be employed.

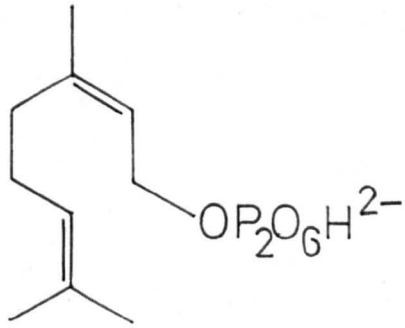
The second dissociation stage of a phosphomonoester is sufficiently nucleophilic to add rapidly to trichloroacetonitrile, to form the reactive intermediate (LXXXI), which is converted into esters (LXXXII; R' = alkyl) in the presence of alcohols, and pyrophosphates (LXXVIII; R' = PO.(OR")OH) with phosphoric acids[143] (Scheme 63).



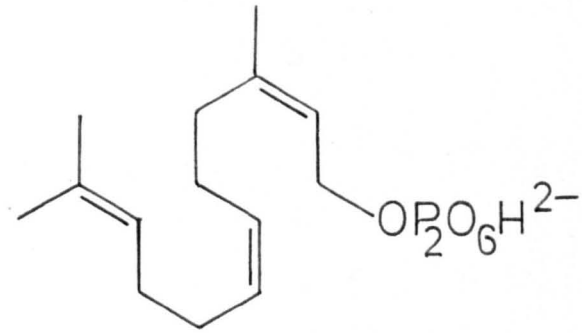
LXXXI LXXXII

Scheme 63

The reaction can also be carried out with unsubstituted orthophosphoric acid, and can also be directed, by addition of either one or two moles of base, so that it stops at either the monoester or diester stage, respectively. Thus the esters geranyl pyrophosphate and farnesyl pyrophosphate (LXXXIV), important in the biosynthesis of carotenoids and steroids, were obtained in yields of 29 and 23% respectively by this method.[144]



LXXXIII



LXXXIV

A recent synthesis of ^{32}P-2-cyanoethyl phosphoric acid utilises trichloroacetonitrile to condense ^{32}P-orthophosphate with 2-cyanoethanol.[145]

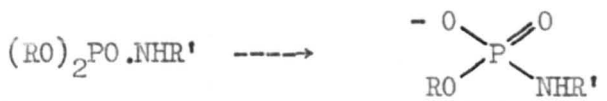
Comparison of the carbodiimide and trichloroacetonitrile methods reveals the following information. With (LXXXI), esterification is strongly favoured in the presence of stoichiometric quantities of alcohol, whereas in the carbodiimide method, pyrophospates are predominantly obtained under these conditions. In the pyrophosphate synthesis, the two reagents behave in a similar manner. Thus as a rule symmetrical and unsymmetrical products are obtained together. The trichloroacetonitrile method is more selective, inasmuch as it can be controlled to give the mono- or diester as desired. Unlike carbodiimide, (LXXXI) reacts with neither diesters of phorphoric acid nor carboxylic acids,

water, etc., only with the second dissociation stage of phosphoric acid, which alone possesses a sufficiently nucleophilic character.[146]

(d) Use of phosphoroamidates.

There are many examples in nature of compounds which contain a P-N bond and which are capable of acting as biological phosphorylating agents. One of these, phosphocreatine, can transfer its phosphate group to adenosine-5'-diphosphate to give the triphosphate *in vivo*. Stokes showed that salts of phosphoramidic acid were stable to base, but underwent very rapid hydrolysis below neutrality,[147] and in addition to orthophosphate, he thought that ammonium metaphosphate also formed, an observation confirmed by Goehring and Sambeth.[148]

The methods of preparation of phosphoramidic diesters have already been described, namely reaction between a phosphorochloridate and a primary or secondary amine, and the reaction between a secondary phosphite, two moles of amine and a polyhalogenalkane. The monoesters are normally prepared from a diester by selective removal of one ester group, (Scheme 64).



Scheme 64

Another method of general applicability, consists, for example, of reacting phenylphosphate, ammonia and dicyclohexylcarbodiimide to

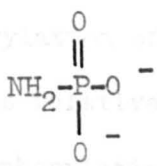
give guanidinium phosphoroamidate[149] (Scheme 65).

$$PhOPO_3H_2 \xrightarrow[NH_3]{DCC} [PhO.PO_2(NH_2)]^- [(C_6H_{11}NH)_2C.NH_2]^+$$

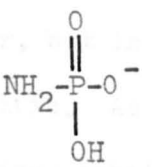
Scheme 65

Phosphoramidic acids are best prepared by hydrogenation of dibenzyl or substituted dibenzyl esters over palladium, or of the diphenyl esters over platinum.[150]

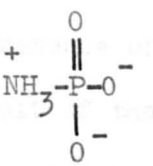
Phosphoramidates possessing at least one free acid function are useful, if weak phosphorylating agents, and will react with other phosphates affording pyrophosphates, but they do not react readily with alcohols.



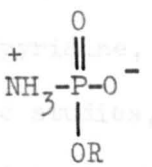
LXXXV



LXXXVI



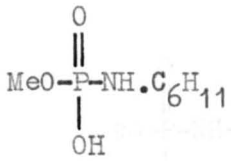
LXXXVII



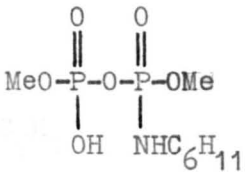
LXXXVIII

The dianion of phosphoramidic acid (LXXXV) is very stable, but is rapidly broken down to orthophosphate in acid, the rate of hydrolysis being proportional to the concentration of the monoanion (LXXXVI and LXXXVIII) in the intermediate pH range. This behaviour is

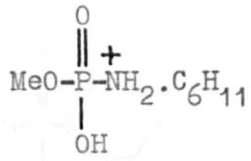
very similar to the hydrolysis of monoalkyl phosphates, the rates in the present case, however, being greater, possibly because the phosphoramidate is mainly in the zwitterionic form (LXXXVII) in solution, as it is in the crystalline state.[151] The monoesters should only exist to a small extent as zwitterions (LXXXVIII) at comparable pH values.



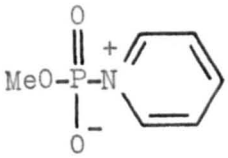
LXXXIX



XC



XCI

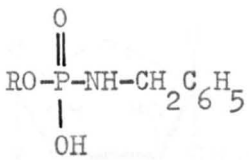


XCII

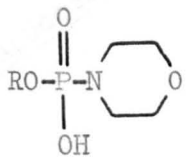
Hamer has studied the phosphorylation reactions of methyl hydrogen N-cyclohexylphosphoramidate (LXXXIX).[152] He found that this phosphoramidate with dilute aqueous ethanol resulted in preferential phosphorylation of the water, but in the presence of pyridine, phosphorylation was relatively unspecific. As a result of these studies, he ruled out phosphorylation by a metaphosphate ester intermediate in the first case, suggesting on the basis of second-order kinetics, that the intermediate (XC) is the phosphorylating entity, and arises from attack of one molecule of (LXXXIX) on a second (presumably in the zwitterionic form) or possibly by attack of the anion of (LXXXIX) on the protonated species (XCI). In the second case he postulated (XCII) as an intermediate, which with monoalkyl dihydrogen phosphates reacts via an intermediate analogous to (XC), but with alcohols and other poor nucleophiles prefe

to react by way of a metaphosphate ester intermediate.

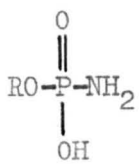
In accordance with a mechanism involving a zwitterionic structure, the reactivity of the amides decreases with the basicity of the amide nitrogen atom. Thus the suitability for pyrophosphate synthesis with the amides given below decreases in the order: (XCIII) and (XCIV) (XCV) (XCVI).[43, 153,154]



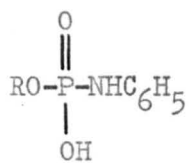
XCIII



XCIV



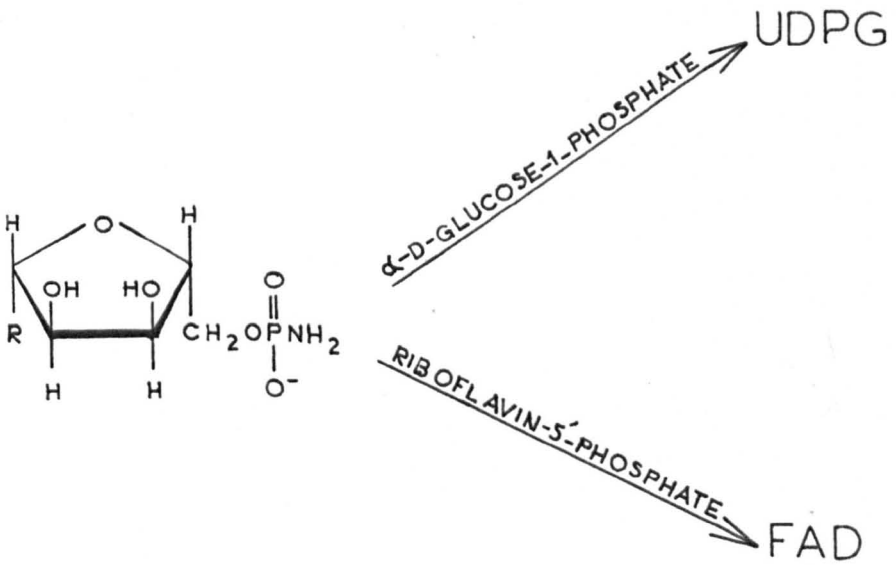
XCV



XCVI

Phosphoramidates have been very successful for the synthesis in good yields of nucleoside polyphosphates, but a considerable advance has resulted from a new method of preparing nucleoside-5'-phosphoramidates (XCVII) in high yield. Chambers discovered that nucleoside-5'-phosphates are condensed with ammonia and dicyclohexylcarbodiimide to yield phosphoramidates, which are isolated in nearly quantitative yields as their dicyclohexyl guanidium salts.[155] Substituting morpholine for ammonia gives the nucleoside-5'-phosphoromorpholidates which have som

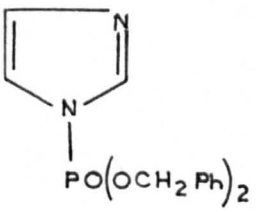
advantages both in reactivity and solubility.[156] Reactions of these phosphoramidates with phosphate monoesters allow synthesis of nucleotide coenzymes, e.g. UDPG, FAD (Scheme 65) and coenzyme A.[43,149,157]



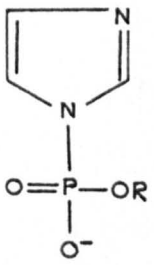
Scheme 66

It is well known that N-phosphoryl guanidine derivatives are phosphorylating agents in vivo, and the imidazole ring of histidine residues in a variety of enzymes has undoubted catalytic function and has been implicated in phosphate transfer reactions. Acetylimidazole is a good acylating agent for alcohols, amines and anions[158,159] and Westheimer has shown that imidazole catalyses the solvolysis of tetrabenzyl pyrophosphate in n-propanol, a protonated species derived from (XCVIII) being a presumed intermediate.[159] Baddiley and co-workers treated imidazole with dibenzyl- and diphenylphosphorochloridate and

obtained the phosphorylated products (e.g. XCVIII), which were highly reactive in the phosphorylation of alcohols, amines and phosphate anions. The monoesterified compounds (IC) have also been prepared, and are said to phosphorylate alcohols more readily than the phosphoramidates,[160] a fact which may be valuable from the synthetic point of view.

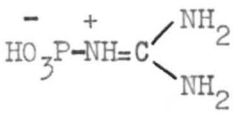


XCVIII

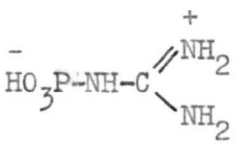


IC

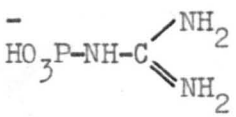
As guanidines are particularly strong bases, the phosphorylation with phosphoroguanidates would be expected to proceed extremely readily. On the other hand, the positive charge in the zwitterion (C) can be distributed mesomerically over 3 atoms (C, CI, CII) with consequent stabilisation of the compound.[146]



C

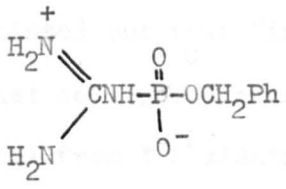


CI



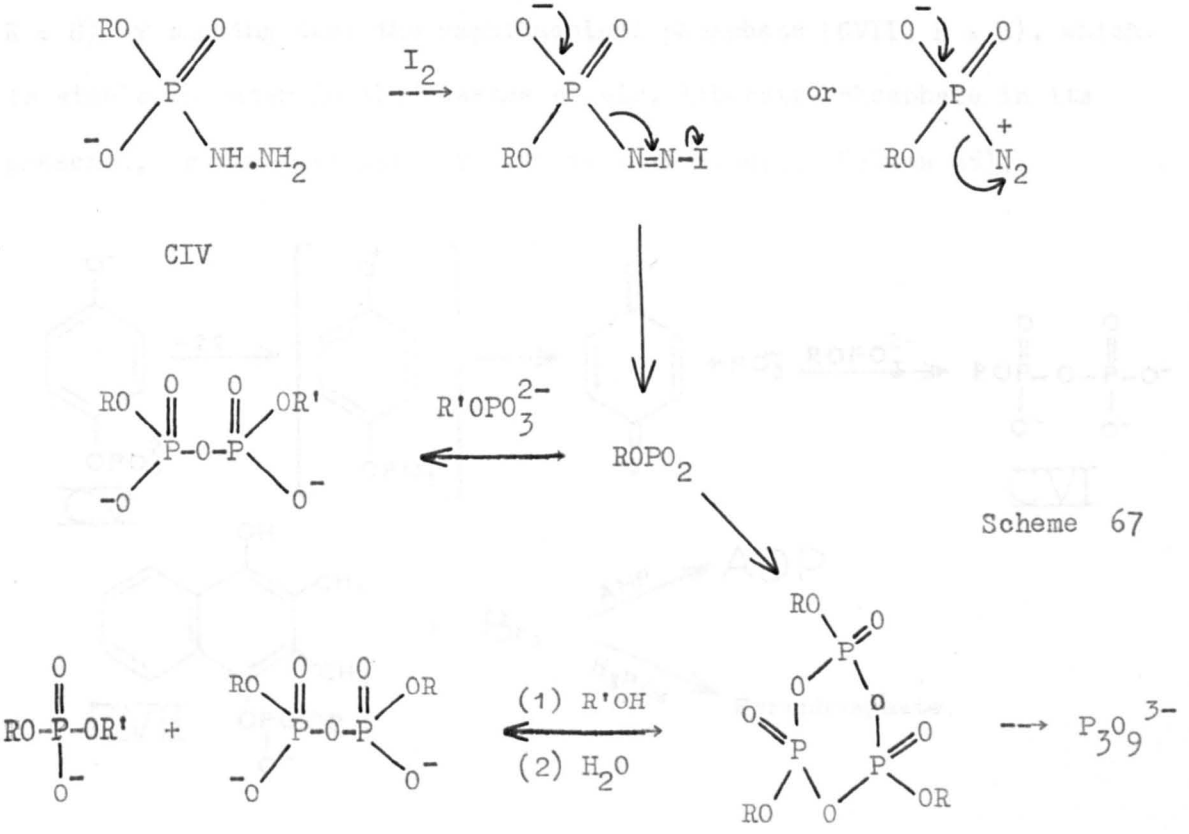
CII

Thus it has been observed that, unlike benzyl hydrogen phosphoramidate, the corresponding guanidate (CIII) does not phosphorylate ADP.[161]



CIII

Brown and co-workers have successfully employed phosphorohydrazidates (CIV) as phosphorylating agents.[162] Thus using oxidation as a means of activation, they suggest the following mechanism (Scheme 67).

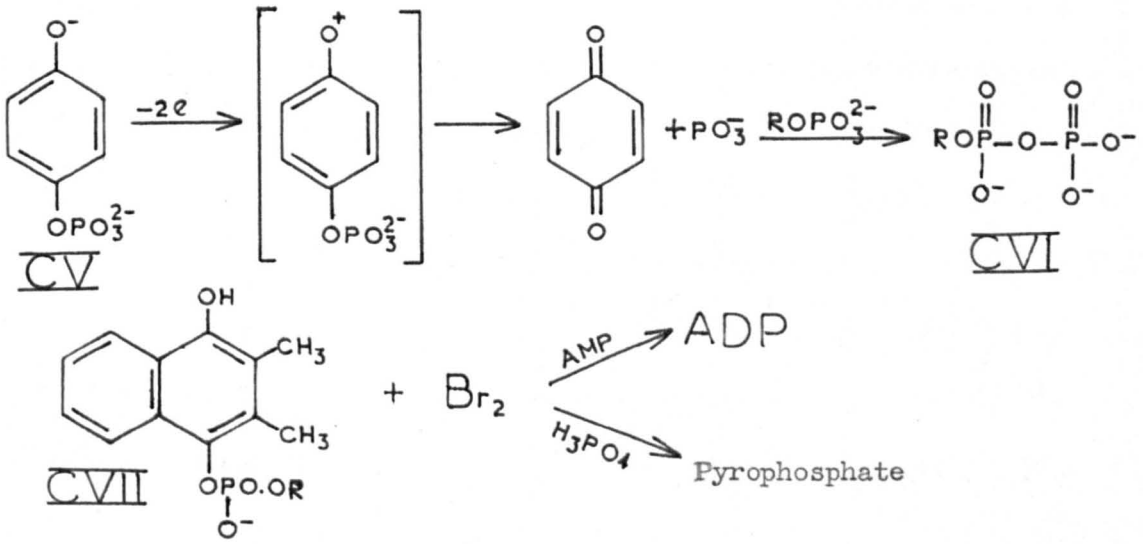


Scheme 67

(e) Oxidative phosphorylation

This is exemplified by the generation of ATP from ADP coupled to the oxidation of carbon-containing substrate molecules. Clark and co-workers have pointed out that fission of the P-X bond which is necessary for $(RO)_2$-POX to act as a phosphorylating agent could be achieved by removal of electrons from X (oxidation).[163] This process is formally equivalent to activation of a phosphoroamidate by protonation of the amino-group.

Biochemical evidence implicates quinones as catalysts in coupling oxidation to phosphorylation in many cases, and it has been suggested that oxidation of a quinol phosphate is an essential step.[163,164,165] The above workers demonstrated the feasability of this process (CV → CVI; R = H) by showing that the naphthaquinol phosphate (CVII; R = H), which is stable in water in the absence of air, liberated phosphate in its presence, or when oxidised by a variety of agents, (Scheme 68).



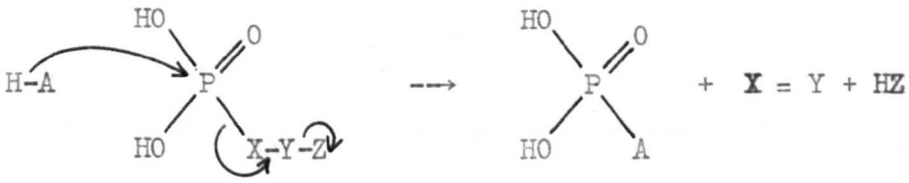
Scheme 68

In every case dimethyl naphthaquinone was formed, and the P-containing products were such as to suggest intermediate metaphosphate formation. Thus in non aqueous solution the naphthaquinol phosphate formed much trimetaphosphate, and its benzyl ester gave rise to P^1,P^2 dibenzyl pyrophoshate. Phosphorylation of an alcohol can also be affected by oxidation of a quinol phosphate by iodine in an alcohol.[166]

Although of general interest, quinolphosphate oxidations are probably of little synthetic value, but oxidation as a means of activation for phosphorylation may have considerable importance in the future.[44]

(f) The P-XYZ system

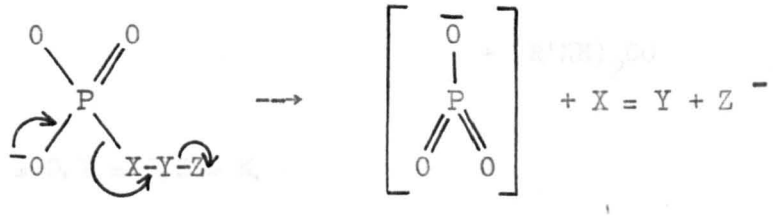
Clark and co-workers have suggested a rationalisation in the design of phosphorylating agents.[46] In a phosphorylating agent in which a P-Z bond is to be broken, fundamentally, whether the leaving group is Z^- or HZ, it must accommodate the electron pair that originally formed the P-Z bond. Any molecule of general formula (CVIII), where X, Y and Z are atoms of any element, but commonly H, C, N, O, S or halogen, is a potential phosphorylating agent if the electrons of the P-X bond can be accommodated on Z (Scheme 68).



CVIII

Scheme 69

Since Z acts as a primary electron-acceptor, it must be strongly electronegative or must become so via attack by an electrophile or an oxidising reagent. The advantages of this system over a simple P-Z reagent is that two extra centres are involved in the transition state of the phosphorylative step, and it is therefore possible to alter the activation energy of the process by varying X and Y for a given Z. The prerequisites for a P-XYZ system to be a phosphorylating agent are that the P-X bond should be weak and Z as electronegative as possible. Therefore, any effect eliminating $p\pi$-$d\pi$ bonding in the P-X bond, or reduction of $p\pi$-$d\pi$ bonding by introducing pure $p\pi$ bonding with an sp^2-hybridised Y atom should weaken the P-X bonding. This mechanism should hold for bimolecular attack on the phosphate (Scheme 69) or unimolecular dissociation into monomeric metaphosphate (Scheme 70).

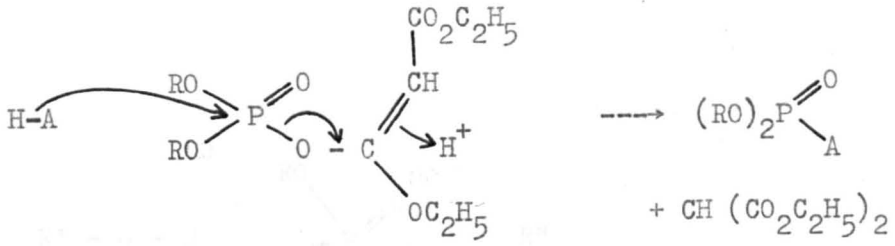


Scheme 70

It should be noted that the entropy change, and probably the entropy of activation, should be positive for both types of reaction.

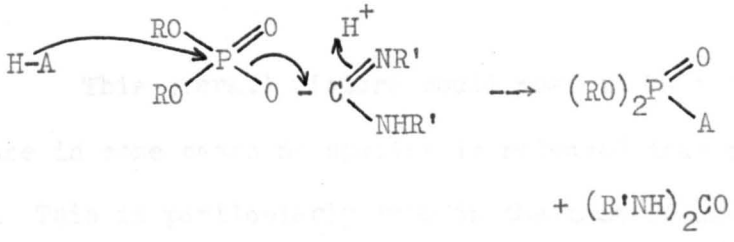
A few examples of P-XYZ systems will be quoted from the phosphorylating agents discussed above. Enol phosphates - under condi

leading to electron withdrawal from the carbon-carbon, for example, are capable of phosphorylating:



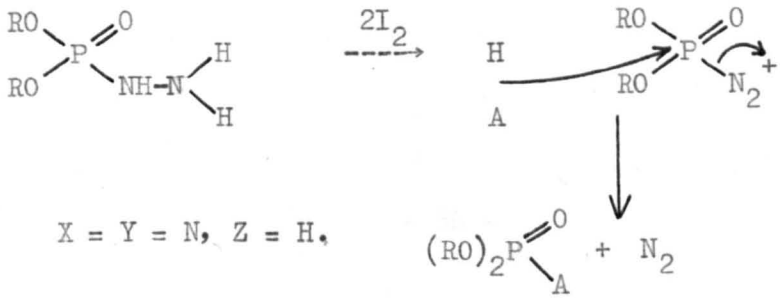
X = O, Y = Z = C.

Imidoyl phosphates:



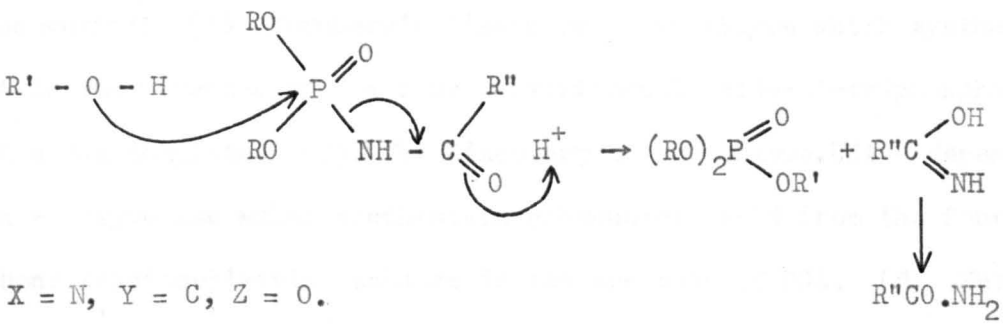
X = O, Y = C, Z = N.

Phosphorohydrazidates:



X = Y = N, Z = H.

N-acylphosphoramidates in contrast to phosphoramidic esters, phosphorylate alcohols in neutral solution. Their enhanced reactivity is due to the decrease in $p\pi$-$d\pi$ bonding between the nitrogen and phosphorus atoms because of the conjugation involving the carbonyl group and a p-orbital on the nitrogen atom.



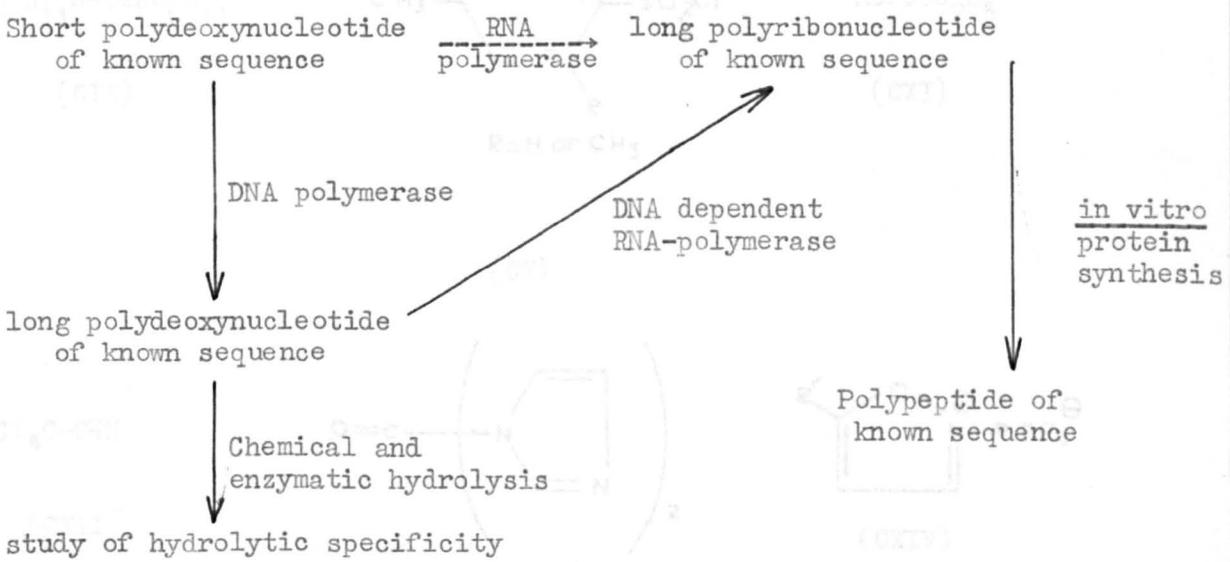
X = N, Y = C, Z = O.

This overall picture would seem to be a little over-generalised, since in some cases no species is released that can be represented by HZ. This is particularly true in the case of phosphorohydrazidates, where, in fact, a molecule of nitrogen is released.

(C) Polymerisation Methods

The last five years have seen a considerable advance in the techniques for the synthesis of polynucleotides. The object of these syntheses has been the preparation of oligonucleotides of known base sequence or random polymerisation in an attempt to prepare polynucleotides containing bases in known ratios. The impetus for the synthesis of oligonucleotides has arisen from three recent biochemical discoveries: (1) Kornberg's discovery of an enzyme which synthesises DNA in the presence of the four deoxyribonucleoside-5'-triphosphates and a DNA template. (2) The discovery of the enzyme, DNA - dependent RNA - polymerase which synthesises ribonucleic acid from the four ribonucleoside-5'-triphosphates in the presence of DNA. (3) The development of an amino acid polymerisation system which brings about in vitro the formation of polypeptide material in response to certain varieties of RNA. The three systems require primers for operation, which also act as code messages controlling the nature of the products. Unfortunately, however, it has not yet proved possible to synthesise templates approaching the length of the natural templates. Relatively short oligonucleotide templates will, however, prime the first two systems, and the products have been found to be polynucleotides of much longer length, those from system (2) proving successful in the priming of system (3). Thus, knowing the base sequences of the primer oligonucleotides has proved to be invaluable for determining the specificity of these systems. The oligo- and polynucleotides of known base sequence

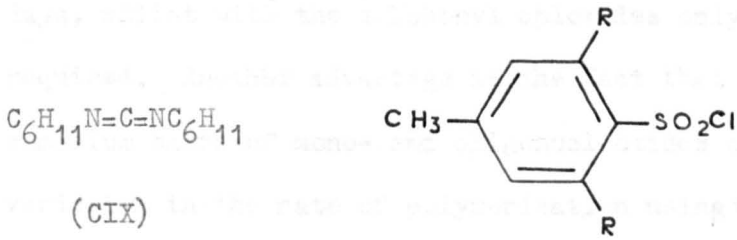
have also been used to study the specificity of enzymatic and chemical hydrolysis of nucleic acids. This is summarised in Scheme 71.



Scheme 71

1. Condensing reagents

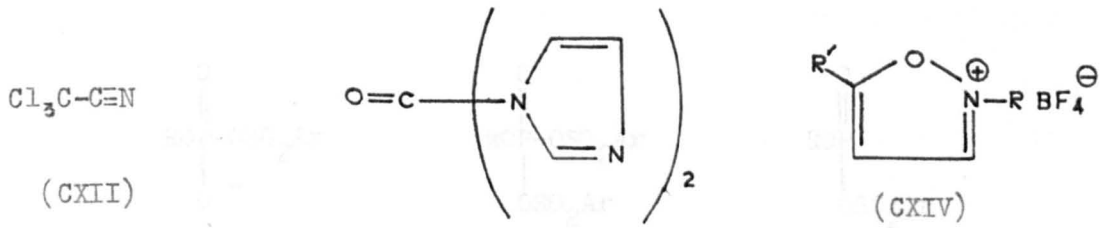
Two reagents have found successful and widespread use for the formation of 3'→5' phosphodiester bonds in oligo- and polynucleotides, namely dicyclohexycarbodiimide (DCC) (CIX) and the aromatic sulphonyl chlorides (CX).[167] Some other reagents that have been used are ethoxyacetylene (CXI), ethyl metaphosphate, trichloroacetonitrile (CXII), carbonylbis(imidazole) (CXIII) and substituted isoxazolium salts (CXIV).



(CIX)

(CX)

(CXI)



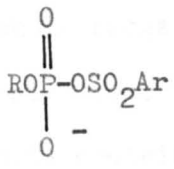
(CXII)

(CXIII)

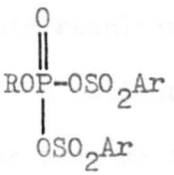
(CXIV)

Khorana has shown that ethoxyacetylene is unsuccessful for the polymer synthesis, the principal product being pyrophosphate (1). This was confirmed during the course of the present work. Polymer synthesis using ethylmetaphosphate produced a complex mixture of products, and there is also danger of glycosidic bond fission and removal of sensitive protecting groups with this reagent. The most promising reagents at the moment thus appear to be (CIX) and (CX), the latter emerging as the most powerful and reactive reagents, which could be a great asset for the stepwise synthesis of long polymers, where rapidity in polymerisation is

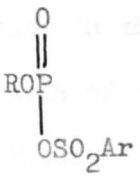
necessary. Thus a typical condensation with DCC takes four or five days, whilst with the sulphonyl chlorides only six or seven hours are required. Another advantage is the fact that the more soluble trialkyl ammonium salts of mono- and oligonucleotides can be utilised. This variation in the rate of polymerisation using the two reagents leads to the conclusion that the mechanisms must be different, and Khorana has suggested that the species (CXV - CXX) might be formed in the presence of an excess of the sulphonyl chloride.[167]



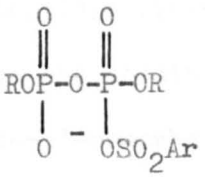
CXV



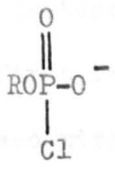
CXVI



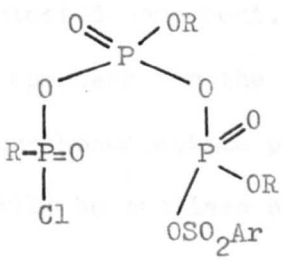
CXVII



CXVIII



CXIX



CXX

Whilst all these would be expected to be reactive phosphorylating entities, lack of information at the moment prevents a definitive mechanism to be deduced. The mechanism of DCC reactions is discussed in a later section.

Two approaches to the synthesis of polydeoxy and polydeoxyribonucleotides (including lower oligomers) have been followed. In the first, nucleotides are added one after another in a strictly stepwise fashion, with isolation of the product at each step, and in the second random polymerisation in which a mixture of mono-, di- or trinucleotides are used, a whole range of products resulting which vary in their degree of polymerisation. It should be obvious that methods of separation of the products contribute to the success of both methods.

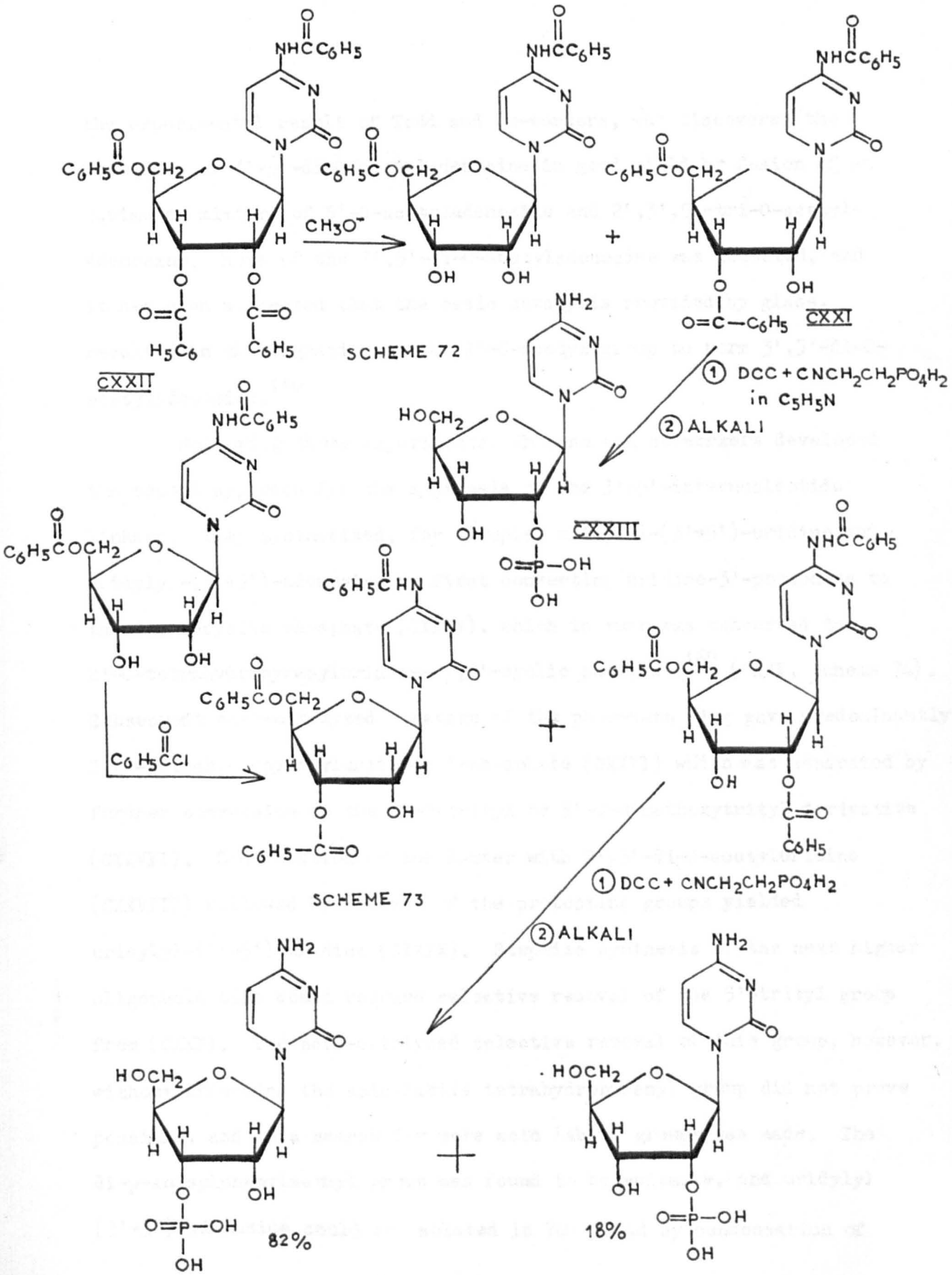
2. Stepwise synthesis

(a) Polyribonucleotides

Two routes to the synthesis of the naturally occurring internucleotidic linkage from two protected nucleoside or nucleotide components are possible.[168] The first is that in which a protected nucleoside-5'-phosphate is condensed with the 3'-hydroxyl group of a second suitably protected component; in the second route, a protected nucleoside-3'-phosphate is condensed with the 5'-hydroxyl group of a second suitably protected component. The former has been found to be the most practical approach for the synthesis of ribonucleotide polymers, the latter for deoxyribonucleotide polymer synthesis. The reasons for these conclusions will be outlined below. One prerequisite for these

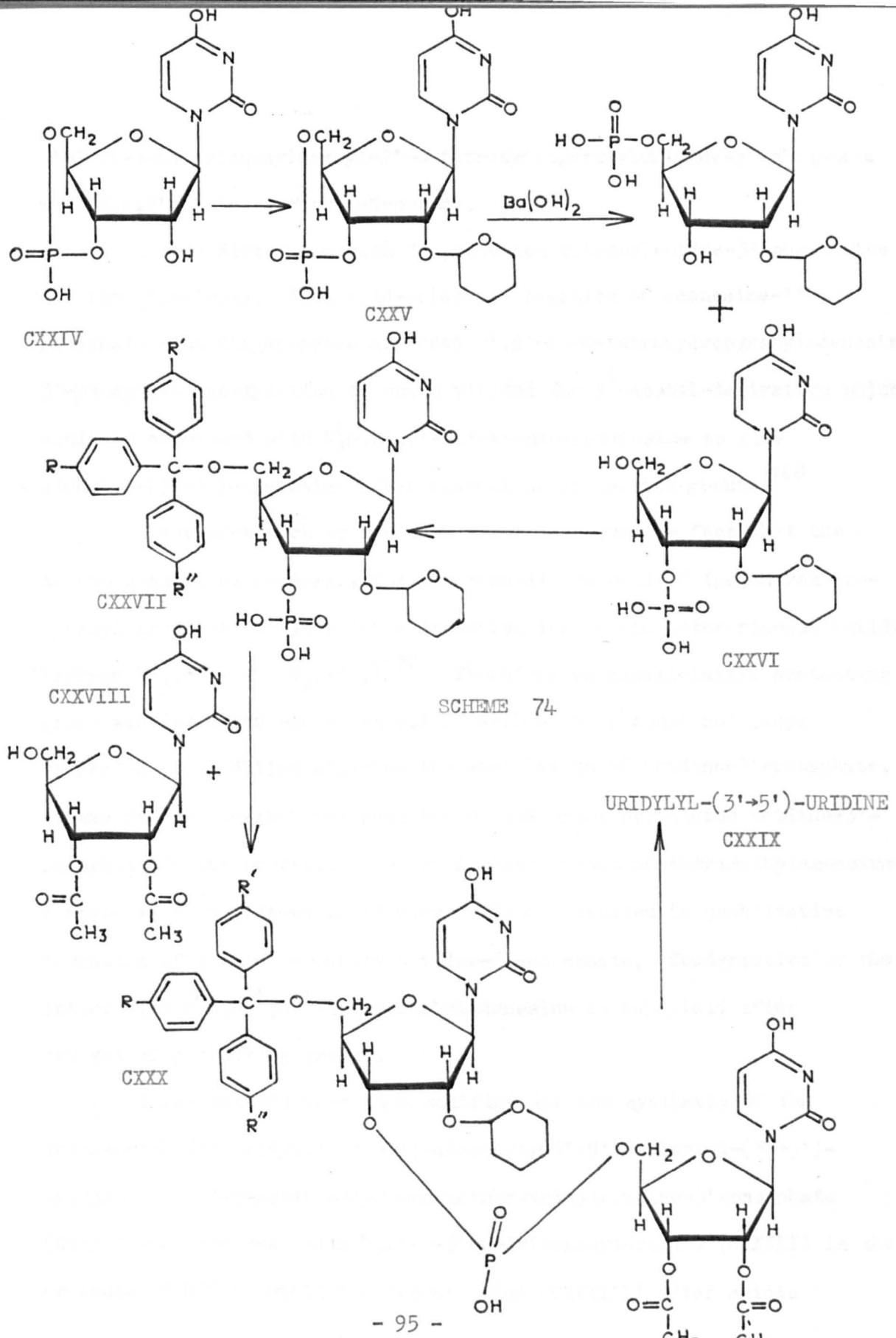
studies has been the development of suitable protecting groups, especially for the ribonucleotide field, where protection of the 2'-hydroxyl group has imposed further problems.

The second approach to polyribonucleotide synthesis was found to be unsuitable because there is a tendency for an acyl group to migrate from the 2'-hydroxyl group to the 3'-hydroxyl group.[168] When $N^6,O^{3'},O^{5'}$-O-tribenzoylcytidine (CXXI), prepared by controlled debenzylation of N^6, $O^{2'}$, $O^{3'}$, $O^{5'}$-tetrabenzoylcytidine (CXXII - Scheme 72), was phosphorylated using a mixture of 2-cyanoethyl phosphate and DCC in pyridine solution with subsequent removal of protecting groups under mildly alkaline conditions, the sole product was cytidine-2'-phosphate (CXXIII). When, however, the isomeric phosphate, $N^6,O^{2'},O^{3'}$-O-tribenzoylcytidine was phosphorylated by the same procedure, the resulting nucleotidic material proved to be a mixture of cytidine-3'-phosphate (82%) and cytidine-2'-phosphate (18%) (Scheme 73). These results show that there is a tendency for an acyl group to migrate from the 2'-hydroxyl group to the 3'-hydroxyl group in ribonucleosides. This migration is apparently acid or base catalysed. Thus during the partial debenzoylation of (CXXII), the only tribenzoyl cytidine obtained was the $N^6,O^{3'},O^{5'}$-tribenzoyl isomer (CXXI), whereas the rates of debenzoylation of the groups on the 2'- and 3'-hydroxyl groups would not be expected to be greatly dissimilar. It is believed that under the basic conditions used, rapid migration of the 2'-O-benzoyl group to the 3'-hydroxyl group occurred. This interpretation is also supported b


CH3O⁻
CXXII
CXXI
SCHEME 72
① DCC + CNCH2CH2PO4H2
in C5H5N
② ALKALI
CXXIII
C6H5COCl
SCHEME 73
① DCC + CNCH2CH2PO4H2
② ALKALI
82%
18%

the experimental result of Todd and co-workers, who discovered the formation of 3'-5'-di-O-acetyladenosine in good yield by fusion of an equimolar mixture of 5'-O-acetyladenosine and 2',3',5'-tri-O-acetyl-adenosine. None of the 2',5'-di-O-acetyladenosine was detected, and it has been suggested that the basic catalysis provided by glass, resulted in the migration of the 2'-O-acetyl group to form 3',5'-di-O-acetyladenosine.[110]

Following these experiments, Khorana and co-workers developed the second approach for the synthesis of the 3'-5'-internucleotide linkage. They synthesised, for example, uridylyl-(3'→5')-uridine and uridylyl-(3'→5')-adenosine by first converting uridine-3'-phosphate to the 3',5'-cyclic phosphate (CXXIV), which in turn was converted to 2'-O-tetrahydropyranyluridine-3',5'-cyclic phosphate[169] (CXXV, Scheme 74). Subsequent base-catalysed cleavage of the phosphate ring gave predominantly 2'-O-tetrahydropyranyluridine-3'-phosphate (CXXVI) which was separated by further conversion to the 5'-O-trityl or 5'-O-dimethoxytrityl derivative (CXXVII). Condensation of the latter with 2',3'-di-O-acetyluridine (CXXVIII) followed by removal of the protecting groups yielded uridylyl-(3'→5')-uridine (CXXIX). Stepwise synthesis of the next higher oligonucleotide would require selective removal of the 5'-trityl group from (CXXX). The acid-catalysed selective removal of this group, however, without affecting the acid-labile tetrahydropyranyl group did not prove possible, and so a search for more acid labile groups was made. The di-p-anisylphenylmethyl group was found to be suitable, and uridylyl (3'→5')-adenosine could be isolated in 70% yield by condensation of


Ba(OH)2
CXXIV
CXXV
CXXVI
CXXVII
CXXVIII
CXXX
URIDYLYL-(3'→5')-URIDINE
CXXIX

SCHEME 74

5'-O-di-p-anisylphenylmethyl-2'-O-tetrahydropyranyluridine-3'-phosphate with N,N,2',3'-tetrabenzoyladenosine.

A more direct approach to protected ribonucleoside-3'-phosphates was then developed. Thus acid-catalysed reaction of adenosine-3'-phosphate with dihydropyran afforded 2',5'-di-O-tetrahydropyranyladenosine-3'-phosphate, acetylation of which yielded the N^6-acetyl-derivative which could be condensed with N^1,N,2',3'-tetrabenzoyladenosine to give adenylyl-(3'→5')-adenosine after removal of protecting groups.[168]

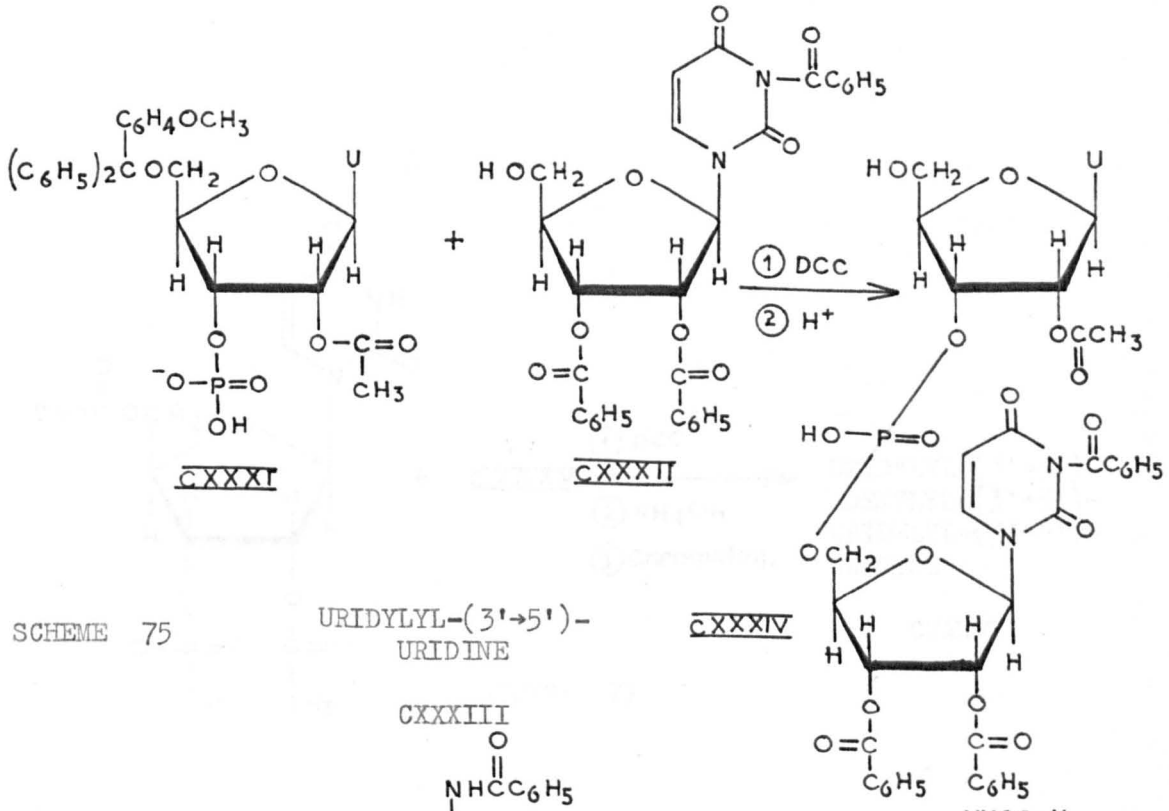
Subsequent work by the same group revealed the fact that the acidic conditions necessary for the complete removal of the tetrahydropyranyl group caused detectable isomerisation of the inter-ribonucleotidic linkage ($C_{3'}\rightarrow C_{5'}$ → $C_{2'}\rightarrow C_{5'}$).[170] Therefore an alkali-labile protecting group was developed since removal of such a group would not cause isomerisation. Whilst studying the acetylation of uridine-3'-phosphate, it was discovered that the reaction of anhydrous pyridinium uridine-3'-phosphate in the presence of an equivalent amount of tetramethylammonium acetate with or without added inert solvent resulted in quantitative formation of 2',5'-di-O-acetyluridine-3'-phosphate. Condensation of the latter with N^1,N,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine in 84% yield after removal of protecting groups.

These methods were then modified for the synthesis of the tetranucleotide uridylyl-(3'→5')-adenylyl-(3'→5')-uridylyl-(3'→5')-uridine.[171] 2'-O-Acetyl-3'-O-monomethoxytrityluridine-3'-phosphate (CXXXI) was condensed with N,2'-O-3'-O-tribenzoyluridine (CXXXII) in the presence of DCC to yield the dinucleotide (CXXXIII) after acidic

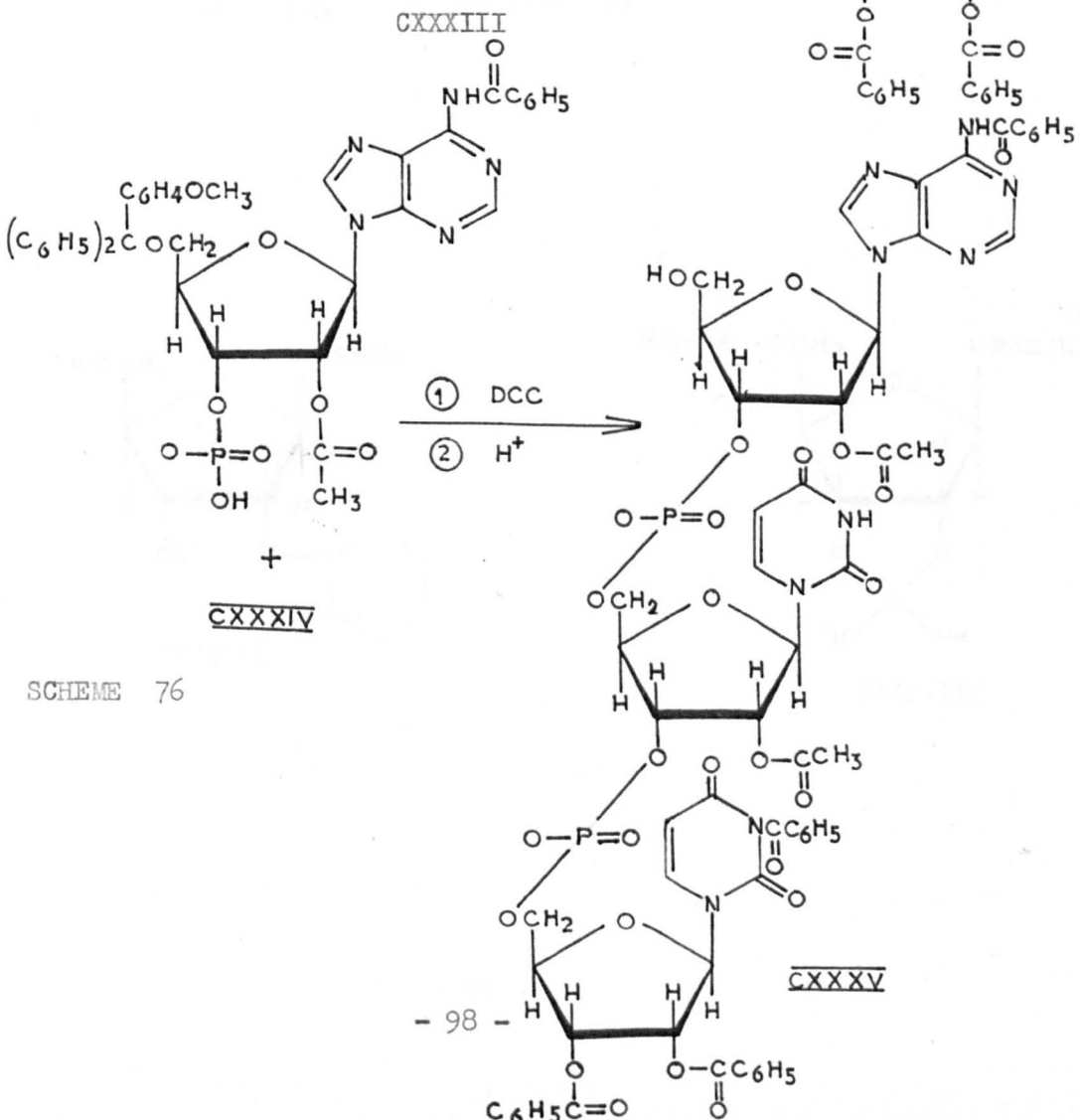
treatment (Scheme 75). This was subsequently condensed with four molar equivalents of N,2'-O,5'-O-triacetyladenosine-3'-phosphate to yield the trinucleotide after treatment with ammonia. To synthesise a tetranucleotide, (CXXXIV) was condensed with 5'-O-monomethoxy trityl-2'-O-acetyl N-benzoyladenosine-3'-phosphate to give the trinucleotide (CXXXV) on mild acid treatment (Scheme 76). (CXXXV) was finally condensed with a sevenfold excess of 2',5'-di-acetyluridine-3'-phosphate, uridylyl-(3'→5')-adenylyl-(3'→5')-uridylyl-(3'→5')-uridine being isolated from the products after treatment with ammonia (CXXXVI; Scheme 77).

Recent work by Smrt and Šorm has resulted in the synthesis of penta-uridylyl-(5'→3')-uridine by similar methods, although this synthesis was achieved by condensation of two trinucleotides.[72] Synthesis of oligonucleotides bearing a 3'-phosphomonoester group is possible by using a nucleotide carrying a 3'-(2-cyanoethyl)phosphate group, or a nucleotide carrying a 2',3'-cyclic phosphate.[173,174] These few examples should be enough to indicate the potentialities of this approach.

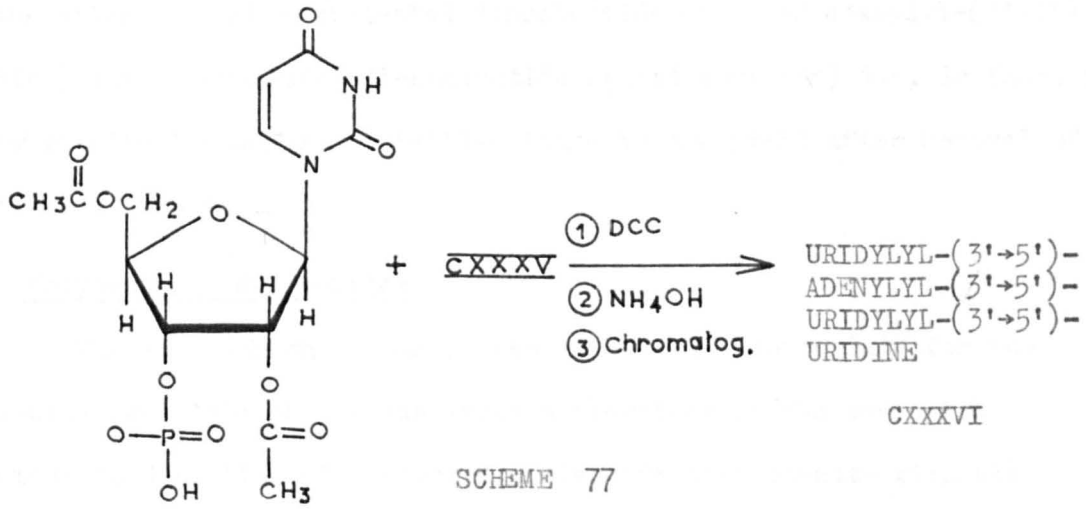
Some success, has, however, been achieved in the synthesis of oligoribonucleotides by the second approach described above.[175] Thus when 2'-O-tetrahydro-5'-O-pivalyladenosine (CXXXVII; R = t-BuCO, R' = H) was condensed with 2',3'-di-O-acetyluridine-5'-phosphate in the presence of DCC, adenylyl-(3'→5')-uridine was isolated from the products in 60% yield after removal of the protecting groups. For the production of higher oligonucleotides, protection of the 2',3'-cis-diol system of the 5'-nucleotide component is necessary, and for this purpose, the acid-labile



SCHEME 75 URIDYLYL-(3'→5')-URIDINE

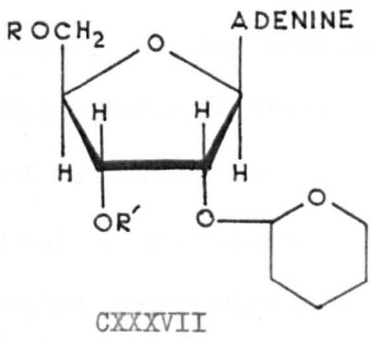


SCHEME 76

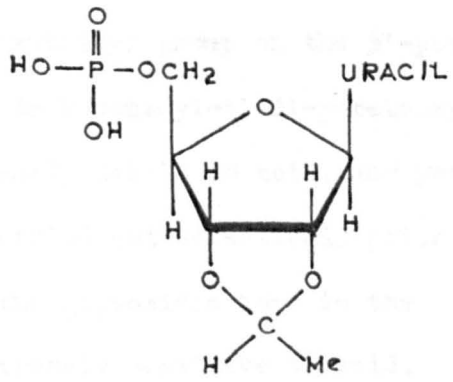


CXXXVI

SCHEME 77



CXXXVII



CXXXVIII

methoxymethylidene group was found to be suitable (CXXXVIII). Condensation of such a protected dinucleotide (e.g. of adenylyl-(3'-5')-uridine) with a protected 3'-nucleotide (first approach) did, in fact, produce uridylyl-(3'→5')-adenylyl-(3'→5')-uridine in 30% yield after removal of protecting groups.

(b) Polydeoxyribonucleotides

The approach which has proved to be most appropriate for the step-wise synthesis of oligodeoxyribonucleotides is the one which involves condensation of protected nucleoside-5'-phosphate with the 3'-hydroxyl group of a second suitably protected component. The considerations leading to this conclusion are:

(1) It is impractical to prepare suitably protected deoxyribonucleoside-3'-phosphates with alkali-labile groups on the ring-amino groups and acid-labile groups, such as the di-p-methoxytrityl group on the 5'-position. For example, the di-p-methoxytrityl group in N-benzoyl-5'-di-p-methoxytritylde oxyadenosine-3'-phosphate is extremely labile to acid, and yet complete removal of the group cannot be carried out selectively prior to removal of the N-benzoyl group, since the glycosidic bond in the N-benzoyldeoxyadenosine moiety is also extremely sensitive to acid.

(2) The ready availability of deoxyribonucleoside-5'-phosphates is of practical significance.

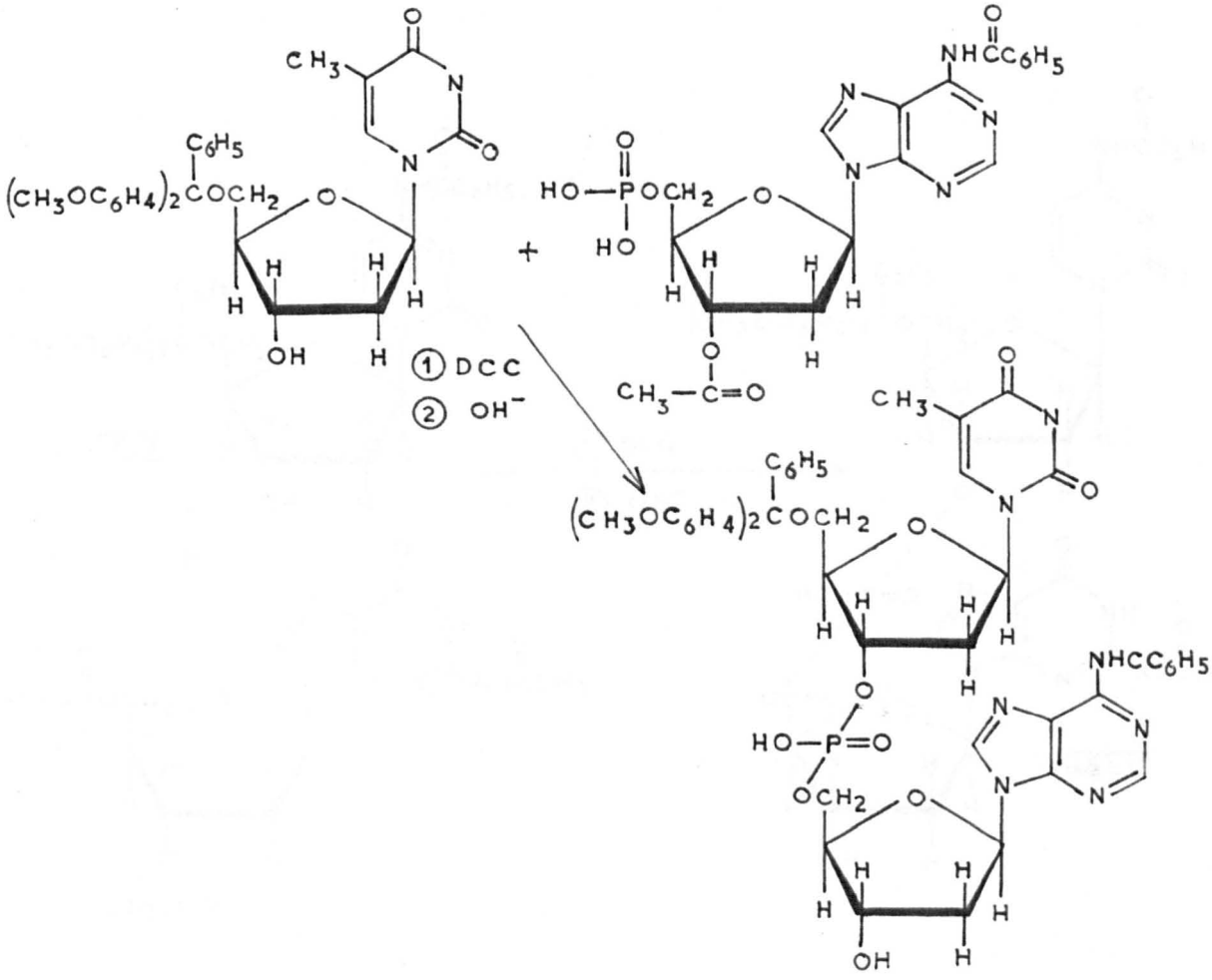
(3) It was found that the yields of internucleotide bonds using stoichiometric amounts of the two components were low when bulky substituents were present in the nucleotide component, as, for example, 5'-O-tritylthymidine-3'-phosphate.[176]

(4) A component bearing the free 5'-hydroxyl group and a preformed diester bond is not completely stable in the presence of DCC. For example, thymidylyl-(3'→5')-3'-O-acetyl thymidine reacted in dry pyridine with DCC to form by-products. Furthermore, triester formation was detected by interaction of an activated diester with the primary hydroxyl group of 3'-O-acetylthymidine.[177] Side reactions of this nature have not been encountered with components which bear the secondary 3'-hydroxyl group.

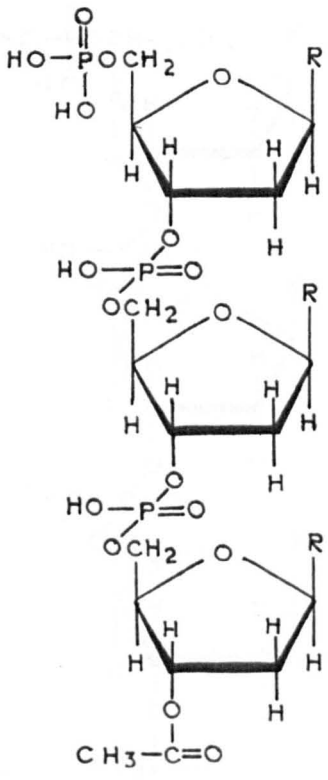
Khorana and co-workers have developed such a range of protecting groups that it is possible to manipulate all of the deoxynucleosides and -nucleotides to synthesise a dinucleotide containing any combination. Selective exposure of the 3'-hydroxyl terminus has also been accomplished by these workers, permitting chain elongation by reaction with a 5'-phosphate end group of mono- or oligonucleotides.[178] The acetyl group has been used extensively to protect the 3'-hydroxyl group, and the di-p-methoxytrityl group for the 5'-hydroxyl group. The acidic lability of the latter is such that it may be removed under conditions which are safe throughout, except for the glycosidic bond in N-benzoyldeoxyadenosine. Only in the latter case was it found necessary to remove first the N-benzoyl group with ammonia, and then the dimethoxytrityl group by treatment with acid. It was also found that the carbodiimide method was uniformly effective, the reaction rates for the various combinations of deoxynucleotide and deoxynucleoside being very similar throughout. Evidence was also found which indicated that while the size of the

protecting groups in the nucleoside has little effect, the size of the groups in the nucleotide has, in contrast, a marked effect on the rate and final yield of the phosphodiester bond.[176] Thus when N,5'-O-bis-dimethoxytrityldeoxyguanosine was condensed with 3'-O-acetylthymidine-5'-phosphate, the rate and yield were "normal", but in the condensation of N,3'-O-bis-dimethoxytrityldeoxyguanosine-5'-phosphate with 5'-O-dimethoxytritylthymidine, the yield was only 12%.

Using these techniques, a pentanucleotide was synthesised.[180] (Scheme 78). N-Benzoyl-3'-O-acetyldeoxyadenosine-5'-phosphate was condensed with 5'-O-di-p-methoxytritylthymidine in the presence of DCC and the dinucleotide (CXXXIX) was isolated after alkaline treatment. This was then condensed with the trinucleotide (CXL) to yield, after removal of protecting groups, the pentanucleotide thymidylyl-(3'→5')-deoxyadenylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidine. Similarly, the condensation of N-benzoyl-5'-O-dimethoxytrityldeoxycytidine (CXLI) with N,3'-O-diacetyldeoxyguanosine-5'-phosphate in the presence of DCC followed by mild alkaline treatment, gave eventually a quantitative yield of (CXLII). (CXLIII), (prepared by acetylation of one of the oligonucleotides obtained from the polymerisation of N-anisyldeoxycytidine-5'-phosphate[181]) was then condensed with the protected dinucleoside phosphate (CXLII) in a mixture of pyridine and dimethylformamide using either DCC or 2,5-dimethylbenzenesulphonyl chloride to yield, after removal of protecting groups, deoxycytidylyl-(3'→5')-deoxyguanylyl-(3'→5')-deoxycytidylyl-(3'→5')-deoxycytidylyl-(3'→5')-deoxycytidine (CXLIV)[182] (Scheme 79).



CXXXIX



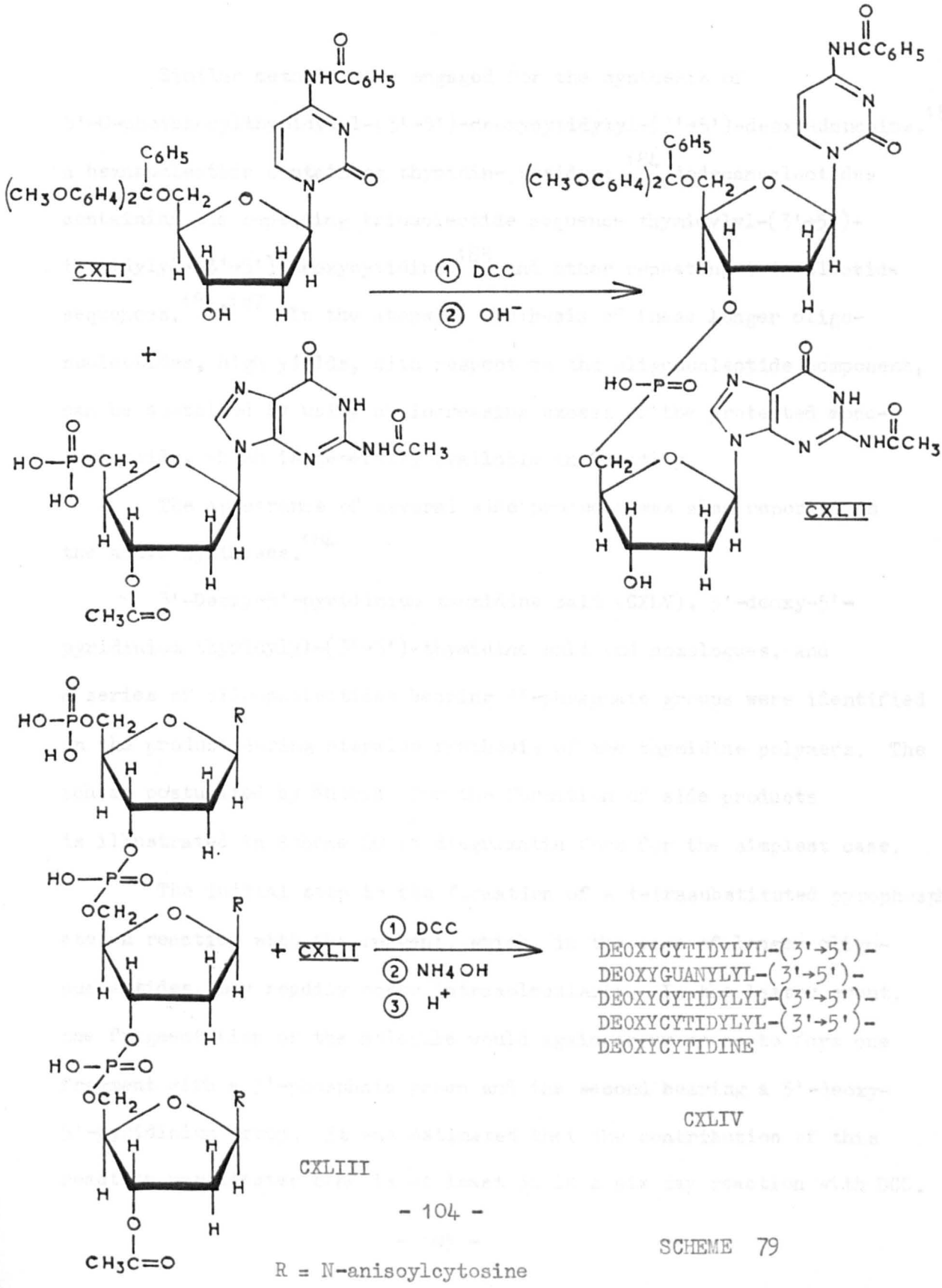
CXL

+ CXXXIX

① DCC
② NH_4OH
③ Chromatog.

THYMIDYLYL-(3'→5')-
DEOXYADENYLYL-(3'→5')-
THYMIDYLYL-(3'→5')-
THYMIDYLYL-(3'→5')-
THYMIDINE

SCHEME 78


CXLI
① DCC
② OH⁻
CXLII
CXLIII
+ CXLII
① DCC
② NH4 OH
③ H+
DEOXYCYTIDYLYL-(3'→5')-
DEOXYGUANYLYL-(3'→5')-
DEOXYCYTIDYLYL-(3'→5')-
DEOXYCYTIDYLYL-(3'→5')-
DEOXYCYTIDINE
CXLIV
R = N-anisoylcytosine

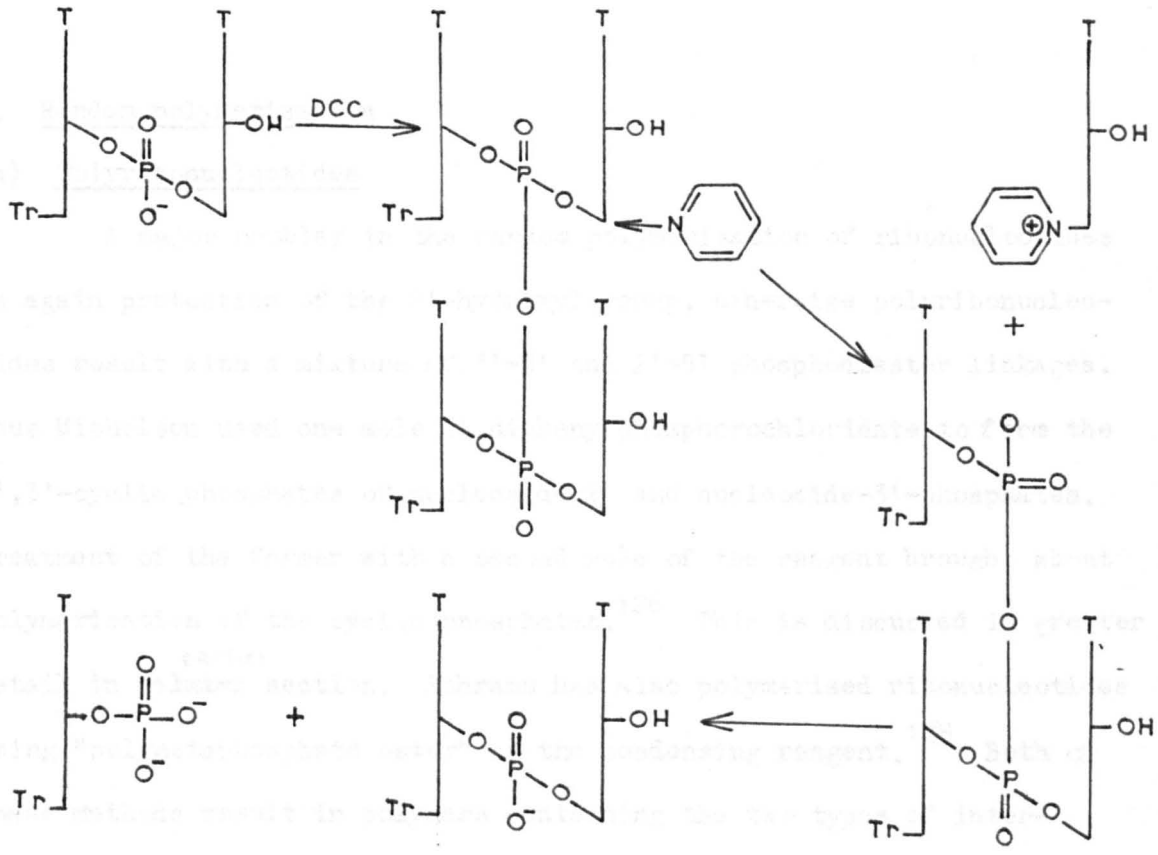
SCHEME 79

Similar methods were engaged for the synthesis of 5'-O-phosphorylthymidylyl-(3'→5')-deoxycytidylyl-(3'→5')-deoxyadenosine,[183] a hexanucleotide containing thymidine residues,[184] dodecanucleotides containing the repeating trinucleotide sequence thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxycytidine,[185] and other repeating trinucleotide sequences.[186,187] In the stepwise synthesis of these longer oligonucleotides, high yields, with respect to the oligonucleotide component, can be sustained by using an increasing excess of the protected mononucleotide, which is generally available in quantity.

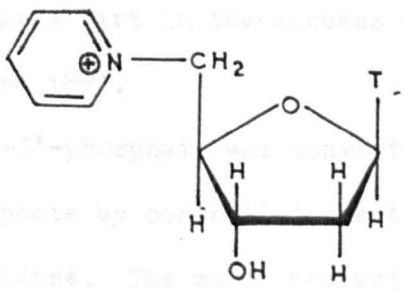
The appearance of several side products was also reported in the above syntheses.[184]

5'-Deoxy-5'-pyridinium thymidine salt (CXLV), 5'-deoxy-5'-pyridinium thymidylyl-(3'→5')-thymidine salt and homologues, and a series of oligonucleotides bearing 3'-phosphate groups were identified in the product during stepwise synthesis of the thymidine polymers. The scheme postulated by Khorana for the formation of side products is illustrated in Scheme 80 in diagramatic form for the simplest case.

The initial step is the formation of a tetrasubstituted pyrophosphate on reaction with the reagent, which, in the case of longer oligonucleotides, may readily occur intramolecularly. In the latter event, the fragmentation of the molecule would again occur so as to form one fragment with a 3'-phosphate group and the second bearing a 5'-deoxy-5'-pyridinium group. It was estimated that the contribution of this reaction per diester bond is at least 5% in a six day reaction with DCC.



SCHEME 80



CXLV

3. Random polymerisation

(a) Polyribonucleotides

A major problem in the random polymerisation of ribonucleotides is again protection of the 2'-hydroxyl group, otherwise polyribonucleotides result with a mixture of 3'→5' and 2'→5' phosphodiester linkages. Thus Michelson used one mole of diphenylphosphorochloridate to form the 2',3'-cyclic phosphates of nucleoside-2' and nucleoside-3'-phosphates. Treatment of the former with a second mole of the reagent brought about polymerisation of the cyclic phosphates.[126] This is discussed in greater detail in an ~~later~~ earlier section. Schramm has also polymerised ribonucleotides using "polymetaphosphate ester" as the condensing reagent.[188] Both of these methods result in polymers containing the two types of internucleotidic linkages mentioned above.

Khorana and co-workers have succeeded in preparing suitably protected ribonucleoside-3'-phosphates which when allowed to react with DCC in pyridine, polymerise to give a mixture of polyribonucleotides of varying chain length, but with 3'→5' phosphodiester bonds exclusively.[170] Recent developments in the techniques for the separation of the products have played a large part in the success of these methods (see for example reference 189).

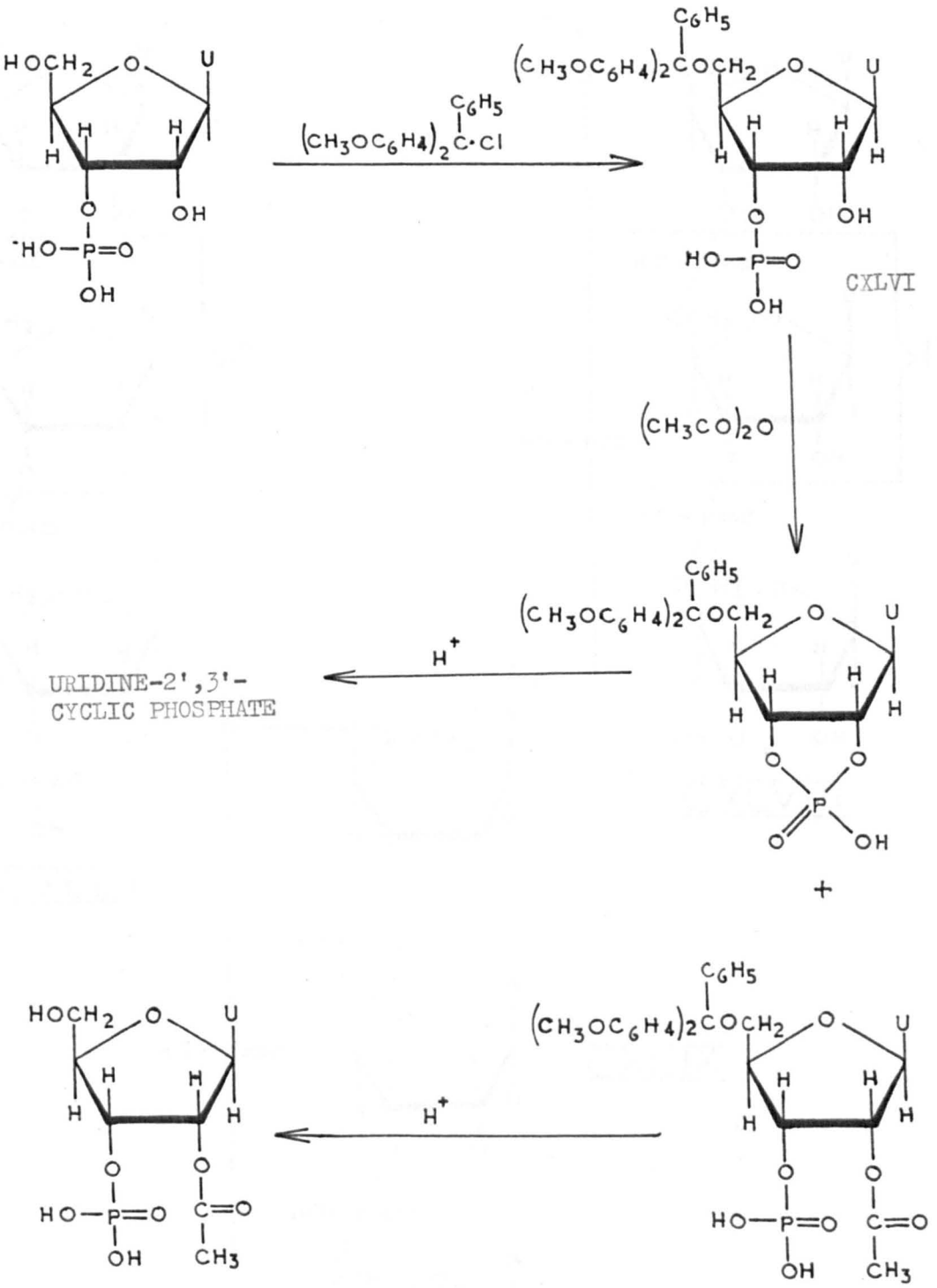
Uridine-3'-phosphate was converted to 5'-O-di-p-methoxytrityl-uridine-3'-phosphate by controlled reaction with di-p-methoxytrityl chloride in pyridine. The major product was the desired one (CXLVI), and this was isolated from the reaction products by chromatography on a DEAE-cellulose column. Reaction of (CXLVI) with acetic anhydride in aqueous

pyridine gave a mixture of the 2',3'-cyclic phosphate and the 2'-O-acetylphosphate. After very brief treatment with acid to remove the di-p-methoxytrytyl group, the productswere separated by partition chromatography on a cellulose column (Scheme 81).

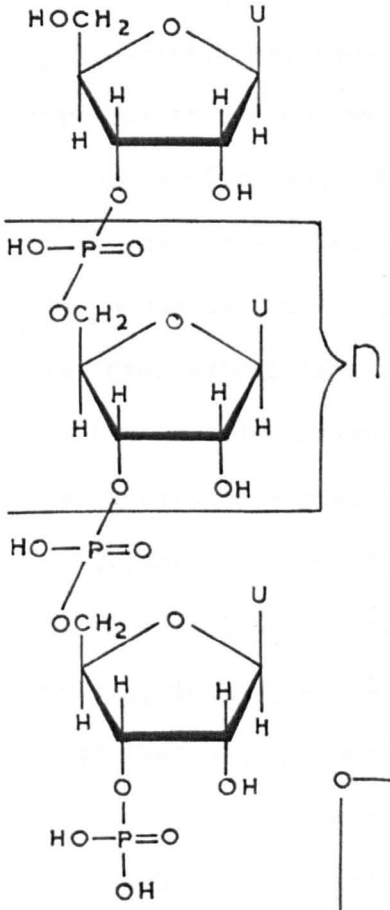
The 2'-O-acetyluridine-3'-phosphate was then polymerised by reaction with DCC in dry pyridine. After removal of the acetyl groups by treatment with ammonia, the products of the reaction were separated by chromatography on a DEAE-cellulose column and proved to be of two types. The first type was a series of homologous linear oligoribonucleotides (general structure (CXLVII), and the second a series of cyclic oligonucleotides (general structure(CXLVIII)). In many cases, the linear oligonucleotides were characterised by identification of the products after enzymatic hydrolysis, the linear trinucleotide being the longest oligonucleotide to be characterised in this way, as it proved difficult to separate the higher homologues. Uridine-3',5'-cyclic phosphate was present in the products to the extent of $7\frac{1}{2}\%$.

(b) Polydeoxyribonucleosides

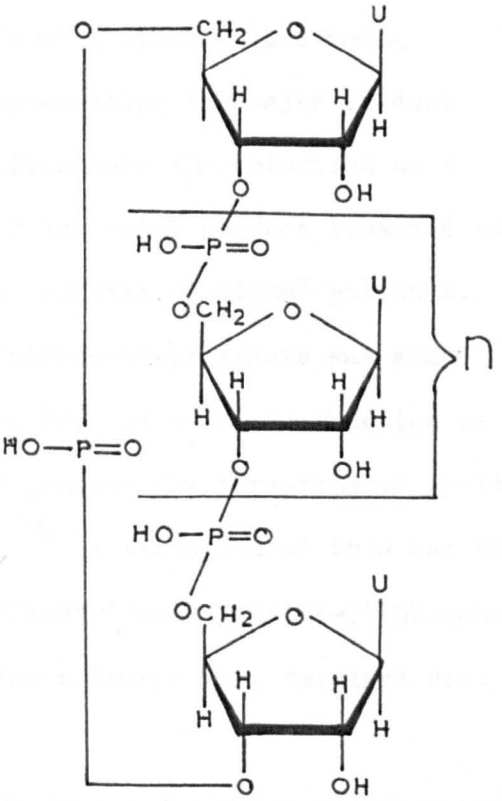
DCC has proved to be the most successful reagent for the polymerisation of monodeoxyribonucleotides, although polymerisation can be effected with p-toluenesulphonyl chloride. It was found, however, that the degree of polymerisation was less with the latter reagent.[190] Thymidine-5'-phosphate was the first mononucleotide to be polymerised using DCC in anhydrous pyridine by Khorana and co-workers.[125] Linear and cyclic oligonucleotides, also identified later in the ribonucleotide


HOCH2
U
H
OH
HO—P=O
OH
(CH3OC6H4)2C·Cl
C6H5
(CH3OC6H4)2COCH2
CXLVI
(CH3CO)2O
H+
URIDINE-2',3'-
CYCLIC PHOSPHATE
+
C=O
CH3

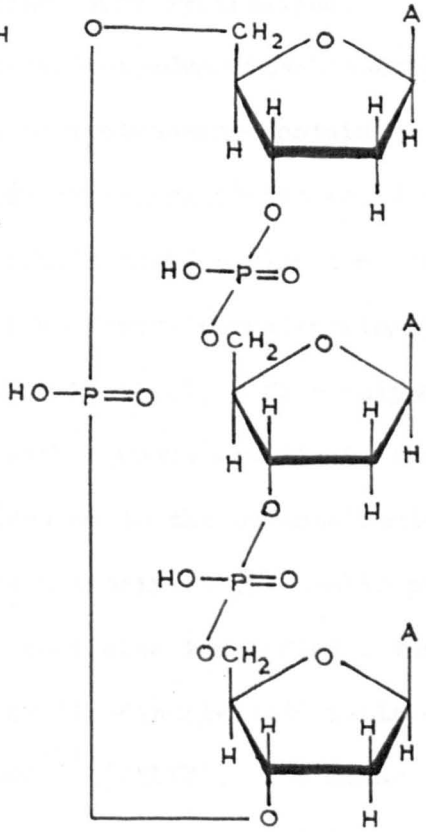
SCHEME 81



CXLVII



CXLVIII



CXLIX

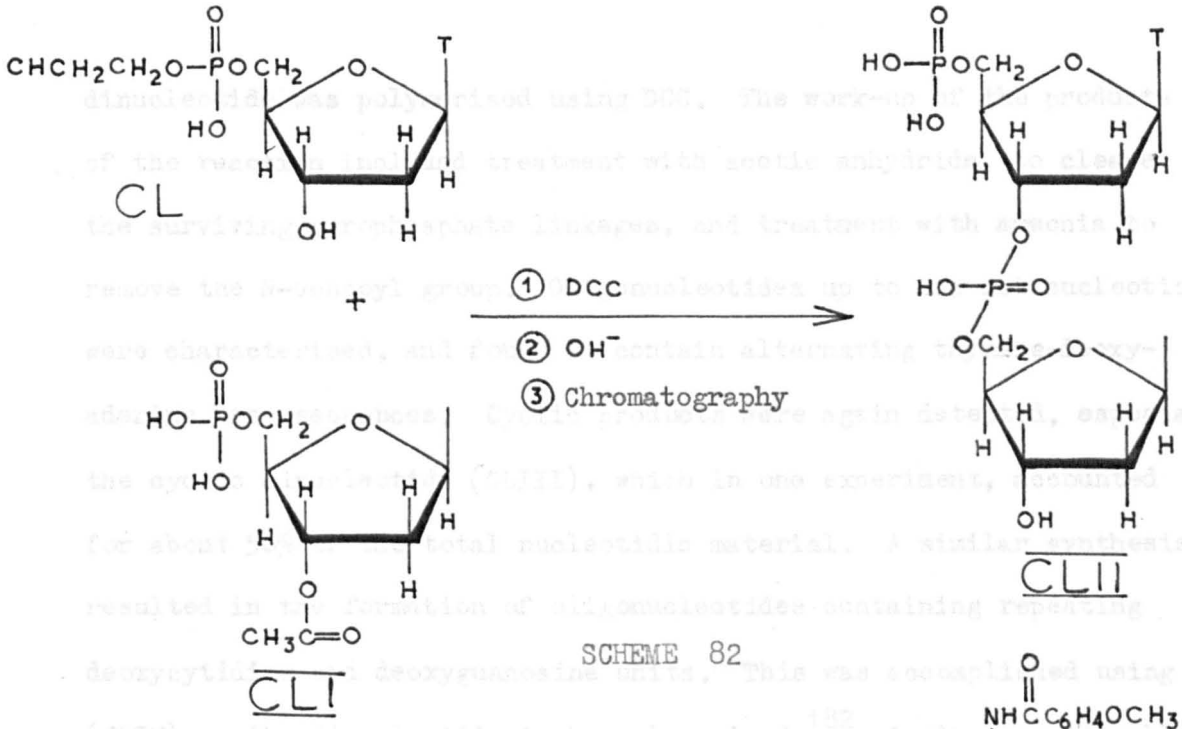
polymerisations, were the products, the cyclic dinucleotide being present to the extent of 15 - 20% and representing the major product of the cyclic type. Linear oligonucleotides were characterised up to the pentanucleotide, but a further 15% of the total product remained on the separating column, and was thought to consist of higher polymers. In later experiments some 3'-O-acetylthymidine-5'-phosphate was added to the reaction mixture. These molecules were expected to function as terminating residues in the polymers and prevent the formation of cyclic products, which was the observed effect.[179] A variation of this was the polymerisation of a mixture of N^6,3'-O-diacetyldeoxycytidine-5'-phosphate and thymidine-5'-phosphate, when thymidine polymers with terminal deoxycytidine residues were synthesised.

N-benzoyldeoxyadenosine-5'-phosphate was the mononucleotide used to synthesise deoxyadenosine-containing polynucleotides, since it was considered that phosphoramidates would result from the reaction of an activated phosphate residue with the purine-ring-amine group. (The preparation of N-benzoyldeoxyadenosine-5'-phosphate is described in the Experimental Section). After polymerisation, the polymeric products were treated with aqueous ammonia to remove the benzoyl group. Linear oligonucleotides up to the octanucleotide were characterised. The cyclic products, deoxyadenosine-3',5'-cyclic phosphate and the cyclic phosphate trinucleotide were also identified , the latter apparently taking the place of the cyclic dinucleotide isolated from the thymidine-5'-phosphate polymerisations[191] (CXLIX). A similar polymerisation of N^6-benzoyldeoxycytidine-5'-phosphate, after removal of the protecting group from

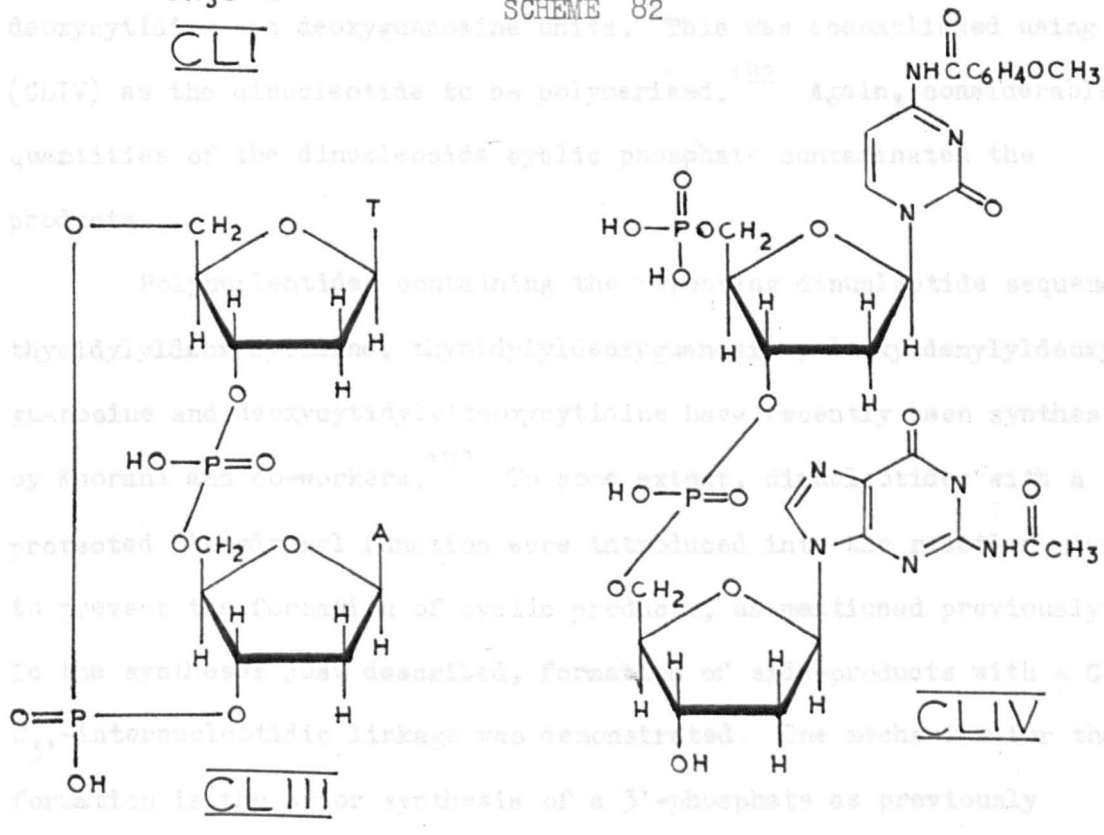
the products, yielded deoxycytidine-containing oligonucleotides (linear and cyclic), whilst polymerisation of N-acetyldeoxyguanosine-5'-phosphate yielded after alkaline hydrolysis to remove the N-acetyl group, deoxyguanosine-containing oligonucleotides.[181,192] A useful outcome of the latter experiment was the discovery that these polymerisations could be effected equally successfully in dry dimethylformamide with a little pyridinium ion-exchange resin present, thus solving many solubility problems.

The above technique was employed to synthesise polydeoxyribonucleotides in the present work. A mixture of thymidine-5'-phosphate and deoxyadenosine-5'-phosphate was polymerised by DCC. In this case, however, the base sequences of the resulting polymers were analysed by the nearest neighbour base frequency method described in a later section.

A natural extension of the random polymerisation experiments with monodeoxyribonucleotides, was the polymerisation of short oligomers of known base sequence. The products expected were polynucleotides containing the repeating base sequence of the reacting oligonucleotides. Polynucleotides containing thymidine and deoxyadenosine in alternating sequence were the first of this type to be synthesised.[180] 2-Cyanoethyl-5'-thymidylate (CL) was prepared from thymidine-5'-phosphate by reaction with DCC in the presence of an excess of 2-cyanoethanol and pyridine. The condensation of (CL) with N-benzoyl-3'-O-acetyldeoxyadenosine-5'-phosphate (CLI) followed by careful alkaline treatment to remove the 3'-O-acetyl and the 2-cyanoethyl group gave the dinucleotide (CLII; Scheme 82) as the major product. After chromatographic purification, this



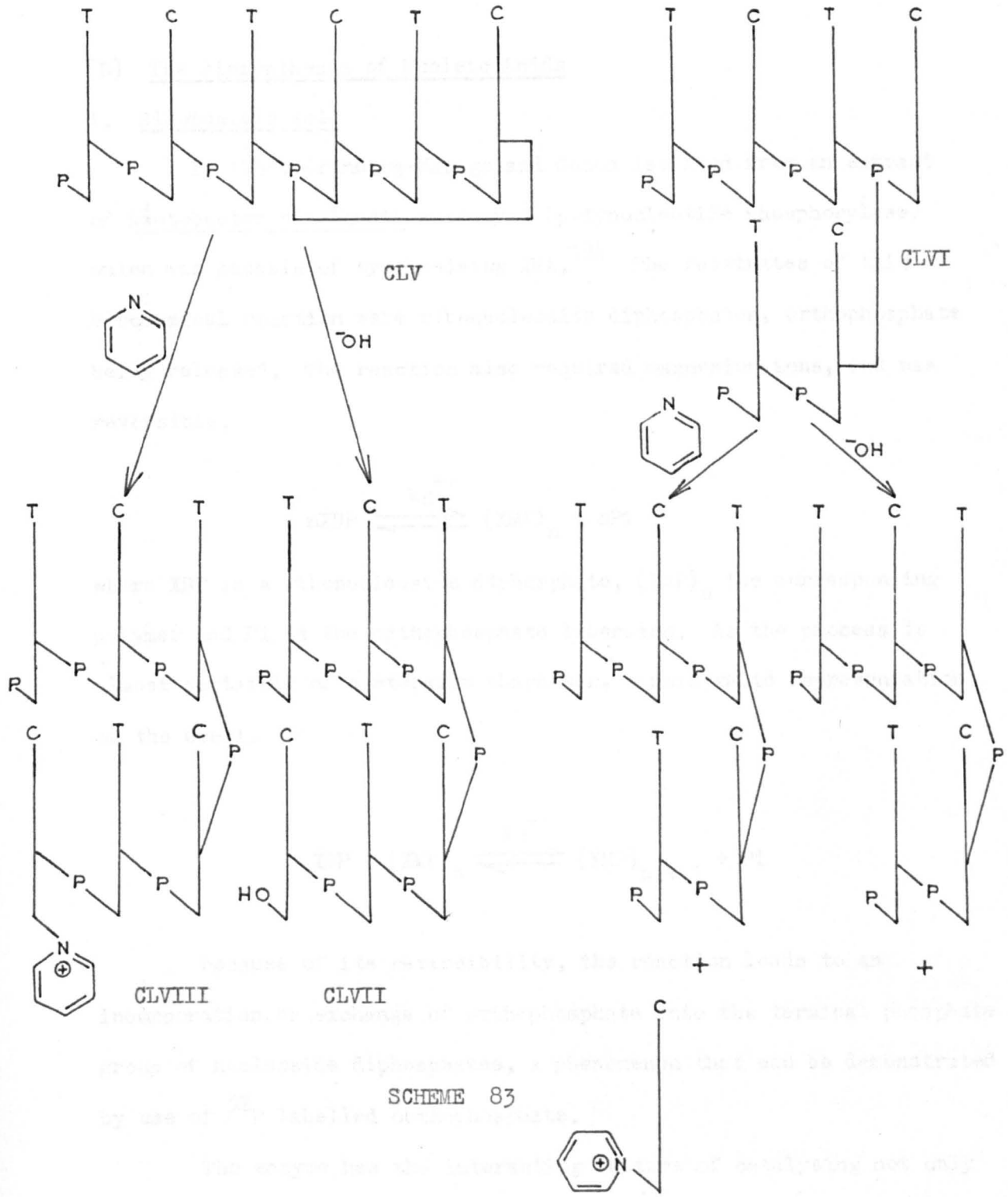
SCHEME 82



dinucleotide was polymerised using DCC. The work-up of the products of the reaction included treatment with acetic anhydride, to cleave the surviving pyrophosphate linkages, and treatment with ammonia to remove the N-benzoyl group. Oligonucleotides up to the octanucleotide were characterised, and found to contain alternating thymine-deoxyadenine base sequences. Cyclic products were again detected, especially the cyclic dinucleotide (CLIII), which in one experiment, accounted for about 50% of the total nucleotidic material. A similar synthesis resulted in the formation of oligonucleotides containing repeating deoxycytidine and deoxyguanosine units. This was accomplished using (CLIV) as the dinucleotide to be polymerised.[182] Again, considerable quantities of the dinucleoside cyclic phosphate contaminated the products.

Polynucleotides containing the repeating dinucleotide sequences thymidylyldeoxycytidine, thymidylyldeoxyguanosine, deoxyadenylyldeoxyguanosine and deoxycytidylyldeoxycytidine have recently been synthesised by Khorana and co-workers.[193] To some extent, dinucleotides with a protected 3'-hydroxyl function were introduced into the reaction mixture to prevent the formation of cyclic products, as mentioned previously. In the syntheses just described, formation of side-products with a $C_{3'}$-$C_{3'}$-internucleotidic linkage was demonstrated. One mechanism for their formation is the prior synthesis of a 3'-phosphate as previously described, and the subsequent activation of this group which would result in the phosphorylation of the 3'-hydroxyl group of an oligonucleotide. Khorana, however, posulates a second scheme which might be

expected to make a greater contribution[193] (Scheme 83). This involves the initial formation of a neutral ester by phosphorylation of the terminal 3'-hydroxyl group with an activated internucleotidic linkage. This esterification could occur intramolecularly (for example (CLV)) or intermolecularly (for example (CLVI)). The triester would then be broken down by pyridine during the reaction, or during the alkaline treatment in the work-up. In each case, attack would be expected to occur at the $C_{5'}$-carbon atom rather than at the disubstituted $C_{3'}$-carbon atom, thereby generating a $C_{3'}$-$C_{3'}$ internucleotidic linkage. The intramolecular reaction would result in the formation of by-products having an even-number of nucleotides, one phosphate end group, and either a 5'-hydroxyl group (CLVII) or a pyridinium residue (CLVIII) at the opposite end of the chain. Such products were not, however, detected. The intermolecular reaction (CLVI) would lead to the formation of compounds with two 5'-phosphomonoester end groups in addition to the 3'-3'-linkage, and would also yield a second fragment bearing 5'-hydroxyl or pyridinium group.



SCHEME 83

(D) The Biosynthesis of Nucleic Acids

1. Ribonucleic acid

In 1955, Grunberg-Manago and Ochoa isolated from an extract of Azotobacter vinelandii an enzyme (polynucleotide phosphorylase) which was capable of synthesising RNA.[194] The substrates of this biochemical reaction were ribonucleoside diphosphates, orthophosphate being released. The reaction also required magnesium ions, and was reversible.

$$nXDP \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} (XMP)_n + nPi$$

where XDP is a ribonucleoside diphosphate, $(XMP)_n$ the corresponding polymer and Pi is the orthophosphate liberated. As the process is almost certainly of a stepwise character, a more valid representation of the events is:

$$XDP + (XMP)_n \overset{Mg^{++}}{\rightleftharpoons} (XMP)_{n+1} + Pi$$

Because of its reversibility, the reaction leads to an incorporation or exchange of orthophosphate into the terminal phosphate group of nucleoside diphosphates, a phenomenon that can be demonstrated by use of ^{32}P labelled orthophosphate.

The enzyme has the interesting feature of catalysing not only the synthesis of "RNA" from mixtures of the four naturally occurring ribonucleoside diphosphates, but also that of non-naturally occurring

polyribonucleotides containing one, two or three different kinds of nucleotides in their chains. The nature of the product depends on the type of nucleoside diphosphate substrates used for the synthesis.[195] Table 3 lists the main types of polyribonucleotides which have been prepared with polynucleotide phosphorylase.

Table 3. Synthetic Polyribonucleotides

SUBSTRATE	POLYMER
ADP	Poly A
GDP	Poly G
UDP	Poly U
CDP	Poly C
IDP	Poly I
Ribothymidine diphosphate	Polyribothymidylic acid
ADP + UDP	Poly AU
GDP + CDP	Poly GC
ADP + GDP + CDP + UDP	Poly AGUC

The various interactions of these synthetic polyribonucleotides have been studied in detail by Doty and co-workers.[196] Chemical and enzymatic degradation of the polymeric products indicated that they consisted of linear chains in which the nucleoside units were interlinked by 3'-5'-phosphodiester bridges.[197] The molecular weight varied from

3×10^4 to 2×10^6, and the synthetic RNA was shown to exhibit biological activity, in that it stimulated the formation of streptolysin S by haemolytic streptococci. Thus the product possessed most of the properties of natural RNA, but no particular base sequence.

It was discovered that large variations in the relative proportions of the different nucleoside diphosphates had a marked influence on the nucleotide composition of the resulting polymer, although when synthetic RNA was prepared from equimolar mixtures of adenosine, guanosine, uridine and cytidine diphosphate, the nucleotide composition of the product was very similar to that of natural Azotobacter RNA. This is illustrated in Table 4.

Table 4. Base ratios of natural and synthetic RNA

Base	Azobacter RNA	Poly AGUC (sample 1)	Poly ACUC (sample 2)
Adenine	1.00	1.00	1.00
Guanine	1.30	1.16	1.25
Uracil	0.73	0.66	0.69
Cytosine	0.90	0.72	0.73

It is thought that polynucleotide phosphorylase tends to be contaminated with quantities of oligoribonucleotides. Even when most highly purified, the enzyme contains a firmly bound oligonucleotide, efforts to remove it having met with no success. This oligonucleotide

consists of about twelve nucleotide residues of adenylic, guanylic, uridylic and cytidylic in roughly the same molar ratios as in Azotobacter RNA. It is believed that these contaminating oligonucleotides serve as primers, since when the enzyme is used in its most highly purified state, a long time-lag occurs before the reaction eventually starts. This time-lag period is reduced by addition of small amounts of oligo- or polynucleotides which appear to act as primers for the reaction. The length of these primers can be quite short, and even the dinucleotide adenylyl-(5'→3')-adenine-5'-phosphate was shown to possess priming ability.

It was discovered that the priming by oligonucleotides was not specific. Thus oligoadenylic acids could prime the synthesis of polyadenylic and polyuridylic acid as well as that of RNA or any of the other polynucleotides. Priming by polynucleotides, on the other hand, was shown to possess a certain degree of spceificity, some primers actually inhibiting polymer synthesis. The priming specificities of various polynucleotides are summarised in Table 5 below.[197] The reason for these specificities is not, at the moment, known.

Table 5. Specificity of Priming by Polynucleotides

Polymer Synthesised	Effect of polymer					
	Poly A	Poly U	Poly C	Poly I	Poly AU	RNA (natural or synthetic)
Poly A	+	-	+	O	+	+
Poly U	-	+	+	O	+	+
Poly C	-	-	+			-
Poly G	O	O	+			
Poly I	-	O	+	+		
Poly AU					+	
Poly AGUC	O	O	+			+

Key: + denotes priming; - denoted inhibition;

O denotes no effect; blank spaces - no information.

If the primer possessed a free 3'-hydroxyl group, the polymeric product was found to be covalently bound to the primer, a fact demonstrated by enzymatic and chemical hydrolysis of the products. Oligonucleotides with a 3'-phosphomonoester group, however, were generally shown to be inhibitory, but where priming ability was demonstrated, the primers were not bound covalently to the polymers. Thus the primers could be recovered quantitatively from the reaction mixture. The details

of this priming activity are not at all clear.

It is difficult to see how polynucleotide phosphorylase can play a physiological role in the synthesis of specific RNA molecules *in vivo*, because it seems to polymerise ribonucleoside-diphosphates in a random fashion, at least *in vitro*. On the other hand, the enzyme must be important because it is found in large quantities in so many different bacteria and also in other types of cells. There is a direct relationship between the amount of RNA synthesis and the amount of the enzyme present at certain stages of the growth cycle. The function of the enzyme could conceivably be that of the breakdown of RNA, that is, the reverse of the polymerisation reaction. This process is slow *in vitro*, but it is thought that this may be because the RNA molecules possess secondary structure in this state, this producing an inhibitory action on polynucleotide breakdown.

Weiss isolated from rat liver a DNA-dependent RNA polymerase, and Hurwitz isolated a similar system from *E. coli*.[198,199] The requirements for this system were the simultaneous presence of the four ribonucleoside triphosphates, a divalent ion (Mg^{++} or Mn^{++}), and double stranded DNA. The omission of one of the triphosphates reduced the incorporation of the others almost to zero, and, as might be expected, the reaction was very sensitive to deoxyribonuclease and ribonuclease.

$$\begin{matrix} \text{nATP} \\ \text{nGTP} \\ \text{nUTP} \\ \text{nCTP} \end{matrix} \xrightarrow[\text{DNA}]{+\ \text{Enzyme}} \left[\begin{matrix} \text{AMP} \\ \text{GTP} \\ \text{UTP} \\ \text{CTP} \end{matrix}\right]_n + 4\ \text{nPPi}$$

Mg^{++}/Mn^{++}

The reversal of this reaction was pyrophosphorolysis of the RNA product, but the reaction was slow and specific for the RNA produced by this enzyme. Thus r-RNA and s-RNA were not attacked, possibly because they possess more secondary structure in solution than does the RNA produced in the enzymatic reaction.

The base composition of the RNA product was found to be determined by that of the primer DNA, which implies that during the *in vitro* reaction, both strands of the DNA are copied (cf. *in vivo*[200]). The DNA primer did not become covalently linked to the polymeric product (cf. polynucleotide phosphorylase), and could be recovered unchanged at the end of the reaction. The reaction was also primed both by single- and by double-stranded ϕx 174 DNA, the base ratios of the resulting RNA being complementary in the first case, and showing Watson-Crick pairing in the second. The base ratio A+T/G+C of double stranded ϕx 174 DNA is 1.31, and the appropriate ratio of the *in vitro* RNA product was also found to be 1.31. This is in contrast with the RNA synthesised *in vivo*, where it has been shown that after T4 'phage infection, the RNA has unequal ratios of A:U and of G:C.[201] In this case, probably only one strand of DNA was being copied.

Nearest neighbour base frequency determinations on the RNA products, indicated that the RNA strands formed *in vitro* were of opposite polarity, as were those of the DNA primer.

Khorana and co-workers have utilised this system to synthesise high-molecular weight RNA with a repeating trinucleotide sequence, which was in turn determined by a short-chain primer DNA with the complementary

base sequence.[202] Thus they used the nonanucleotide d-$(TTC)_3$, which contained the repeating sequence thymidylyl-thymidylyl-deoxycytidylate, as a template for the DNA-dependent RNA-polymerase system to bring about the synthesis of the complementary polyribonucleotide containing the repeating sequence adenylyl-adenylyl-guanylate, the substrates for the reaction being adenosine- and guanosine-5'-triphosphates. The length of the polymeric products was estimated to be in the range of 150 - 200 nuclectide units, and nearest-neighbour base frequency analysis confirmed the repeating base sequences in the product. It has also been demonstrated that this enzyme catalyses the synthesis of polyadenylate from single-stranded DNA in the presence of ATP above.[203] The conclusion that short runs of thymidylate residues were responsible for this synthesis was supported by the work of Khorana. The latter observed the stimulation of polyadenylate synthesis in the presence of ATP as above using d-$(TTC)_3$ as the template. It is believed that the non-pairing base (cytidine) present between the pairs of thymidylate residues can loop out so as to permit the synthesis of continuous polyadenylate. Thus it does not appear to be essential to have continuous runs of one base in the DNA template.

There are now many systems known which are capable of synthesising polyribonucleotides, from ribonucleotide di- or triphosphates. The function of these systems <u>in vivo</u>, however, is not at all clear at the moment.

2. Deoxyribonucleic acid

When the four common deoxyribonucleoside-5'-triphosphates were incubated with an extract of thymus, bone marrow or E. coli, no net synthesis of DNA was detectable. The dominant process in this system would be expected to be the destruction of DNA by the extracts. When, however, the substrates were labelled on the β-phosphorus atom by ^{32}P, it was found by Kornberg and co-workers, that out of 10^6 cpm added, some 50 cpm were incorporated into acid insoluble products.[204] This represents a very small amount of DNA synthesis, but probably most of the product was broken down by the nucleases that were undoubtedly present in these crude extracts. Purification of this DNA synthesising enzyme, revealed the following system:

$$\left.\begin{matrix} \text{nTTP} \\ \text{ndGTP} \\ \text{ndATP} \\ \text{ndCTP} \end{matrix}\right\} + \text{DNA} \longrightarrow \text{DNA} - \left[\begin{matrix} \text{TMP} \\ \text{dGMP} \\ \text{dAMP} \\ \text{dCMP} \end{matrix}\right]_n + 4n\text{PP}$$

The requirements for this system were - the presence of all four common deoxyribonucleoside-5'-triphosphates, a highly polymerised DNA primer and Mg^{++} ions. If one of the nucleotide substrates was omitted, the extent of the reaction was diminished by a factor of about 10^2. Even so, a significant, though small, incorporation could be detected, and in this so-called limited reaction, it was shown that the nucleotides were in fact added onto the ends of the primer, in a manner governed by base-pairing rules. Thus, when each of the four nucleoside

-5'-triphosphates with radioactive labelling on the β-phosphorus atom, was incubated with the enzyme system and a DNA primer, it was discovered that the radioactivity sedimented at the same rate as the DNA, and that the two could not be separated by heat denaturation. Enzymatic degradation by Micrococcal DNAse and calf-spleen phosphodiesterase, to produce the 3'-mononucleotides, showed that whichever nucleoside-5'-^{32}P-triphosphate was originally incorporated, the radioactivity was shared by all of the four 3'-deoxyribonucleotides of DNA. Kornberg[205] has suggested that the DNA-primer could possess ends of slightly unequal length, and that the shorter end could then add on a few nucleotides complementary to the nucleotides of the longer sister chain. There is, as yet however, no experimental proof to support this hypothesis.

The products, derived to the extent of 95% or more from the substrates, of these biosyntheses have been characterised by a number of methods. Physical measurements indicated the product to be similar to high molecular weight, double stranded DNA isolated from natural sources. On the basis of measurements of its sedimentation coefficient and reduced viscosity, the molecular weight was estimated to be 6×10^6. It also had a sharp "melting point", and degradation of the product by specific enzymes was as expected for DNA. (The "melting point" is a temperature at which a drastic change occurs in a number of physical properties of a solution of DNA, for example, optical density, viscosity and optical rotation, and is a consequence of the breakdown of the helical structure of the DNA). Heat denatured DNA, existing in the single

stranded conditions, proved to be an excellent primer for the reaction, as was single stranded ϕx 174 DNA. It has been discovered, moreover, that when highly purified preparations of the enzyme were used, DNA in the single-stranded condition was the only suitable primer.

DNA polymerase was found to incorporate analogues of the common deoxynucleotide-5'-triphosphates into a polymer. For example, deoxy-UTP can take the place of TTP, but not of the other three nucleotides. The various substitutions are summarised in Table 6 below.

Table 6

Replacement of Natural Bases by Analogues in the Enzymatic synthesis of DNA[205]

Analogue used in the Form of the Deoxynucleoside Triphosphate	Deoxynucleoside Triphosphate replaced by the Analogue			
	TTP	dATP	dCTP	dGTP
	% of Control Value			
Uracil	54	0	0	0
5-Bromouracil	97(100)	0(0)	0(0)	0(0)
5-Fluorouracil	32(9)	0(0)	0(0)	0(0)
5-Hydroxymethylcytosine	(0)	(0)	(98)	(0)
5-Methylcytosine	0	0	185	0
5-Fluorocytosine	0(0)	0(0)	63(67)	0(0)
N-Methyl-5-fluorocytosine	0	0	0	0
Hypoxanthine	0	0	0	25
Xanthine	0	0	0	0

Values in brackets were measured with T_2 polymerase; the others were measured with E. coli polymerase. Zero values are inserted where there was less than 2% and where this did not differ significantly from the background.

Chemical analysis of the base composition of the enzymatic product disclosed the fact that [A] = [T] and [G] = [C] as was the case in the primer DNA. Moreover, the ratio [A] + [T]/[G] + [C] was equal to the ratio that was characteristic of the primer DNA. These results are summarised in Table 7 below.

Table 7

Nucleotide Composition of Enzymatically Synthesised DNA[205]

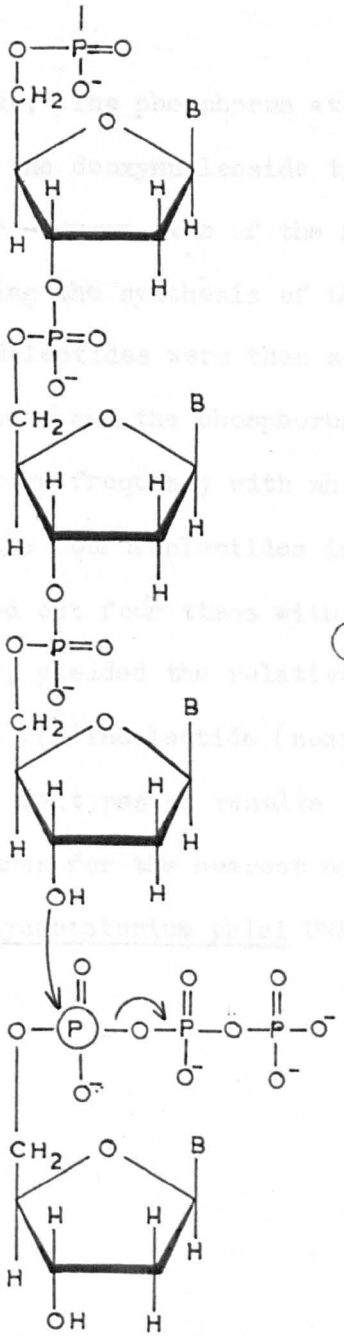
Primer DNA	Ap	Tp	Gp	Cp	Product $\frac{A+G}{T+C}$	Product $\frac{A+T}{G+C}$	Primer $\frac{A+T}{G+C}$
Micrococcus lysodeikticus	0.147	0.145	0.354	0.354	1.00	0.41	0.39
E. coli	0.248	0.254	0.249	0.249	0.99	1.01	0.97
Calf Thymus	0.286	0.283	0.214	0.217	1.00	1.26	1.29
Bacteriophages T_2, T_4 and T_6	0.319	0.318	0.184	0.179	1.01	1.76	1.84
dAT copolymer	0.500	0.500	0.002	0.002	1.00	>250	>40

Thus the nucleotide sequence of the priming DNA is reproduced in the synthetic DNA product. Moreover, the characteristic [A] + [T]/[G] + [C] ratio is observed in the product whether the extent of synthesis is

small, say 10% by weight of the primer added, or whether synthesis is extensive in the range 10 to 20 fold. This phenomenon is thought to indicate that during the limited replication of double-stranded DNA, only a small proportion of the primer molecules are copied, but completely so. This is in contrast with the limited replication of single-stranded ϕx 174, in which about 20% of each primer molecule is copied.

An important demonstration of the fact that the polymer synthesised by the DNA polymerase system was a faithful copy of the primer DNA, was obtained by the method of nearest neighbour base frequency determination, developed by Kornberg and co-workers.[206] This is the nearest approach yet obtained to a complete base sequence determination of a high molecular weight nucleic acid. (The base sequence determination by Holley and co-workers was on a relatively low molecular weight sRNA molecule.) This method involved the enzymatic synthesis of DNA using one nucleoside triphosphate labelled with phosphorus-32 in the α position, the other three substrates being unlabelled. The radioactive phosphorus, attached by a phosphodiester linkage to the 5'-carbon atom of the deoxyribose, then became the bridge between that substrate molecule and the nucleotide at the growing end of the chain with which the substrate reacted (Scheme 84).

At the end of the synthetic reaction, (after some 10^{16} diester bonds had been synthesised) the DNA was isolated and digested enzymatically with Micrococcal DNase and calf spleen phosphodiesterase. This treatment cleaved the phosphodiester linkages specifically to yield quantitatively

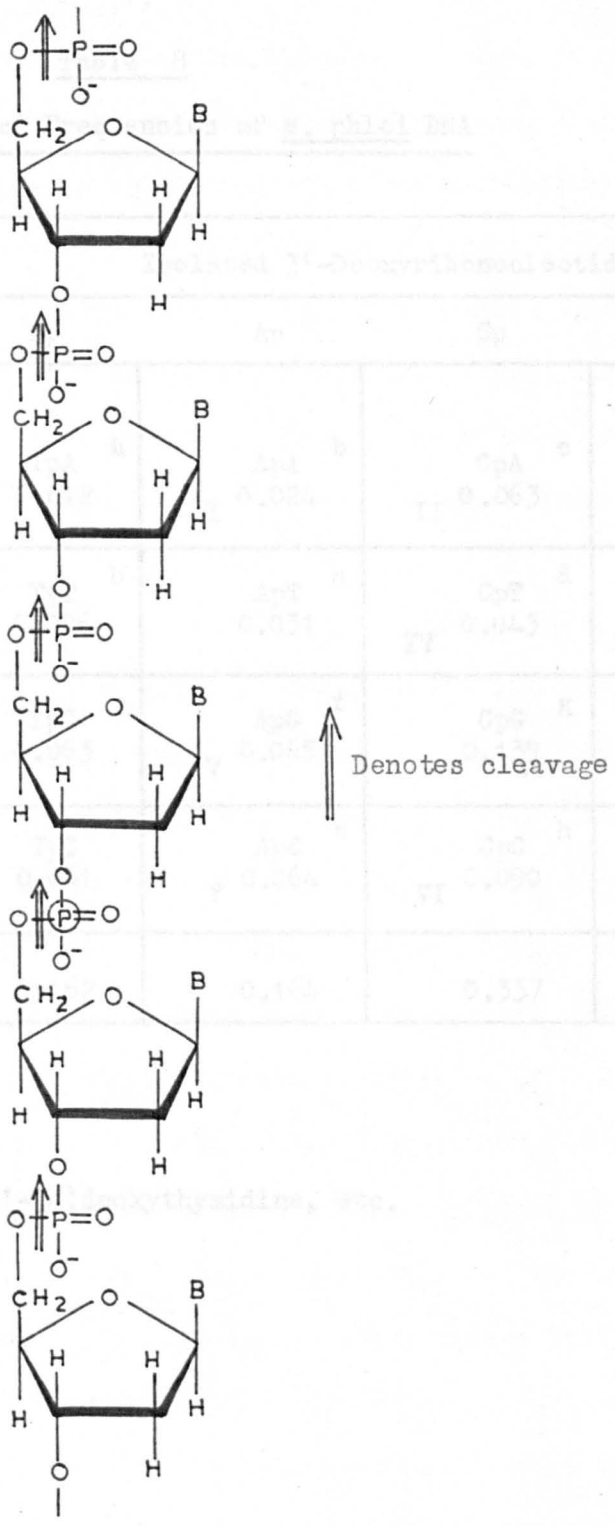


SCHEME 84

the 3'-deoxynucleotides. The phosphorus atom formerly attached to the 5'-oxygen atom of the deoxynucleoside triphosphate substrate was then attached to the 3'-oxygen atom of the nucleotide with which the substrate reacted during the synthesis of the DNA chains (Scheme 85).

The 3'-deoxynucleotides were then separated and isolated by paper electrophoresis, and the phosphorus-32 content of each was a measure of the relative frequency with which a particular substrate reacted with each of the four nucleotides in the synthesis of the DNA. This procedure, carried out four times with a differently labelled substrate in each case, yielded the relative frequencies of all the sixteen possible kinds of dinucleotide (nearest-neighbour) sequencies.

To illustrate the types of results that can be obtained from such studies, the figures for the nearest neighbour frequencies of the DNA synthesised with Mycobacterium phlei DNA primer will be considered in detail (Table 8).



SCHEME 85

Table 8

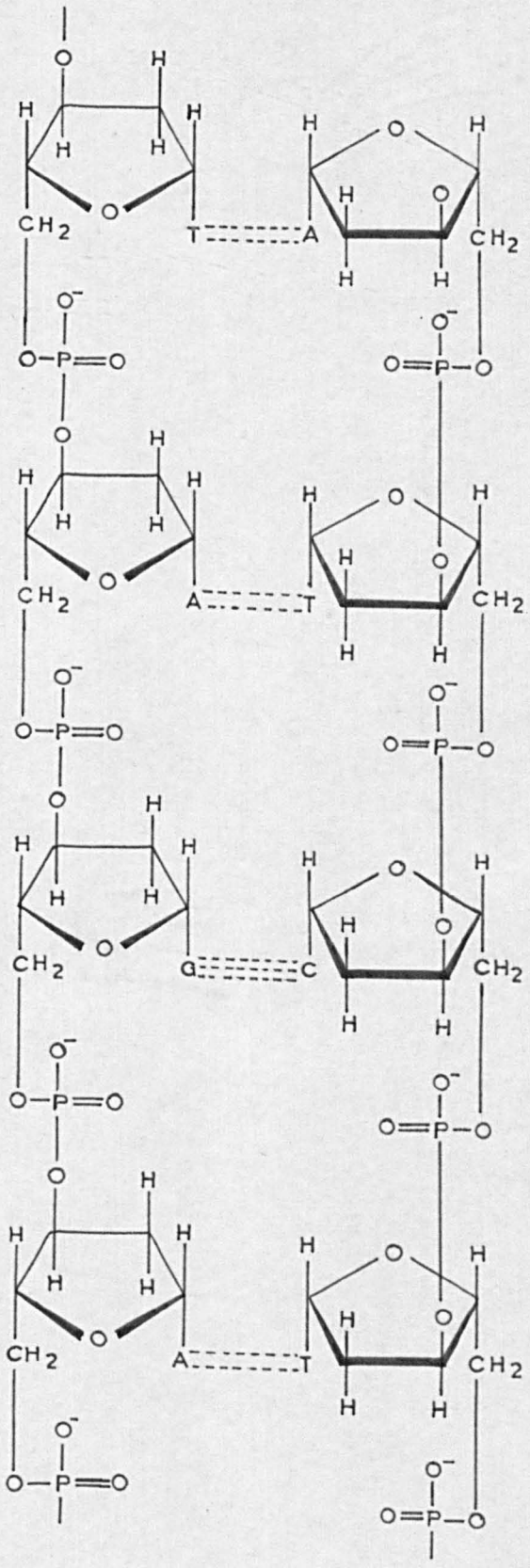
Nearest Neighbour Frequencies of M. phlei DNA

Reaction No.	Labelled Triphosphate	Isolated 3'-Deoxyribonucleotide			
		Tp	Ap	Cp	Gp
1	dATP^{32}	TpA^{a} 0.012	ApA^{b} I 0.024	CpA^{c} II 0.063	GpA^{d} III 0.065
2	dTTP^{32}	TpT^{b} I 0.026	ApT^{a} 0.031	CpT^{d} IV 0.045	GpT^{c} V 0.060
3	dGTP^{32}	TpG^{e} II 0.063	ApG^{f} IV 0.045	CpG^{g} 0.139	GpG^{h} VI 0.090
4	dCTP^{32}	TpC^{f} III 0.061	ApC^{e} V 0.064	CpC^{h} VI 0.090	GpC^{g} 0.122
	SUMS	0.162	0.164	0.337	0.337

TpA denotes deoxyadenylyl-(5'→3')deoxythymidine, etc.

The sums of each of the four columns show that incorporation of deoxyadenylate is equal to thymidylate, and deoxyguanylate is equal to deoxycytidylate. The incorporation of purine nucleotides to pyrimidine nucleotides is exactly that of the chemical composition of the primer DNA as isolated from nature (molar proportions of thymine, deoxyadenine, deoxycytosine and deoxyguanine of 0.165, 0.162, 0.335 and 0.338 respectively). These results indicate that in each of the experiments, faithful replication of the overall composition of the primer DNA was achieved.

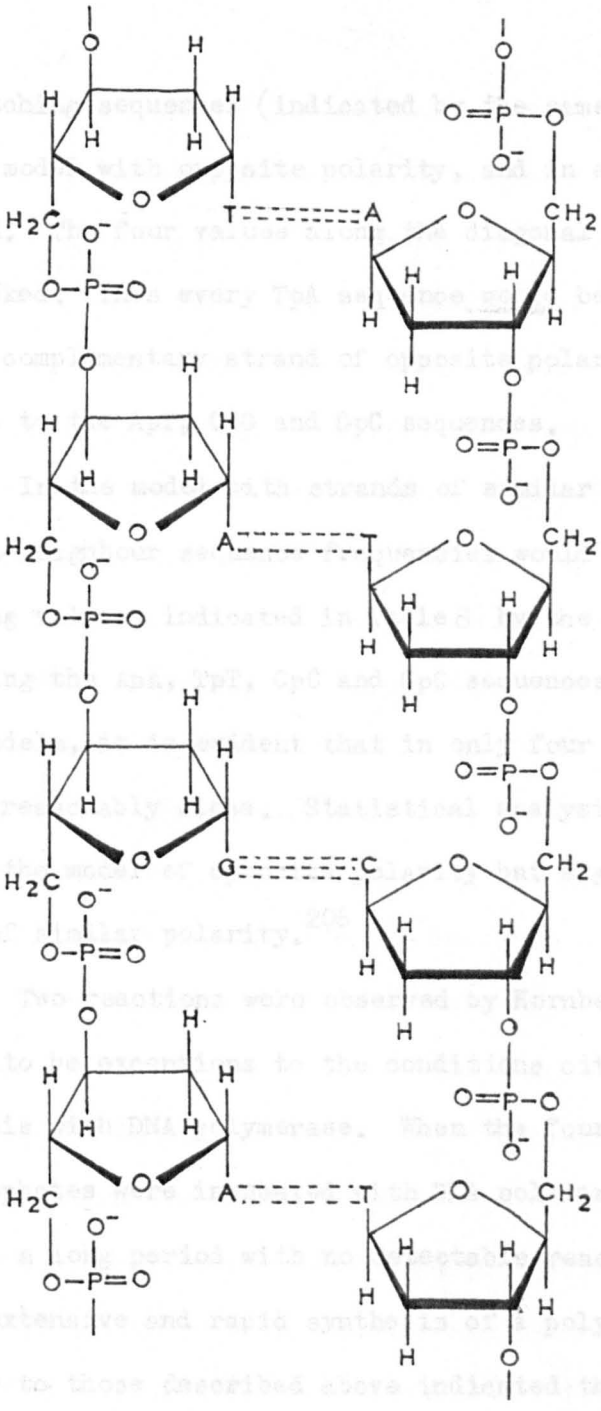
These results also establish in the enzymatically synthesised DNA the base-pairing of the Watson and Crick model, and the opposite polarity of the two strands. Table 8 shows that the amounts of ApA and TpT sequences are equivalent, as are the frequencies of CpC and GpG sequencies. The matchings of the other sequence frequencies depend on whether the strands of the double helix are of similar or opposite polarity. Schemes 86 and 87 compare strands of similar polarity with strands of opposite polarity. Thus on examination of the first base sequence T → A (TpA) on the left-hand strand of either double helix, it can be observed that the matching sequences predicted by the two models are different. In the opposite polarity model the matching sequence is T → A (TpA), whereas in the similar polarity model, the matching sequence in A → T (ApT). In each of the three sequences shown in Scheme 87 (T → A, A → G, G → A) the values are matched by the sequences of the opposite polarity model, but not by those of the similar polarity model. Examination of all the entries in Table 8 shows that there are



Similar Polarity

TpA (0.012) = ApT (0.031)
ApG (0.045) = TpC (0.061)
GpA (0.065) = CpT (0.045)

SCHEME 86



Opposite Polarity

TpA (0.012) = TpA (0.012)
ApG (0.045) = CpT (0.045)
GpA (0.065) = TpC (0.061)

SCHEME 87

six matching sequences (indicated by the same Roman numeral) predicted by the model with opposite polarity, and in each instance the agreement is good. The four values along the diagonal are independent and cannot be checked. Thus every TpA sequence would be matched by a TpA sequence in the complementary strand of opposite polarity; the same constraint applies to the ApT, CpG and GpC sequences.

In the model with strands of similar polarity, the sixteen nearest-neighbour sequence frequencies would fall into eight pairs of matching values, indicated in Table 8 by the same lower case letter. Excluding the ApA, TpT, CpC and GpG sequences, which match similarly in both models, it is evident that in only four of twelve instances are the values reasonably close. Statistical analysis of the data confirmed a good fit to the model of opposite polarity but significant deviation from the model of similar polarity.[206]

Two reactions were observed by Kornberg and co-workers, which proved to be exceptions to the conditions cited above for polynucleotide synthesis with DNA polymerase. When the four common deoxyribonucleoside triphosphates were incubated with DNA polymerase but without any DNA primer, a long period with no detectable reaction was observed, after which extensive and rapid synthesis of a polymer occurred.[207] Analyses similar to those described above indicated that the product had the following characteristics.

1. It contained the bases thymine and adenine only.
2. These bases were arranged in an alternating sequence; that is, it was an alternating copolymer of adenosine and thymine (dAT).

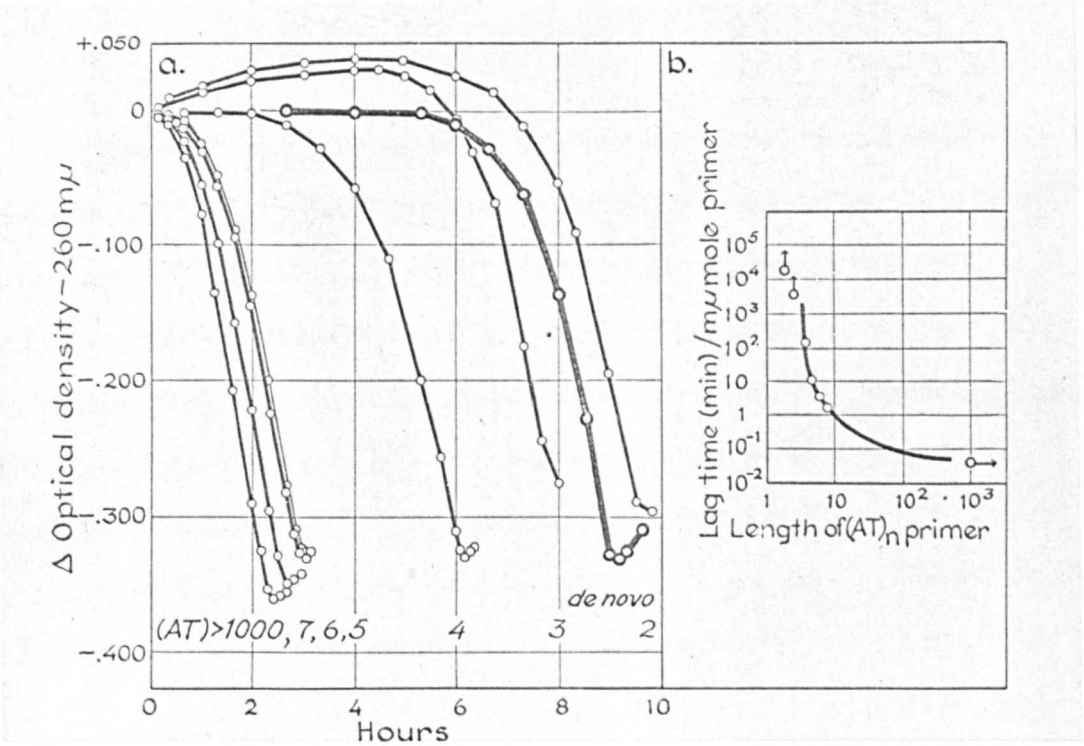
3. It consisted of macromolecules organised as relatively stiff particles with molecular weights from 2 to 8 x 10^6, and "melted" sharply at 71°. Unlike natural DNA, however, the "melting" of the dAT copolymer upon heating and subsequent reformation on cooling were completely reversible, a reflection of its simple base sequence.

When dAT copolymer was isolated from the unprimed reaction and incubated with DNA polymerase, dATP, dTTP and Mg^{2+}, there was a prompt synthesis of polymer again a dAT copolymer. The primer dAT copolymer was thus found to act as a template for the DNA polymerase system.

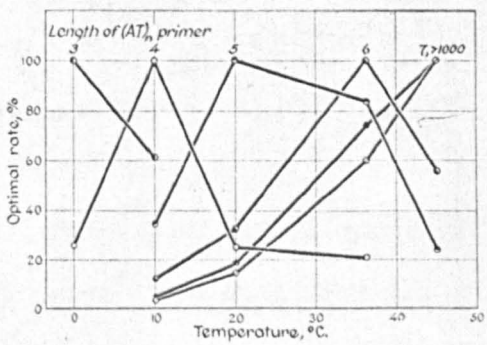
The sensitivity of the physical methods used was inadequate for the detection of synthesis much below 1% of the total reaction, and therefore for studying the lag period. However, by measuring the development of priming or lag-reducing activity, Kornberg and co-workers were able to demonstrate a progressive reaction during the lag period.[207] Thus an aliquot taken at the beginning of the lag period showed no priming activity (no reduction of the lag period), whilst an aliquot taken at the end of the lag period produced no further lag period, an aliquot taken in the middle of the lag period showing intermediate priming activity. Characterisation of the products during the lag period suggested that a dAT copolymer of large size was synthesised during the first fifth of the period, and that the further time course of synthesis followed largely from the autocatalytic replication of a few macromolecules produced de novo. Thus the number of molecules of polymer in the reaction mixture increased, whereas the average size remained constant. It was also found that the length of the lag period was a

function of the primer concentation, and it was calculated that the reaction was exponential until the enzyme ceased to be in excess of the polymer concentration.[208]

Kornberg and Khorana combined forces to study the influence of primer chain-length on the DNA polymerase reaction.[209] They used dAT copolymer primers synthesised by chemical methods, essentially as described in a previous section. Denoting these copolymers by the trivial notation $(AT)_n$, they studied the series $(AT)_2$ to $(AT)_7$, and obtained the results shown in Scheme 88. Polymer synthesis occurred promptly when $(AT)_7$, $(AT)_6$ or $(AT)_5$ were used, and considerably earlier than the de novo reaction or when $(AT)_4$ was used. No significant effect on the lag time of the de novo reaction could be attributed to the use of $(AT)_3$ or $(AT)_2$. This priming effect of the oligomers $(AT)_{4-7}$ was indistinguishable from that of dAT in requiring the presence of both dATP and TTP as well as DNA polymerase and Mg^{++}. The product of these polymerisations was a large molecule with an alternating base sequence of deoxyadenine and thymine. It is interesting to note that the kinetics of dAT synthesis were previously described as exponential,[208] but this relationship was not observed in the above experiments. This was attributed to the absence of an endonuclease, longer molecules were produced, and new priming points were not generated. In support of this, addition of small amounts of an endonuclease to the system, resulted in exponential kinetics for the reaction.

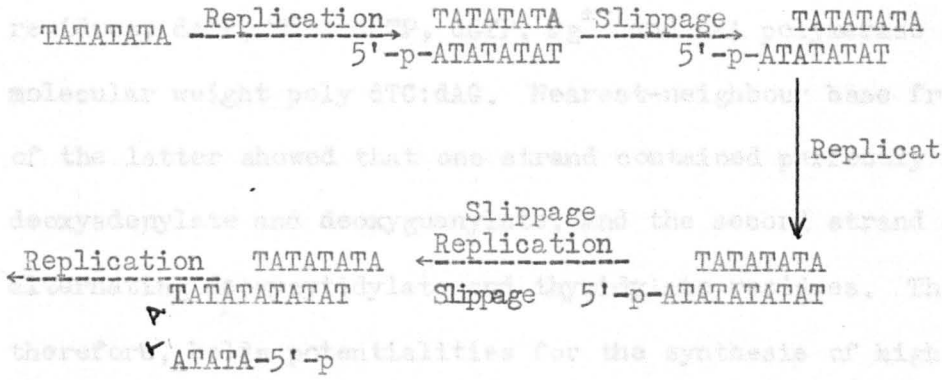


SCHEME 88 - Priming of dAT synthesis by $(AT)_n$ oligomers at 37°C. (a) The reaction mixtures were primed with AT, $(AT)_7$, $(AT)_6$, $(AT)_5$, $(AT)_4$, $(AT)_3$ or $(AT)_2$ and compared with a typical de novo synthesis curve at 37°C. (b) The length of $(AT)_n$ polymer refers to the number (n) of AT dinucleotide residues in the polymer. The symbols with an arrow attached are undetermined values that are at least greater than as represented on the graph.



SCHEME 89 - Optimal temperatures of priming by $(AT)_n$ oligomers.

Studies on the influence of temperature on the priming ability of the primers $(AT)_{2-7}$ produced the interesting observation that each oligomer had an optimal temperature for priming (Scheme 89). Thus, for example, $(AT)_4$ primed optimally at 10°C, whilst $(AT)_7$ was virtually inert at that temperature. As a result of these studies, Kornberg and Khorana proposed the mechanism illustrated in Scheme 90 for the reiterative replication of oligonucleotides.



Scheme 90

They also postulated that it was the slippage step of the reaction that was critically temperature dependent. Thus an $(AT)_4$ template, after slippage, would be linked to its replica by only six hydrogen bonds, which would not be expected to be stable at 37°C, but might be far more stable and effective at 10°C.

Khorana and co-workers have recently extended their studies on polynucleotide synthesis by the DNA polymerase system by using synthetic oligodeoxyribonucleotides as templates.[210] Thus a mixture of the enzyme, Mg^{++}, $(T)_{11}$, $(dA)_7$ and dATP led to the synthesis of polydeoxyriboadenylate, which was much longer than the template. A mixture of $(T)_{11}$, $(dA)_7$, dATP, TTP, Mg^{++} and DNA polymerase led to extensive formation of the homopolymers, polydeoxyadenylate and polythymidylate, the molecular weight of the polymer being several million. $d\text{-}(TC)_5$, containing alternating thymidylate and deoxycytidylate residues, $d\text{-}(AG)_5$, containing alternating deoxyadenylate and deoxyguanylate residues, dATP, TTP, dCTP, dGTP, Mg^{++} and DNA polymerase synthesised high molecular weight poly dTC:dAG. Nearest-neighbour base frequency analysis of the latter showed that one strand contained perfectly alternating deoxyadenylate and deoxyguanylate, and the second strand contained alternating deoxycytidylate and thymidylate residues. This system therefore, holds potentialities for the synthesis of high molecular weight polydeoxynucleotides with simple repeating base sequences.

Kornberg also discovered another unprimed polynucleotide synthesis of the DNA polymerase system.[211] Using high concentrations of the enzyme and the substrates deoxyguanosine and deoxycytidine-5'-triphosphates, the synthesis of a high molecular weight product was realised. This product consisted of two homopolymers, poly-dG and poly-dC, in the form of a double helix. A lag period before extensive polymer synthesis was again observed, and the reaction possessed all the characteristics of the unprimed dAT copolymer synthesis.

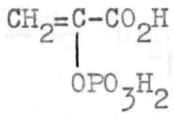
This peculiar specificity of the products formed by the unprimed reactions provided the germ for some of the work described in this thesis on the chemical synthesis of polydeoxynucleotides using nucleotide substrates containing the bases adenine and thymine. The results (to be discussed in more detail in the Experimental Discussion) indicate that it is the enzyme DNA polymerase that is responsible for this specificity and not the configurations of the two nucleotides. The polymeric products were analysed by the nearest-neighbour base frequency method, described above.

Other polynucleotide synthesising systems extractedfrom cells of various origins have now been discovered, and Berg and co-workers have discovered that by use of Mn^{++} instead of Mg^{++} in the DNA polymerase system from E. coli, ribonucleotides can be incorporated into polymers, mixed polynucleotides containing ribo- and deoxyribonucleotide residues resulting.[212]

PART II

A. Enol Esters and their Properties

Whilst investigating the enzymatic conversion of D-glyceric acid-3-phosphate to pyruvate and orthophosphate Meyerhof and Lohmann succeeded in isolating a new intermediate and characterised it as phosphoenol pyruvate,[213] (CLIX).

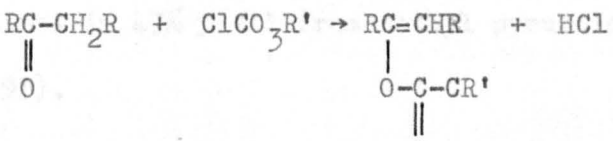


CLIX

Thus phosphoenol pyruvate represents the last phosphorylated three-carbon-atom compound both in glycolysis and fermentation, and also seems to play a role in carbon dioxide fixation.[214] The biochemical interest in phosphoenol pyruvate provided an impetus for its synthesis, and that of other enol phosphates which, it was predicted, would act as phosphorylating reagents.

1. General methods of preparation of enol esters

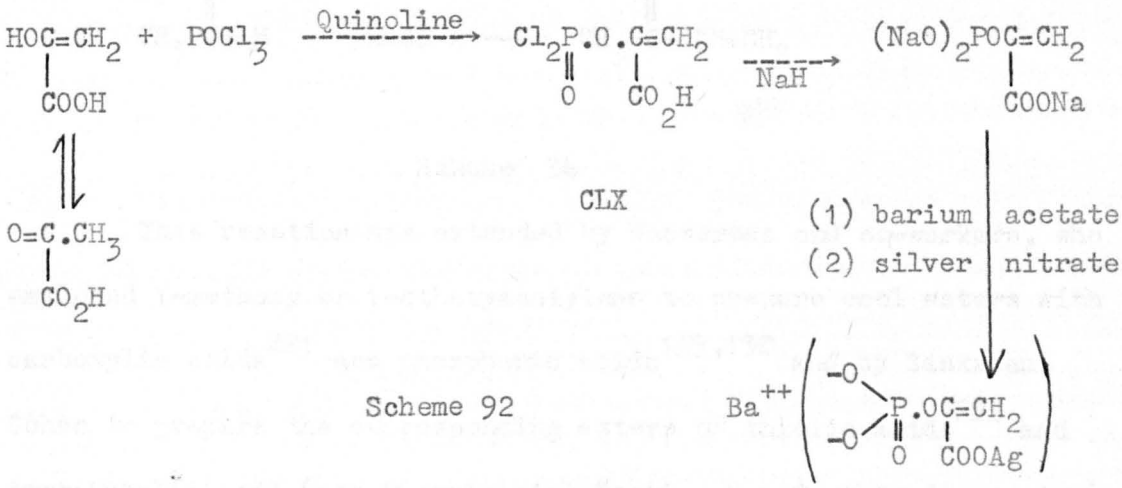
(a) Carbonyl compounds, which are capable of existing to an appreciable extent in the enol form, when refluxed with acyl chlorides or acid anhydrides yield enol esters (Scheme 91).



Scheme 91

If the carbonyl compound cannot exist to any appreciable extent as the enol, for example acetone, this reaction is not possible.

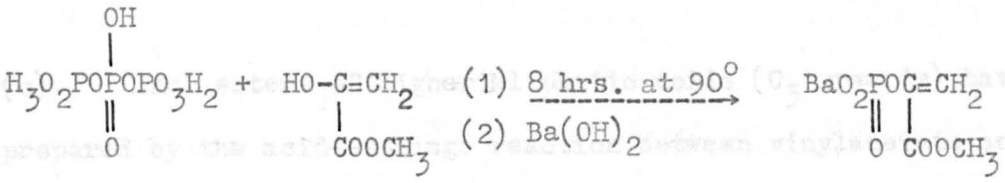
This method was first applied in the phosphate field to the synthesis of phosphoenol pyruvate. Thus the reaction of pyruvic acid with phosphorus oxychloride in quinoline, followed by alkaline hydrolysis of the intermediate (CLX) resulted in the synthesis of phosphoenol pyruvate (as the silver barium salt)[215,216] (Scheme 92).



Scheme 92

The yield, however, did not exceed 9%, despite later improvements.[217,218,219] Similarly the barium salt of 2-carboxy-1-methylvinyl dihydrogen phosphate was prepared from ethyl sodioacetoacetate and phosphorus oxychloride.[220]

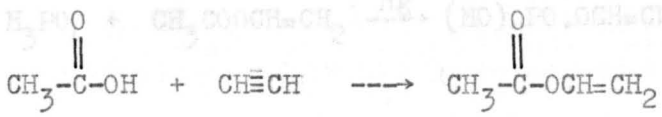
Phosphorylation of an enol can also be accomplished by using polyphosphoric acid. Thus 1-carbomethoxyvinyl barium phosphate (CLXI) was obtained in 47% yield from methyl pyruvate and triphosphoric acid.[112] (Scheme 93).



CLXI

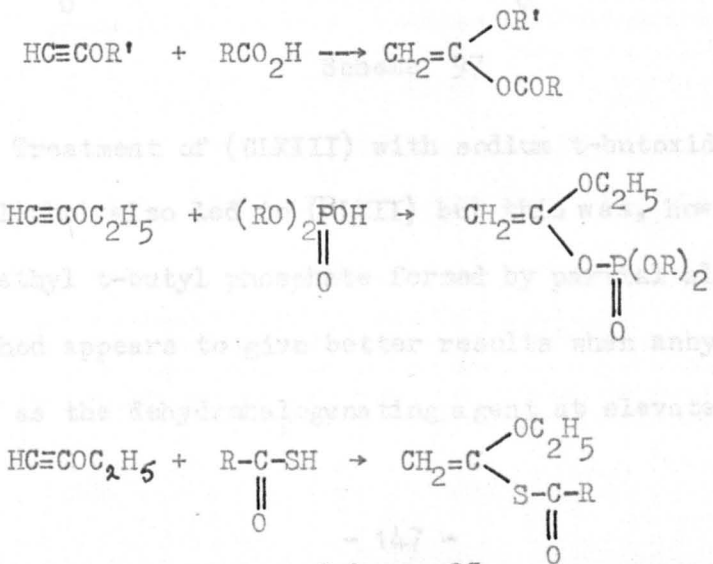
Scheme 93

(b) Acetic acid adds to acetylene in the presence of mercuric salts or acetylsulphuric acid to give vinyl acetate. (Scheme 94).



Scheme 94

This reaction was extended by Wasserman and co-workers, who employed 1-methoxy or 1-ethoxyacetylene to prepare enol esters with carboxylic acids[221] and phosphoric acids[129,130] and by Banks and Cohen to prepare the corresponding esters of thiolic acids[222] and terephthalic acid (see Experimental Section for further discussion). (Scheme 95).



Scheme 95

(c) Enol esters of higher aliphatic acids (C_3 upwards) have been prepared by the acid-exchange reaction between vinylacetate and an excess of the aliphatic acid in the presence of a mercuric-ion catalyst.[223,224] When extended to the preparation of enol phosphates, for example, vinyl dihydrogen phosphate from vinyl acetate and phosphoric acid, the yield of the pyridinium salt was 26%.[225] (Scheme 96).

$$H_3PO_4 + CH_3COOCH{=}CH_2 \xrightarrow{Hg} (HO)_2PO.OCH{=}CH_2 + CH_3COOH$$

Scheme 96

(d) Dehydrohalogenation of 2-haloalkyl phosphates has been found to have limited use in the synthesis of vinyl phosphates. Thus diethyl vinyl phosphate (CLXII) was prepared by the dehydrobromination of diethyl--2-bromoethylphosphate (CLXIII) with sodium hydride in ether[226] (Scheme 97).

$$(C_2H_5O)_2\overset{\overset{O}{\|}}{P}OCH_2CH_2Br + NaH \rightarrow (C_2H_5O)_2\overset{\overset{O}{\|}}{P}OCH{=}CH_2 + NaBr + H_2$$

Scheme 97

Treatment of (CLXIII) with sodium t-butoxide in tertiary butyl alcohol also led to (CLXII) but this was, however, contaminated with diethyl t-butyl phosphate formed by partial alcoholysis of (CLXII).[226] The method appears to give better results when anhydrous sodium carbonate is used as the dehydrohalogenating agent at elevated temperatures.[227]

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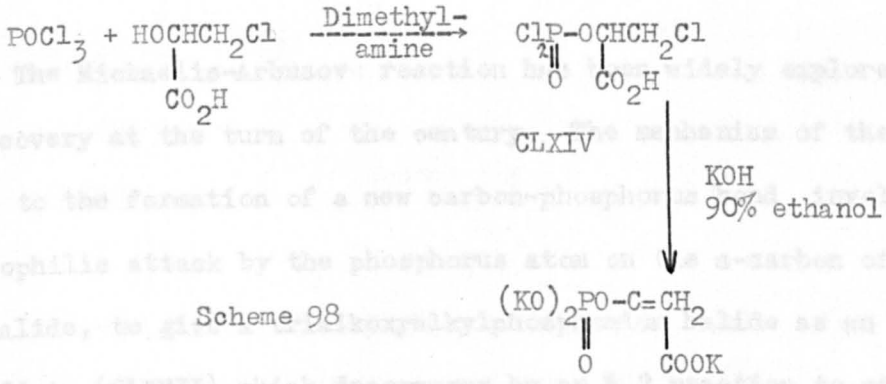
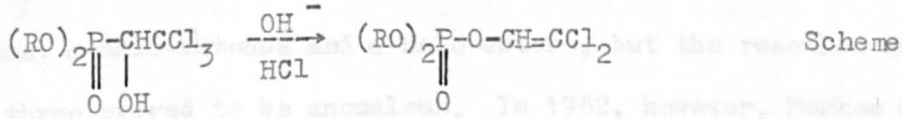
This method has been utilised for the preparation of phosphoenolpyruvate.[228,229] Reaction of β-chlorolactic acid with phosphorus oxychloride gave the intermediate 2-chloroalkyl phosphorus compound (CLXIV), which was treated with ethanolic potassium hydroxide to give the potassium salt of phosphoenol pyruvate (Scheme 98).



Scheme 98

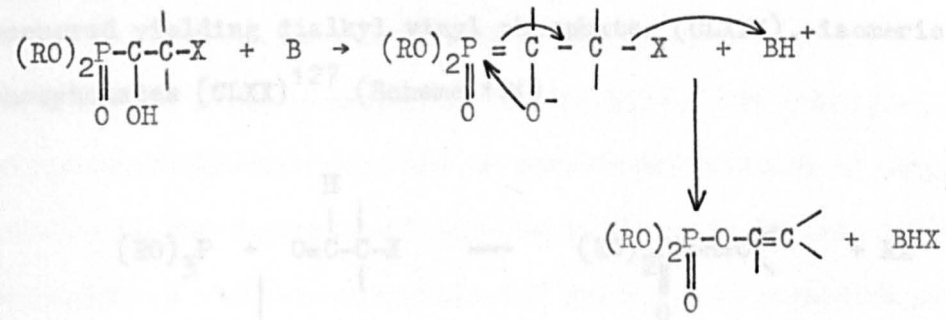
The overall yield did not, however, exceed 10%, calculated from the isolation of the silver barium salt.

(e) Phosphonates of type (CLXV), having a 2,2,2-trichloro-1-hydroxyethyl grouping, were found to undergo rearrangement on treatment with alkali, leading to the corresponding enol phosphates (CLXVI) by elimination of hydrogen halide and fission of the phosphorus-carbon bond.[230,231] (Scheme 99).



Scheme 99

The mechanism has been formulated as (23):



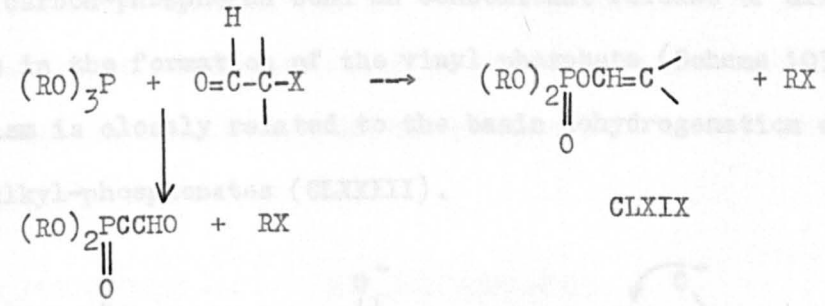
(f) The Michaelis-Arbusov reaction has been widely explored since its discovery at the turn of the century. The mechanism of the reaction leading to the formation of a new carbon-phosphorus bond involves a nucleophilic attack by the phosphorus atom on the α-carbon of the alkyl halide, to give a trialkoxyalkylphosphonium halide as an intermediate (CLXVII) which decomposes by an S_N2 reaction to give a dialkyl phosphonate (CLXVIII) and an alkyl halide (Scheme 100).

$$(RO)_3P + R'X \rightarrow \underset{\text{CLXVII}}{[(RO)_3\overset{+}{P}R']X^-} \rightarrow \underset{\text{CLXVIII}}{(RO)_2P(O)R'} + RX$$

Scheme 100

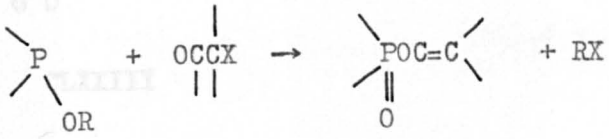
Aromatic and aliphatic acid chlorides were also found to undergo the Michaelis-Arbusov reaction, and attempts have been made to use α-halo-aldehydes, α-halo-ketones and α-halo-esters, but the reaction with the latter three proved to be anomalous. In 1952, however, Perkow discovered that α-halo aldehydes did react with trialkyl phosphates according to

the Michaelis-Arbusov reaction, but that a new type of rearrangement occurred yielding dialkyl vinyl phosphates (CLXIX), isomeric with the phosphonates (CLXX)[127] (Scheme 101).



Scheme 101

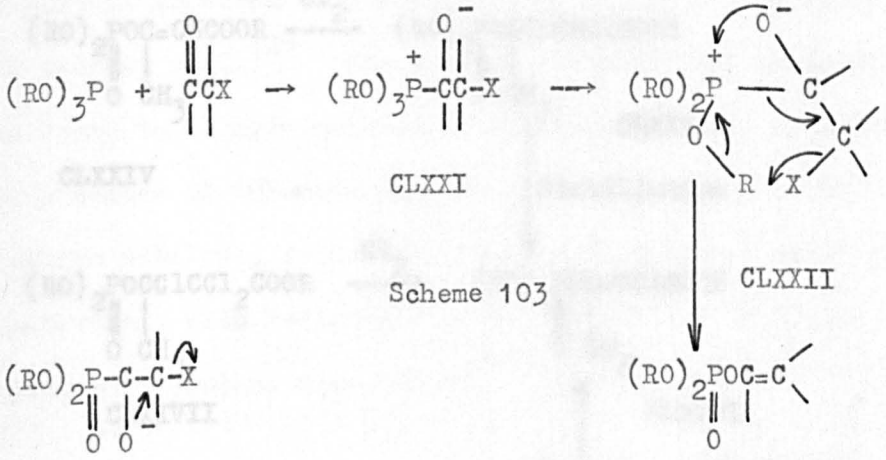
The most general formulation of this reaction is given in Scheme 102, and more recent work has shown that α-halo-ketones and in some case α-halo-esters are also able to undergo this reaction.



Scheme 102

Various mechanisms have been proposed for the Perkow reaction, and although some seem rather improbable, the experimental evidence available does not allow the mechanism to be established with certainty. The mechanism proposed independently by Allen and Johnson,[232] and Kharasch and Bengelsdorf[233] seems to comply with all the experimental data available, and seems to be the most reasonable one.[44,128,234] The mechanism involves an initial attack by the phosphorus atom on the

carbonyl carbon to give the adduct (CLXXI), and a similar attack by the negatively charged oxygen on the phosphorus atom with intermediate formation of a three-membered ring (CLXXII). The subsequent cleavage of the carbon-phosphorus bond on concomitant release of alkyl halide results in the formation of the vinyl phosphate (Scheme 103). This mechanism is closely related to the basic dehydrogenation of 1-hydroxy-2-haloalkyl-phosphonates (CLXXIII).



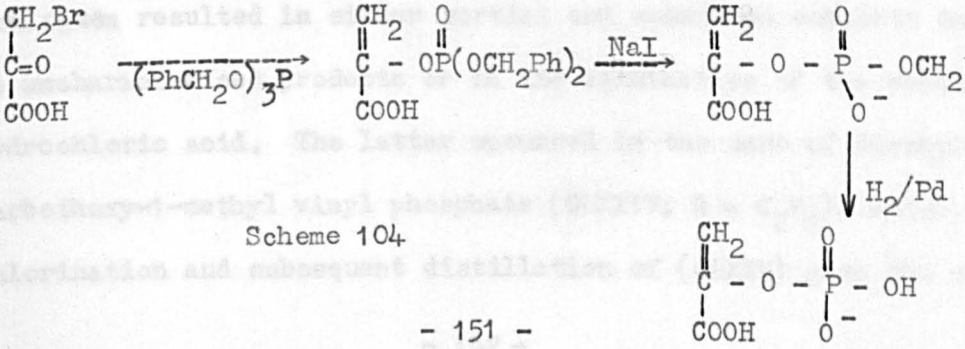
CLXXI

CLXXII

Scheme 103

CLXXIII

Phosphoenol pyruvate has been prepared by reaction of bromopyruvic acid with tribenzyl phosphite, followed by anionic debenzylation and subsequent hydrogenolysis[235] (Scheme 104).

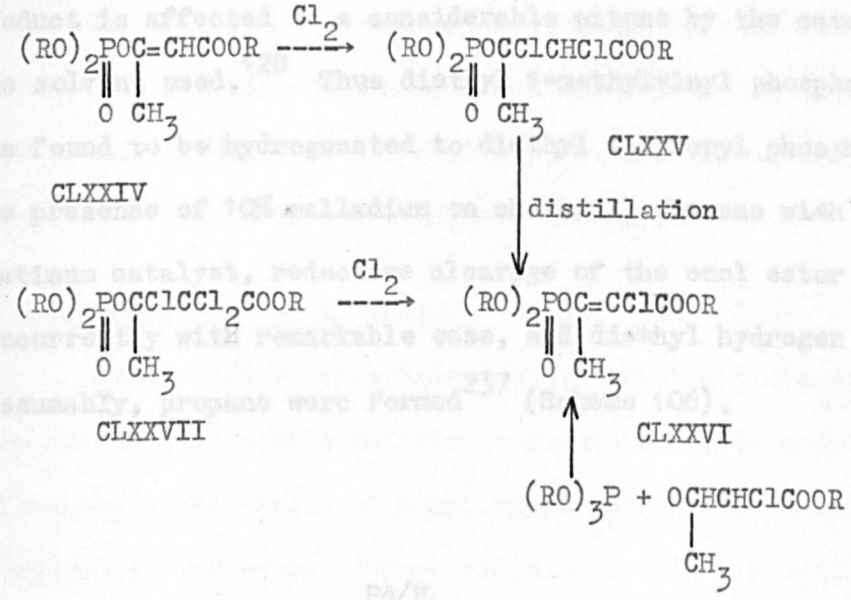


Scheme 104

2. Properties of enol esters

(a) Halogenation

The addition of chlorine or bromine to the ethylenic double bond of dialkyl vinyl phosphates usually proceeds with great ease. Chlorination can be accomplished by introducing the calculated amount of chlorine into a solution of the phosphate in carbon tetrachloride (Scheme 105).



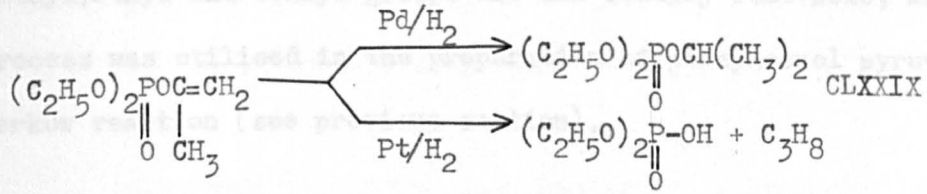
Scheme 105

Distillation of the chlorine addition products of higher analogues resulted in either partial and sometimes complete decomposition to uncharacterised products or in the elimination of the elements of hydrochloric acid. The latter occurred in the case of diethyl-2-carbethoxy-1-methyl vinyl phosphate (CLXXIV; R = C_2H_5), which upon chlorination and subsequent distillation of (CLXXV) gave the vinyl

phosphate (CLXXVI) identical with the compound prepared directly from triethyl phosphate and ethyl α-chloroacetoacetate (CLXXVI) could be converted to the trichloro derivative (CLXXVII) by further action of chlorine.[236]

(b) Hydrogenation

Only a limited amount of data is available concerning the hydrogenation of vinyl phosphates, but it appears that the nature of the product is affected to a considerable extent by the catalyst and the solvent used.[128] Thus diethyl 1-methylvinyl phosphate (CLXXVIII) was found to be hydrogenated to diethyl isopropyl phosphate (CLXXIX) in the presence of 10% palladium on charcoal, whereas with the use of a platinum catalyst, reductive cleavage of the enol ester group took place concurrently with remarkable ease, and diethyl hydrogen phosphate and, presumably, propane were formed[237] (Scheme 106).



CLXXVIII

Scheme 106

Such a reaction is analogous to the reductive cleavage of vinyl carboxylates using platinum catalyst.[238] Benzyl ester groups in vinyl phosphates can, however, be selectively removed by hydrogenolysis if

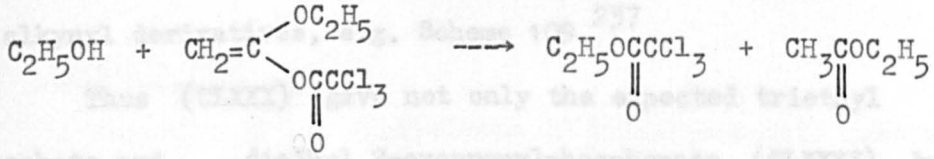
the latter is stopped before reduction of the double-bond takes place.[235] Thus debenzylation of the dibenzyl ester of phosphoenol pyruvate and of the sodium salt of the monobenzyl ester gives good yields of phosphoenol pyruvate when palladium on charcoal in 50% aqueous methanol was used and the hydrogenation was stopped after the consumption of the theoretical quantity of hydrogen. If the reaction was allowed to proceed beyond this point, the reduction of the ethylenic double bond took place at a rate one-twentieth that of the reductive debenzylation. However, an increase in the methanol content of the solvent caused a more rapid reduction of the double bond, and consequently the yields of phosphoenol pyruvate were seriously impaired.

(c) Monodealkylation by anions

Dialkyl vinyl phosphates undergo nucleophilic monodealkylation when heated with alkali halides in suitable solvents for 5 - 20 minutes, giving the alkali salts of alkyl vinyl hydrogen phosphates in good yield. Methyl, ethyl and benzyl groups are all readily removable, and this process was utilised in the preparation of phosphoenol pyruvate by the Perkow reaction (see previous section).

(d) Reaction with alcohols

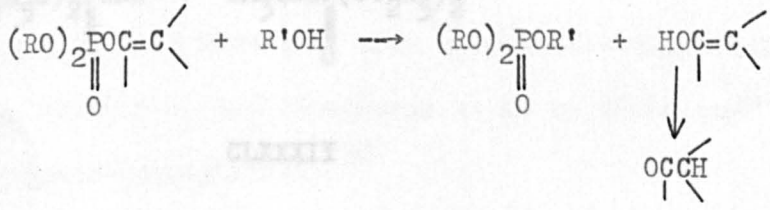
Arens and co-workers were able to isolate 1-ethoxyvinyl trichloroacetate from trichloroacetic acid and ethoxyacetylene,[239] and were able to obtain from the reaction of this ester with an alcohol, the corresponding ester in 89% yield (Scheme 107).



Scheme 107

Wasserman and co-workers extended this type of reaction to synthesise a series of esters prepared from 1-methoxy- and 1-ethoxyvinyl carboxylic esters, and 1-ethoxyvinyl phosphates with various alcohols.[130,221] Thus these 1-ethoxyvinyl esters appear to be efficient acylating and phosphorylating agents towards alcohols.

Most enol phosphates, even the reactive ketone acylals, prepared via the Perkow reaction appear, however, to be inefficient in phosphorylation of alcohols. It has been reported, however, that when dialkyl vinyl phosphates are heated in absolute ethanol in the presence of catalytic amounts of sodium alkoxide or p-toluene sulphonic acid, transesterification of the enol ester linkage does occur, and the enol is liberated as a carbonyl compound (Scheme 108).

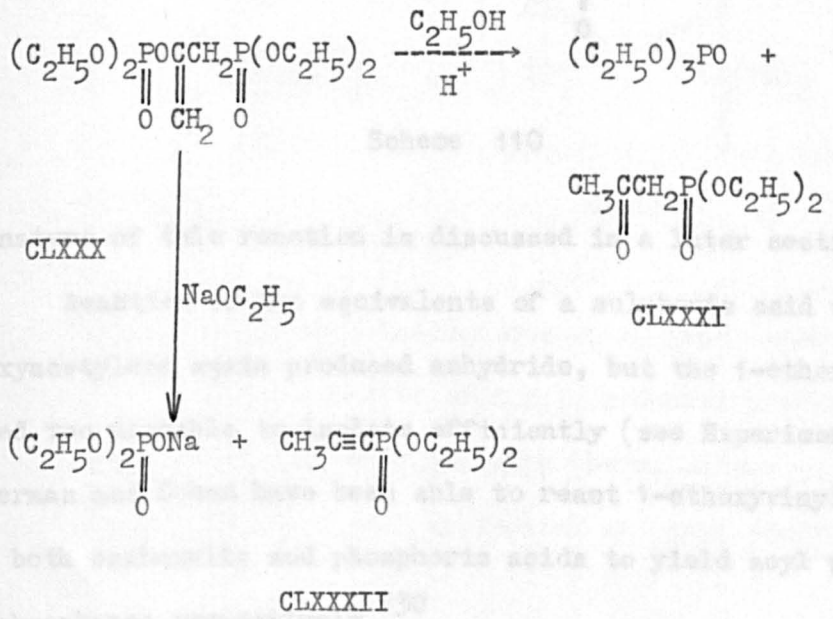


Scheme 108

In the presence of larger amounts of sodium alkoxide, however, the alcoholysis can take an alternative course leading to formation

of alkynyl derivatives, e.g. Scheme 109.[237]

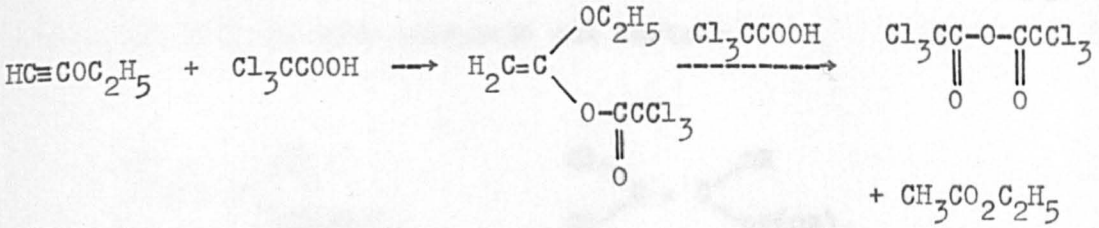
Thus (CLXXX) gave not only the expected triethyl phosphate and -diethyl 2-oxopropylphosphonate (CLXXXI) but also diethyl hydrogen phosphate and diethyl propynylphosphonate (CLXXXII).



Scheme 109

(e) Reaction with acids

Reaction of two equivalents of trichloroacetic acid with one of ethoxyacetylene led to the formation of trichloroacetic anhydride (Scheme 110) and it was postulated that the intermediate 1-ethoxyvinyl trichloroacetate was immediately attacked by a second molecule of the acid.[239] Thus if the 1-ethoxyvinyl trichloroacetate was isolated and reacted with trichloroacetic acid, the anhydride again resulted.



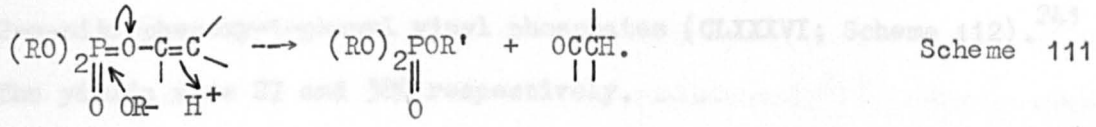
Scheme 110

The nature of this reaction is discussed in a later section.

Reaction of two equivalents of a sulphonic acid with one of ethoxyacetylene again produced anhydride, but the 1-ethoxyvinyl sulphonate proved too unstable to isolate efficiently (see Experimental Discussion). Wasserman and Cohen have been able to react 1-ethoxyvinyl phosphates with both carboxylic and phosphoric acids to yield acyl phosphates and pyrophosphates respectively.[130]

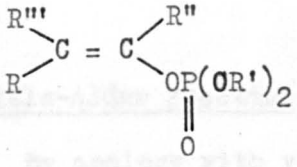
Dialkyl vinyl phosphates prepared via the Perkow reaction have also been used for the synthesis of acyl phosphates, pyrophosphates and (indirectly) for peptide synthesis by reaction with carboxylic, phosphoric

or an appropriately protected amino acid (Scheme 111).

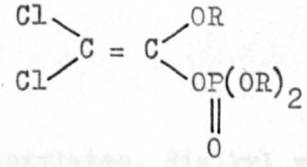


Scheme 111

Cramer, however, found that the enol phosphate analogues of pyruvates (CLXXXIII; R' = C_2H_5, R" = $CO_2C_2H_5$, and R"' = H) would not phosphorylate phosphoric esters,[240] but the enol phosphate synthesised from trichloroacetic acid and triethyl phosphate (CLXXXIVA R = C_2H_5) would phosphorylate both phosphate and acetate.



CLXXXIII



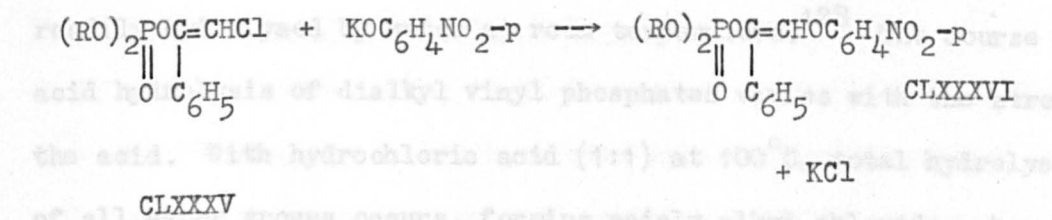
CLXXXIV

He also found that derivatives of malonic ester (CLXXXIII; R = H, R' = C_2H_5, R" = OC_2H_5 and R"'= $CO_2C_2H_5$), prepared similarly from bromomalonic ester, were extremely reactive (being ketene acylals) and formed pyrophosphates, acetyl phosphates and acyl phosphates with protected amino acids.

(f) Substitution of halogen in the vinyl ester group

Halogen in the 2-position of the vinyl group can be substituted without cleavage of the vinyl ester linkage. Thus dimethyl and diethyl

2-chloro-1-phenyl vinyl phosphates (CLXXXV; R = CH_3, C_2H_5) gave on heating with potassium p-nitrophenoxide, the corresponding 2-p-nitrophenoxy-1-phenyl vinyl phosphates (CLXXXVI; Scheme 112).[241] The yields were 27 and 38% respectively.



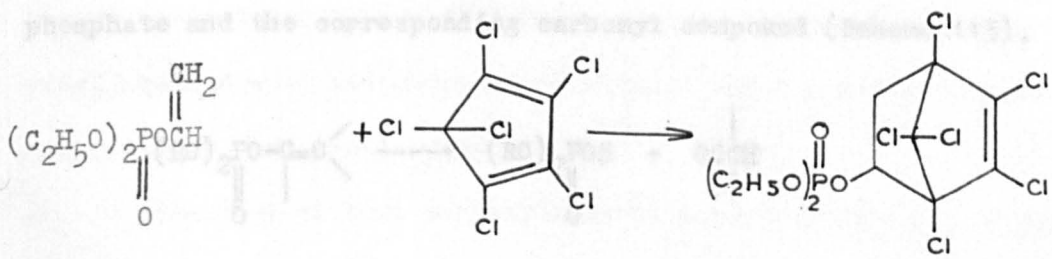
CLXXXV

CLXXXVI

Scheme 112

(g) Diels-Alder reaction

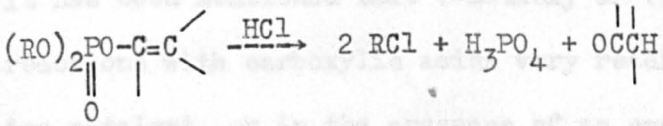
By analogy with vinyl carboxylates, dialkyl vinyl phosphates may participate in the Diels-Alder Reaction, acting as the dienophile. Thus reaction of diethyl vinyl phosphate with hexachlorocyclopentadiene gave the adduct (CLXXXVII; Scheme 113) in 65% yield.[242]



Scheme 113

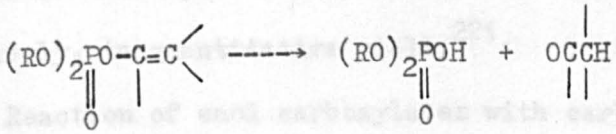
(h) Hydrolysis

Dialkyl vinyl phosphates appear to be remarkably stable towards water. Thus diethyl vinylphosphate is hydrolysed to the extent of only 11% after 20 hours at 100°C in 30% aqueous ethanol.[243] 1-Ethoxyvinyl esters are, however, very susceptible to hydrolysis (see Experimental Discussion). For example 2-carbethoxy-1-ethoxyvinyl phosphate is readily hydrolysed by water at room temperature.[128] The course of acid hydrolysis of dialkyl vinyl phosphates varies with the strength of the acid. With hydrochloric acid (1:1) at 100°C, total hydrolysis of all ester groups occurs, forming mainly alkyl chloride, phosphoric acid and the corresponding ketone.



Scheme 114

Under milder conditions however, selective hydrolysis of the enol ester group can be effected, yielding the dialkyl hydrogen phosphate and the corresponding carbonyl compound (Scheme 115).



Scheme 115

Alkaline hydrolysis of various dialkyl vinyl phosphates with 0.5 N potassium hydroxide at 80°C was found to cleave the enol ester linkage only. Diethyl 2-carbethoxy-1-methylvinyl phosphate, however, consumes two moles of base owing to simultaneous hydrolysis of the carbethoxy group.[236]

B. Specific Reactions of Enol Esters

Enol esters, owing to their ease of attack by nucleophiles act as acylating agents and have been used for many important chemical and biological syntheses, some of which will now be discussed in detail.

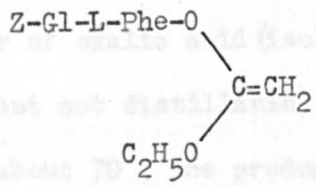
1. Enol carboxylates

It has been mentioned that 1-methoxy or 1-ethoxyacetylenes undergo reactions with carboxylic acids very readily in the presence of mercuric ion catalyst, or in the presence of an excess of the acetylene, to yield the corresponding enol esters. The latter may then undergo reaction with a nucleophile. Thus Arens and co-workers[239] prepared ethyl trichloroacetate by the reaction of 1-ethoxyvinyl trichloroacetate with ethanol. Similarly, Wasserman and Wharton prepared p-nitro benzyl acetate and 2,4-dinitrophenyl acetate by the reaction of 1-methoxy-vinyl acetate with p-dinitrobenzyl alcohol and 2,4-dinitrophenol, respectively, in quantitative yield.[221]

Reaction of enol carboxylates with carboxylic acids yields the corresponding anhydride. Thus 1-ethoxyvinyl trichloroacetate with trichloroacetic acid resulted in the formation of trichloroacetic anhydride and ethyl acetate. Mixed anhydrides can be prepared by reaction

of a 1-ethoxyvinyl ester of an acid with another acid.

Amines also react with enol carboxylates in a similar manner, to yield amides. The vigorous reaction of 1-methoxyvinyl acetate with benzylamine led to the isolation of N-benzylacetamide in quantitative yield.[221] This has led to the development of alkoxyvinyl esters as coupling reagents in the synthesis of peptides. From the reaction of benzyloxycarbonylglycyl-L-phenylalanine (Z-Gly-L-Phe-OH) with ethoxyacetylene the corresponding 1-ethoxyvinyl ester could be isolated (CLXXXVIII) which was condensed with glycine ethyl ester to yield the tripeptide in c. 50% yield, racemisation occurring only to the extent of about 3%.[244]



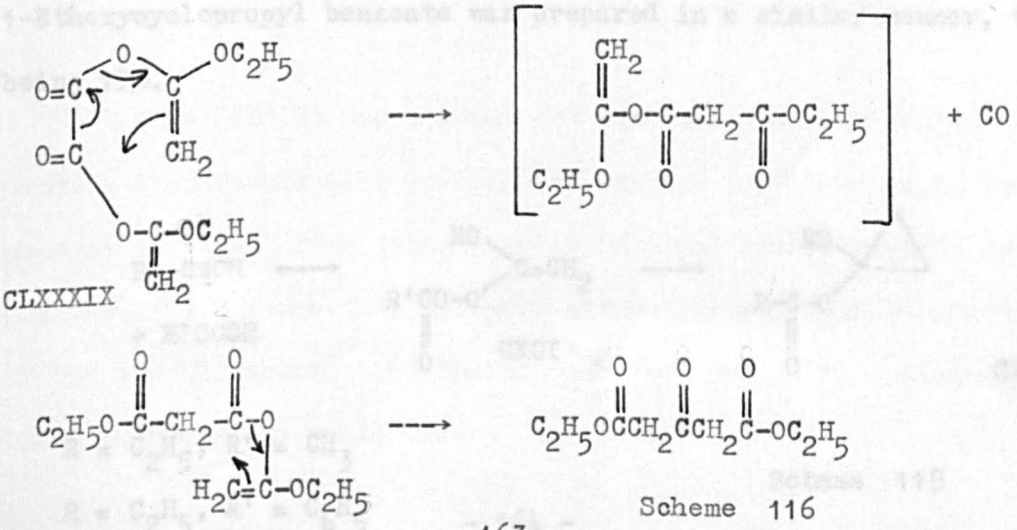
CLXXXVIII

Sheehan and Hlavka were able to isolate 1-ethoxyvinyl phthaloyl-glycinate from the reaction of ethoxyacetylene with phthaloylglycine and glycine ethyl ester in aqueous solution.[245] Heating the adduct with glycine ethyl ester in dioxane yielded the dipeptide phthaloylglycyl-glycine ethyl ester in good yield. More recently, Arens and co-workers have prepared several 1-ethoxyvinyl esters of phthaloyl amino-acids and of benzyloxycarbonyl-glycine. Reaction of the latter with glycine ethyl ester resulted in the synthesis of the dipeptide ester, and these

workers also synthesised amino-acid anhydrides.[246] Thus the method appears to show considerable potentialities for the preparation of peptides.

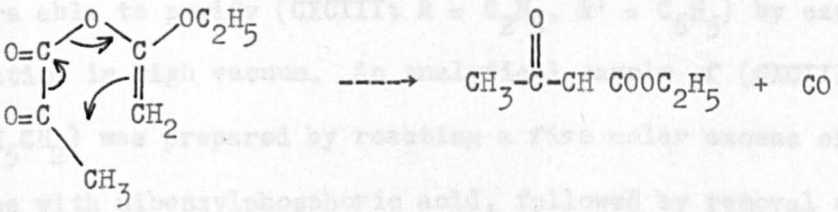
Rearrangements:-

Several interesting rearrangements of 1-ethoxylvinyl carboxylates have been discovered recently. Zwanenburg heated 1-ethoxyvinyl tri- and dichloroacetates to 170°C, at which temperature rearrangement occurred, the products of the reaction being ethyl 6-chloro-β-(chloroacetoxy)-crotonate and ethyl 6-trichloro-β-(trichloroacetoxy)crotonate, respectively. The mechanism of this rearrangement is discussed in the Experimental Discussion. Somewhat similar rearrangements of some 1-ethoxyvinyl carboxylates were discovered in the present work. Thus the di-ethoxyvinyl ester of oxalic acid (isolated and identified by its infrared spectrum, but not distillable) (CXXXXIX) underwent rearrangement on heating to about 70°, the product being acetone dicarboxylate. A five-centre- succeeded by a four-centre-rearrangement has been postulated as the reaction mechanism (Scheme 116).



Scheme 116

1-Ethoxyvinyl pyruvate (CXC) has also been found to undergo a thermal rearrangement, a five centre rearrangement being the suggested mechanism once again (Scheme 117). The product of this rearrangement was ethylacetoacetate.

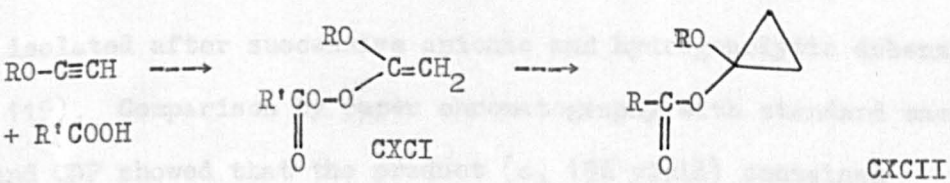


CXC

Scheme 117

Cyclopropanols:-

1-Ethoxyvinyl carboxylates have been used for the synthesis of 1-substituted cyclopropanols (Scheme 118) by Wasserman and co-workers.[247] Addition of (CXCI) and methylene iodide to a zinc-copper couple in glyme resulted in the formation of 1-ethoxycyclopropyl acetate (CXCII). 1-Ethoxycyclopropyl benzoate was prepared in a similar manner, the yield being 19%.



CXCI

CXCII

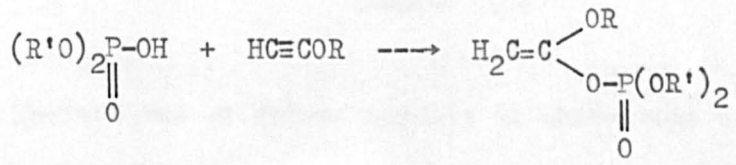
$R = C_2H_5$, $R' = CH_3$

$R = C_2H_5$, $R' = C_6H_5$

Scheme 118

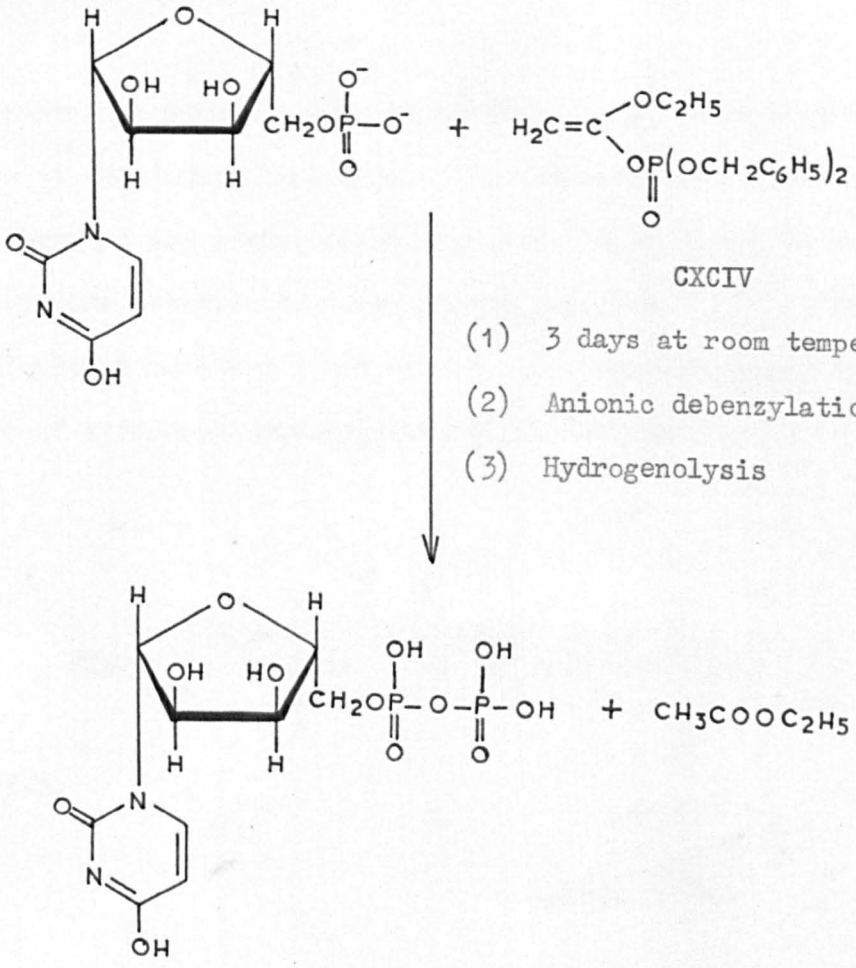
2. Enol phosphates

Attempts by Arens and co-workers to isolate 1-ethoxyvinyl phosphates (CXCIII) by reaction of a phosphoric acid with ethoxyacetylene were unsuccessful.[248] Using a mercuric ion catalyst or an excess ethoxyacetylene, however, Wasserman and Cohen were able to isolate such compounds as oils which tended to polymerise on heating.[129,130] Thus they were able to purify (CXCIII; R = C_2H_5, R' = C_6H_5) by cautious distillation in high vacuum. An analytical sample of (CXCIII; R = C_2H_5, R' = $C_6H_5CH_2$) was prepared by reacting a five molar excess of ethoxyacetylene with dibenzylphosphoric acid, followed by removal of solvent and excess ethoxyacetylene. These compounds were found to phosphorylate alcoholic, phenolic and nucleosidic hydroxyls, phosphoric acids, amines and carboxylic acids, the only other product being ethyl acetate.



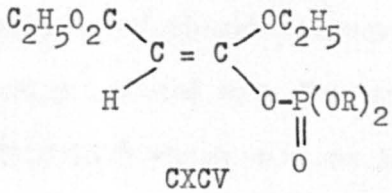
CXCIII

Thus (CXCIV) was allowed to react with the pyridinium salt of uridine-5'-monophosphate at room temperature for three days, and the product isolated after successive anionic and hydrogenolytic debenzylation (Scheme 119). Comparison by paper chromatography with standard samples of UMP and UDP showed that the product (c. 15% yield) contained UDP and UMP in the ratio 9:1.



SCHEME 119

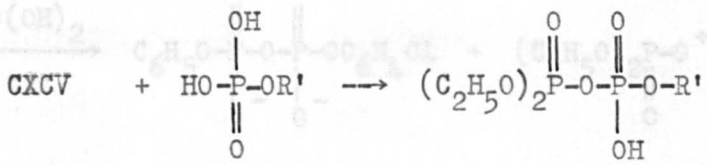
Derivatives of ketene acylals of phosphoric acid have proved to be good phosphorylating agents in a similar manner (CXCV).[249]



CXCV

Thus the reaction of (CXCV; R = C_2H_5) with adenosine-5'-monophosphate results in the formation of the diethyl ester of

adenosine-5'-diphosphate. When nucleotides, such as thymidine-3'-phosphate or adenosine-3'-phosphate are condensed with (CXCV), the diethyl thymidyl and adenyl pyrophosphates formed initially can undergo further reaction to form oligonucleotides.[250,251] The reaction of (CXCV) with monoesters of phosphoric acid enables unsymmetrical triesters of pyrophosphoric acid to be synthesised (Scheme 120).[116]

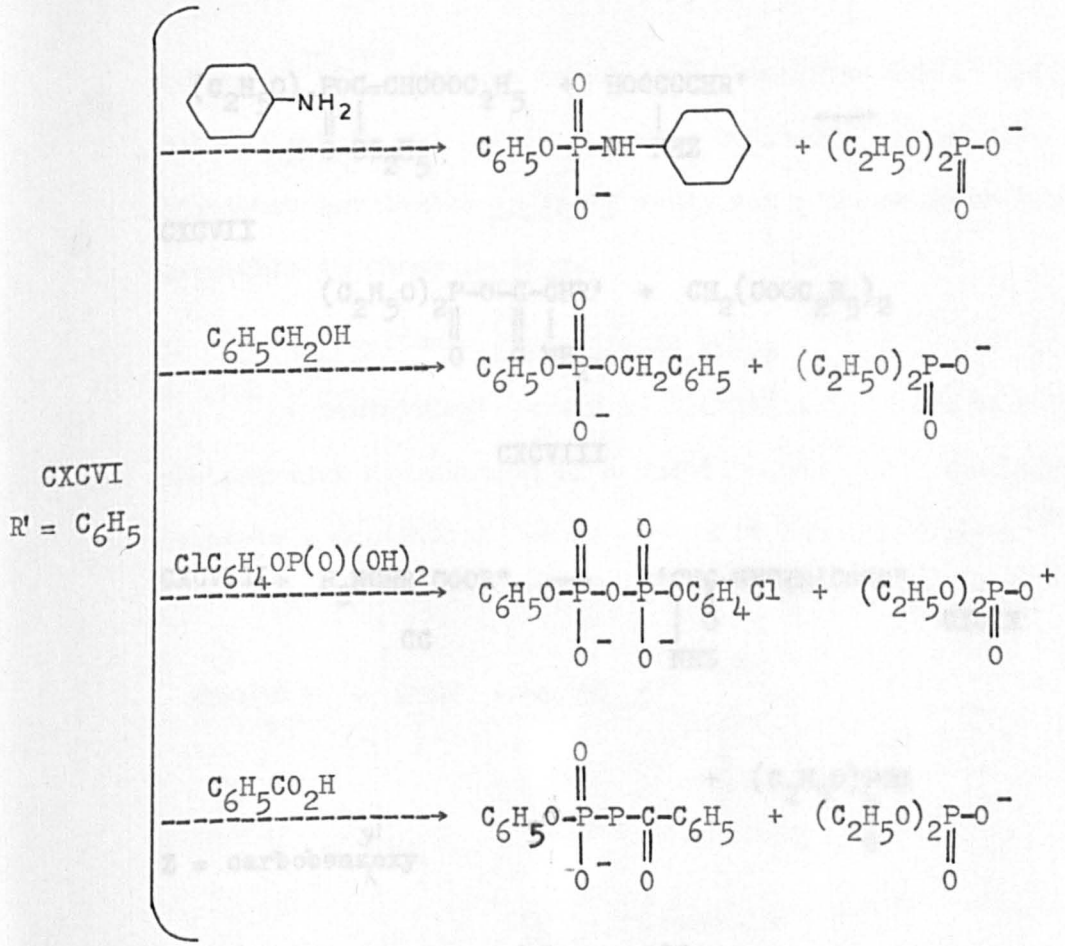


CXCVI

+ malonic ester

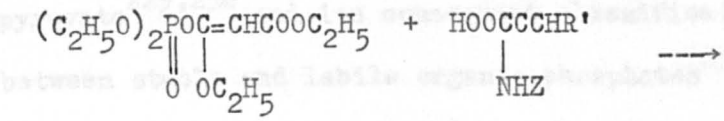
Scheme 120

(CXCVI) will react with acids, amides, alcohols and phosphoric acid monoesters to give acyl phosphates, amides of phosphoric acid, unsymmetrical phosphoric acid esters and unsymmetrical pyrophosphates, respectively (Scheme 121) and thus (CXCVI) is a valuable phosphorylating agent.

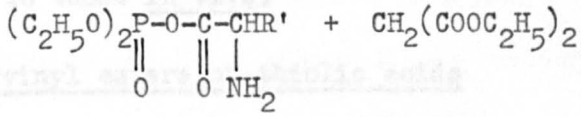


Scheme 121

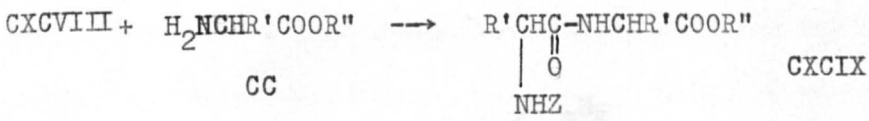
Ketene acylals have also been the starting point of a peptide synthesis, which by analogy with the enzymatic synthesis,[252,253] involves formation of anhydrides of phosphoric acid and amino-acids.[254,255] Thus (CXCVII) readily reacted with N-protected amino-acids, giving the mixed anhydride (CXCVIII) which without isolation yielded dipeptide derivatives (CXCIX) in high yields on subsequent aminolysis with esters or salts of other amino-acids (CC) (Scheme 122).

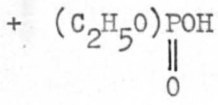


CXCVII



CXCVIII





Z = carbobenzoxy

Scheme 122

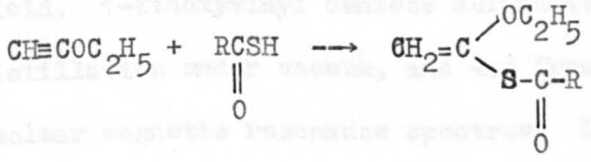
N-carbobenzyloxyglycylglycine and N-carbobenzyloxyglycyl-D,L-phenylalanine, for example, were synthesised in yields of 82 and 61% by this method, using the corresponding amino-acid derivatives.[254,255] Similar results are obtained using ethoxyvinyl dibenzyl phosphate in the Anderson-Callahan systems.

The reaction of phosphoenol pyruvate with phosphoric acids, although having a very important role in carbohydrate metabolism - namely the enzymatic transphosphorylation of adenosine-5'-monophosphate to form adenosine-5'-diphosphate and pyruvic acid - has not yet been achieved <u>in vitro</u>. The rather slow acid hydrolysis of phosphoenol

pyruvate[229,256] and its consequent classification as intermediate between stable and labile organic phosphates[257] indicate that transphosphorylation *in vitro* would not proceed under conditions comparable to those *in vivo*.

3. Ethoxyvinyl esters of thiolic acids

1-Ethoxyvinyl esters of thiolic acids have been prepared in the present work by reaction of a thiolic acid with ethoxyacetylene with a mercuric ion catalyst, or an excess of ethoxyacetylene[222] (Scheme 123).



R = CH_5 or C_4H_5

Scheme 123

These esters were also demonstrated to be good acylating agents. Thus acetanilide was isolated from the reaction of the 1-ethoxyvinyl thiolacetate and aniline in good yield. In this case the other product of the acylation was ethylthionacetate. This reaction was confirmed by Wasserman and co-workers, who, in addition to the normal product, observed anti-Markownikow addition of the acid to ethoxyacetylene due to free-radical inducing contaminants in the acid used, this being avoided in the present work by careful distillation of the acid.

4. Enol sulphonates

Arens was unable to isolate 1-ethoxyvinyl sulphonates from the

reaction of ethoxyacetylene with sulphonic acids.[298] Difficulty in isolating these esters was also experienced in the present work, even using a mercuric-ion catalyst or an excess of the ethoxyacetylene in the reaction between the sulphonic acid and ethoxyacetylene. The esters were very unstable, and immediate anhydride formation was observed on exposure to the atmosphere. Even when crude 1-ethoxyvinyl methylsulphonate was distilled under vacuum the distillate still contained some 20% anhydride. This product, however, underwent reaction with aniline, methane sulphonanilide being isolated from the product in 63% yield. 1-Ethoxyvinyl benzene sulphonate was, however, purified by distillation under vacuum, and was free from anhydride according to its nuclear magnetic resonance spectrum. It was, however, again too unstable for a successful elemental analysis. From the products of the reaction between this ester and aniline, benzene sulphonanilide was isolated in 72% yield, illustrating the acylating properties of these enol esters once again.

C. Non-isolatable Intermediates

1. Ethoxyacetylene

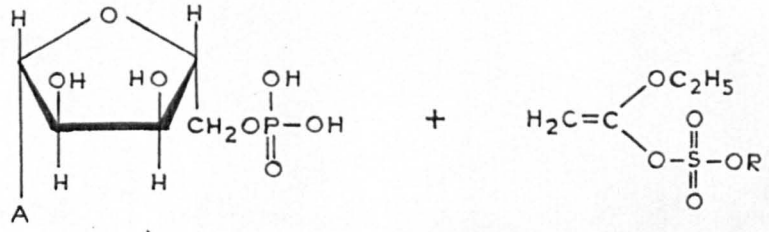
Many interesting nucleotide and coenzyme syntheses have been accomplished by reaction of monoesters of phosphoric and other acids with ethoxyacetylene. The intermediate 1-ethoxyvinyl ester has not been isolated owing to instability, but has been reacted with a nucleophile *in situ*. That such an intermediate has been formed has been demonstrated in many cases by the infrared spectrum of the reaction

products, the spectra of which displayed peaks characteristic of 1-ethoxyvinyl esters at c. 5.7 and 5.95μ. For carboxylic esters, the 5.7μ band represents C=O stretching for an enol ester, and that at 5.95 has been ascribed to $>C=CH_2$ stretching. Thus in 1-substituted vinyl acetates and ketene acetals, for example, the latter band appears at approximately 6.0μ.[258] In 1-ethoxyvinyl phosphates, sulphonates and sulphates, however, the band at 5.7μ cannot be ascribed to $>C=O$ stretching, and awaits identification.

This method of synthesis was utilised for the preparation of adenosine-5'-sulphatophosphate (CCI) described in detail in the Experimental Section. The 1-ethoxyvinyl esters generated by reaction of ethoxyacetylene with benzyl- or 2-cyanoethyl sulphates could not be purified for elemental analysis by distillation under high vacuum, and so, after removal of excess ethoxyacetylene and solvents, they were condensed with pyridinium adenosine-5'-phosphate. The sulphatophosphate was formed in good yields after hydrogenolytic debenzylation or alkaline hydrolysis respectively. (Scheme 124).

The only product from the reaction of phthalic acid and excess ethoxyacetylene was phthalic anhydride. When the reaction was performed in the presence of an amino-acid ester, however, the phthaloyl protected amino-acid ester (CCII) could be isolated in good yield.

This reaction could proceed by initial formation of the anhydride (CIII) and subsequent attack by the amino-acid ester, followed by reaction of the product (CCIV) with ethoxyacetylene to give (CCV)

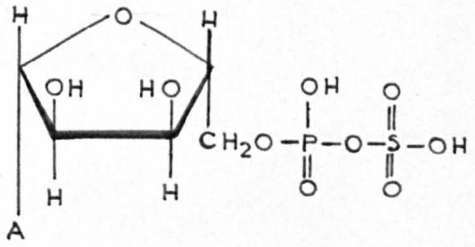


(1) 5 days

(2) Hydrogenolysis (R = $C_6H_5CH_2$) or alkaline hydrolysis (R = $NCCH_2CH_2$)

A = adenine

R = $C_6H_5CH_2$ or $NCCH_2CH_2$

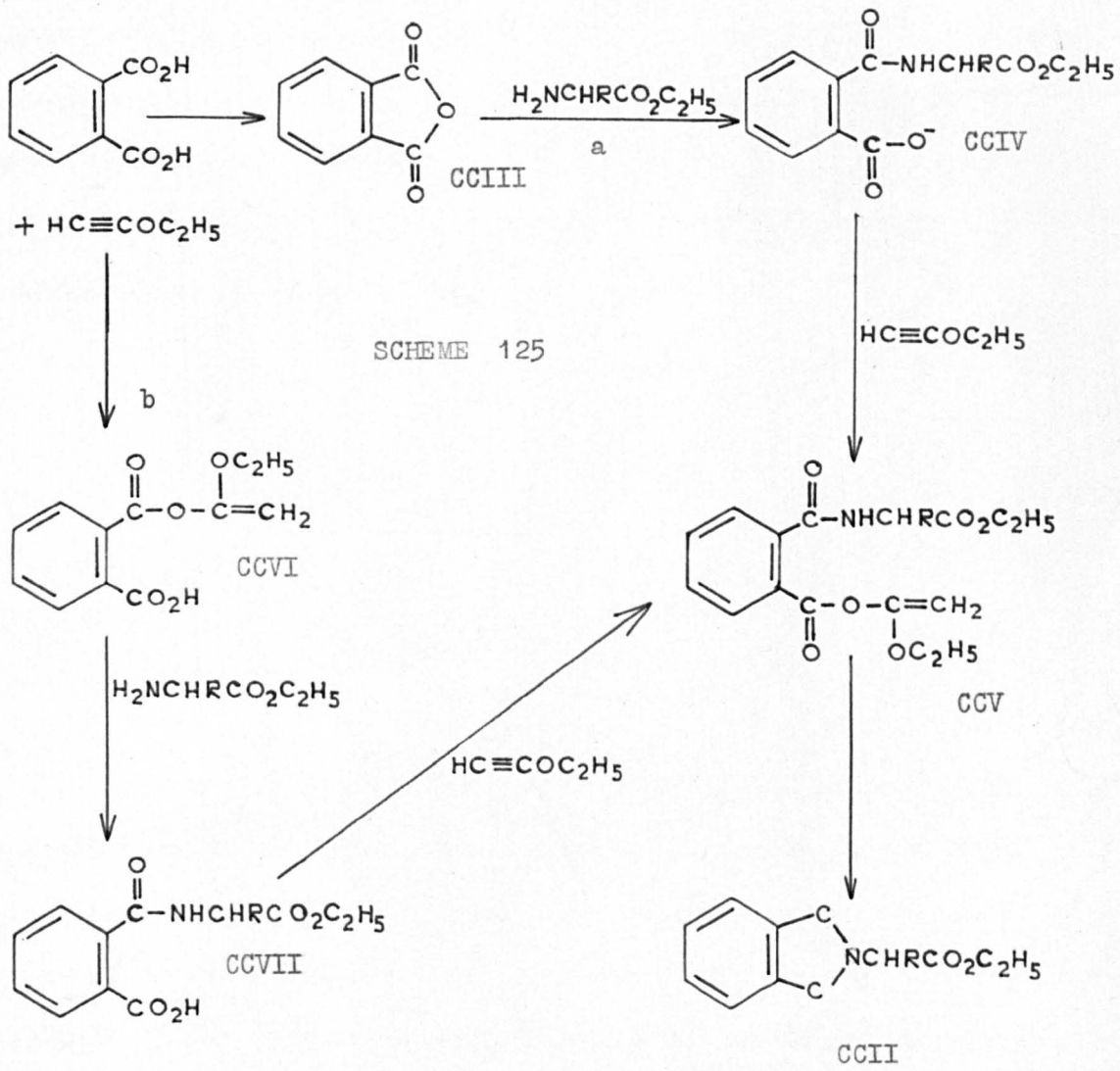


CCL

SCHEME 124

which could then form the protected amino-acid (CCII) (Path a, Scheme 125). Alternatively, one mole of ethoxyacetylene could react with phthalic acid, the resulting 1-ethoxyvinyl ester (CCVI) then being attacked by amino-acid ester to form (CCVII), followed by the reaction of the second carboxylic acid group with ethoxyacetylene to give (CCV) and subsequent formation of the protected amino-acid ester (CCII; Path b,

Scheme 125).

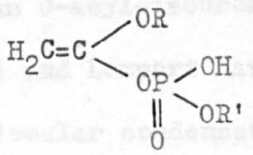


SCHEME 125

No racemisation was detected if an optically-active amino-acid ester was used, and this appears to be a promising method for end-group labelling or protecting peptides (see Experimental Section and Discussion).

The fact that some 1-ethoxyvinyl phosphates proved to be too unstable for isolation and purification has been mentioned previously.

However, Wasserman and Cohen were able to synthesise nucleoside derivatives and coenzymes by reaction of theappropriate 1-ethoxyvinyl phosphate with a nucleophile *in situ*.[129,130] Thus adenosine-5'-phosphate as either the pyridinium or the triethylammonium salt was allowed to react with ethoxyacetylene to produce (CCVIII; R = C_2H_5; R' = adenosine-5', not isolated) which in methanol solution was slowly converted to the corresponding monomethyl ester.



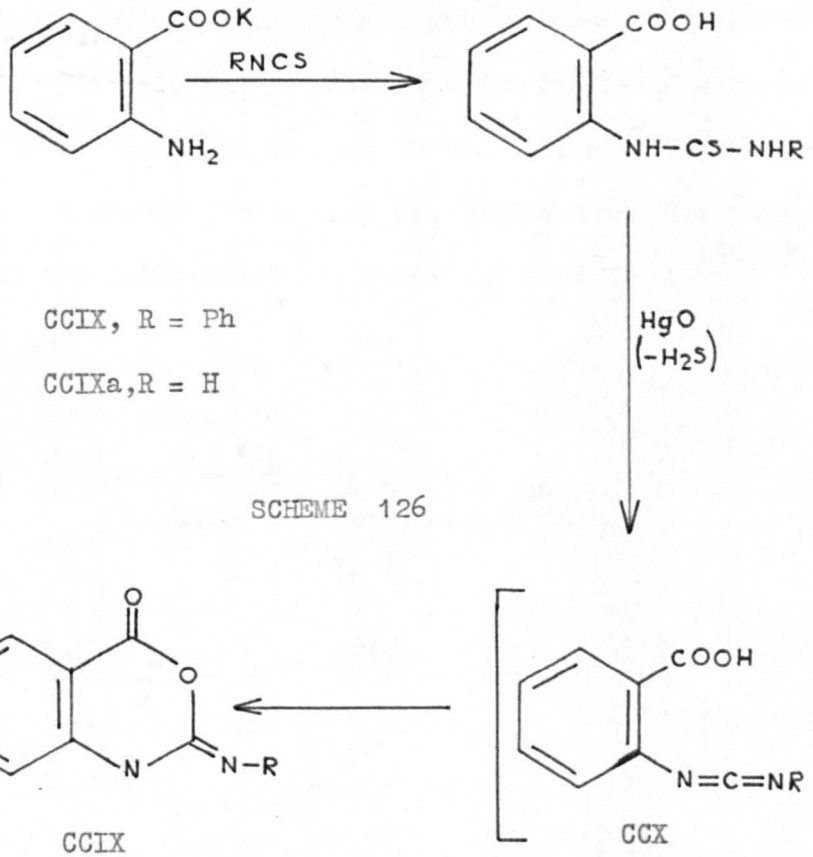
CCVIII

This method also proved to be useful in the synthesis of internucleotidic linkages. When pyridinium 3'-acetylthymidine-5'-phosphate was allowed to react with an excess of ethoxyacetylene, followed by 5'-tritylthymidine, thymidine-3'-thymidine-5'-phosphate was isolated in 33% yield after detritylation and deacetylation. Finally, the method proved suitable for a synthesis of FAD. (CCVIII; R = C_2H_5, R' = Adenosine-5') was condensed with riboflavin-5'-monophosphate, and after isolation and purification of the product, FAD was obtained in 10 - 15% yield. The advantage of this method over the dicyclohexylcarbodiimide method, stems from the fact that the adenosine-5'-phosphoric acid could be activated and excess ethoxyacetylene removed before the addition of

riboflavin phosphate. Thus formation of the cyclic-4',5'-riboflavin phosphate (the major product in the carbodiimide route[259]) was kept to a minimum. Furthermore, this method retained the simplicity of the carbodiimide route in that the protecting groups used by earlier workers[108] were not needed, thus avoiding losses experienced in debenzylation, deacetylation, etc.

2. Carbodiimides

In the condensation of acids with nucleophiles in the presence of carbodiimides, an O-acyl-isourea has been postulated as an intermediate, although Doleschall and Lempert have isolated a related cyclic O-acyl-isourea by intramolecular condensation of an O-carboxyphenyl-carbodiimide. Examination of its reactions with different nucleophiles, gives support to the above mechanism.[260] Reaction of potassium anthranilate with phenyl isothiocyanate formed O-phenylthioureido-benzoic acid. This, on treatment with mercuric oxide in acetone readily loses one mole of hydrogen sulphide yielding 2,1-H-phenylimino-3,1,4H-benzoxazin-4-one (CCIX) by cyclisation of the expected 2-carboxy-diphenylcarbodiimide (CCX) (Scheme 126).

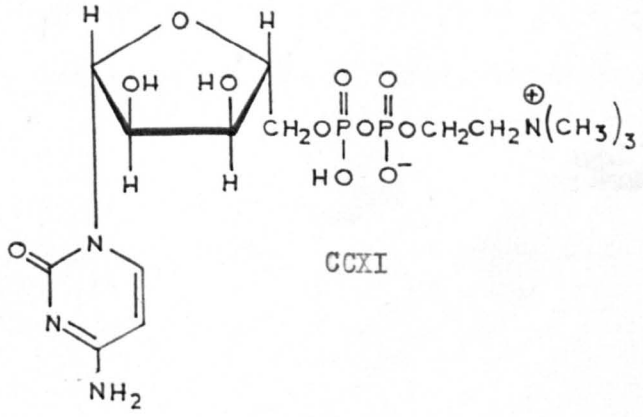


SCHEME 126

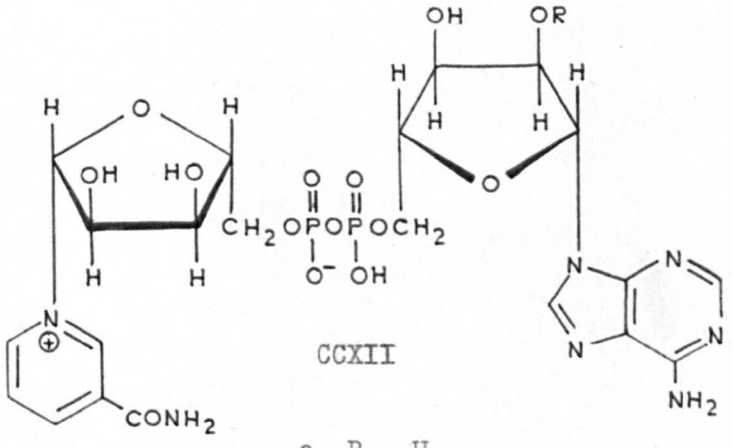
(CCIXa) and its hydrochloride reacted with alcohols, leading to O-ureido-benzoic esters. It should be noted that the hydrochloride was found to react more readily than the free base,[261] thus proving the susceptibility of these reactions towards acid catalysis, as was postulated by Khorana.[134]

Since the use of dicyclohexyl carbodiimide as a condensing reagent in the synthesis of oligo- and polynucleotides has already been described in detail, a few examples from the coenzyme field will

now be discussed. Kennedy has synthesised cytidine diphosphate choline (CCXI) from cytidine-5'-phosphate and choline phosphate in the presence of carbodiimide in good yield. The symmetrical dicholine phosphate was not formed. Similarly nicotinamide-adenine dinucleotide (NAD) (CCXIIa) was synthesised in relatively good yield.[135,136,262]



CCXI



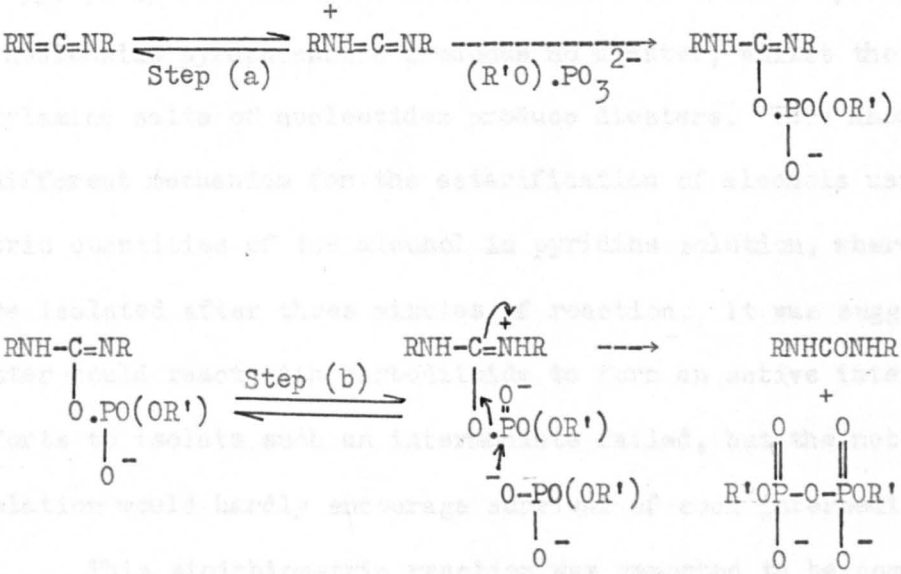
CCXII

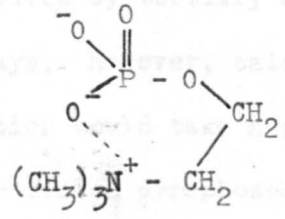
a, R = H

b, R = PO_3H_2

The synthetic route was also shown to be capable of extension to NAD^+P (CCXIIb).

The reason for the predominance of the unsymmetrical pyrophosphate arising from these reactions has been the subject of considerable discussion. Thus Khorana suggests that step (a) (Scheme 127) is dependent on the nucleophilicity of the attacking reagent whilst step (b) is insensitive to this nucleophilicity, although the mechanism in both cases must be similar. Thus he proposed that cylidylic acid, the stronger nucleophile, competes effectively in step (a), but that in step (b) both cytidylic acid and choline phosphate are equally effective.





CCIX

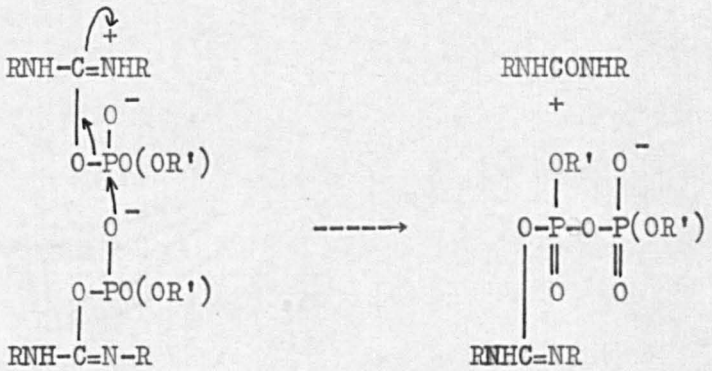
Scheme 127

Cohen, however, has provided an alternative more reasonable hypothesis to accommodate these results.[102] Thus a rapid preferential formation of (CCXIII) by cytidylic acid could occur followed by competition for the protonated isourea by choline phosphate (CCIX) and the comparably nucleophilic monoanion of (CCXIII).

Khorana also supports the view that step (b) (Scheme 127) is insensitive to nucleophilicity by quoting the formation of methyl esters of nucleotides from the reaction of nucleotides in methanol in the presence of carbodiimides. He maintains that this reaction cannot proceed via pyrophosphate formation since addition of tri-n-butylamine salts of nucleoside pyrophosphate produces no diester, whilst the tri-n-butylamine salts of nucleotides produce diesters. Thus Khorana postulates a different mechanism for the esterification of alcohols using stoichiometric quantities of the alcohol in pyridine solution, where pyrophosphates were isolated after three minutes of reaction. It was suggested that the latter would react with carbodiimide to form an active intermediate. Efforts to isolate such an intermediate failed, but the methods of attempted isolation would hardly encourage survival of such intermediates.

This stoichiometric reaction was reported to be completely inhibited by tertiary base, the only product being pyrophosphate even after 12 days. However, calculations have led to the conclusion that the reaction could take a period of years for completion.[102] If, however, an activated pyrophosphate (Scheme 128) (CCX) was formed, even in the presence of a tertiary base, this could be attacked very slowly by alcohols, so that reaction is only observed when the alcohol is present in

a large excess.



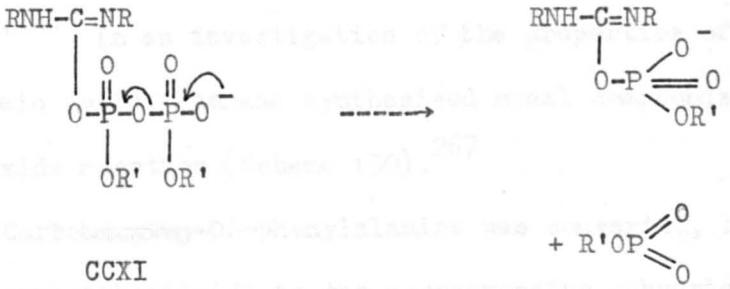
CCX

Scheme 128

If a tertiary-base is absent, attack by the alcohol would be more rapid, and in the reaction of a nucleotide with carbodiimide, polymerisation is observed (see earlier section). Thus this observed difference in reactivity of the alcohol and phosphate anion tends to disprove the suggestion that step (b) (Scheme 127) is insensitive to the nucleophilicity of the attacking reagent. It was also discovered that from the reaction of di-p-nitrophenyl phosphoric acid with carbodiimide in methanol, a methyl isourea was obtained, suggesting that the alcohol was competing successfully for the protonated carbodiimide[263] i.e. the nucleophilicities were comparable.

Involvement of metaphosphate has also been suggested in this type of mechanism[86,102] Thus (CCXI), which is in effect the anhydride of a monoester of phosphoric acid with a much stronger acid, could yield

metaphosphate, which would be the phosphorylating entity (Scheme 129).



CCXI

Scheme 129

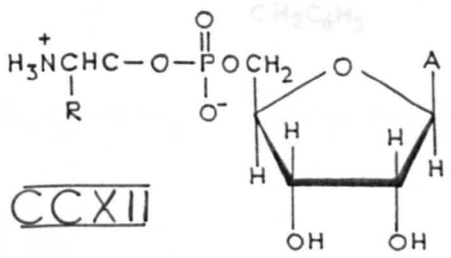
This mechanism could also explain the fact that in the presence of a tertiary base monoesters of phosphoric acid undergo pyrophosphate formation, whilst diesters do not, (Scheme 128) being open to the former, but not the latter.

Uridine diphosphate glucose, and FAD have also been synthesised by the carbodiimide method by condensation of the two phosphate entities but the method proved to be very unsatisfactory, since cyclic phosphate formation is the preferred reaction, and yields of the coenzymes were exceedingly low.[259,264] Adenosine-5'-sulphatophosphate, has also been synthesised by condensation of adenosine-5'-phosphoric acid with sulphuric acid using dicyclohexylcarbodiimide in pyridine.

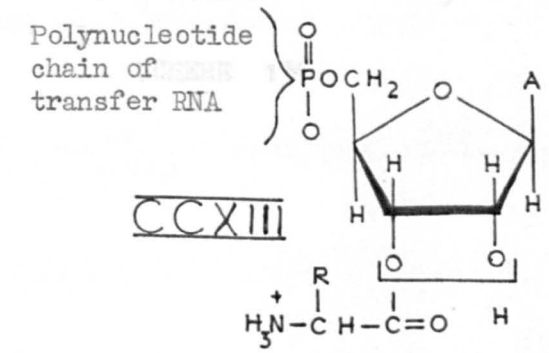
The first steps in the enzymatic synthesis of a polypeptide chain are the activation of α-amino-acids by reaction with adenosine-5'-triphosphate

to form the mixed anhydrides (CCXII) with AMP and pyrophosphate, and the subsequent transfer of the amino-acid groups to the terminal adenosine residues (CCXIII) of a transfer-RNA molecule. The aminoacyl group has been shown to be linked to the 2'-O or 3'-O of a terminal adenosine unit.[265,266] In an investigation of the properties of amino-acyl-ribonucleic acids, Khorana synthesised model compounds using a carbodiimide reaction (Scheme 130).[267]

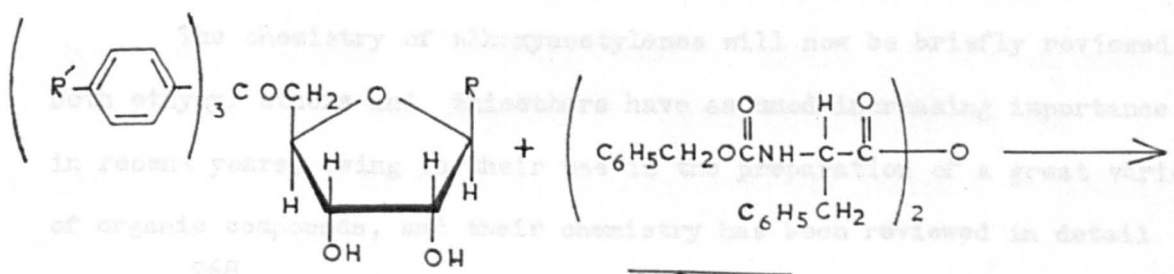
Carbobenzyloxy-DL-phenylalanine was converted, by reaction with dicyclohexylcarbodiimide to the corresponding anhydride (CCXIV). The products of the reaction of the latter with 5'-O-tri-p-methoxytrityl-uridine in pyridine was treated to remove protecting groups and the required amino-acyl nucleoside isolated by partition chromatography.



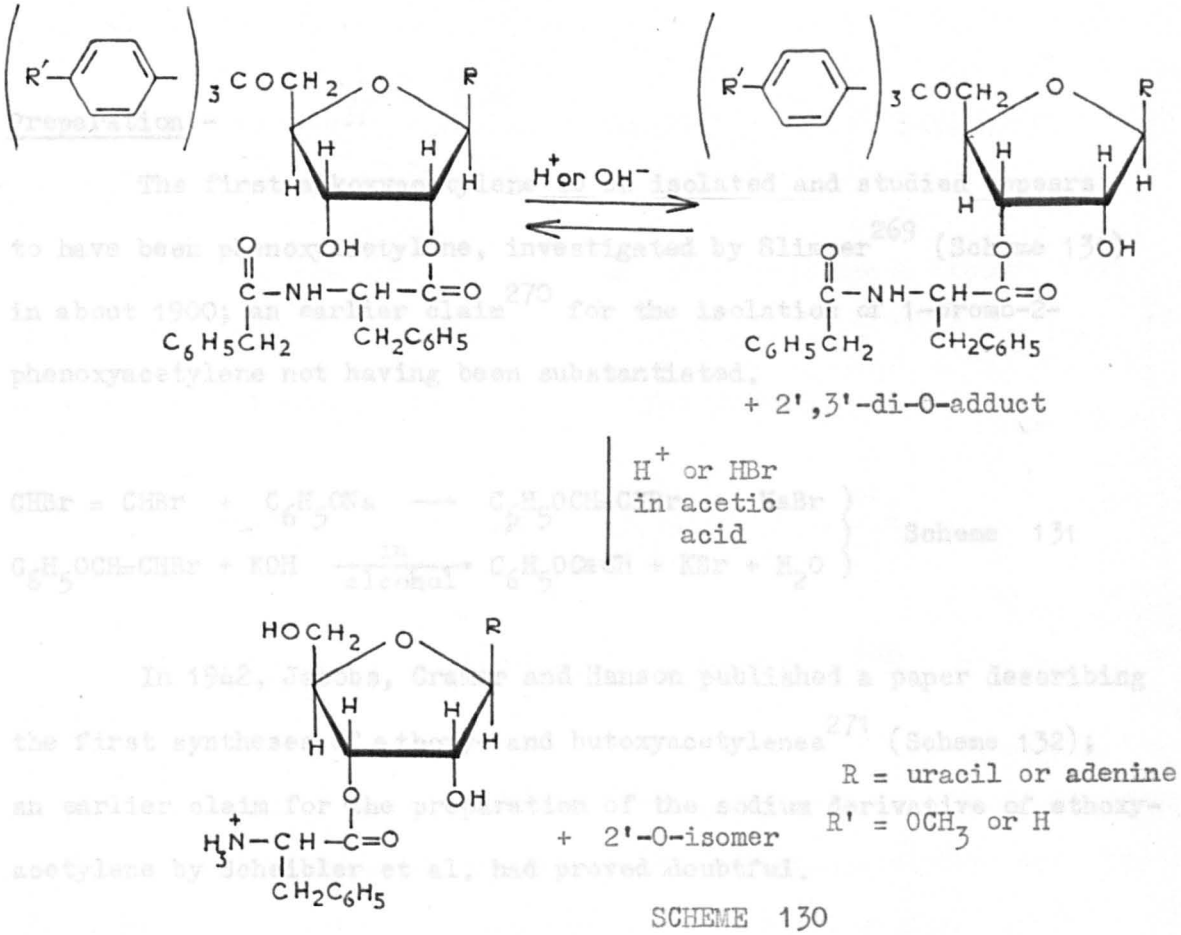
CCXII



CCXIII



CCXIV



SCHEME 130

D. The Chemistry of Alkoxyacetylenes

The chemistry of alkoxyacetylenes will now be briefly reviewed. Both ethynyl ethers and thioethers have assumed increasing importance in recent years, owing to their use in the preparation of a great variety of organic compounds, and their chemistry has been reviewed in detail by Arens.[268]

Preparation:-

The first alkoxyacetylene to be isolated and studied appears to have been phenoxyacetylene, investigated by Slimmer[269] (Scheme 131) in about 1900; an earlier claim[270] for the isolation of 1-bromo-2-phenoxyacetylene not having been substantiated.

$$CHBr = CHBr + C_6H_5ONa \longrightarrow C_6H_5OCH{=}CHBr + NaBr$$
$$C_6H_5OCH{=}CHBr + KOH \xrightarrow[\text{alcohol}]{\text{in}} C_6H_5OC{\equiv}CH + KBr + H_2O$$

Scheme 131

In 1942, Jacobs, Cramer and Hanson published a paper describing the first syntheses of ethoxy- and butoxyacetylenes[271] (Scheme 132); an earlier claim for the preparation of the sodium derivative of ethoxy-acetylene by Scheibler et al. had proved doubtful.

$$BrCH_2CH(OR)_2 \xrightarrow{Br_2} Br_2CHCH(OR)_2 \xrightarrow[\text{alcohol}]{Zn} BrCH{=}CHOR \xrightarrow{KOH} HC{\equiv}OR$$

Scheme 132

A method developed by Eglington, Jones, Shaw and Whiting[273] for the elimination of HX (X = halogen) and ROH from α,β-halogeno-ethers in the presence of sodamide in liquid ammonia (Scheme 133) was extended to the preparation of alkoxy-acetylenes.[274] Thus ethoxyacetylene was prepared in 60% yield using diethyl chloroacetal and sodamide in liquid ammonia (Scheme 134).

$$R'CH(OR)\text{-}CHXR'' \xrightarrow{NaNH_2} R'C{\equiv}CR''$$

Scheme 133

$$ClCH_2\text{-}CH(OC_2H_5)_2 \xrightarrow[NH_3]{NaNH_2} CH{\equiv}COC_2H_5$$ Scheme 134

Aldehydes are the starting point of a preparation (Scheme 135) discovered by Ficini,[275] which was developed by Arens[276,277] for the preparation of a whole spectrum of alkoxyacetylenes.

$$R'CH_2CHO + ROH + HCl \longrightarrow R'CH_2CHCl(OR) \xrightarrow[5\text{-}10^\circ]{Cl_2}$$

$$R'CHClCHCl(OR) \xrightarrow[\text{or pyridine}]{\text{Diethylaniline}} R'CCl{=}CHOR \xrightarrow{KOH} R'C{\equiv}COR$$

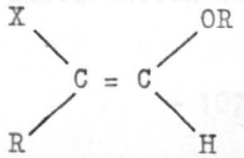
Scheme 135

The method used for the preparation of ethoxyacetylene in the present work is a modification (see Experimental Section) of the one developed by Nazarov, Krasnaia and Vinogradov[278] (Scheme 136).

$$CH_2{=}CHOC_2H_5 \xrightarrow[-30^\circ]{Br_2} CH_2Br\text{-}CHBr(OC_2H_5) \xrightarrow{\text{Diethylaniline}}$$

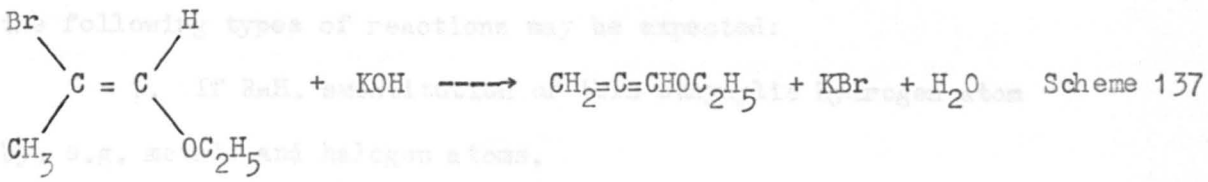
$$CHBr{=}CHOC_2H_5 \xrightarrow{KOH} HC{\equiv}COC_2H_5$$ - Scheme 136

The β-halogeno α,β-unsaturated ethers that result in the last two preparations are obtained as mixtures of the cis and trans isomers, the latter (CCXV) predominating.



CCXV

The pure isomers can be isolated by careful fractional distillation.[278] It is very fortunate that the original mixtures are rich in the product with trans α-hydrogen and β-halogen, because this configuration, in accordance with the general rule for trans elimination during dehydrohalogenations with bases, favours the formation of alkoxyacetylenes. The cis isomer reacts sluggishly and can be recovered from the reaction residues, although Ficini[275] has reported that a cis-rich mixture of β-bromopropenyl ethyl ether, on prolonged heating with potassium hydroxide under distillation conditions yields ethoxypropyne contaminated with much ethyl allenyl ether (Scheme 137).



Scheme 137

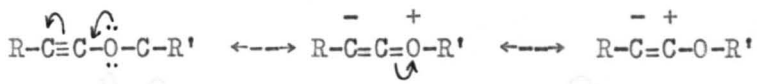
Properties:-

The alkoxyacetylenes, HC≡COR and 2-aralkyl-1-alkoxy-1-alkynes, RC≡COR', are mobile, colourless liquids. The lower members have objectionable, musty odours, the higher ones smelling sweeter. Phenoxyacetylene is rather unstable and polymerises at room temperature to a deep red liquid and then to a solid. The alkoxyacetylenes are more stable and, when pure and sealed in tubes, can be stored at 0°C for some time. On exposure to air, a yellow and finally a brown colour develops, but by distillation most of the material can be recovered

unchanged. Ethoxyacetylene is toxic, inhalation of its vapours causing headaches, and may be dangerous. Jacobs, Cramer and Hanson[271] reported that mice are killed, even on short exposure to low concentrations of ethoxyacetylene.

Reactions:

From a consideration of the structure of the alkoxyacetylenes shown in (CCXVI)



CCXVI

the following types of reactions may be expected:

1. If R=H, substitution of this ethynylic hydrogen atom by, e.g. metals and halogen atoms.

2. Attack by electrophilic reagents on the 2-carbon atom.

3. Attack by nucleophilic reagents on the 1-carbon atom.

4. If R = alkyl group, allenic rearrangements.

5. Reactions of the ether function.

6. Polymerisations.

7. Ring closures involving both acetylenic carbon atoms of ethoxyacetylene.

All seven types of reactions are known, as well as some free-radical additions.

This situation may be briefly contrasted with that for ethynyl thioethers, in which there are several indications that, owing to the

ability of the sulphur atom on the one hand to share its lone pairs of electrons with electron deficient groups and on the other hand to accommodate a decet of electrons in its valence shell, the -SR group can act as an electron donor or as an acceptor, according to the nature of the other reacting species. Electrophilic reagents will invoke electron release from the -SR group, and will therefore become attached to the β-carbon atom; nucleophilic reagents, by inducing the reverse polarisation, also become attached to the β-carbon atom. This is summarised in (CCXVII)[279,280].

Polarisation of ethynyl thioethers during reactions with electrophilic reagents

Polarisation of ethynyl thioethers during reactions with nucleophilic reagents

CCXVII

Some of the above seven types of reactions of alkoxyacetylenes will now be considered in greater detail.

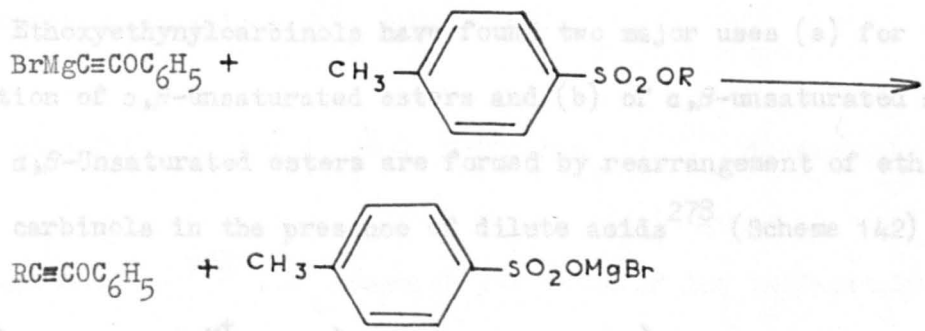
1. Reactions of the ethynylic hydrogen atom. - The ethynylic hydrogen atom is easily replaceable by heavy metals, e.g. silver, copper and mercury. The most useful metallic derivatives of alkoxyacetylenes are, however, those which are readily formed by reaction with Grignard reagents or lithium compounds, usually in ethereal solution (Schemes 138 and 139).

$$HC{\equiv}COR + C_2H_5MgBr \longrightarrow BrMgC{\equiv}COR + C_2H_6$$ - Scheme 138

$$HC{\equiv}COR + C_6H_5Li \longrightarrow LiC{\equiv}COR + C_6H_6$$ - Scheme 139

The Grignard derivatives form oily complexes insoluble in ether but soluble in benzene, whereas the lithium analogues are ether soluble to a certain extent. The sodium derivative of phenoxyacetylene has also been obtained by direct reaction of the ethynyl ether with finely divided sodium in ether.[269,281] The sodium derivative of ethoxyacetylene, prepared from cis- or trans-ethyl β-chlorovinyl ether with sodamide in liquid ammonia, is of considerable preparative importance because the solution can be used directly for reactions with carbonyl compounds.[282]

These metal derivatives can be used for the synthesis of alkynated products of the type RC≡COR'. Thus 1-phenoxybutyne and 1-phenoxy-1-hexyne are obtained from phenoxyacetylene magnesium bromide and ethyl or butyl p-toluenesulphonate in ether[281] (Scheme 140).



Scheme 140

Attempts to prepare halogenated alkoxyacetylenes by reaction of the Grignard reagent with free halogen or hypohalite were not fully successful, probably because of the pronounced instability of the products.[268,283]

One of the most important use of metallated ethynyl ethers is for the synthesis of carbinols by reaction with carbonyl compounds[268,278] (Scheme 141).

$$RR'C{=}O \;+\; MC{\equiv}COC_2H_5 \longrightarrow RR'C(OM)C{\equiv}COC_2H_6$$

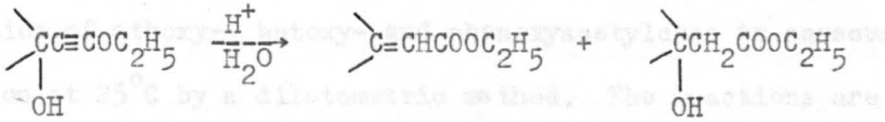
$$\xrightarrow[H_2O\ (M = Na \text{ or } Li)]{NH_4Cl\ (M = MgBr)} RR'C(OH)C{\equiv}COC_2H_5$$

Scheme 141

The Grignard derivatives of ethoxyacetylene cannot usually be employed for the preparation of secondary carbinols from aldehydes[284] because these compounds react further with the initially formed magnesium bromide carbinolates. Such complications are avoided by using ethoxyacetylenelithium in ether[285] or the sodium derivative in liquid ammonia[282] provided that the aldehyde is stable in ammonia.

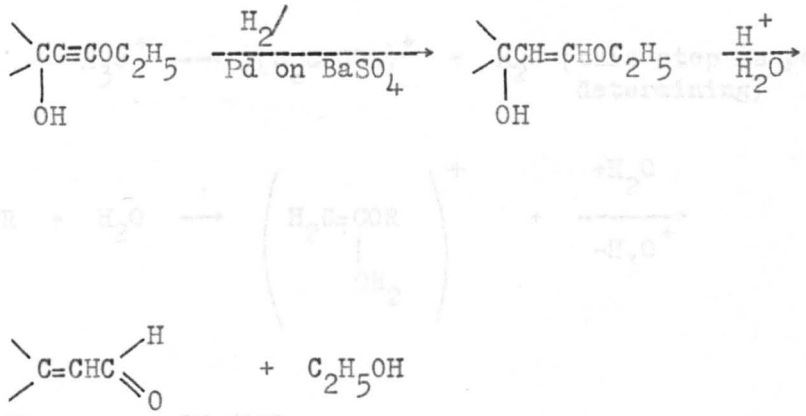
Ethoxyethynylcarbinols have found two major uses (a) for the preparation of α,β-unsaturated esters and (b) of α,β-unsaturated aldehydes.

α,β-Unsaturated esters are formed by rearrangement of ethoxyethynyl carbinols in the presence of dilute acids[278] (Scheme 142).



Scheme 142

Partial reduction of the triple bond by hydrogen and a palladium catalyst on barium sulphate, followed by acid treatment gives the α,β-unsaturated aldehydes.[278] (Scheme 143).



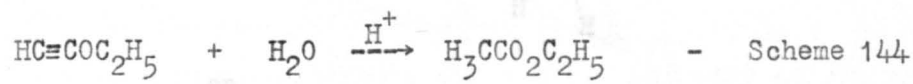
Scheme 143

This method has been used for the synthesis of all-trans and of a cis-Vitamin A aldehyde.

2. Attack by Electrophilic Reagents

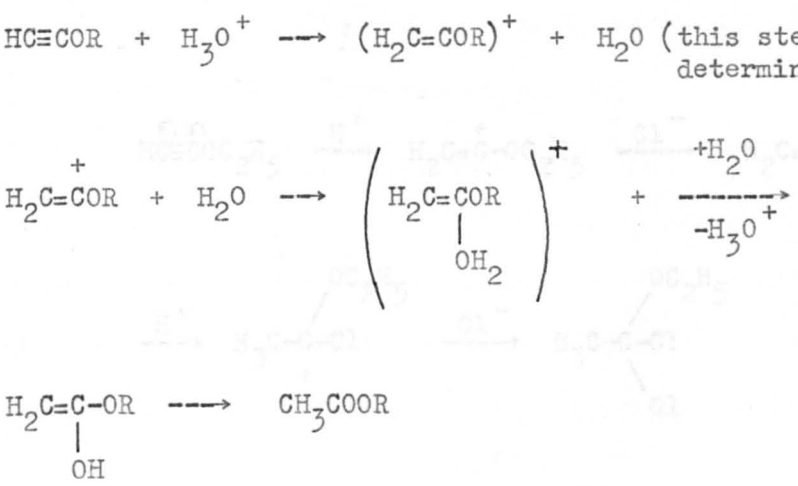
(a) Acid-catalysed hydration

Shaking alkoxyacetylenes with dilute mineral acids results in their hydration to esters.[271] (Scheme 144).



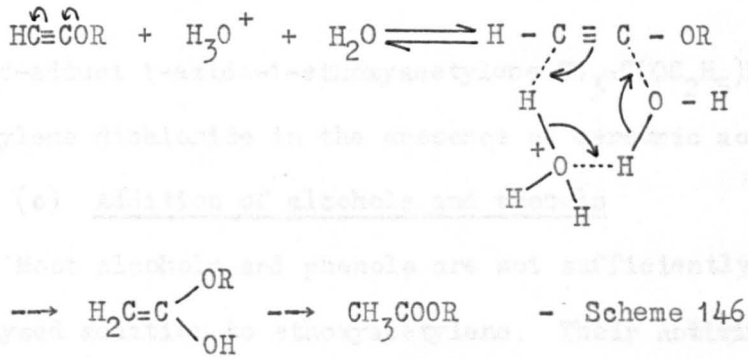
Scheme 144

Jacobs and Searles[286] have measured the rates of the acid-catalysed hydration of ethoxy-, butoxy- and phenoxyacetylenes in aqueous alcoholic solution at 25°C by a dilatometric method. The reactions are first order with respect to the alkoxyacetylene and hydrogen ion concentration, and these kinetics are consistent with the following mechanism (Scheme 145):



Scheme 145

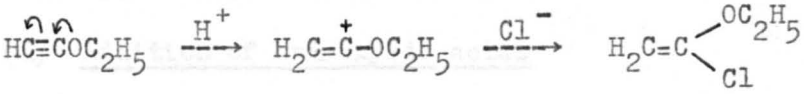
However, a concerted mechanism would also provide a satisfactory rationalisation of the data. (Scheme 146).

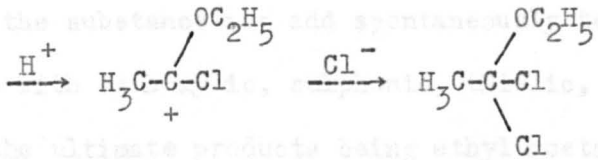


Scheme 146

(b) Addition of anhydrous hydrogen halides and of hydrazoic acid

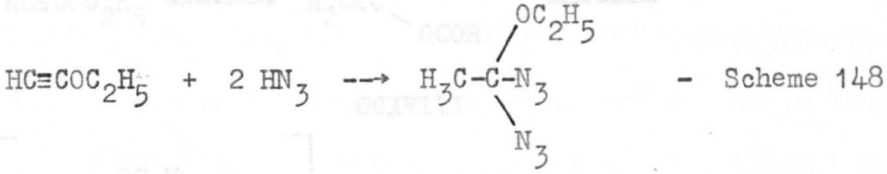
Anhydrous hydrogen halides in ethereal solution are added easily at room temperature to form initially α-halogenvinyl ethers[287] and then saturated α,α-dihalogenoethers[288] (Scheme 147).





- Scheme 147

Hydrazoic acid reacts similarly to give the di-adduct[289] (Scheme 148).

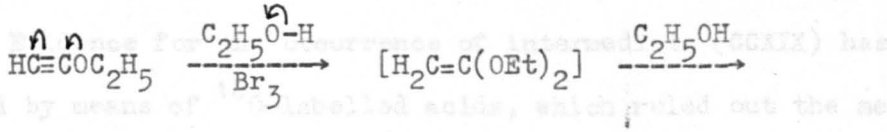


- Scheme 148

The mono-adduct 1-azido-1-ethoxyacetylene $CH_3=C(OC_2H_5)N_3$ can be isolated in methylene dichloride in the presence of mercuric acetate.[268]

(c) Addition of alcohols and phenols

Most alcohols and phenols are not sufficiently acidic to undergo uncatalysed addition to ethoxyacetylene. Their activity can be increased, however, by addition of boron fluoride, occasionally combined with mercuric oxide, and under these conditions addition readily occurs.[271] The catalyst, however, converts ethoxyacetylene into a polymer, and the yields are therefore rather unsatisfactory (Scheme 149).

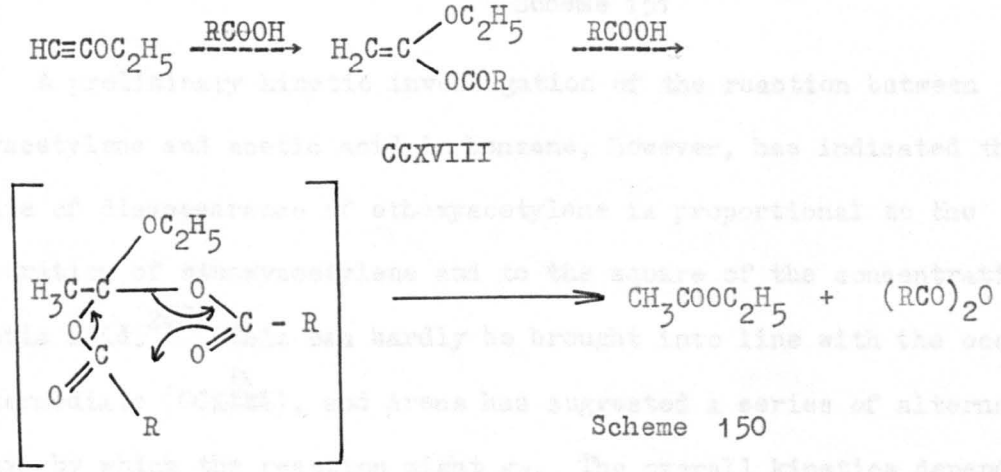


- Scheme 149

Neither **ketene**acetal, postulated as the first intermediate, nor the ortho-ester have been isolated.

(d) Addition of hydroxylic acids

When the hydrogen atom of an -OH group becomes sufficiently acidic, the substance can add spontaneously to alkoxyacetylenes. This is the case with carboxylic, sulphonic, thiolic, sulphuric and phosphoric acids, the ultimate products being ethyl acetate and the anhydride[239,248,273,275] (Scheme 150).



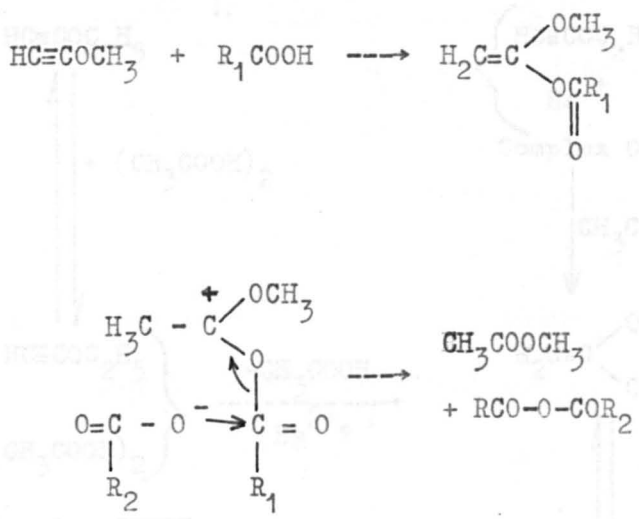
Scheme 150

not isolated

CCXIX

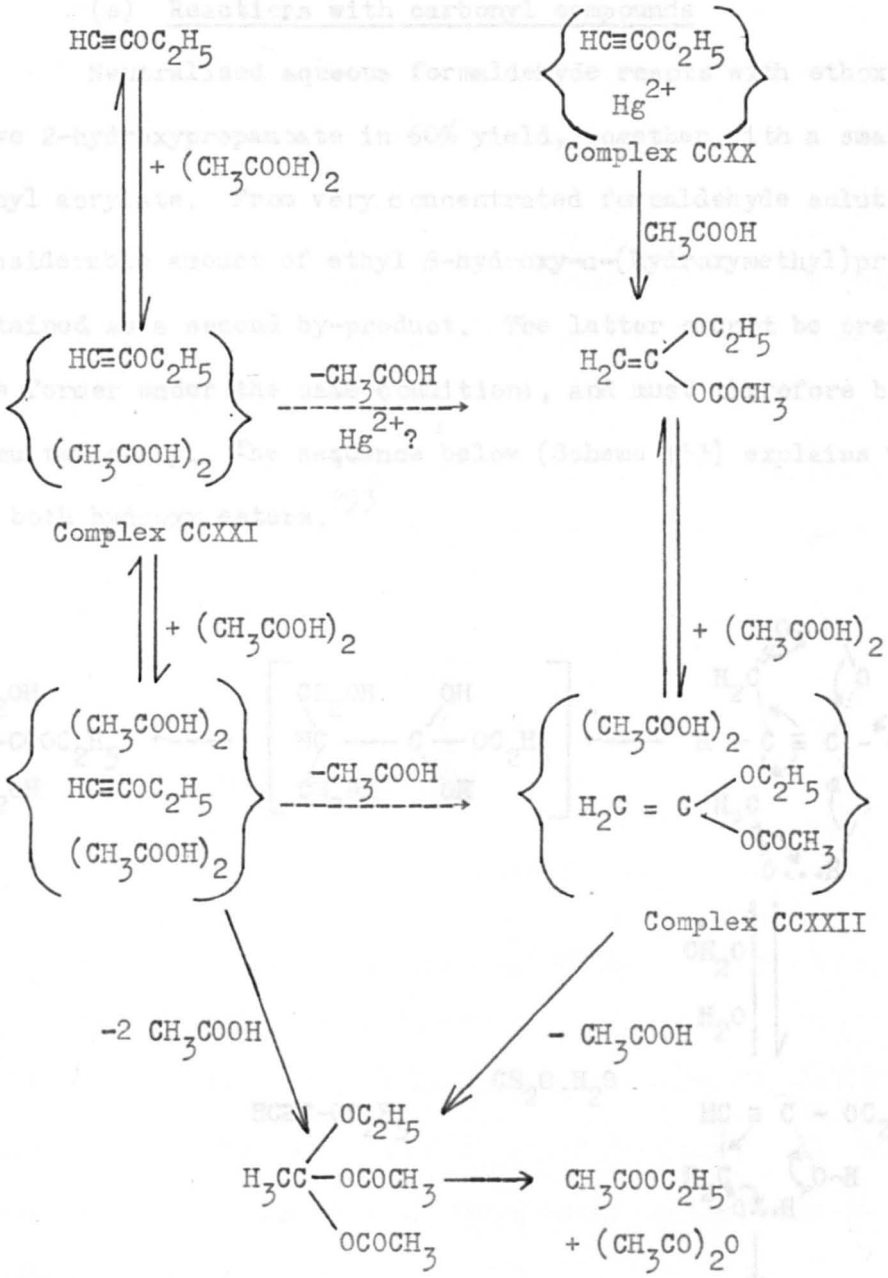
The monoadduct (CCXVIII) has been isolated, either by performing the reaction with an excess of ethoxyacetylene or using a mercuric acetate catalyst.[129,130,221,222,239,244,290,291] These intermediates act as acylating agents, the chemistry of which has been discussed earlier.

Evidence for the occurrence of intermediate (CCXIX) has been acquired by means of ^{18}O-labelled acids, which ruled out the mechanism below[292] (Scheme 151).



Scheme 151

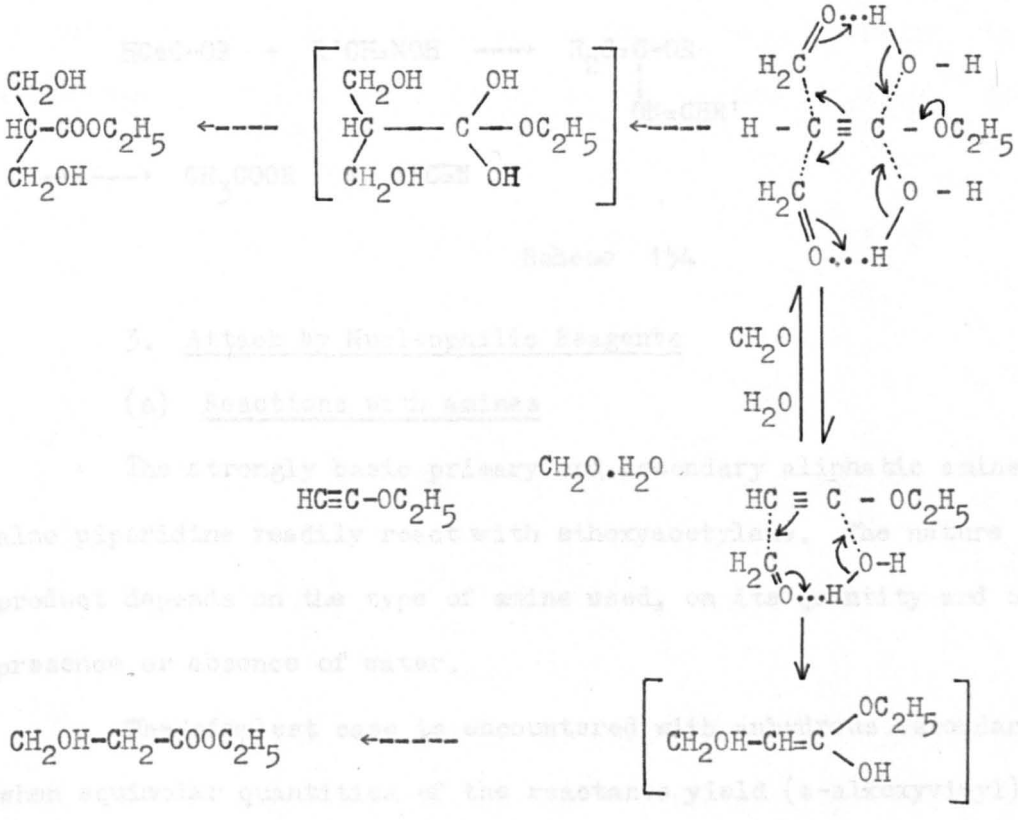
A preliminary kinetic investigation of the reaction between ethoxyacetylene and acetic acid in benzene, however, has indicated that the rate of disappearance of ethoxyacetylene is proportional to the concentration of ethoxyacetylene and to the square of the concentration of acetic acid.[268] This can hardly be brought into line with the occurrence of intermediate (CCXIX), and Arens has suggested a series of alternative pathways by which the reaction might go. The overall kinetics depend on the relative rates of the several steps. These, in turn, determine whether or not (CCXXII) will be an intermediate product. Mercuric ions possible catalyse the irreversible transformation of complex (CCXXI) into (CCXXII) or give rise to the formation of (CCXXII) via complex (CCXX) (Scheme 152).


HC≡COC2H5
+ (CH3COOH)2
HC≡COC2H5
Hg2+
Complex CCXX
CH3COOH
HC≡COC2H5
(CH3COOH)2
-CH3COOH
Hg2+?
H2C=C
OC2H5
OCOCH3
Complex CCXXI
+ (CH3COOH)2
+ (CH3COOH)2
(CH3COOH)2
HC≡COC2H5
(CH3COOH)2
-CH3COOH
(CH3COOH)2
H2C = C
OC2H5
OCOCH3
Complex CCXXII
-2 CH3COOH
- CH3COOH
OC2H5
H3CC—OCOCH3
OCOCH3
CH3COOC2H5
+ (CH3CO)2O

Scheme 152

(e) Reactions with carbonyl compounds

Neutralised aqueous formaldehyde reacts with ethoxyacetylene to give 2-hydroxypropanoate in 60% yield, together with a small amount of ethyl acrylate. From very concentrated formaldehyde solutions a considerable amount of ethyl β-hydroxy-α-(hydroxymethyl)propanoate is obtained as a second by-product. The latter cannot be prepared from the former under the same conditions, and must therefore be formed simultaneously. The sequence below (Scheme 153) explains the formation of both hydroxy esters.[293]



Scheme 153

Analagous reactions have been performed with aqueous solutions of other aliphatic aldehydes and with phenylacetaldehyde,[294] but the yields rapidly decrease with increasing chain length. Ketones and aromatic aldehydes do not react.

(f) Mukaiyama and Hata have shown that it is possible to dehydrate aldoximes using ethoxyacetylene,[295] although attempts by the author to repeat this experiment were unsuccessful. No nitrile, the product of this reaction, was isolated or present in the reaction mixture as demonstrated by vapour chromatography of the reaction mixture (Scheme 154).

$$HC{\equiv}C\text{-}OR + R'CH{=}NOH \longrightarrow H_2C{=}\underset{\displaystyle ON{=}CHR'}{\underset{|}{C}}\text{-}OR \longrightarrow CH_3COOR + R'C{\equiv}N$$

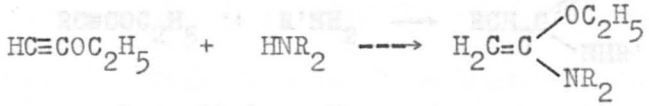
Scheme 154

3. Attack by Nucleophilic Reagents

(a) Reactions with amines

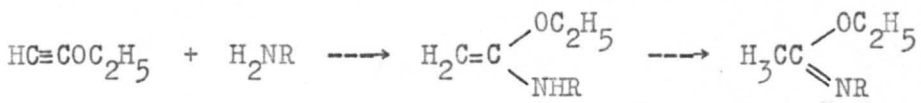
The strongly basic primary and secondary aliphatic amines and also piperidine readily react with ethoxyacetylene. The nature of the product depends on the type of amine used, on its quantity and on the presence or absence of water.

The simplest case is encountered with anhydrous secondary amines, when equimolar quantities of the reactants yield (α-alkoxyvinyl)dialkylamines[296] (Scheme 155).



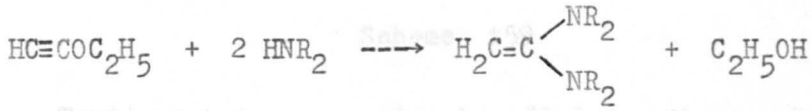
Scheme 155

If anhydrous primary amines are used, the initial products tautomerise to imino-ethers[296] (Scheme 156).



Scheme 156

If excess amine is used, these reactions may be followed by others, replacement of the $-OC_2H_5$ group occurring to form amidines[296] (Scheme 157).

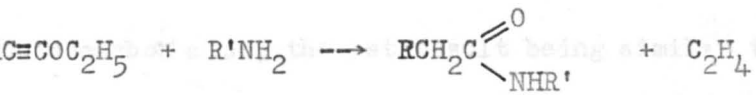


R = alkyl

R = alkyl or aryl

Scheme 157

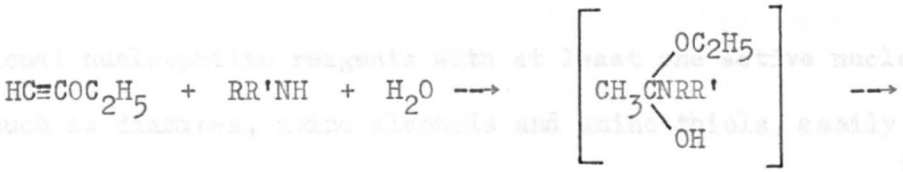
If the reactions require prolonged refluxing, the acetyl derivative of amines are also formed[268] (Scheme 158).



R = alkyl or H

Scheme 158

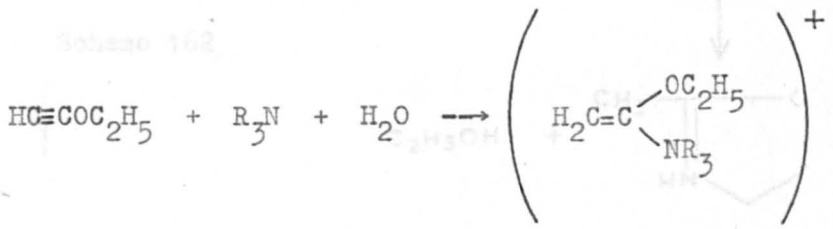
Primary and secondary amines in the presence of water give rise to substituted acetamides[297] (Scheme 159).



R and R' = alkyl or H

Scheme 159

Tertiary amines can also be added to ethoxyacetylene if water is present, the product being a vinyl substituted quaternary base[298] (Scheme 160).



(b) Reactions with alcohols

Alcohols are not only added to ethoxyacetylene with BF_3 catalysis, but also (although slowly) as alcoxide.[299] The latter reaction can be considered as a nucleophilic addition of $OC_2H_5^-$

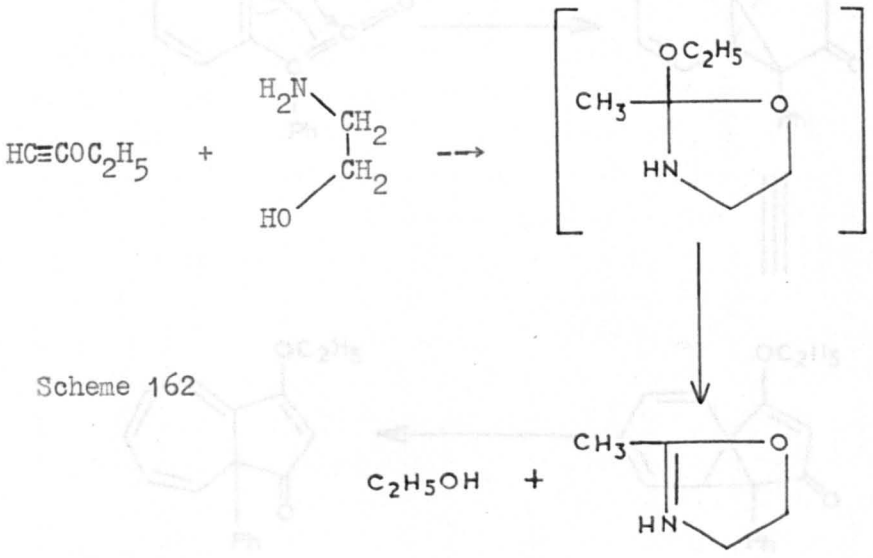
ions to the α-carbon atom, the net result being similar to the BF_3 catalysed reaction, but the ortho-ester can now be isolated (Scheme 161).

$$HC{\equiv}COC_2H_5 + \overset{-}{O}C_2H_5 \longrightarrow \overset{-}{H}C{\equiv}C(OC_2H_5)_2 \xrightarrow{C_2H_5OH}$$

$$H_2C{=}C(OC_2H_5)_2 \text{ etc.} \longrightarrow H_3C{-}C(OC_2H_5)_3$$

Scheme 161

Bifunctional nucleophilic reagents with at least one active nucleophilic group, such as diamines, amino alcohols and amino thiols, easily yield heterocyclic addition products when warmed with ethoxyacetylene.[300]



Scheme 162

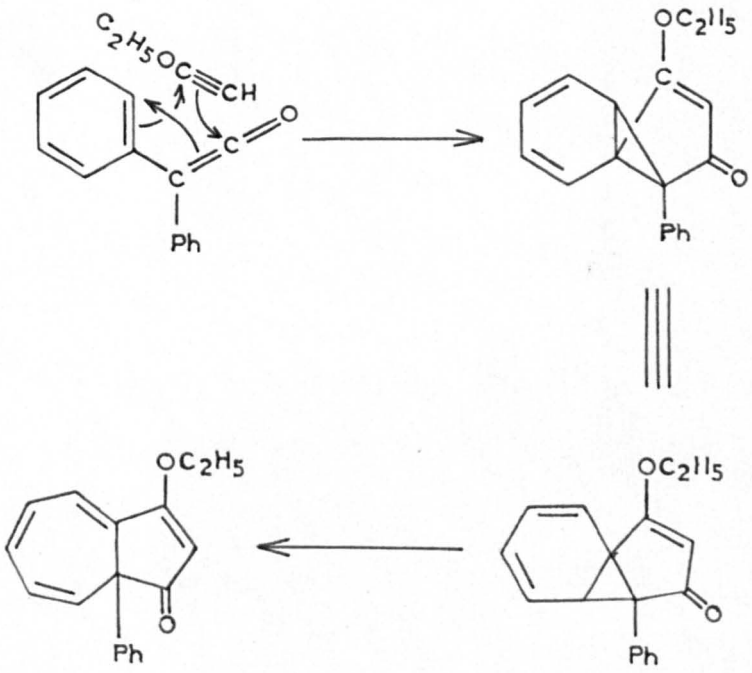
(c) Ring closures involving both acetylenic carbon atoms of ethoxyacetylene

Arens reported that diphenylketene and ethoxyacetylene reacted in nitromethane at low temperature to yield an adduct formulated on

sound analogy as 3-ethoxy-4,4-diphenylcyclolactenone[301] (Scheme 163).

Scheme 163

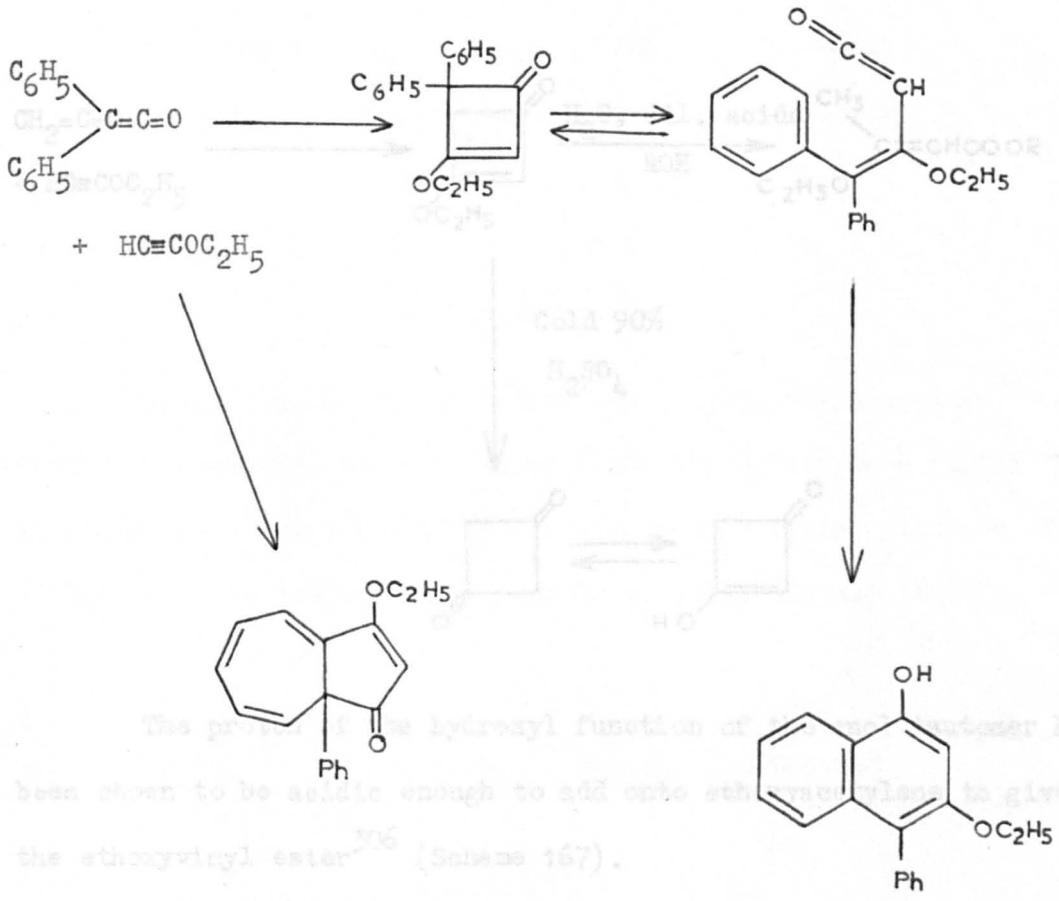
Arens[268] later withdrew this proposal without an alternative one. This was, however, furnished unequivacally by Barton et al[302] (Scheme 164).



Scheme 164

Woodward et al. were, however, able to demonstrate that another mode of cycloaddition involved the formation of an alkoxy-cyclobutenone

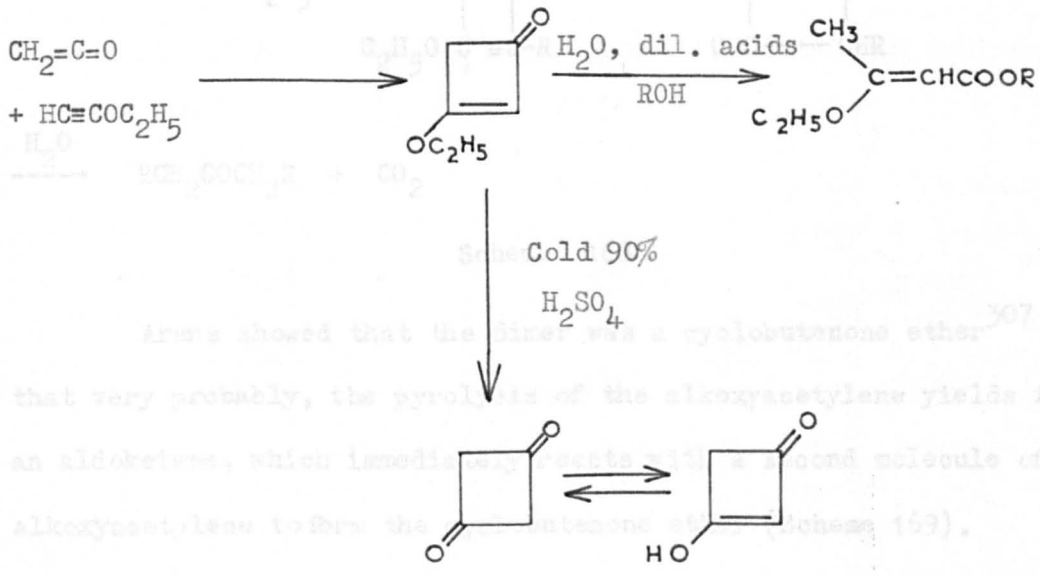
but was obscured by the formation of rearrangement products.[303] The pathways which produced these various products are summarised below. (Scheme 165).



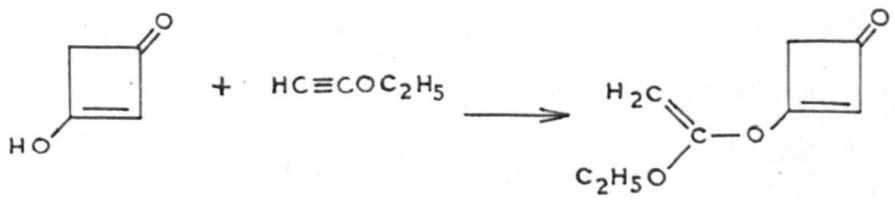
Scheme 165

Hasek and Martin then demonstrated that reaction of dimethylketene and ethoxyacetylene gave 3-ethoxy-4,4-dimethyl-2-cyclobuten-1-one.[304]

Similarly Wasserman and Dehmlow reacted ketene with ethoxyacetylene to yield 1-ethoxycyclobuten-3-one, which yielded the parent cyclobuta-1,3-dione on treatment with cold concentrated sulphuric acid[305] (Scheme 166).

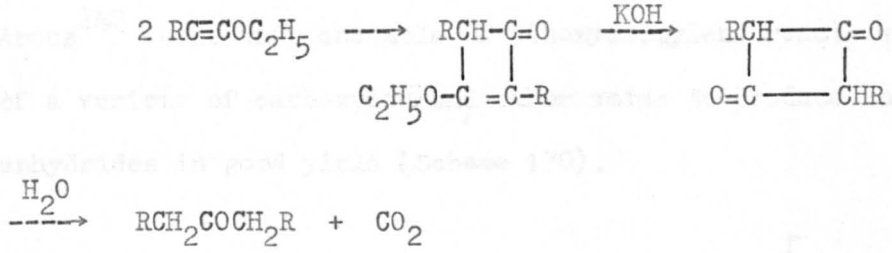


The proton of the hydroxyl function of the enol tautomer has been shown to be acidic enough to add onto ethoxyacetylene to give the ethoxyvinyl ester[306] (Scheme 167).



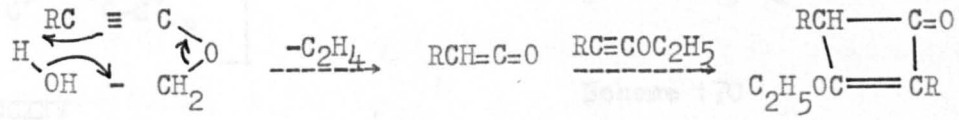
(d) Reactions of the ether function

Ficini observed that ethoxyheptyne on being heated to 120° evolved ethene and forms a product which is a dimer minus C_2H_9, hydrolysis of the product giving dihexyl ketone[275] (Scheme 168).



Scheme 168

Arens showed that the dimer was a cyclobutenone ether[307] and that very probably, the pyrolysis of the alkoxyacetylene yields first an aldoketene, which immediately reacts with a second molecule of the alkoxyacetylene to form the cyclobutenone ether (Scheme 169).



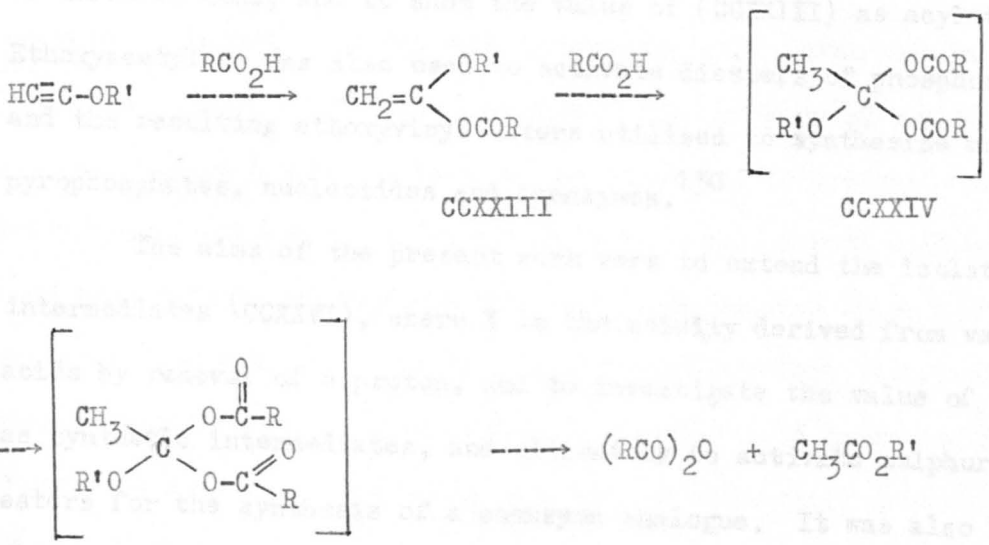
Scheme 169

PART III

EXPERIMENTAL DISCUSSION

A. Formation of Ethoxyvinyl Esters of Acids

During investigations on the reactions of alkoxyacetylenes, Arens[248] found that one mole of ethoxyacetylene reacts with two moles of a variety of carboxylic and other acids to produce the corresponding anhydrides in good yield (Scheme 170).



CCXXIII CCXXV

CCXXV

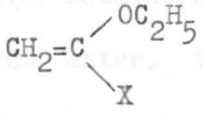
Scheme 170

This reaction has been assumed to occur by the intermediate formation of 1-alkoxyvinyl esters and 1-alkoxyethylidene diesters (CCXXIV), anhydride formation resulting from decomposition of (CCXXIV) via the transition state (CCXXV),[292] Early attempts to isolate intermediate (CCXXIII) in the case of the reaction of acetic acid with ethoxyacetylene, were unsuccessful using equimolar quantities or an excess

of ethoxyacetylene. However, Arens reported the facile formation of (CCXXIII) (R = CCl_3, R' = C_2H_5) as well as (CCXXIII) (R = $CHCl_2$, R' = C_2H_5) from trichloroacetic and dichloroacetic acid respectively, although monochloroacetic acid failed to yield (CCXXIII) (R = CH_2Cl, R' = C_2H_5).[239]

Wasserman was able to extend the formation of (CCXXIII) by the reaction of various carboxylic acids with an excess of methoxy- or ethoxyacetylene in a variety of solvents, or by using the catalytic effect of mercuric ions, and to show the value of (CCXXIII) as acylating agents.[221] Ethoxyacetylene was also used to activate diesters of phosphoric acid,[129] and the resulting ethoxyvinyl esters utilised to synthesise unsymmetrical pyrophosphates, nucleotides and coenzymes.[130]

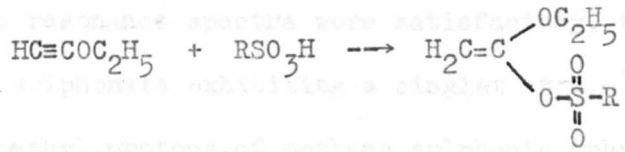
The aims of the present work were to extend the isolation of intermediates (CCXXVI), where X is the moiety derived from various acids by removal of a proton, and to investigate the value of the esters as synthetic intermediates, and ultimately to activate sulphuric acid esters for the synthesis of a coenzyme analogue. It was also hoped to activate nucleotides to promote polymerisation.



CCXXVI

1. Sulphonic acids

Arens has reacted ethoxyacetylene with sulphonic acids to product the anhydrides in good yield, and so an attempt was made to isolate the corresponding ethoxyvinyl esters if possible (Scheme 171).



Scheme 171

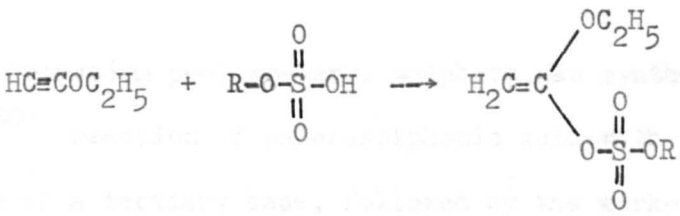
R = CH_3- and C_6H_5-

Methane sulphonic acid and benzene sulphonic acid were the two acids used, and care had to be taken to ensure that they were completely anhydrous, otherwise anhydride was the primary product. Thus methane sulphonic acid was purified by distillation in vacuo, and benzene sulphonic acid by azeotropic distillation with benzene. The sulphonic acids were reacted with the ethoxyacetylene using either an excess of the latter or mercuric ions as a catalyst. When methane sulphonic acid was the reactant, the reaction had to be cooled to acetone - dry ice temperature, otherwise the anhydride was formed, almost explosively at room temperature, whereas the reaction using benzene sulphonic acid was not nearly so vigorous. In each case the ethoxyvinyl ester was isolated in good yield and could be purified by distillation under reduced pressure. However, as shown by the nuclear magnetic resonance spectrum, it was never possible to remove c. 20% methane sulphonic anhydride from the 1-ethoxyvinyl methane sulphonate, the former apparently co-distilling with the ester. 1-Ethoxyvinyl benzene sulphonate was purified successfully, but owing to the extreme tendency to form anhydride when exposed to the atmosphere for even a minute or so, it proved impossible to secure satisfactory elemental analyses. The infrared spectrum, however, showed in each case peaks at c. 1740 and 1670 $cm.^{-1}$, characteristic of 1-ethoxyvinyl esters, and the nuclear

magnetic resonance spectra were satisfactory, that of 1-ethoxyvinyl methane sulphonate exhibiting a singlet at 6.6τ, which corresponds to the methyl protons of methane sulphonic anhydride. In each case the two methylenic and two vinylic protons were superimposed, complex splitting resulting at c. 6.0τ. The 1-ethoxyvinyl methane sulphonate and benzene sulphonate both proved to be good sulphonating agents (cf. carboxylic and phosphoric esters). Thus, reaction of the esters with aniline gave methane sulphonanilide and benzene sulphonanilide respectively in good yield.

2. Sulphuric acids

It was hoped to extend these syntheses of ethoxyvinyl esters of sulphur containing acids, to monoesters of sulphuric acid (Scheme 172).



Scheme 172

R = $C_6H_5CH_2$-,
$O_2NC_6H_4$-,
or $NCCH_2CH_2$-

It was expected that the resulting active ethoxyvinyl sulphate ester should be capable of acting as a good sulphating agent, if its properties were analagous to the corresponding esters of carboxylic and phosphoric acids, a property which could be utilised in the

preparation of adenosine-5'-sulphatophosphate from adenosine-5'-phosphoric acid. It was necessary that the group R- should be capable of removal by mild treatment without damage to the rest of the coenzyme analogue. Many such protecting groups have been used in nucleotide chemistry. Of these the benzyl group, p-nitrophenyl group and 2-cyanoethyl group were used since the former may be removed by mild catalytic hydrogenation, and the latter two from diesters by mild alkaline treatment.

Sodium benzyl sulphate was prepared essentially by the method devised by Bacon and Daggart[308] but difficulty was experienced in isolating the sodium salt of benzyl sulphuric acid from the reaction mixture because of alkaline hydrolysis of the product to give sodium sulphate and benzyl alcohol. However, suitable modifications were devised which yielded sodium benzyl sulphate, although in rather low yield.

Potassium p-nitrophenyl sulphate was synthesised by a similar method;[309] reaction of chlorosulphonic acid with p-nitrophenol in the presence of a tertiary base, followed by the work-up described in the Experimental Section.

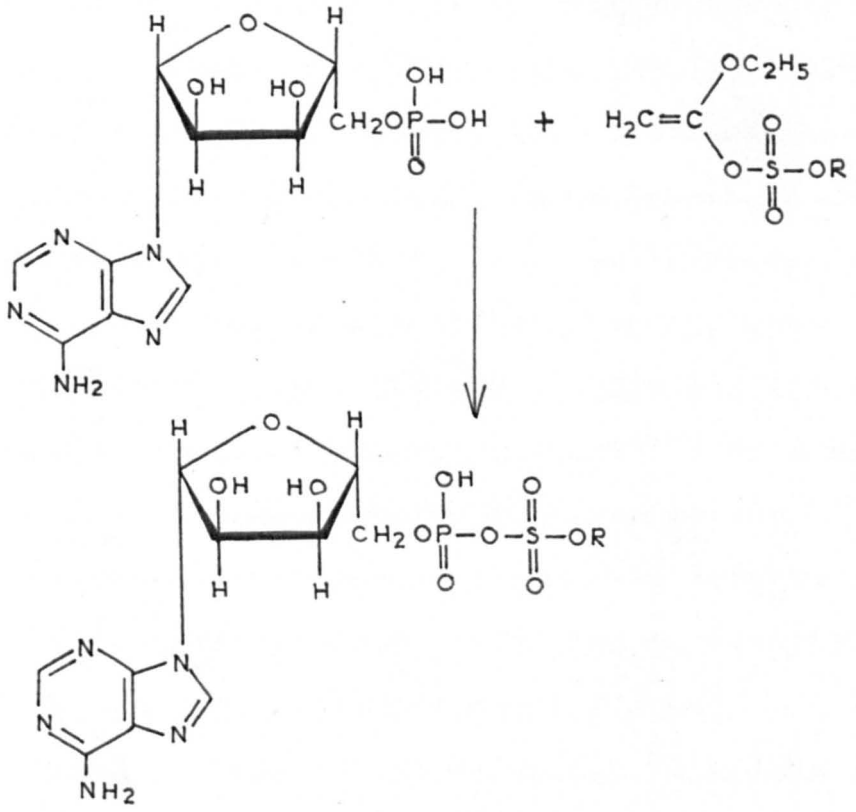
To prepare barium-2-cyanoethyl sulphate, chlorosulphonic acid was reacted with ethylene cyanohydrin in the presence of tertiary base, but difficulty was again encountered in isolating the barium salt, the result of adding alkali being hydrolysis and production of barium sulphate. However by a means of a suitable work-up, a sample that was mainly barium-2-cyanoethyl sulphate contaminated, very probably, with inorganic material was isolated. Many attempts were made to purify

the required salt. Thus it was recrystallised six times, chromatographed on an alumina column which was eluted with ethanol with a water gradient, and finally recrystallised twice further, but the elemental analysis figures even then indicated contamination by inorganic salts. A sample of this barium salt was converted to the acid and the latter titrated against alkali to pH 7 on an automatic titrator. Evaporation of the resulting solution gave sodium 2-cyanoethyl sulphate as a rather deliquescent white solid, of which elemental analysis proved difficult. The titration figures were, however, extremely satisfactory.

The reaction of pyridinium benzylsulphate with ethoxyacetylene yielded a brown gum and a small amount of a light brown coloured solid, suspected to be pyrosulphate, which could be filtered from a solution of the crude residue in methylene dichloride. The infrared spectrum of the gum exhibited peaks at 1725 and 1650 $cm.^{-1}$, which characterised it as the ethoxyvinyl ester of benzyl sulphuric acid. Attempts to purify this ester by distillation in vacuo failed, a charred solid remaining in the distillation flask. The ethoxyvinyl esters of p-nitrophenyl and 2-cyanoethyl sulphuric acids were similarly prepared and characterised, although attempts to distil these also failed.

As attempts to purify these 1-ethoxyvinyl esters of sulphates failed, it was decided to use them in the crude condition after evaporation of solvents and excess ethoxyacetylene from the reaction mixture. If the reactivity of these esters paralleled that of carboxylic, phosphoric, etc. acids it was expected that they should be good sulphating agents and, therefore, provide a useful path for the synthesis of

adenosine-5'-sulphatophosphate, the analogue of "active sulphate" discussed in the Introduction (Scheme 173),



Scheme 173

followed by removal of the protecting group R.

Adenosine-5'-sulphatophosphate has been synthesised by Baddiley, Buchanan and Letters[310] who allowed adenosine-5'-phosphoric acid to react with a pyridine-sulphur trioxide complex, and independently by Reichard and Ringertz[311] who condensed adenosine-5' phosphoric acid and inorganic sulphate using dicyclohexylcarbodiimide, yields being

low in each case. The former route was followed to prepare an authentic sample for comparison purposes by chromatography.

The crude 1-ethoxyvinyl benzyl sulphate and anhydrous pyridinium adenosine-5'-phosphate were dissolved in dry methylene dichloride and dimethylformamide, the resulting solutions being left in the dark at room temperature for five days. Benzyl groups were removed from the residue by catalytic hydrogenation using a palladium-charcoal catalyst. Chromatography of the resulting solution using two solvent systems, and electrophoresis using buffer of two different pH values, indicated formation of adenosine-5' sulphatophosphate in 35% yield. A phosphorus determination of a solution of the coenzyme eluted from the paper was satisfactory. Other products identified in the reaction mixture were adenosine-5'-phosphoric acid, adenosine, and probably adenosine-5'-sulphato-phosphate-(2',3')-sulphate, an identification that was confirmed from the preparation using pyridinium p-nitrophenyl sulphate.

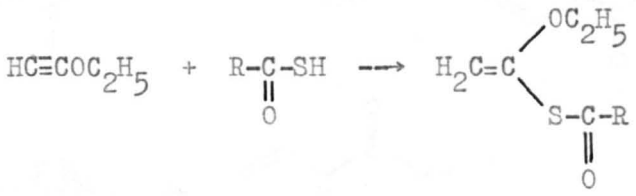
In a second synthesis, crude 1-ethoxyvinyl 2-cyanoethyl sulphate and anhydrous pyridinium adenosine-5'-phosphate were dissolved in dry methylene dichloride and dimethylformamide, the resulting solution being left in the dark at room temperature for five days. The 2-cyanoethyl protecting groups were removed by alkaline hydrolysis. Samples were extracted from the reaction mixture so that the progress of hydrolysis could be followed. Hydrolysis in each sample was terminated by cooling and addition of Amberlite IR 120 (H^+ form) to neutrality. Chromatography of each of the samples using two solvent systems followed by elution of strips of paper parallel to the direction of development indicated

synthesis of adenosine-5'-sulphatophosphate in 45% yield, the optimum time for hydrolysis at 100° being two minutes. The only other products were adenosine-5'-phosphoric acid and adenosine.

The product of the reaction of 1-ethoxyvinyl p-nitrophenyl sulphate and pyridinium adenosine-5'-phosphoric acid was subjected to alkaline hydrolysis at 100°C to remove p-nitrophenyl groups. Samples were extracted at intervals to follow the progress of hydrolysis, the latter being terminated by cooling andaddition of Amberlite IR 120 (H^+ form) ion-exchange resin. p-Nitrophenol was then removed by extraction in ether. Each sample was chromatographed using two solvent systems, and electrophoresis at pH 5.5. No adenosine-5'-sulphatophosphate resulted in the samples where alkaline hydrolysis had not exceeded two hours; whilst removal of p-nitrophenyl groups was complete after five minutes of alkaline hydrolysis. The major product of the reaction was adenosine-5'-sulphatophosphate-(2',3')-sulphate. This was characterised by elution from the paper, the optical density at 260 mμ of the resulting solution determined, followed by removal of sulphate groups by acid hydrolysis, and isolation of sulphate as the insoluble barium salt, which could be isolated, dried and weighed. The results indicated that each molecule of nucleotide contained two sulphate groups. A trace ($< 2\%$) of adenosine-5'-sulphatophosphate resulted from those samples where alkaline hydrolysis had exceeded two hours, which would suggest that this was a secondary product resulting from the alkaline hydrolysis of the (2',3')-sulphate.

3. Thiolic acids

It has also proved possible to isolate 1-ethoxyvinyl esters of thiolic acids. Thiolacetic and thiobenzoic acids were purified and dried by careful fractional distillation in vacuo, and reacted with ethoxyacetylene in the usual manner. 1-Ethoxyvinyl thiolacetate and 1-ethoxyvinyl thiolbenzoate, respectively, were the products which could be distilled to yield relatively stable golden-coloured liquids[222] (Scheme 174).



Scheme 174

R = CH_3- or C_6H_5-

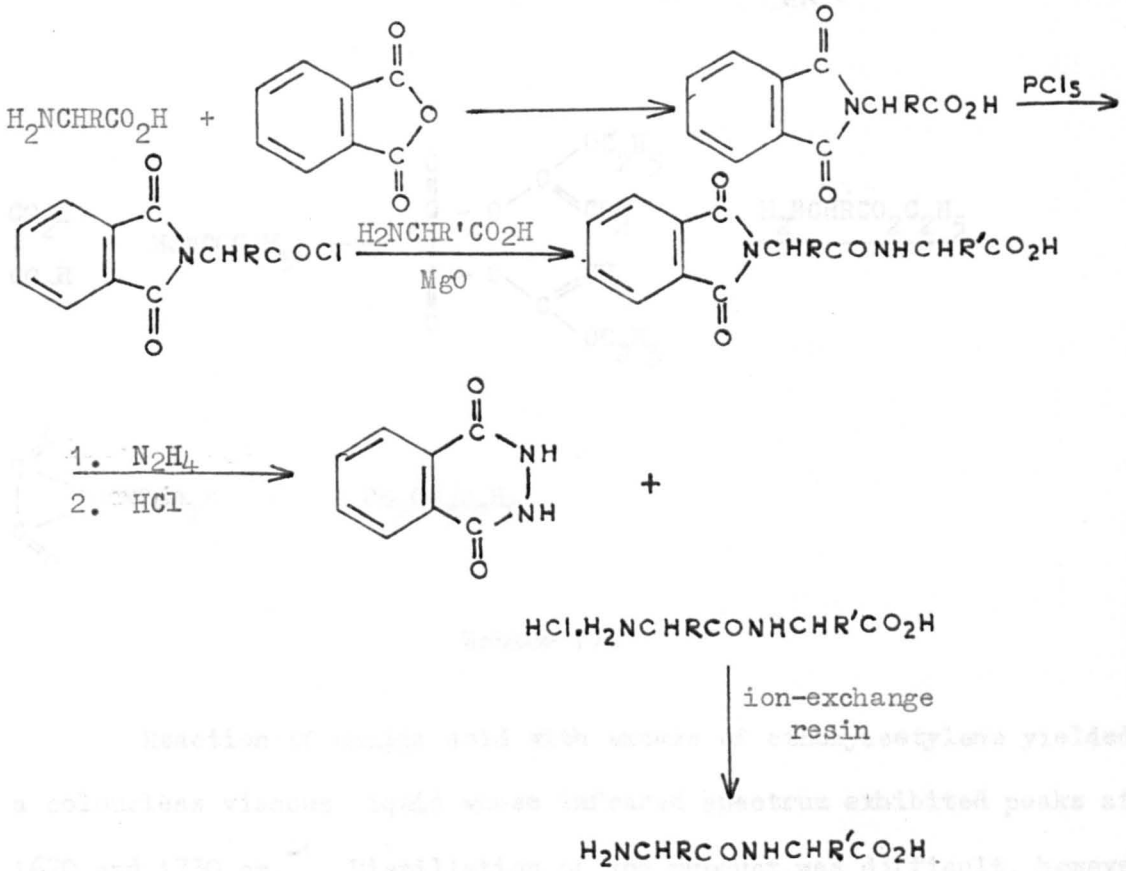
The infrared spectra of these esters also exhibited peaks at c. 1740 and 1670 $cm.^{-1}$

The reaction of 1-ethoxyvinyl thiolacetate with aniline yielded acetanilide in good yield, ethyl thionacetate being detected as a reaction product by vapour-phase chromatography of a sample of the reaction mixture. The ethyl thionacetate peak was characterised by comparing its retention time with that of an authentic sample, this being repeated using another column packing. 1-Ethoxyvinyl thiolbenzoate was similarly shown to be a good acylating agent.

4. Dicarboxylic and α-keto acids

The phthaloyl group has been used extensively for protection of

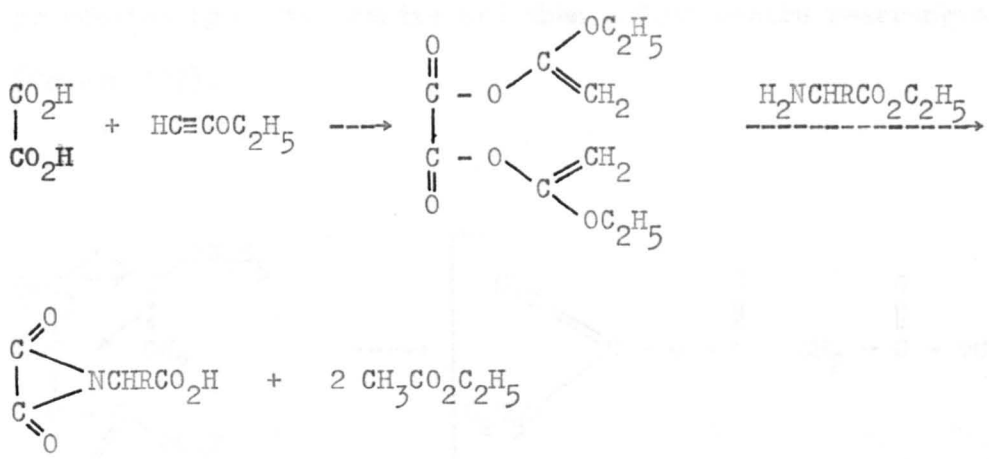
the amino function in amino-acids and peptides during the stepwise synthesis of peptides[312] (Scheme 175).



Scheme 175

The carboxyl function may also be converted to an anhydride, azide or ester for coupling with the second amino acid or peptide. The preparation of the phthaloyl protected amino acid or peptide involves considerable heating which could easily destroy many labile oligopeptides. Thus an attempt was made to isolate the diethoxyvinyl esters of some

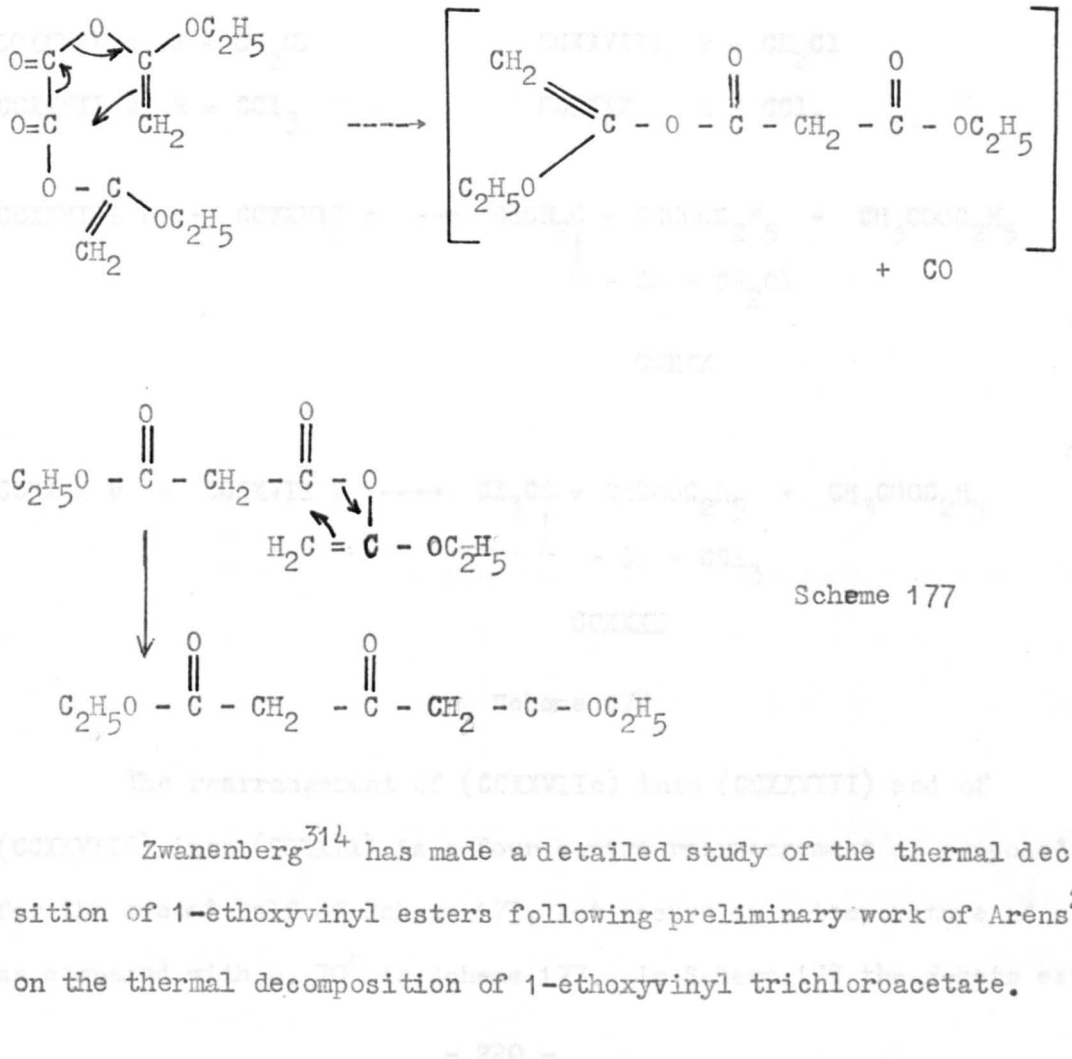
dicarboxylic acids, which might then react with the amino group of an amino-acid to protect it. (Scheme 176).



Scheme 176

Reaction of oxalic acid with excess of ethoxyacetylene yielded a colourless viscous liquid whose infrared spectrum exhibited peaks at 1670 and 1730 cm.$^{-1}$ Distillation of the product was difficult, however, owing to the evolution of gas, and a rearrangement was suspected. Hence the experiment was carried out under controlled conditions. The diethoxyvinyl ester was heated carefully to c. 70°, when a gas was evolved, which was collected over water in a measuring cylinder after first passing through a water condenser, and identified as carbon monoxide by the formation of molybdenum blue.[313] A little ethyl acetate also condensed in the latter and was collected. The reaction

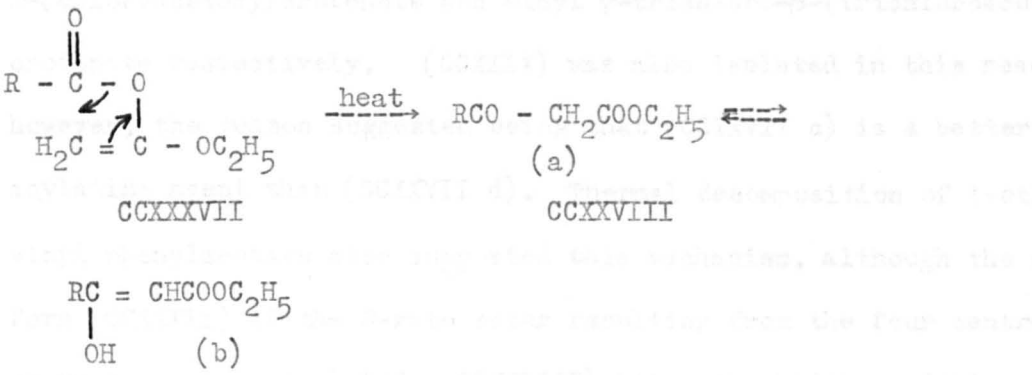
was extremely exothermic so that control by cooling the reaction flask was necessary. The brown liquid remaining in the flask was distilled in vacuo to give acetone dicarboxylate in good yield, and was identified by elemental analysis, infrared spectrum and formation of a derivative - the copper salt. The rearrangement was envisaged as proceeding by a five centre and then a four centre rearrangement (Scheme 177).



Scheme 177

Zwanenberg[314] has made a detailed study of the thermal decomposition of 1-ethoxyvinyl esters following preliminary work of Arens[239] on the thermal decomposition of 1-ethoxyvinyl trichloroacetate.

Zwanenberg proposed the following scheme (Scheme 178) to account for the products of the decomposition:

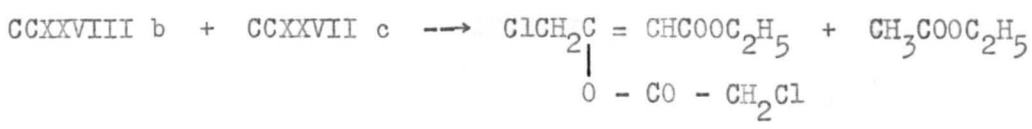


CCXXXVII CCXXVIII

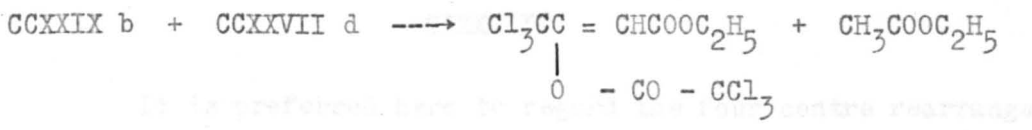
CCXXVIII (b)

CCXXVII c R = CH_2Cl CCXXVIII R = CH_2Cl

CCXXVII d R = CCl_3 CCXXIX R = CCl_3



CCXXX

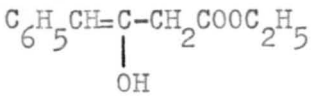


CCXXXI

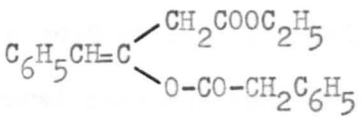
Scheme 178

The rearrangement of (CCXXVIIc) into (CCXXVIII) and of (CCXXVIId) into (CCXXIX) is a four centre rearrangement as proposed for the second half of Scheme 177, but occurs at a temperature of c. 170° as compared with c. 70° in Scheme 177. In Scheme 178 the β-keto ester,

(CCXXVIII) or (CCXXIX) is acylated in the enolic form, b, with liberation of ethyl acetate and formation of the products ethyl γ-chloro-β-(chloroacetoxy)crotonate and ethyl γ-trichloro-β-(trichloroacetoxy)-crotonate respectively. (CCXXIX) was also isolated in this reaction, however, the reason suggested being that (CCXXVII c) is a better acylating agent than (CCXXVII d). Thermal decomposition of 1-ethoxy-vinyl phenylacetate also supported this mechanism, although the enol form (CCXXXII) of the β-keto ester resulting from the four centre rearrangement is acylated, (CCXXXIII) being the major product.



CCXXXII

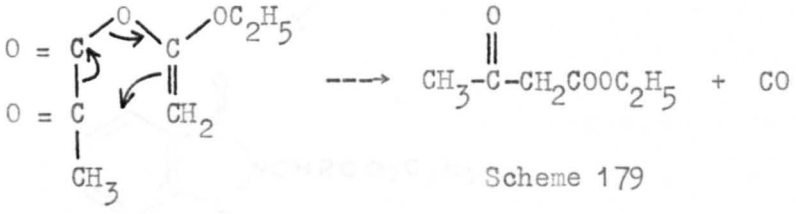


CCXXXIII

It is preferred here to regard the four centre rearrangement as occurring by nucleophilic attack by the π-electron system of the double bond on the electron deficient carbon atom of the $\rangle C = \ddot{O}$ group (Scheme 177 cf. Scheme 178).

The fact that in the present rearrangement (Scheme 177) a small amount of ethyl acetate resulted could be explained if the product of the five centre rearrangement is acylated in one of its enol forms by the 1-ethoxyvinyl ester.

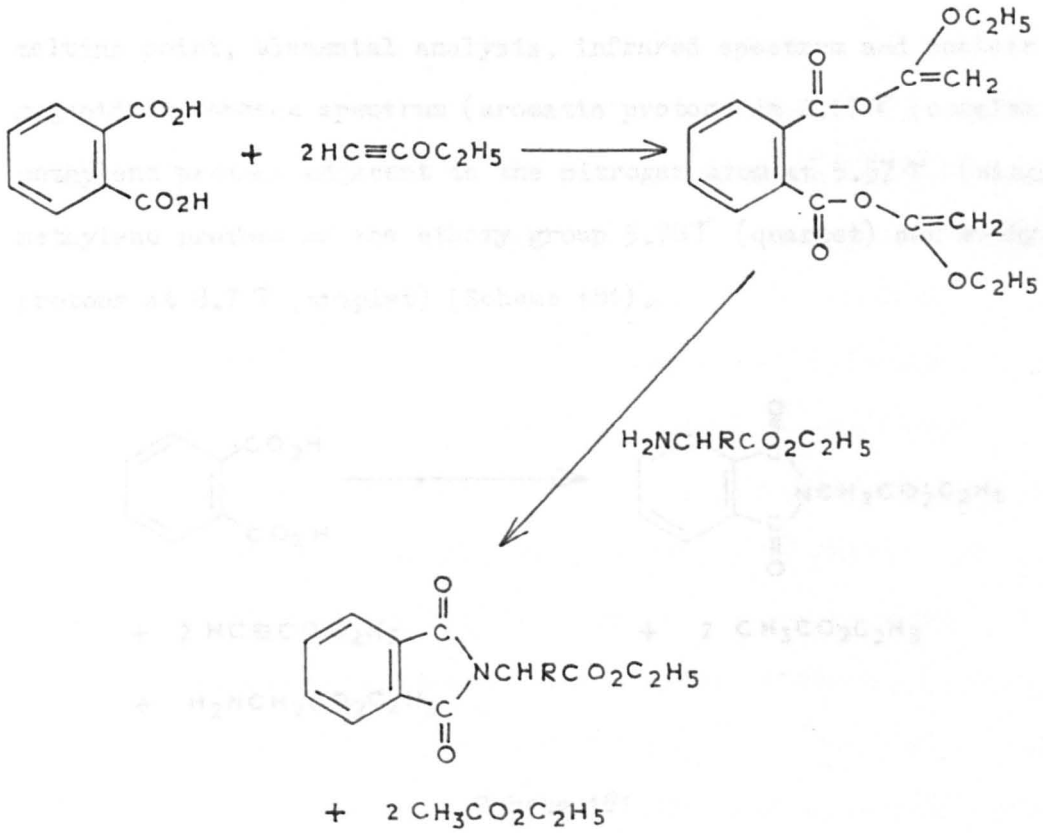
Further evidence for such a five centre rearrangement was obtained by use of pyruvic acid. Reaction of anhydrous pyruvic acid with ethoxyacetylene again yields the 1-ethoxyvinyl ester of this acid (characterised by peaks in the infrared spectrum at 1670 and 1730 cm.$^{-1}$) and this again underwent rearrangement on heating to c. 70°C, carbon monoxide again being evolved (Scheme 179).



Scheme 179

The product of this rearrangement could then not undergo a further four centre rearrangement as in Scheme 177; and ethyl acetoacetate was isolated in good yield. The latter was characterised by elemental analysis, infrared spectrum and formation of a derivative - the 2,4-dinitrophenylhydrazone. Production of some ethyl acetate can be explained as above.

The possibility of preparing diethoxyvinyl esters of other dicarboxylic acids was then considered, the acid chosen for preliminary investigations being phthalic acid, which would lead to the synthesis of an amino-acid or peptide protected by the conventional phthaloyl group (Scheme 180).

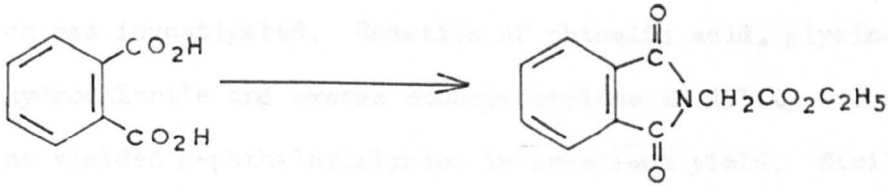


Scheme 180

Reaction of anhydrous phthalic acid with ethoxyacetylene, however, yielded only phthalic anhydride, using either an excess of ethoxyacetylene or catalysis with mercuric ions, this result being due presumably to the close proximity of the carboxylic functions. Possible mechanisms for this reaction were discussed in an earlier section.

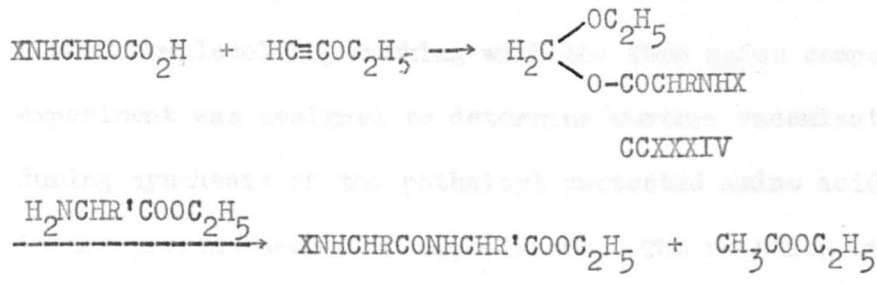
Anhydrous phthalic acid in tetrahydrofuran was added to glycine ethyl ester and ethoxyacetylene in dry methylene dichloride. On removal of solvents and excess ethoxyacetylene, a gum remained, which, on trituration with aqueous ethanol yielded needle-like crystals of N-phthaloylglycine ethyl ester in good yield, identified by its

melting point, elemental analysis, infrared spectrum and nuclear magnetic resonance spectrum (aromatic protons at 2.17 τ (complex splitting), methylene protons adjacent to the nitrogen atom at 5.57 τ (singlet), methylene protons of the ethoxy group 5.78 τ (quartet) and methyl protons at 8.7 τ (triplet) (Scheme 181).



Scheme 181

Arens[315] has used ethoxyacetylene to prepare peptides by refluxing an N-protected amino-acid or peptide with an amino-acid or peptide ester (or as the hydrochloride) in moist ethyl acetate (Scheme 182).



X = protecting group

Scheme 182

The 1-ethoxyvinyl esters (CCXXXIV) of the N-protected amino acids and peptides have been isolated by a number of workers,[244,245,246] Sheehan and Hlavka isolating one such intermediate in aqueous solution.

In view of the importance of the phthaloyl group for the protection of the amino group of amino-acids and peptides, the possibility of using ethoxyacetylene to form such protected amino-acids in aqueous solution was investigated. Reaction of phthalic acid, glycine ethyl ester hydrochloride and excess ethoxyacetylene in dilute aqueous pyridine yielded N-phthaloylglycine in excellent yield. Similarly, reaction of phthalic acid, glycine and excess ethoxyacetylene in very dilute aqueous pyridine yielded N-phthaloylglycine in moderate yield. Glycine and phthalic anhydride were other products recovered in small quantities from the reaction mixture, but no 1-ethoxyvinyl phthaloyl glycinate was detected as might be expected on the basis of Sheehan and Hlavka's experiments.

Very little racemisation occurs during the synthesis of dipeptides by means of ethoxyacetylene, but occasionally racemisation has been observed during the synthesis of higher peptides when the hydrochlorides of the amino components were used. This complication can be avoided almost completely by working with the free amino components.[312] An experiment was designed to determine whether racemisation occurred during synthesis of the phthaloyl protected amino acids or peptides in the present series of experiments. The reaction of phthalic acid, L-phenylalanine methyl ester hydrochloride and excess ethoxyacetylene in dilute aqueous pyridine solution gave N-phthaloyl-L-phenylalanine

methyl ester in good yield. The phthaloyl group was next removed using the technique of Sheehan and Frank. N-phthaloyl-L-phenylalanine methyl ester in ethanol was refluxed with alcoholic hydrazine hydrate solution. After removal of solvent, hydrochloric acid was added and the flask held at 50°C for five minutes. Phthaloyl hydrazide was removed by filtration from the cooled solution, the filtrate evaporated to dryness, and L-phenylalanine methyl ester hydrochloride crystallised by trituration of the residue. The specific rotation of the product was compared with that of the starting product, no racemisation being observed.

Thus this method appears to be an excellent one for the protection of the amino group of amino-acids or peptides during peptide synthesis in which mild techniques must be employed. No heating is necessary (which could denature higher peptides) and no racemisation appears to occur.

To discover whether the di-1-ethoxyvinyl ester of a dicarboxylic acid could be isolated, ethoxyacetylene was allowed to react in the presence of a mercuric ion catalyst with terephthalic acid, in which the stereochemistry of the two carbonyl groups prevents intramolecular anhydride formation. The reaction was very slow, due, no doubt, to the insolubility of the terephthalic acid in the solvent system so that the acid had only completely disappeared after being stirred at room temperature for two days. After evaporation of the resulting solution to dryness and recrystallisation of the residue from methylene dichloride-hexane di-1-ethoxyvinyl terephthalate was obtained in good yield as stable pale yellow crystals. This product was characterised by its

infrared spectrum, nuclear magnetic resonance spectrum (aromatic protons at 1.98 τ (singlet)), olefinic and methylenic protons at 6.1 τ (complex splitting) and methyl protons at 8.65 τ (triplet), and elemental analysis.

B. Base Sequences of Chemically Synthesised Polynucleotides

The synthesis of ribo- and deoxyribonucleic acids by Ochoa and Kornberg respectively using enzymatic techniques has already been described (see Introduction) in detail. It is as a result of the Kornberg experiments on deoxyribonucleic acid synthesis that the present sequence studies on synthetic polydeoxyribonucleotides were initiated. In summary, Kornberg isolated an enzyme from Escherichia coli, DNA polymerase, that was capable of synthesising deoxyribonucleic acid from the four common deoxyribonucleoside-5'-triphosphates. There were two stringent conditions required by the system, namely (1) all four of the deoxyribonucleoside-5'-triphosphates had to be present (2) high molecular weight deoxyribonucleic acid had to be present as a primer.

Kornberg later discovered that if the DNA primer was omitted from the reaction mixture, there was a long lag period when no detectable reaction could be observed, after which there occurred a rapid synthesis of a polymer which contained only deoxyadenylate and -thymidylate. The synthesis of this polymer did not require deoxycytidine triphosphate or deoxyguanosine triphosphate and proceeded in the same manner in their absence. The product was found to be a rigid, double stranded macromolecule like deoxyribonucleic acid, and by nearest neighbour analysis techniques was shown to be a copolymer in which deoxyadenylate and

thymidylate was incorporated in equal proportions and in alternating sequence (Poly dAT).

A second, similar synthesis was shown to occur if deoxycytidine-5'-triphosphate and deoxyguanosine-5'-triphosphate was incubated with DNA polymerase. The product isolated after an initial lag period, was again found to be a rigid, double stranded macromolecule. This time, however, the product was a mixture of the two homopolymers (Poly dG and Poly dC), not always in equal amounts.

The reason for this interesting specificity in the structure of the polymers from the unprimed enzymatic synthesis using the two pairs of deoxyribonucleoside-5'-triphosphates could be (a) a result of the molecular configurations of the nucleotides themselves or (b) a result of the enzyme stereochemistry, i.e. only the enzyme determines the base order if no primer is present. In order to determine which factor was operative, it was necessary to eliminate one of the above factors, and this could be accomplished conveniently by synthesising the polymers synthetically i.e. using a chemical condensing reagent, which would thus eliminate any enzyme stereochemistry factor.

It was hoped to develop ethoxyacetylene as a condensing reagent for the synthesis of polydeoxyribonucleotides, in a similar manner to the use of dicyclohexylcarbodiimide. Khorana[167] has, however, shown that the only product from the reaction of pyridinium thymidine-5'-phosphate and ethoxyacetylene in anhydrous pyridine was P^1,P^2-dithymidine. pyrophosphate, and a similar experiment performed during the present work confirmed this result. Thus, it appears that whereas the activation

of a phosphomonoester may occur with ethoxyacetylene, the resulting pyrophosphate lacks the nucleophilicity to undergo further reaction with the ethoxyacetylene, a step which is essential for conversion of the initially formed symmetrical pyrophosphate to a phosphorylating agent.

Dicyclohexylcarbodiimide has been used successfully for the polymerisation of deoxyribonucleotides and this reagent was therefore employed in the present series of experiments. The base sequences of the polymeric products were analysed by the nearest-neighbour frequency technique described in the Introduction. The two deoxyribonucleotides chosen for polymerisation by dicyclohexylcarbodiimide were thymidine-5'-phosphate and deoxyadenosine-5'-phosphate, which, it will be remembered, were the components of a Poly dAT copolymer in the unprimed enzymatic syntheses. The nearest-neighbour base frequency analysis involves the use of ^{32}P labelled deoxyribonucleotides; in this case, thymidine-5'-phosphate-P-32 and deoxyadenosine-5'-phosphate-P-32. Both of these were successfully synthesised, thymidine-5'-phosphate-P-32 being also obtained from the Radiochemical Centre, Amersham, who were, however, unable to synthesise the deoxyadenosine-5'-phosphate-P-32.

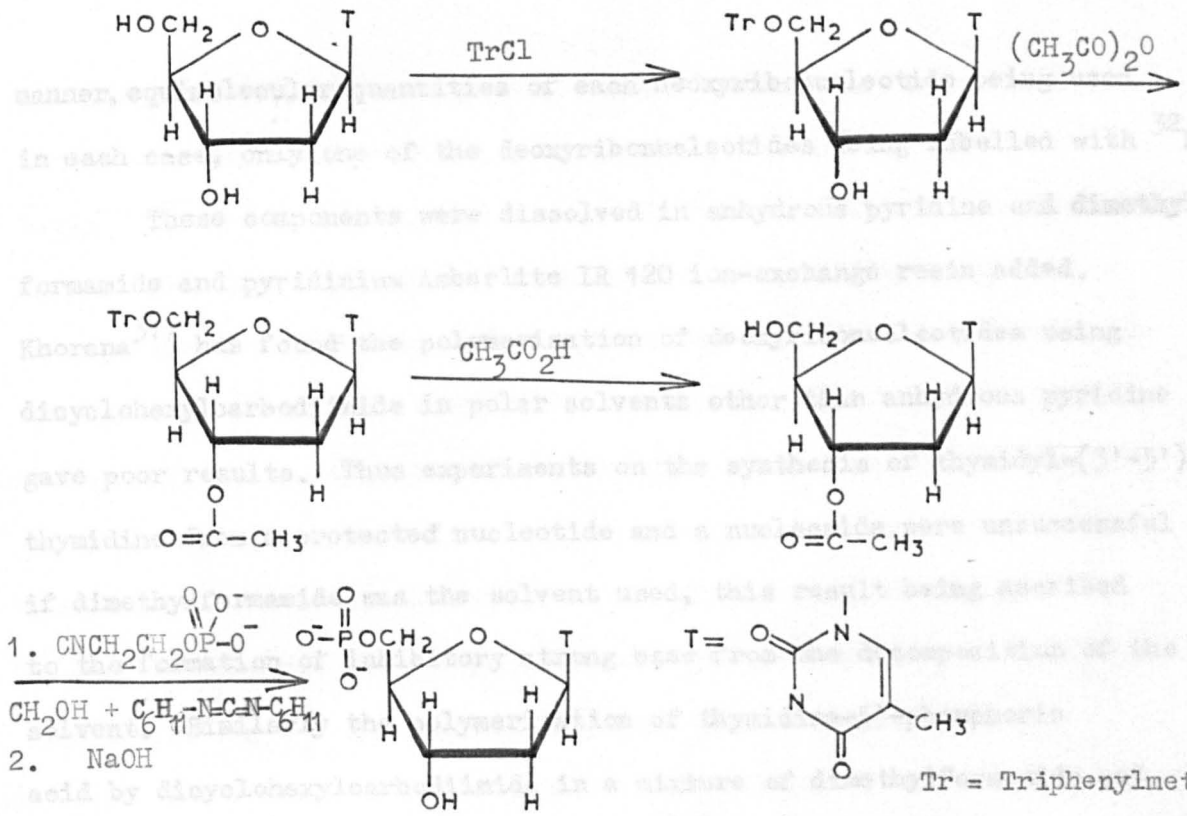
Thymidine-5'-phosphate-P-32 was synthesised by the method used by Michelson and Todd[98] and modified by Tener.[138] Thymidine was tritylated in the 5'-primary hydroxyl position, and this protected nucleoside acetylated in the 3'-secondary hydroxyl position. The 5'-trityl group was then removed by treatment with acid, and the exposed 5'-primary hydroxyl group phosphorylated using pyridinium 2-cyanoethyl phosphate-P-32 and dicyclohexylcarbodiimide. The former was synthesised by the Radiochemical Centre, Amersham, using the technique of Pfitzner and

Moffatt.[145] Acetyl and 2-cyanoethyl groups were finally removed by alkaline hydrolysis to yield thymidine-5'-phosphate-P-32 (Scheme 183).

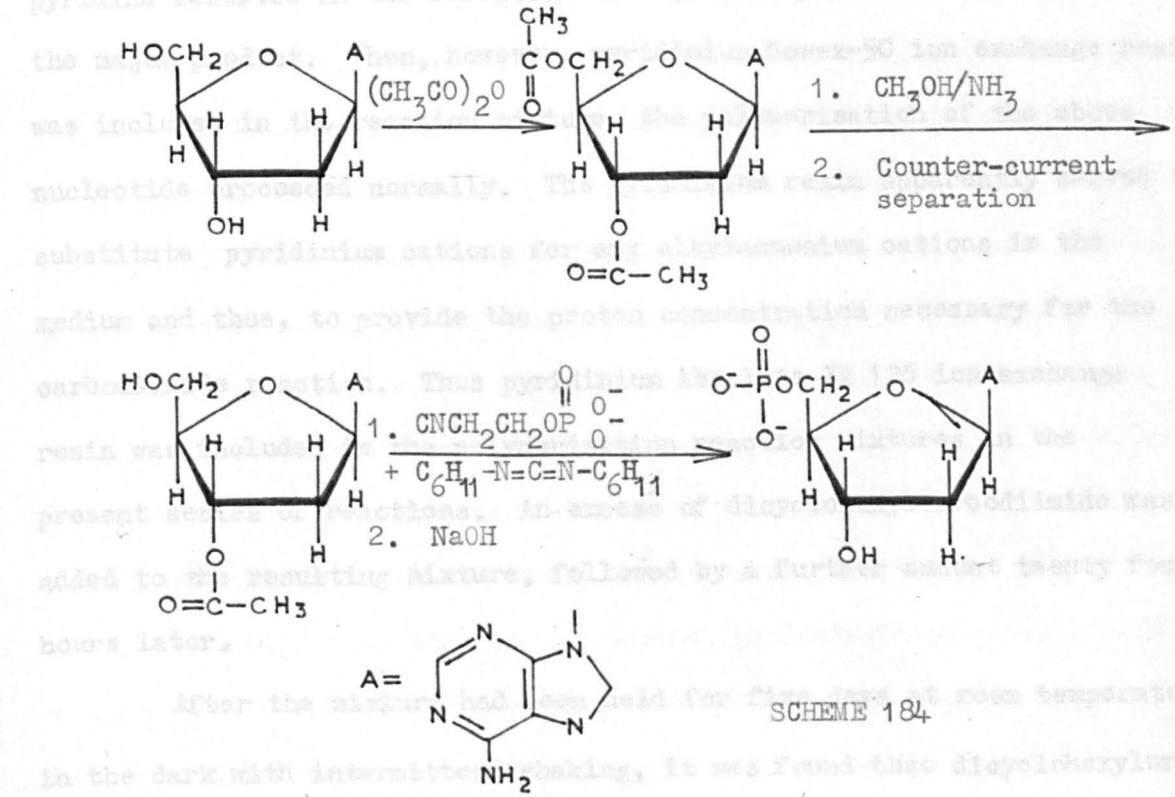
The above procedure could not be applied to the synthesis of deoxyadenosine-5'-phosphate-P-32 owing to the acid lability of the glycosidic linkage in purine nucleosides. Two methods of synthesis of this nucleotide were employed. The first developed by Anderson, Hayes Michelson and Todd[99,316] involved the diacetylation of the 3'- and 5'-hydroxyl groups of deoxyadenosine followed by partial deacylation of the product to yield a mixture of 3'-acetyldeoxyadenosine, 5'-acetyldeoxyadenosine, 3',5'-diacetyldeoxyadenosine and deoxyadenosine. These four products were then separated by counter-current distribution, the products identified by paper chromatography of the fractions. The isolated 3'-acetyldeoxyadenosine was then phosphorylated using pyridinium 2-cyanoethyl phosphate-P-32 and dicyclohexylcarbodiimide, followed by removal of acetyl and 2-cyanoethyl groups by alkaline hydrolysis to yield the desired ^{32}P-nucleotide (Scheme 184).

The second method of synthesis of deoxyadenosine-5'-phosphoric acid-P-32 involved the direct phosphorylation of deoxyadenosine using pyridinium 2-cyanoethylphosphate-P-32 and dicyclohexylcarbodiimide in anhydrous pyridine.[138] The required nucleotide was then isolated by chromatography on an ion-exchange column (Dowex 1 - X8, chloride) using a water into hydrochloric acid linear gradient. The overall yield from deoxyadenosine using this method was better than that for the former method.

Each polymerisation experiment was performed in an identical



SCHEME 183



SCHEME 184

manner, equimolecular quantities of each deoxyribonucleotide being used in each case, only one of the deoxyribonucleotides being labelled with ^{32}P.

These components were dissolved in anhydrous pyridine and dimethylformamide and pyridinium Amberlite IR 120 ion-exchange resin added. Khorana[317] has found the polymerisation of deoxyribonucleotides using dicyclohexylcarbodiimide in polar solvents other than anhydrous pyridine gave poor results. Thus experiments on the synthesis of thymidyl-(3'→5')-thymidine from a protected nucleotide and a nucleoside were unsuccessful if dimethylformamide was the solvent used, this result being ascribed to the formation of inhibitory strong base from the decomposition of the solvent. Similarly the polymerisation of thymidine-5'-phosphoric acid by dicyclohexylcarbodiimide in a mixture of dimethylformamide and pyridine resulted in the formation of P^1,P^2-dithymidine-5'-pyrophosphate as the major product. When, however, pyridinium Dowex-50 ion exchange resin was included in the reaction mixture, the polymerisation of the above nucleotide proceeded normally. The pyridinium resin apparently served to substitute pyridinium cations for any alkylammonium cations in the medium and thus, to provide the proton concentration necessary for the carbodiimide reaction. Thus pyridinium Aberlite IR 120 ion exchange resin was included in the polymerisation reaction mixtures in the present series of reactions. An excess of dicyclohexylcarbodiimide was added to the resulting mixture, followed by a further amount twenty four hours later.

After the mixture had been held for five days at room temperature in the dark with intermittent shaking, it was found that dicyclohexylurea

had separated out. Water was then added to destroy any pyrophosphates formed simultaneously during polymerisation, the aqueous solution being extracted with ether to remove dicyclohexylurea, and the pH of the aqueous solution adjusted to 7 with alkali. The residue resulting on evaporation to dryness was taken up in water and filtered to remove the ion-exchange resin. Mononucleotides and lower oligonucleotides were removed by two techniques - (1) by dialysis against water (2) by paper chromatography on ion-exchange paper, on which the polynucleotides were held back near to the origin, although the upper limit of the polynucleotides was not always distinct. The polydeoxyribonucleotides were then ready for the nearest neighbour base sequence analysis.

The latter was accomplished by evaporation of the polynucleotide solution to dryness, and subsequent solution of the residue in Tris buffer and calcium chloride solution. This solution was then incubated with the enzymes Micrococcal DNase, followed by Calf Spleen phosphodiesterase at 37°C, which degraded the polymers into deoxyribonucleoside-3'-phosphates. These were separated by paper chromatography, each ultraviolet absorbing band being cut out and the nucleotide eluted from the paper with water. A suitable aliquot of this solution was transferred onto an aluminium planchette and evaporated to dryness under an infrared lamp. The activity of the sample was determined using scintillation counting techniques, the scintillator being an anthracene crystal.

Khorana[125] was able to polymerise thymidine-5'-phosphoric acid using dicyclohexylcarbodiimide very successfully, but when he attempted to extend this technique to the polymerisation of deoxyadenosine-5'-phosphoric

acid difficulties were encountered owing to the reactivity of the amino group present on the purine ring.[191] This presumably involves phosphorylation of the amino group to form phosphoramidates with concomitant loss of the nucleotides involved in chain lengthening. Tener[138] has studied the rates of phosphorylation of the various functional groups on deoxyribonucleosides, namely, the 3'- and 5'-hydroxyl groups and the pyrimidine and purine ring amino groups. This was studied by allowing pyridinium 2-cyanoethyl phosphate plus dicyclohexylcarbodiimide to compete for the groups in the presence of a large excess (ninefold) of the nucleoside, and then to isolate and determine the amount of each phosphorylated product. The results are reproduced in Table 9.

Table 9

Ratio of Products from Direct Phosphorylation of Nucleosides

	3'-Phosphate	5'-Phosphate	N-Phosphate
Thymidine	1	7	-
Deoxycytidine	1	7	6
Deoxyadenosine	1	16	Not detected
Isopropylideneadenosine	-	4	1
Isopropylideneguanosine	-	9	1
Isopropylidenecytidine	-	7	6

With nucleosides containing amino groups on the pyrimidine or purine rings, the mononucleotide fraction was heated at 100° for an hour to break the phosphoamide linkages. The hydroxylate was then

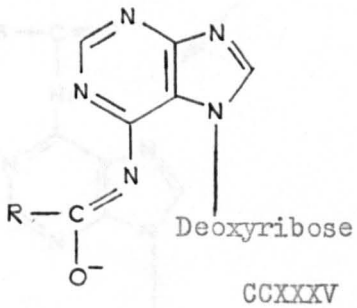
rechromatographed and the ratio of nucleoside to nucleotide determined. This ratio showed the rate of attack of the phosphorylating agent on the ring amino group relative to the hydroxyl groups. Even in the most unfavourable case, that of the cytosine nucleosides, the loss of the phosphorylating agent due to phosphorylation of the amino group is less than 50%, and of interest in the present case for deoxyadenosine, no N-phosphate could be detected.

In order that the molecular configurations of the nucleotides should be as close as possible to those in the unprimed enzymatic syntheses, no protecting group was used for the purine ring amino group of the deoxyadenosine-5'-phosphoric acid in the majority of the present chemical polymerisations.

On the basis of Tener's studies, however, phosphorylation of the ring amino group should not have occurred to any great extent. As a precaution, however, runs were performed in which the ring amino group of both labelled and unlabelled deoxyadenosine-5'-phosphoric acid was protected.

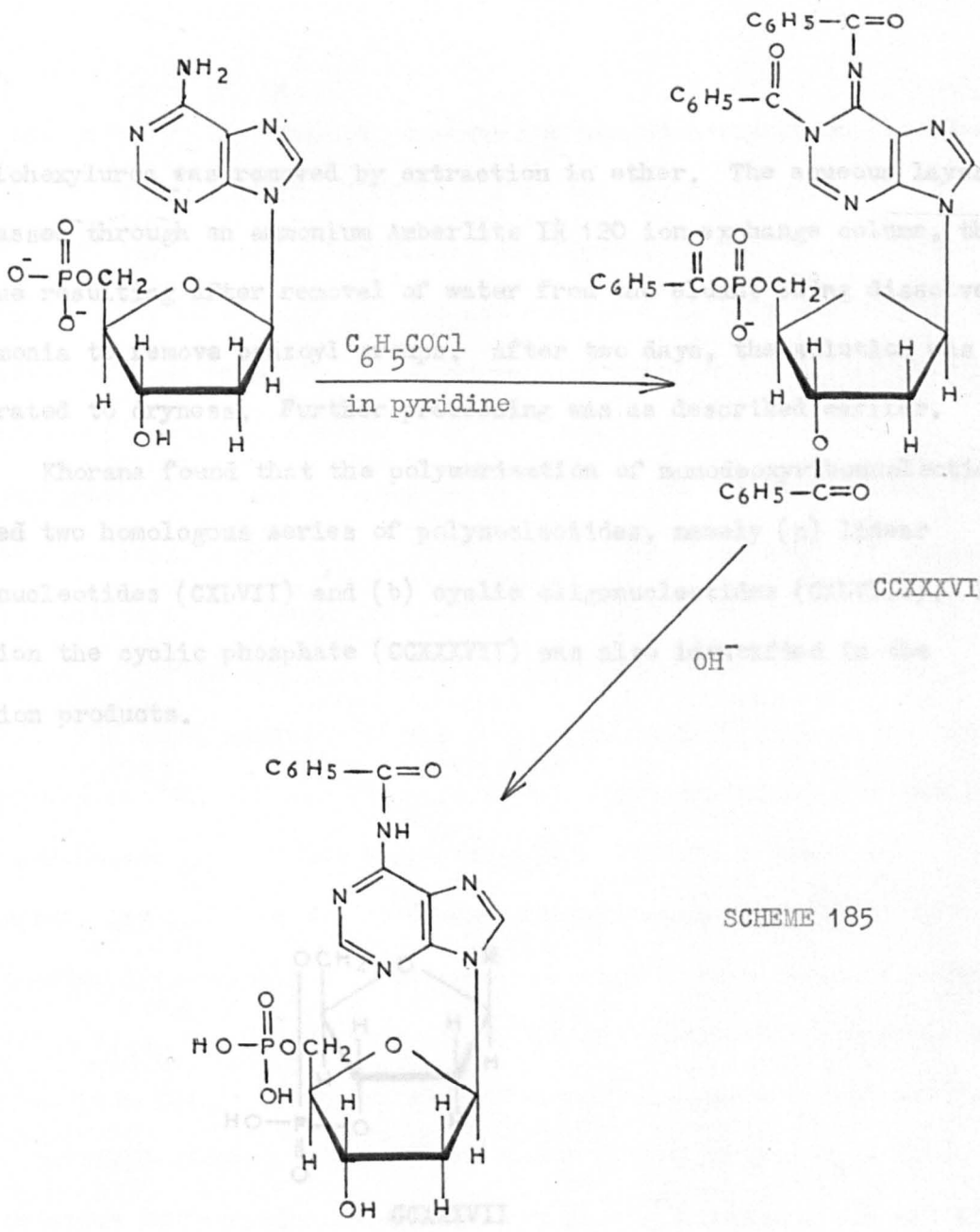
Khorana[191] in his polymerisations of deoxyadenosine-5'-phosphate utilised the benzoyl group as a protecting group for the 6-amino function, and N-benzoyldeoxyadenosine-5'-phosphoric acid was successfully synthesised by his method. Khorana found that the weakly basic ring amino groups could not be selectively acylated in the presence of free hydroxyl groups in the deoxyribose moiety of the nucleoside, the only exception being that of the amino group on the cytosine ring.[318] Additional complications arise using limited amounts of the acylating agents in the case of nucleotides

due to anhydride exchange reactions.[319] Consequently, the general approach taken in the preparation of N-acylmonodeoxyribonucleotides has been to acylate fully the nucleotide and then to remove all the acyl groups except that on the ring amino group. It was found by Khorana that there are marked differences in the rates of O- and N-deacetylation in rather strongly alkaline (c. 1 N sodium hydroxide) solutions. The cause of the stability of the N-acyl groups at high pH values is thought to be the ionisation of the amide group, shown in (CCXXXV) this ionisation according to spectrophotometric determination occurring at about pH 11.



CCXXXV

Using these observations, Khorana developed a highly satisfactory synthesis of N-benzoyldeoxyadenosine-5'-phosphoric acid. Pyridinium deoxyadenosine-5'-phosphate was treated with benzoyl chloride in dry pyridine, and the nucleotide product (CCXXXVI), was extracted in chloroform. The gum remaining after removal of chloroform was treated with alkali, then neutralised with ion-exchange resin. The benzoic acid which appeared in evaporation of the resulting solution was removed. Lyophilisation of the remaining aqueous solution yielded N-benzoyldeoxy-adenosine-5'-phosphate as a white powder (Scheme 185).

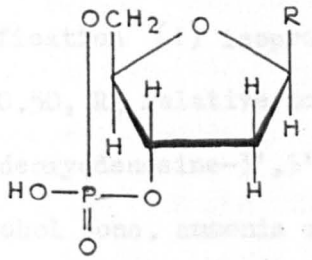


SCHEME 185

Polymerisation experiments using N-benzoyldeoxyadenosine-5'-phosphoric acid were carried out essentially as described previously for the unprotected nucleotide, although the work-up was slightly modified to remove the benzoyl groups from the polymerisation products. Water was added to the reaction mixture and after two hours at room temperature,

dicyclohexylurea was removed by extraction in ether. The aqueous layer was passed through an ammonium Amberlite IR 120 ion exchange column, the residue resulting after removal of water from the eluant being dissolved in ammonia to remove benzoyl groups. After two days, the solution was evaporated to dryness. Further processing was as described earlier.

Khorana found that the polymerisation of monodeoxyribonucleotides yielded two homologous series of polynucleotides, namely (a) linear oligonucleotides (CXLVII) and (b) cyclic oligonucleotides (CXLVIII). In addition the cyclic phosphate (CCXXXVII) was also identified in the reaction products.



CCXXXVII

During the polymerisation of each of the four deoxyribonucleotides, the corresponding deoxyribonucleoside-3',5'-phosphate was identified, but the distribution of higher homologues depended on the type of deoxyribonucleotide used in the polymerisation. Thus, if pyrimidine deoxyribonucleotides were polymerised, the cyclic dinucleotide (XII, n = 0)

was the major cyclic product, whereas for purine deoxyribonucleotides, the cyclic trinucleotide (CXLVIII n = 1) was the major one.

In the present experiments, the formation of a cyclic deoxyribonucleotide as a major product was suspected in only one polymerisation experiment, that being for deoxyadenosine-5'-phosphoric acid-P-32 and thymidine-5'-phosphoric acid. When the reaction products were chromatographed on ion-exchange paper to separate mononucleotides from polynucleotides, a fast moving, ultraviolet absorbing band was visible on inspection of the paper by ultraviolet light, of R_F value 0.9. This band was therefore cut out, and the nucleotide eluted from the paper with water. The total activity of the sample was 8700 c.p.s. and the optical densities at 250, 260 and 280 mμ gave the following absorption ratios - $\frac{250}{260} = 0.85$ and $\frac{280}{260} = 0.24$, suggesting that the base present was adenine. The solution was rechromatographed using two solvent systems to confirm identification (1) isopropyl alcohol conc. ammonia water (7:1:2 v/v) $R_F = 0.50$, R_F relative to deoxyadenosine-5'-phosphoric acid = 2.5 (Khorana for deoxyadenosine-3',5'-cyclic phosphate 0.38 and 2.6). (2) n-Propyl alcohol conc. ammonia water (55:10:35 v/v) $R_F = 0.66$, R_F relative to deoxyadenosine-5'-phosphate = 1.3 (Khorana 0.6 and 1.43). This evidence points to deoxyadenosine-3',5'-cyclic phosphate as a relatively major product in this particular polymerisation, but this behaviour was not observed in apparently identical polymerisations. The factors which could have varied in this particular experiment are water content and nucleotide concentration. Great care was always taken to exclude moisture, and the volume of solvent was similar to that used in other polymerisation experiments. However, this polymerisation is the only one in which

undiluted pyridine was used as solvent (dimethylformamide + pyridine + pyridinium ion-exchange resin being used in other experiments). Hence, if all of the nucleotide material did not go into solution immediately, the effect would be to dilute the reaction mixture and encourage the formation of intramolecular cyclic phosphate.

In the nearest neighbour base sequence analysis, the phosphorus -32 content of the 3'-deoxyribonucleotides, isolated by ion-exchange paper chromatography is a measure of the relative frequency with which one 5'-deoxyribonucleotide reacted with another during the synthesis of the polynucleotides. Thus in the polymerisation of thymidine-5'-phosphoric acid-P-32 with deoxyadenosine-5'-phosphoric acid, the ratio of active 3'-deoxyadenylate to active 3'-thymidylate derived from the polymer had a mean value of 1:5.44, with a standard deviation in the last figure of 2.6. In the polymerisation of deoxyadenosine-5'-phosphoric acid-P-32 with thymidine-5'-phosphoric acid, the same ratio had the mean value of 5.05:1, with a standard deviation in the first figure of 1.17. This means that polymers contain TpT linkages and dApT linkages in a ratio of $5.44 \pm 2.6:1$. (The following abbreviations will be made: dA for deoxyadenosine and T for thymidine; the symbol p in dApT implies a phosphate linkage from the 3' carbon of deoxyadenosine to the 5' carbon atom in thymidine). The polymers also contain dApdA linkages, and TpdA linkages in the ratio of $1:5.05 \pm 1.17$. If, as in Kornberg's unprimed enzymatic synthesis of deoxyribonucleic acid from deoxyadenosine- and thymidine-5'-triphosphates, a perfectly alternating copolymer had been the product in the present experiments, then all the phosphorus-32

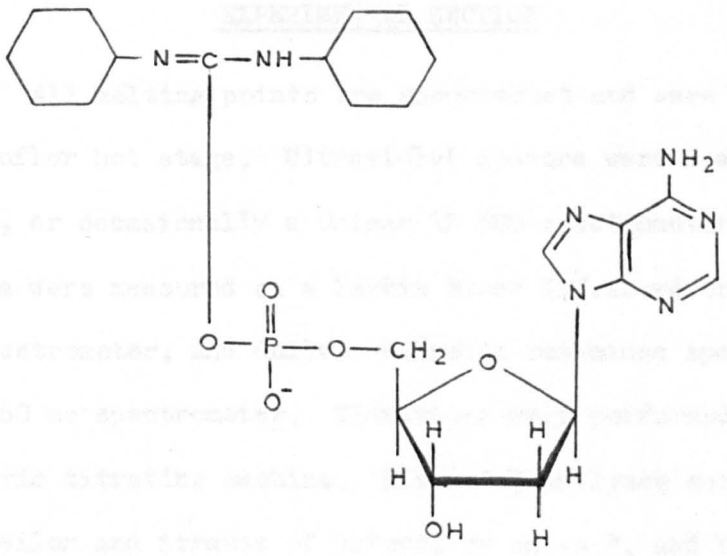
activity would have been transferred to the initially unlabelled deoxyribonucleotide. On the other hand, if, as in Kornberg's unprimed enzymatic polymerisations of deoxycytosine- and deoxyguanosine-5'-triphosphates, homopolymers had been the products, then no activity would be transferred.

The above results therefore, indicate that it is the stereochemistry of E. coli DNA polymerase which determines the base sequences of the polymers which it synthesises in the absence of a primer, and not the molecular configurations of the nucleotides themselves. The results also show that the polymerisation of the deoxyribonucleotides by dicyclohexylcarbodiimide was not a purely random process, since in this case, 50% of the phosphorus-32 activity would have been transferred to the initially unlabelled nucleotide. Thus if the assumption (which is strictly true only for infinitely long or circular molecules) is made that the number of TpdA linkages is equal to the number of dApT linkages, it is clear that the polymers are neither homopolymers nor perfectly alternating sequences, but probably random sequences of nucleotides with the number of thymidylic residues predominating over deoxyadenylic in a ratio of mean value 27.5:1.

The assumption that the number of dApT linkages is equal to the number of TpdA will not hold in reality, since the polydeoxyribonucleotide products are neither infinitely long nor circular. This ratio should, however, be perturbed only by a small factor, the perturbation decreasing with increase in polymer length, since it is only the terminal nucleotide units that affect this ratio. Thus in the polynucleotide

pTpTpTpdA.....pTpTpdA, there should be only one more TpdA linkage that dApT linkage, and the contribution of this to the ratio for the whole polymer is length dependent. This extra TpdA linkage would obviously be cancelled out if the other end of the polymer pdA was the determinating unit. The number of polymer chains having terminal dA units must, however, be relatively small owing to the predominance of thymidylic residues in the chains.

This relative lack of incorporation of deoxyadenosine-5'-phosphoric acid into the polymers could be accounted for by the greater ease of phosphorylation of the thymidine-3'-hydroxyl group, or, when dicyclohexylcarbodiimide is used as the condensing agent, by the lower susceptibility to nucleophilic attack of the hypothetical active intermediate (CCXXXVIII), analogous to the isolatable intermediates formed when ethoxyacetylene is used.[129] Evidence pointing to the former as the important factor comes from work by Tener,[138] who, when phosphorylating deoxyribonucleoside using 2-cyanoethylphosphoric acid and dicyclohexylcarbodiimide, observed that the 3'-hydroxyl group of deoxyadenosine was much less susceptible to phosphorylation than the corresponding group in either thymidine or deoxycytidine.



CCXXXVIII

The ratios of TpT to dApT linkages obtained when thymidine-5'-phosphoric acid-P-32 and N-benzoyldeoxyadenosine-5'-phosphoric acid were polymerised, and similarly those of dApdA to TpdA when thymidine-5'-phosphoric acid and N-benzoyldeoxyadenosine-5'-phosphoric acid-P-32 were polymerised, were not as consistent as those from polymerisations using unprotected deoxyadenylic residues. Thus the figures for the ratio dApdA:TpdA 65:1 and 1:1.15. The ratios 67:1 and 65:1 were derived from experiments in which unreacted mononucleotides were separated from polymers by dialysis, and if dialysis was inefficient, some labelled mononucleotides remaining in the polymer fraction, such high ratios might be expected. The ratios obtained when mononucleotides were separated by paper chromatography are, however, in general agreement with those obtained when no amino protecting group was used.

PART IV

EXPERIMENTAL SECTION

All melting points are uncorrected and were determined on a Kofler hot stage. Ultraviolet spectra were measured on a Unicam SP 800, or occasionally a Unicam SP 500 spectrometer, whilst infrared spectra were measured on a Perkin Elmer Infracord or Perkin Elmer 221 spectrometer, and nuclear magnetic resonance spectra on a Perkin Elmer 60 mc spectrometer. Titrations were performed on a Radiometer automatic titrating machine. Elemental analyses were carried out by Drs. Weiler and Strauss of Oxford, or on an F. and M. carbon/hydrogen/nitrogen analyser (Model 180). Phosphorus-32 activity was determined using Panax Modular equipment.

Pyridine, methylene dichloride and dimethylformamide were all distilled before use, pyridine being stored over calcium hydride or molecular sieve, methylene dichloride over potassium hydroxide pellets and dimethylformamide over molecular sieve. All evaporations were carried out under reduced pressure on a rotary evaporator at a temperature below 40°C.

Tetrahydrofuran was purified and dried by refluxing over sodium hydroxide pellets for three hours, distilled and refluxed over sodium wire for six hours, and finally distilled from the sodium.

Paper Chromatography. - The ascending technique was used at room temperature with Whatman No. 1, No. 4 and diethylaminoethyl

cellulose ion exchange (Whatman DE-20) papers. The solvent systems used were:

Solvent A, n-propanol - 0.88 ammonia - water (6:3:1, v/v); Solvent B, iso-butyric acid - 0.5 N ammonia (5/3, v/v); Solvent C, iso-propanol - 0.88 ammonia - water (7:1:2, v/v); Solvent D, saturated aqueous butanol; Solvent E, 0.3 M ammonium formate; Solvent F, isopropanol - 0.5 M ammonium acetate (pH 6) (5:2, v/v); Solvent G, iso-propanol -0.88 ammonia - acetic acid - water (4:1:2:2, v/v); Solvent H, Ethanol - 1.0 M ammonium acetate (pH 7.5) (7:3).

Paper Electrophoresis. - This was done on Whatman No. 4 paper for 3 hours at a voltage of 500 v.

Nucleotides were located by inspection under ultraviolet light, phosphate esters were detected with the ammonium molybdate/perchloric acid spray,[320] and sulphate by the barium chloride/rhodizonic acid spray.[310]

The ethoxyacetylene used in this work was obtained from Koch-Light Laboratories and distilled before use, or prepared by a modification of the procedure used by Nazarov and co-workers.[278]

1,2-Dibromo-1-ethoxyethane. - Ethyl vinyl ether (205 g.) was slowly added to stirred and cooled (-20° to -30°C) bromine (454 g.). The resulting 1,2-dibromoethoxyethane was used without further purification for the preparation of 2-bromoethoxyethylene.

2-Bromo-1-ethoxyethylene. - Diethylaniline (1000 g.) was stirred and warmed to 95°C under a pressure of 16 - 20 mm. The 1,2-dibromoethoxyethane was added dropwise over the course of three hours, during which the 2-bromoethoxy ethylene distilled over into an ice-cooled flask. On fractional distillation of the product 2-bromoethoxyethylene (280 g.) distilled over at b.p. 45-50° and 18 mm. pressure.

Ethoxyacetylene. - 2-Bromo-1-ethoxyethylene (280 g.) and powdered potassium hydroxide (560 g.) was stirred and heated, and at about 110°C ethoxyacetylene rapidly distilled. The product was fractionally distilled again before use to give ethoxyacetylene (100 g. - 51% from the ethyl vinyl ether) b.p. 51-53°C.

A. Experiments with Sulphonic acids and Ethoxyacetylene

1-Ethoxyvinyl methyl sulphonate. - Methane sulphonic acid was distilled in an atmosphere of dry nitrogen gas, the fraction b.p. 168.5-169°C at 14 mm. pressure being collected and used. Distilled methane sulphonic acid (2.96 g.) in dry methylene dichloride (30 mls.) was added over the course of one hour to cooled (acetone-dry ice bath) and magnetically stirred ethoxyacetylene (6.4 g.). The burgundy-red coloured solution was stirred at room temperature for four hours, when excess ethoxyacetylene and solvent were removed by evaporation under reduced pressure to yield crude golden-yellow coloured 1-ethoxyvinyl methyl sulphonate (4.00 g., 78%). The latter was fractionally distilled, colourless 1-ethoxyvinyl methyl sulphonate distilling at b.p. 86° at 2.5 mm. pressure. The product was too unstable to give a satisfactory elemental analysis but the infrared spectrum showed peaks at 1670 and 1735 $cm.^{-1}$, characteristic of ethoxyvinyl esters, and $\nu_{as}\ SO_2$ at 1385, $\nu_{s}\ SO_2$ at 1175, $\nu_{as}\ CH_3$ 2960, $\nu_{s}\ CH_3$ 2900, $\delta_{as}\ CH_3$ 1470 and $\delta_{s}\ CH_3$ 1385 $cm.^{-1}$ (liquid film). The nuclear magnetic resonance spectrum exhibited peaks at 6.0 τ (complex splitting), 6.8 τ (singlet) and 8.6 τ (triplet) integrating as 4:2.8:2.5 (theory 4:3:3) and a singlet at 6.6 τ which was found to correspond to the methyl groups of methane sulphonic anhydride (comparison with an authentic sample) and represented an impurity of 23%.

Aniline (0.31 g.) was added to 1-ethoxyvinyl methyl sulphonate

(0.38 g.) in a tube, the latter becoming hot and a solid crystallising out of solution. The solid was filtered off, recrystallised from ethanol to give crystals of m.p. greater than 200°C, which were very soluble in water. It was concluded that this product was the anilinium salt of the sulphonic acid (60 mg.). The filtrate was triturated with 95% ethanol when a solid crystallised out of the solution, and this proved to be methane sulphonanilide (0.24 g., 63%) m.p. 98.5 - 99.5°C (lit., 101°).

1-Ethoxyvinylbenzene sulphonate.[139] - Commercial benzene sulphonic acid (10 g.) in benzene (100 mls.) was dehydrated by azeotropic distillation using a Dean-Stark apparatus. When no more water collected, benzene was distilled off until the volume was c. 30 mls., when the resulting solution was frozen in an ice-salt bath. The solid was melted until a few crystals remained in the solution, which was stored overnight at 0-5°C. The benzene was decanted and the crystals of anhydrous benzene sulphonic acid were washed with cold (0°C) dry benzene and finally dried over phosphorus pentoxide in vacuo.

Anhydrous benzene sulphonic acid (3.2 g.) in dry benzene (35 mls.) was added over the course of one hour to cooled (-15°C) and magnetically stirred ethoxyacetylene (7.1 g.). The burgundy-red solution was then stirred for two hours at room temperature when excess ethoxyacetylene and solvents were removed by evaporation under reduced pressure to yield crude 1-ethoxyvinyl benzene sulphonate (4.19 g., 92%) as a brown oil. The latter was distilled to give colourless 1-ethoxyvinyl benzene

sulphonate b.p. 148-150°C at 0.4 mm. pressure. The product was too unstable to give a satisfactory elemental analysis, but the infrared spectrum showed peaks at 1745 and 1675 $cm.^{-1}$ characteristic of 1-ethoxyvinyl esters and ν_{as} SO_2 at 1390, ν_s SO_2 at 1190 $cm.^{-1}$ (carbon tetrachloride solution). The nuclear magnetic resonance spectrum showed peaks at 2.3 τ (complex splitting), 6.2 τ (complex splitting) and a triplet at 8.7 τ, integrating as 5.6:4:2.9 (theory 5:4:3).

Aniline (50 mg.) was added to 1-ethoxyvinyl benzene sulphonate (112 mg.) in a tube, the latter becoming hot and a yellow-white solid forming almost immediately. This solid was recrystallised from methanol to yield benzene sulphonanilide (66 mg. - 72%) m.p. 111°C, (lit. 110°C).

B. The Activation of Sulphuric Acid Esters Using Ethoxyacetylene in the Synthesis of Adenosine-5'-sulphatophosphate.[321]

Sodium benzyl sulphate.[308] - Chlorosulphonic acid (32 mls.) was added to cooled (0°C) and stirred benzyl alcohol (51 mls.) and triethylamine (138 mls.) in chloroform (100 mls.) over the course of seven hours. The reaction was very vigorous and triethylamine hydrochloride was deposited as a powder in the condenser and reaction vessel. A thick cream coloured emulsion remained after stirring for two hours at room temperature, and was washed twice with water (50 ml. aliquots). The chloroform layer was then neutralised with 30% aqueous sodium hydroxide, the resulting solution being concentrated by evaporation in vacuo when inorganic material precipitated out of solution. After removal of this material by filtration, the filtrate was left at 0°C overnight during which triethylamine hydrochloride crystallised out. This was filtered from the chloroform solution, 30% aqueous sodium hydroxide was again added to the latter to pH 8, when a solid precipitated out of solution. This was removed by filtration and recrystallised from 95% ethanol to give silvery leaflets of sodium benzyl sulphate (26 g., 23%), m.p. 194-196°C (lit., 183.5 - 184.5°C).

[Found: C, 40.1; H, 3.7; S, 14.7. Calc. for $C_7H_7O_4SNa$: C, 40.0; H, 3.4; S, 15.25%].

Hydrogenation of the sodium benzyl sulphate. - Sodium benzyl sulphate (104 g.) was hydrogenated using a 10% palladium on charcoal catalyst.

Uptake of hydrogen was rapid at first, but then virtually stopped, conceivably due to poisoning of the catalyst. More catalyst was then added (15 - 20 mg.) and hydrogenation continued for 12 hours, when the total uptake of hydrogen was 12.2 mls. at N.T.P. The theoretical uptake was 11.2 mls. of hydrogen at N.T.P.

Titration of monobenzyl sulphuric acid with alkali. - Sodium benzyl sulphate (210 mg.) was converted to the acid by passage through an Amberlite IR-120 (H^+ form) ion exchange column, and the eluant titrated against 0.95 N aqueous sodium hydroxide on the automatic titrator. The volume of of alkali used (1.05 ml.) corresponded to 210 mg. of sodium benzyl sulphate.

Barium-2-cyanoethyl sulphate. - Ethylene cyanohydrin (10 g.) in dry pyridine (22.4 g.) was slowly added to a solution of chlorosulphonic acid (16.4 g.) in dry ether (150 mls.) cooled to -12°C. The solution was briskly stirred, the temperature of the contents of the flask being held below -10°C during the addition and for one hour after completion of the addition. The ether was then decanted from the white sludge to which water was carefully added until a clear solution resulted. This solution was cooled to 0°C, and barium hydroxide (12.1 g. as a saturated aqueous solution) added. Evaporation of the solution to c. 20 mls. resulted in the deposition of an inorganic white solid, which was filtered off, the filtrate being then evaporated to dryness, leaving a white solid, which was recrystallised three times from

aqueous ethanol. Analysis of the product indicated inorganic contamination, and it was subjected therefore to chromatographic purification on an alumina column. The contaminated barium-2-cyanoethyl sulphate (700mg.) was chromatographed on alumina with gradient elution in ethanol-water. The aqueous concentration of the ethanol was increased by 5% using 10 mls. of each concentration, and 2 ml. aliquots of the eluant were collected and evaporated to dryness in vacuo. Fractions 5, 6 and 7 yielded 588 mg. of a white solid, which was then recrystallised twice from ethanol.

The analysis figures for this compound were consistently lower than those calculated for barium-2-cyanoethyl sulphate, but infrared ($\nu_{as}\ SO_2$, 1450, $\nu_{s}SO_2$ 1200, ν CN 2270 $cm.^{-1}$) and nuclear magnetic resonance data (two triplets at 5.6 and 6.9 τ integrating as 1:1) indicated its presence, probably contaminated by barium sulphate. [Thus found: C, 12.2; H, 1.7; N, 5.0.

$C_6H_8N_2O_8S_2Ba$ requires C, 16.5; H, 1.8; N, 6.45%].

Titration of 2-cyanoethyl sulphuric acid with alkali. - An aqueous solution of the contaminated barium-2-cyanoethyl sulphate (466 mg.) was converted to the acid by passage through an Amberlite IR 120 (H^+ form) ion exchange column. The eluant was evaporated to a transparent gum in vacuo, and this subjected to 0.4 mm. pressure for four hours, when 52.3 mg. of the 2-cyanoethyl sulphuric acid as a gum remained. This was then titrated against 0.23 N aqueous sodium hydroxide solution to pH 7 using the automatic titrator, 14.2 mg. of sodium hydroxide being

taken up. 52.3 mg. of the 2-cyanoethyl sulphuric acid required 13.9 mg. of sodium hydroxide for neutralisation. The solution of the sodium 2-cyanoethyl sulphate was evaporated to a white deliquescent crystalline residue (58 mg.), the weight expected on the base of the titration being 61 mg. These results are tabulated below.

Weight of 2-cyanoethyl sulphuric acid	52.3 mg.
Weight of sodium hydroxide taken up	14.2 mg.
Weight of sodium hydroxide to neutralise 52.3 mg. 2-cyanoethyl sulphuric acid	13.9 mg.
Weight of sodium 2-cyanoethyl sulphate	58 mg.
Weight calculated of sodium 2-cyanoethyl sulphate based on titration with sodium hydroxide	61 mg.

The sodium 2-cyanoethyl sulphate was so deliquescent that successful elemental analysis proved unsuccessful.

Potassium p-nitrophenyl sulphate.[309] - Triethylamine (30 mls.) in dry ether (25 mls.) was added to cooled (-10°C) and stirred chlorosulphonic acid (11.8 g.). To the resulting stirred slurry p-nitrophenol (14 g.) in dry ether (25 mls.) was added over the course of one hour, stirring being continued at room temperature for a further hour. The solution was then refluxed for thirty minutes.

The ether was decanted from the white sludge. Water (50 mls.) was then added to the latter, followed by 10% aqueous potassium hydroxide to pH 8. The golden yellow solution was extracted with

ether (three x 50 mls.) and the aqueous layer adjusted to pH 3 with 1 N sulphuric acid. This solution was extracted with ether (three x 50 ml.) to remove excess p-nitrophenol and the resulting aqueous layer adjusted to pH 11 with 10% aqueous potassium hydroxide. This aqueous solution was diluted with four volumes of acetone, cooled to c. -5°C the resulting white inorganic precipitate being filtered off. The filtrate was left at -5°C overnight, fine yellow needle-like crystals were deposited after 24 hours. These were filtered off, washed with acetone and dried in vacuo. The infrared spectrum (ν_{as} SO_2, 1450, ν_s SO_2 1210 and δ CH for two adjacent hydrogen atoms 840 $cm.^{-1}$) was consistent with potassium p-nitrophenyl sulphate (15 g. - 58%).

Adenosine-5'-sulphatophosphate. -

[1] An authentic sample was prepared by the method used by Baddiley et al.[310] Chlorosulphonic acid (9.5 mls.) was added dropwise to pyridine (23 mls.) in chloroform (100 mls.). Stirring was continued for c. one hour after addition of the acid, when the pyridine-SO_3 complex formed as a white solid precipitate, the pyridine hydrochloride being soluble in chloroform. The complex was filtered, wahsed with chloroform and dried under vacuum.

Pyridine-SO_3 complex (44.4mg.) was added to a stirred solution of adenosine-5'-phosphoric acid in an aqueous sodium hydrogen carbonate solution (1.2 mls. of a solution of a 7% aqueous sodium hydrogen carbonate solution). The temperature of the solution was maintained at 30 - 40°C., and after fifteen minutes the solution was cooled to 0°C. Paper

chromatography and electrophoresis was performed on this solution and the spots characteristic of the products were used as markers in the following experiments.

[2] Sodium benzyl sulphate (415 mg.) was converted to the pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion-exchange column. The eluant was evaporated in vacuo to a gum which was dissolved in dry pyridine (10 mls.), this solution being evaporated to a yellow gum once again. This process was repeated, and the resulting material dissolved in dry methylene dichloride (3 mls.). This solution was added over the course of thirty minutes to a cooled ($0^{\circ}C$) and stirred solution of ethoxyacetylene (430 mg.) in methylene dichloride (2 mls.). After three hours at room temperature, excess ethoxyacetylnene and solvent were removed by evaporation in vacuo. An orange coloured gum remained. This was subjected to 0.05 mm. pressure at $25^{\circ}C$ for several hours. An infrared spectrum of this gum exhibited peaks at 1725 and 1650 $cm.^{-1}$, characteristic of ethoxyvinyl esters. Attempts to purify the product by distillation under reduced pressure were unsuccessful, thus it was used in this crude state for the preparation of benzyl adenosine-5'-sulphatophosphate.

Adenosine-5'-phosphoric acid (20 mg.) was converted to its pyridinium salt by passage through an Amberlite 120 (pyridinium form) ion exchange column, and the eluant evaporated to dryness under reduced pressure, leaving a white solid. This was dissolved as far as possible in dry pyridine, the resulting suspension being evaporated to dryness

once again. The process was repeated, the solid finally being dissolved as far as possible in dry methylene dichloride (5 mls.) and dimethylformamide (5 mls.). The crude ethoxyvinyl benzyl sulphate (40 mg.) in dry methylene dichloride (4 mls.) was then added. The yellow solution which resulted on vigorously shaking was left in the dark in a tightly stoppered flask for five days at room temperature. Solvents were then removed by evaporation under reduced pressure, and the brown viscous residue remaining was dissolved in water (20 mls.) and ethanol (1 ml.). The benzyl group was removed by catalytic hydrogenolysis using a 10% palladium-charcoal catalyst. After the theoretical amount of hydrogen had been taken up, the catalyst was removed by filtration and the filtrate evaporated to small volume in vacuo. Paper chromatography and electrophoresis of this solution indicated formation of adenosine-5'-sulphatophosphate in 35% yield.

Paper Chromatography: Ascending front chromatography on Whatman No. 4 paper.

Solvent System A. - R_F values:-

COMPOUND	Adenosine-5'-phosphoric acid marker	Experiment [1] Authentic Preparation	Experiment [2]
Adenosine-5'-phosphoric acid	0.15	0.15	0.16
Adenosine-5'-sulphatophosphate		0.26	0.25
Adenosine			0.63
Unknown compound containing no phosphate or sulphate			0.80

Solvent System B. - R_F values:-

COMPOUND	Adenosine-5'-phosphoric acid marker	Experiment [1] Authentic Preparation	Experiment [2]
Adenosine-5'-phosphoric acid	0.57	0.55	0.53
Adenosine-5'-sulphatophosphate		0.37	0.38
Adenosine			0.73
Adenosine-5'-sulphatophosphate-(2',3')-sulphate?		0.23	0.23
Unknown compound containing no phosphate or sulphate			0.84

The adenosine-5'-sulphatophosphate spot which resulted from chromatography using Solvent B, was cut out, eluted from the paper, and the nucleotide chromatographed using Solvent A. It then had the same R_F value as the authentic adenosine-5'-sulphatophosphate chromatographed in Solvent A.

Paper Electrophoresis. - Electrophoresis was carried out on Whatman No. 4 paper impregnated with 0.5 M ammonium acetate buffers at two different pH values. Experiments were run for three hours at 500 volts pH 5.5:

COMPOUND	CMS. towards ANODE	CMS. towards CATHODE
Adenosine-5'-phosphoric acid	3.0	
Adenosine-5'-sulphatophosphate	5.5	
Adenosine-5'-sulphatophosphate-(2',3')-sulphate (?)	81.	
Adenosine		0.7
Unknown compound containing no phosphate or sulphate		8.2

These values were identical with those using the authentic preparation, and adenosine-5'-phosphoric acid and adenosine markers.

pH 8.8.

COMPOUND	CMS. towards ANODE	CMS. towards CATHODE
Adenosine-5'-phorphoric acid	4.2	
Adenosine-5'-sulphatophosphate	5.7	
Adenosine-5'-sulphatophosphate-(2',3')-sulphate	7.5	
Adenosine	10.8	0.9
Unknown compound containing no phosphate or sulphate		9.8

These values were identical with those using the authentic preparation, and adenosine-5'-phosphoric acid and adenosine markers.

Estimation of phosphorus content. - The adenosine-5'-sulphatophosphate spot resulting from chromatographic separation using Solvent B was cut out and the nucleotide eluted from the paper. The phosphorus content was estimated colorimetrically as phosphomolybdate (12), the total nucleotide concentration being estimated by the ultraviolet absorption. [Found: P, 7.1. Calc. for $C_{10}H_{16}N_5O_{10}PS$: P, 7.25%].

[3] - The crude barium-2-cyanoethyl sulphate (210 mg.) was converted to the pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion exchange column. Evaporation of the eluant under reduced pressure gave a yellow oil, which was dissolved in pyridine. The resulting solution was evaporated to an oil once again, and this process repeated, the oil being finally dissolved in dry methylene dichloride (4 mls.). This solution was added over the course of thirty minutes to cooled (0°C) and magnetically stirred ethoxyacetylene (470 mg.) in dry methylene dichloride (3 mls.). The resulting orange coloured solution was stirred for three hours at room temperature, when excess ethoxyacetylene and solvent were removed under reduced pressure. The oil which remained showed peaks in the infrared spectrum at 1740 and 1660 $cm.^{-1}$ characteristic of ethoxyvinyl esters.

Adenosine-5'-phosphoric acid (21 mg.) as its anhydrous pyridinium salt was dissolved as far as possible in dry methylene dichloride (2 mls.) and dimethylformamide (2 mls.) and to this solution the above ethoxyvinyl ester (100 mg.) in dry methylene dichloride (2 mls.) was added. The solution resulting after vigorous shaking was kept in

the dark with exclusion of moisture for five days. Solvents were removed by evaporation under reduced pressure, and 0.5 N aqueous sodium hydroxide solution (5 mls.) added to the remaining brown coloured oil. The resulting solution was maintained at 100°C for four minutes, samples (0.25 mls.) being extracted at one minute intervals. Each sample was immediately cooled in an ice-bath and neutralised with Amberlite IR 120 (H^+ form) ion exchange resin. Chromatography in Solvents A and B followed by elution of $\frac{1}{4}$ x 1" strips of the paper parallel to the direction of development indicated formation of adenosine-5'-sulphato-phosphate in 45% yield after treatment with the sodium hydroxide for 2 minutes.

Paper Chromatography.

COMPOUND	Solvent A	Solvent B
Adenosine-5'-phosphate	0.12	0.46
Adenosine-5'-sulphatophosphate	0.23	0.37
Adenosine	0.58	0.68

These values were similar to those using the authentic preparation, and adenosine-5'-phosphoric acid and adenosine markers.

[4] Potassium p-nitrophenyl sulphate (228 mg.) was converted to its pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion-exchange column, the eluant being evaporated to a brown solid in vacuo. The residue was dissolved in dry pyridine and the solution

evaporated to dryness. The process was repeated, the solid being finally dissolved in dry methylene dichloride (2 mls.). This solution was added over the course of one hour to a cooled ($0^{\circ}C$) and stirred solution of ethoxyacetylene (240 mg.) in dry methylene dichloride (2 mls.). A burgundy coloured solution resulted which turned a golden colour on stirring the solution for two hours at room temperature. Excess ethoxyacetylene and solvent were removed under reduced pressure, the resulting brown viscous liquid being subjected to 0.2 mm. pressure for several hours. An infrared spectrum of the remaining gum exhibited peaks at 1750 and 1670 $cm.^{-1}$, characteristic of ethoxyvinyl esters.

Adenosine-5'-phosphoric acid (21.6 mg.) as its pyridinium salt was dissolved as far as possible in dry methylene dichloride (3 mls.) and dimethylformamide (3 mls.) and to this solution the above ethoxyvinyl ester (150 mg.) in dry methylene dichloride (2 mls.) was added. The suspension was shaken vigorously and the resulting solution kept in the dark with exclusion of moisture for five days. Solvents were then removed by evaporation under reduced pressure, and 1 N aqueous potassium hydroxide (10 mls.) added to the resulting oil. This solution was maintained at $100^{\circ}C$ for three hours, samples (0.5 ml.) being extracted at minute intervals for the first ten minutes, and half hourly thereafter. Each sample was cooled in an ice bath and neutralised with Amberlite IR 120 (H^{+} form) ion exchange resin, the resin then being filtered off and p-nitrophenol removed by extraction with ether (three x 5 ml.), the aqueous solution then being chromatographed using Solvents A, B and electrophoresis at pH 5.5.

It was found that alkaline hydrolysis of the p-nitrophenyl groups was complete after five minutes of the alkaline treatment at 100°C, and with samples where hydrolysis had not exceeded two hours, the main products being unchanged adenosine-5'-phosphoric acid and adenosine-5'-sulphatophosphate-(2',3')-sulphate (identified below), and no adenosine-5'-sulphatophosphate. However, with samples which had been subjected to hydrolysis for more than two hours, a trace of adenosine-5'-sulphatophosphate was detected. All the nucleotides moved at the rates expected in all three systems, authentic markers being used. The adenosine-5'-sulphatophosphate-(2',3')-sulphate isolated by chromatography using Solvent B was cut out and the nucleotide eluted from the paper. The absorption of the resulting solution was determined at 260 mμ, the water evaporated off, and the residue treated with 0.5 N hydrochloric acid at 100°C for five minutes. A saturated solution of barium chloride was added, and the resulting barium sulphate centrifuged down, the supernatant liquid decanted off, and the inorganic residue dried and weighed. The hydrolysis of 0.932 mg. of nucleotide gave 0.952 mg. barium sulphate, and so the molar ratio of nucleotide to sulphate was 1:0.45, indicating two sulphate groups per nucleotide molecule.

C. Experiments with Thiolic Acids and Ethoxyacetylene.[222]

1-Ethoxyvinyl thiolacetate. - Thioacetic acid (2.23 g.) in dry methylene dichloride (4 mls.) was added over the course of a half hour to cooled (0°C) and magnetically stirred ethoxyacetylene (7.3 g.) in dry methylene dichloride (5 mls.). The resulting yellow-green solution was then stirred at room temperature for four hours, when solvent and excess ethoxyacetylene were removed by evaporation from the golden coloured solution, to give 1-ethoxyvinyl thiolacetate (3.05 g., 66%). This product was distilled, b.p. 58-62°C at 3.5 mm. pressure. $\nu_{max.}$ 1720, 1680 cm.$^{-1}$.

[Found: C, 49.2; H, 6.7; S, 22.1. $C_6H_{10}O_2S$ requires: C, 49.3; H, 6.9; S, 21.9%].

Ethyl thionacetate.[322] - Dry hydrogen chloride gas was passed through a solution of acetonitrile (30.4 g.), (first dried over anhydrous magnesium sulphate for two hours), and absolute ethanol (29.8 g.) in dry ether (40 mls.) for eight hours. The saturated solution was left overnight, and when, next day, no crystals of ethyl acetimidate hydrochloride had appeared, dry ether (20 mls.) was added and the solution was shaken vigorously to drive off excess hydrogen chloride gas. The crystals of ethyl acetimidate hydrochloride which then appeared were filtered, more crystals appearing in successive filtrates. The combined fractions of crystals were then shaken with 20% aqueous potassium hydroxide (175 mls.) and ether (100 mls.), and the ethyl acetimidate extracted with the ether layer. Further extractions of the aqueous

layer with ether (100, 50 and 50 ml. portions) were performed, and the ether evaporated from the combined ether extracts. Ethyl acetimidate (27 g., 42%) remained.

A solution of the ethyl acetimidate in ether (30 mls.) was cooled (0°C), and dry hydrogen sulphide bubbled through this solution for one hour. Two layers had then formed, and water was added to the two layers. The aqueous layer was extracted with ether (20 and 10 ml. aliquots) and hydrogen sulphide passed through the combined cooled (0°C) ether extract for a further two hours, when the ether was evaporated off. Ethyl thionacetate, a yellow coloured, pungent smelling and fuming liquid remained (9.8 g., 31%), along with crystals of thioacetamide (6.5 g.) m.p. 112-113°C, authentic m.p. 112.5°C. These crystals were filtered from the ethyl thionacetate, whose infrared spectrum was found to be identical to that of the authentic compound[323] as was its ultraviolet spectrum in ethenol, $\lambda_{max.}$ = 241 mμ, logε = 3.9

1-Ethoxyvinyl thiobenzoate. - Thiobenzoic acid (2.71 g.) in dry methylene dichloride was added over the course of a half hour to a cooled (0°C) and magnetically stirred solution of ethoxyacetylene (4.23 g.) in dry methylene chloride (5 mls.). Stirring was then continued for four hours at room temperature, when solvent and excess ethoxyacetylene were removed by evaporation from the golden-coloured solution, 1-ethoxyvinyl thiolbenzoate remaining (3.57 g., 92%). This was then distilled, b.p. 88-90°C at 0.4 mm. pressure. $\nu_{max.}$ 1740, 1675 cm.$^{-1}$ [Found: C, 64.0; H, 5.5; S, 15.3. $C_{11}H_{12}O_2S$ requires C, 63.45; H, 5.8; S, 15.8%].

Aniline (0.35 g.) was added to a solution of 1-ethoxyvinyl thiolbenzoate (0.39 g.) in dry methylene dichloride (3 mls.), and the resulting solution refluxed for twenty hours. The solvent was evaporated off, and the resulting solid recrystallised from absolute ethanol to yield flat crystals of benzanilide (0.27 g., 73%) m.p. 163°C, mixed m.p. with an authentic sample 160-161°C.

Ethyl thionacetate could again be identified on the vapour-phase chromatograms of a mixture of 1-ethoxyvinyl thiolbenzoate and aniline using two different columns. An ultraviolet spectrum of this reaction mixture had $\lambda_{max.}$ = 241 mμ.

D. Experiments with α-keto and di-carboxylic Acids and Ethoxyacetylene.[291]

The reaction of pyruvic acid with ethoxyacetylene. - Pyruvic acid (Eastman Kodak) was fractionally distilled before use, the fraction b.p. 64°C at 11 mm. pressure being used.

Ethoxyacetylene (10.8 g.) was added slowly to mercuric acetate (435mg.) in dry methylene dichloride (50 mls.), the resulting solution being cooled (-15°C) and magnetically stirred. To this solution, the distilled pyruvic acid (4.5 g.) in dry ether (50 mls.) and dry methylene dichloride (10 mls.) was added dropwise from a pressure equalising funnel over the course of one hour. The resulting burgundy-red solution was stirred at room temperature for a further four hours, solvents and excess ethoxyacetylene being removed from the resulting golden-yellow coloured solution by evaporation. 1-Ethoxyvinyl pyruvate (8.1 g., 91%) remained, its infrared spectrum exhibiting peaks at 1670 and 1730 $cm.^{-1}$.

This ethoxyvinyl ester was then stirred and heated carefully, and when the temperature had reached c. 80°C, an exothermic reaction occurred, so that control was necessary by cooling the reaction flask. Volatile products were collected by passage through a water condenser, and other gases were collected in a measuring cylinder immersed in water. Heating was continued for two hours.

Ethylacetate (130 mg.) was collected from the receiver, and carbon monoxide collected in the measuring cylinder (650 mls. at N.T.P. -65%), and this was identified by the formation of molybdenum blue.[313]

The dark-brown residue remaining in the reaction flask was then fractionally distilled to give ethyl acetoacetate (3.8 g., 55%)

b.p. 61-62°C at 10 mm. pressure. A comparison of its infrared spectrum with that of an authentic sample was satisfactory.

[Found: C, 55.4; H, 7.7. Calc. for $C_6H_{10}O$: C, 55.2; H, 7.8%].

The 2,4-dinitrophenylhydrozone was also prepared m.p. 93.5-94°C, mixed m.p. 94-95°C. 3.0 g. of black solid residue remained in the distillation flask.

The reaction of oxalic acid with ethoxyacetylene. - Ethoxyacetylene (10.7 g.) was added slowly to mercuric acetate (444 mg.) in dry methylene dichloride (50 mls.) and the solution cooled (-15°C) and magnetically stirred. Anhydrous oxalic acid (4.4 g.) in dry ether (35 mls.) and dry methylene dichloride (15 mls.) was added dropwise over the course of one hour, and then stirred for a further three hours, when solvents and excess ethoxyacetylene were removed by evaporation, to give ethoxyvinyl oxalate (9.6 g., 85%). This ester was then heated carefully as previously, ethylacetate (960 mg.) and carbon monoxide (880 mls. at N.T.P. - 86%) being collected. Fractional distillation of the residue gave diethyl acetone dicarboxylate (4.9 g., 58%) b.p. 74-76°C at 10 mm. pressure.

[Found: C, 53.0; H, 7.3. Calc. for $C_9H_{14}O_5$: C, 53.5; H, 7.0%].

The copper salt of acetone dicarboxylate was prepared, m.p. 147-148°C. Mixed m.p. with an authentic sample 146.5-147.5°C. A comparison of the infrared spectrum with that of an authentic sample of diethyl acetone dicarboxylate was entirely satisfactory. 2 g. of solid black residue remained in the distillation flask.

The reaction of phthalic acid with ethoxyacetylene. - Mercuric acetate (416 mg.) and ethoxyacetylene (10.6 g.) were added to cooled (-10°C) and magnetically stirred dry methylene dichloride. Anhydrous phthalic acid (8.42 g.) in tetrahydrofuran (50 mls.) was added over the course of one hour to this solution, and stirring continued for four hours at room temperature. Solvents and excess ethoxyacetylene were removed by evaporation, when white needles crystallised out of solution. These were filtered off, washed with ether dried in vacuo, and identified as phthalic anhydride (7.2 g., 92%), m.p. 133.5-134°C mixed m.p. 133.5-134°C.

E. The Protection of amino-acids by Phthaloylation using Ethoxyacetylene.

N-Phthaloylglycine ethyl ester. - Glycine ethyl ester hydrochloride (6 g.) was dissolved in a minimum volume of water, and solid potassium hydroxide added to the cooled solution (0°C) until pH 9. This solution was then extracted with ether (three x 50 ml.), the combined ether extracts being dried over anhydrous magnesium sulphate. This solution was filtered, the ether being evaporated off the filtrate leaving glycine ethyl ester (2.23 g.) as a brown oil.

Glycine ethyl ester (2.23 g.) was run into a cold (0°C) solution of ethoxyacetylene (18.2 g.) in dry methylene dichloride (50 mls.) and the solution magnetically stirred. Anhydrous phthalic acid (3.59 g.) in tetrahydrofuran (30 mls.) was added to this solution over the course of one hour, stirring being continued for a further six hours at room temperature. On removing solvents and excess ethoxyacetylene, a brown gum remained, from which transparent needles crystallised on trituration with aqueous ethanol. These were identified as N-phthaloylglycine ethyl ester, (4.08 g., 80%) m.p. 112.5 - 113.5°C. (Beilstein m.p. 111-113°C). [Found: C, 62.0; H, 4.8; N, 5.7. Calc. for $C_{12}H_{11}NO_4$: C, 61.8; H, 4.8; N, 6.0%].

The infrared spectrum was as expected and the nuclear magnetic resonance spectrum consisted of peaks at:- 2.17 τ , complex splitting; 5.57 τ , unsplit; 5.78 τ , quartet; 8.7 τ , triplet, and integrated in the ratio 4:2:2:3.

This experiment was repeated using glycine ethyl ester hydrochloride with pyridine in aqueous solution, when N-phthaloylglycine

ethyl ester was again isolated in 85% yield.

N-Phthaloylglycine. - A solution of glycine (0.75 g.) and phthalic acid (1.66 g.) in pyridine (2 mls.) and water (15 mls.) was dripped over the course of one hour into briskly stirred ethoxyacetylene (5.42 g.). The reaction was markedly exothermic, the solution becoming hot during the addition. After stirring for six hours, the two phase solution was evaporated down to a golden oil, which was triturated with absolute ethanol when a fine white solid was precipitated. This was filtered off, washed with ethanol to give glycine (122 mg.) m.p. 258-260°C mixed m.p. 260-262°C. On evaporation of the filtrate, a further 78 mg. of glycine crystallised out of solution, and was filtered off. The filtrate was evaporated to dryness, when phthalic anhydride (78 mg.) sublimed round the neck of the flask, m.p. 133-134°C, mixed m.p. 132.5-134°C. The solid residue remaining in the flask was dissolved in a small amount of 95% ethanol and triturated with benzene/40-60 petroleum ether, when N-phthaloylglycine crystallised (1.1 g., 54%). m.p. 192-194°C (Beilstein m.p. 192°C).

[Found: C, 58.2; H, 3.5; N, 7.1. Calc. for $C_{10}H_7NO_4$: C, 58.5; H, 3.4; N, 6.8%].

The infrared and nuclear magnetic resonance spectra were as expected.

Investigation for racemisation during formation of phthaloyl protected amino acids.[272] - Phthalic acid (1.26 g.) in pyridine (2 mls.) and water (20 mls.) was dripped over the course of thirty minutes into L-phenylalanine

methyl ester hydrochloride (1.64 g.), ethoxyacetylene (13.8 g.) and water (5 mls.). The solution became quite hot and was stirred for a further two hours. The resulting solution was evaporated in vacuo to a brown oil, from which N-phthaloyl-L-phenylalanine methyl ester was crystallised by trituration with benzene/40-6- petroleum ether (2.1 g., 84%) m.p. 113-114°C. [Found: C, 70.2; H, 5.1; N, 4.7. Calc. for $C_{28}H_{15}NO_4$: C, 69.9; H, 4.9; N, 4.5%].

The N-phthaloyl-L-phenylalanine methyl ester was dissolved in absolute ethanol (30 mls.), and alcoholic hydrazine hydrate solution (10 mls. of a solution of hydrazine hydrate (4.1 g.) in ethanol (100 mls.)) added. The resulting solution was refluxed for one hour, when solvent was removed by evaporation in vacuo and c. 2 N hydrochloric acid (30 mls.) added to the brownish residue. The flask was warmed to 50°C for five minutes and cooled slowly to room temperature. Phthalyl hydrazide was removed by filtration, and the filtrate evaporated to a gum in vacuo. L-Phenylalanine methyl ester hydrochloride was recrystallised from ethanol (1.05 g., 81%).

$[\alpha]_D^{20°}$ of L-phenylalanine methyl ester hydrochloride = -3.9°.

$[\alpha]_D^{20°}$ of L-phenylalanine methyl ester hydrochloride after phthaloylation and removal of the protecting group = -4.08°.

Di-1-ethoxyvinyl terephthalate. - A suspension of anhydrous terephthalic acid (4.16 g.) in dry methylene dichloride (50 mls.) was added over the course of thirty minutes to magnetically stirred ethoxyacetylene (14.14 g.)

and mercuric acetate (200 mg.) in tetrahydrofuran (50 mls.)s. After stirring for two days, the suspension had disappeared, a greenish coloured solution resulting. Excess ethoxyacetylene and solvents were removed by evaporation under reduced pressure, when a brown crystalline compound remained. This was recrystallised three times from dry methylene dichloride - hexane to yield di-1-ethoxyvinyl terephthalate (3.5 g., 50 %), m.p. 82.5-83.5°C.

[Found: C, 62.6; H, 6.1. $C_{16}H_{18}O_6$ requires: C, 62.7; H, 5.9%].

The infrared spectrum exhibited peaks at 1740 and 1675 $cm.^{-1}$ characteristic of 1-ethoxyvinyl esters, and the nuclear magnetic resonance spectrum exhibited peaks at 1.98τ (singlet), 6.1τ (complex splitting), 8.65τ (triplet) integrating as 4:8:6.

F. Sequence Specificity in Synthetic Polydeoxyribonucleotides.[325,326]

Preparation of P-32-deoxyribonucleotides.

Preparation of nucleosides and nucleotides. - Deoxyadenosine was obtained from Nutritional Biochemical Corporation, Calbiochem. (as the monohydrate); thymidine from Calbiochem; deoxyadenosine-5'-phosphoric acid from Calbiochem. (as the monoammonium salt) and Pabst Laboratories, and thymidine-5'-phosphoric acid from Pabst Laboratories (as the sodium salt).

Enzymes. - Calf Spleen phosphodiesterase was obtained from Koch-Light Laboratories, and Micrococcal Deoxyribonuclease (extracted from Micrococcus pyogenes v. aureus) as a dry ammonium sulphate cake from the Worthington Biochemical Corporation, New Jersey.

Thymidine-5'-phosphoric acid-P-32. (A) - Barium thymidine-5'-phosphate-P-32 (18 mg.) which had been prepared by the Radiochemical Centre, Amersham, was chromatographed on an Amberlite IR120 (pyridinium form) ion exchange column. Eight 10 ml. fractions of the eluant were collected, and a sample of each chromatographed on paper using Solvent C. Some separation of the required nucleotide had been achieved by the ion exchange chromatography, and so the first two fractions of the eluant were bulked and evaporated down to a white residue in vacuo. This residue was used in the polymerisations.

B.- Thymidine-5'-phosphoric acid-^{32}P. - This was prepared by the method of Michelson and Todd[98] followed by that of Tener.[138]

5'-Tritylthymidine. - Anhydrous thymidine (210 mg.) was tritylated by trityl chloride (282 mg.) in anhydrous pyridine (1.5 mls.), and 5'-tritylthymidine was isolated in 76% yield (175 mg., m.p. 125°, lit., 128°).

3'-Acetyl 5'-tritylthymidine. - 5'-tritylthymidine (175 mg.) was treated with freshly distilled acetic anhydride (0.4 mls.) in dry pyridine (1.5 mls.). The resulting 3'-acetyl 5'-trityl thymidine was recrystallised from benzene-petroluem ether (40/60) to give the product as needles in 91% yield (168 mg.; m.p. 104°, lit., 105°).

3'-Acetylthymidine. - 3'-Acetyl 5'-tritylthymidine (168 mg.) was detritylated using acetic acid (1 ml. of 80%). 3-Acetylthymidine, recrystallised from acetone-petroleum ether (40/60) was isolated in 58% yield (53 mg., m.p. 172-174°, lit., 176°).

Thymidine-5'-phosphoric acid-^{32}P. - A stock solution of 2-cyanoethyl phosphoric acid-^{32}P was made up as follows. Barium 2-cyanoethyl phosphate (688 mg.) and barium 2-cyanoethyl phosphate-^{32}P (16.6 mg.) was converted to the acid form by passage through an Amberlite IR 120 (H^+ form) ion exchange column, the eluant being evaporated to c. 20 mls. volume under reduced pressure. Pyridine (20 mls.) was added to this

solution, and the latter reduced to c. 10 mls. by evaporation. The solution was made up to 50 mls. with pyridine in a graduated flask.

3'-Acethylthymidine (18 mg.) was phosphorylated using 2-cyanoethyl phosphoric acid-^{32}P (5 mls. of the above stock solution) and dicyclohexylcarbodiimide (95.6 mg.) in anhydrous pyridine solution (2 mls.). During the work up, the acetyl groups was removed by alkaline hydrolysis (1 N aqueous sodium hydroxide solution). After filtration of the resulting solution, the filtrate was passed through an Amberlite IR 120 (H^+ form) ion-exchange column. Paper chromatography using Solvent C indicated that the only ultraviolet absorbing product was thymidine-5'-phosphoric acid -^{32}P (12 mg., 60%).

Deoxyadenosine-5'-phosphoric acid-^{32}P.

(A) The method of Todd and co-workers was used for this preparation,[99,316] followed by that of Tener.[138]

3',5'-Diacetyldeoxyadenosine. - Anhydrous deoxyadenosine (2 g.) was acetylated using freshly distilled acetic anhydride (5 mls.) in dry pyridine (10 mls.). 3',5'-Diacetyldeoxyadenosine was recrystallised from ethyl acetate-petroleum ether (40/60) (1.9 g., 78%; m.p. 138-142°, lit., 151-152°).

3-Acetyldeoxyadenosine. - 3',5'-diacetyldeoxyadenosine (1.9 g.) was partially deacetylated using a solution of saturated methanolic ammonia (60 mls.). The products were separated on an automatic countercurrent distribution apparatus. With ethyl acetate-water as

the solvent system, 190 transfers were completed. The distribution of products was determined by running paper chromatograms of samples taken from every fifth tube, the solvent system for the chromatograms being Solvent D. Samples were then taken at more frequent tube intervals to determine the product boundaries exactly. The following products were isolated:

Tubes 1 - 20	Deoxyadenosine	306 mg.
Tubes 24 - 47	5'-acetyldeoxyadenosine	425 mg.
Tubes 56 - 96	3'-acetyldeoxyadenosine	236 mg.
Tubes 120 - 150	3',5'-diacetyldeoxyadenosine	1.17 g.

The 3'-acetyldeoxyadenosine was recrystallised from ethanol to give 216 mg. of the nucleoside (m.p. 215-216°, lit., 214-216°).

Deoxyadenosine-5'-phosphoric acid-^{32}P. - 3-Acetyl deoxyadenosine (15 mg.) was phosphorylated using 2-cyanoethyl phosphoric acid-^{32}P (5 mls. of the stock solution) and dicyclohexylcarbodiimide (86.5 mg.) in anhydrous pyridine (1 ml.). The acetyl group was removed by alkaline hydrolysis during the work up. Deoxyadenosine-5'-phosphoric acid-^{32}P, identified by paper chromatography on Whatman Chromedia DE81 and Solvent E using a nucleotide marker, was isolated successfully (16.9 mg.).

(B) The method of Tener[138] was used in this case.

Deoxyadenosine (81 mg. as the monohydrate) was phosphorylated using 2-cyanoethyl phosphoric acid-^{32}P (from barium 2-cyanoethyl

phosphate (384 mg.) + barium 2-cyanoethyl phosphate-^{32}P (37.5 mg.)) and dicyclohexylcarbodiimide (211 mg.) in dry pyridine (2 mls.). The 2-cyanoethyl group was removed during the work up by treatment with ammonia, and the products of the reaction were separated by chromatography on a Dowex 1-X8 (chloride, 300-400 mesh) ion-exchange column. Elution was carried out using a linear gradient of one litre of 0.01 N hydrochloric acid into one litre of water. Unreacted deoxyadenosine came off first, followed closely by a second unidentified nucleotidic product. The deoxyadenosine-5'-phosphoric acid-^{32}P came off the column in fractions 46-70 (1 fraction = 15 mls.) (23 mg., 25%, activity 5,928 x 10^3 c.p.s.).

N-Benzoyldeoxyadenosine-5'-phosphoric acid. - This was prepared by the method of Khorana and co-workers.[191] The product was isolated as a dry white powder by lyophilisation in 80% yield.

N-Benzoyldeoxyadenosine-5'-phosphoric acid-P-32. - Deoxyadenosine-5'-phosphoric acid-P-32 was converted to its pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion-exchange column, the eluant was evaporated to a gum under reduced pressure, and water (5 mls.) added to the gum. The nucleotide was lyophilised, but rather unsatisfactorily since a semi-solid gum remained. This residue was dissolved in freshly distilled benzoyl chloride (0.3 mls.) in dry pyridine (3 mls.). The resulting solution was shaken in the dark at room temperature for one hour, when the reddish coloured solution was

poured into chloroform (10 mls.) and ice-water (10 mls.). The heterogeneous solution was shaken and left for thirty minutes, when the chloroform layer was decanted and the aqueous layer extracted with chloroform (two 10 ml. aliquots). The combined chloroform extracts were evaporated to a gum under reduced pressure, and the gum dissolved in pyridine (2 mls.) and ice-water (1 ml.) and cold 2 N aqueous sodium hydroxide solution (2.5 mls.). The flask was immersed in an ice-bath for nine minutes when an excess of Amberlite IR 120 (pyridinium form) ion-exchange resin was added rapidly to neutralise and remove sodium ions. To ensure complete removal of the latter, the solution was washed through an Amberlite IR 120 (pyridinium form) column. The eluant was concentrated to c. 10 mls. when benzoic acid largely crystallised out. The mixture, which was kept cold in an ice-bath was repeatedly extracted with ether (eight x 30 ml.) to remove benzoic acid. The pH of the final solution was around 4, and a little pyridine (c. 5 mls.) was added and this solution evaporated under reduced pressure to give a yellow gum of pyridinium N-benzoyl deoxyadenosine phosphate (12 mg., 46%). Paper chromatography using Solvent F exhibited only one ultraviolet absorbing product, R_F 0.6 (deoxyadenosine-5'-phosphoric acid marker 0.2).

The polymerisation of thymidine-5'-phosphoric acid-P-32 with deoxyadenosine-5'-phosphoric acid.[191] - Sodium thymidine-5'-phosphate (17.9 mg.) and diammonium thymidine-5'-phosphate-P-32 (4.8 mg., of activity 29,125 x 10^3 c.p.s.) were converted to the pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion-exchange column, the eluant being concentrated to c. 5 mls. by evaporation under reduced pressure. Deoxyadenosine-5'-phosphoric acid (21 mg. of the monohydrate) were added, and the resulting solution evaporated to dryness. Dry pyridine (10 mls.) was added to the semi-solid residue, the resulting suspension being evaporated to dryness. This process was repeated with 10, 5 and 2 ml. portions of dry pyridine. The residue was finally dissolved in dry dimethylformamide (1 ml.) and dicyclohexylcarbodiimide (52 mg.) added, followed by dry pyridinium Amberlite IR 120 ion-exchange resin. The solution was tightly stoppered and kept in the dark at room temperature for 24 hours. Dicyclohexylurea crystallised out, and dry dimethylformamide (1 ml.) and dicyclohexylcarbodiimide (54 mg.) were added to the mixture.

After five days in the dark with exclusion of moisture at room temperature and frequent shaking, water (10 mls.) was added, and the mixture left at room temperature for two hours. The aqueous solution was extracted with ether (six x 30 ml.) and the aqueous layer was then evaporated to dryness under reduced pressure. The residue was dissolved in water (10 mls.) and this solution adjusted to pH 7 with saturated aqueous lithium hydroxide solution. The resulting solution was evaporated to dryness, water (10 mls.) was added to the residue and

the pH of the solution adjusted to 7 as previously to ensure complete removal of pyridine. The resulting solution was evaporated to dryness, water (10 mls.) added and the solution filtered, the filtrate being divided in half, and the mononucleotides and lower oligonucleotides removed from each half as below.

(i) One half of the above solution was made up to c. 15 mls. with water, and dialysed against water for 48 hours, the resulting solution being evaporated to dryness under reduced pressure.

(ii) The other half of the solution was concentrated to c. 1 ml. by evaporation under reduced pressure, and this solution chromatographed on Whatman Chromedia DE81 paper using Solvent E. Polynucleotides and higher oligonucleotides were held near the origin, and this band was cut out and eluted into water, the eluant being evaporated to dryness under reduced pressure.

Nearest Neighbour base sequence analysis.[206] - The same technique was used for the polymeric gum from each of the two methods of mononucleotide and lower oligonucleotide separation. The gum was dissolved in Tris buffer solution (0.2 mls. ≡ 2 μ moles - 12.2 mg. in 10 mls. water), aqueous calcium chloride solution (0.2 mls. ≡ 1 μ mole - 5.5 mg. in 10 mls. water) and water (0.2 mls.). Micrococcal DNase (4.5 mg. ≡ 450 - 1250 units) was added, and the resulting solution incubated at 37°C for two hours, when the solution was adjusted to pH 7 using 0.1 N hydrochloric acid. Calf spleen phosphodiesterase (c. 1 unit) was added, and the solution incubated at 37°C for one hour, when

phosphodiesterase (c.1 unit) was again added, followed by another unit after another hour's incubation. The solution was incubated for a total time of three hours with phosphodiesterase, the resulting solution then being concentrated to c. 0.3 ml. by evaporation under reduced pressure. The solution was chromatographed using Whatman Chromedia DE 81 and Solvent E to separate thymidine-3'-phosphoric acid from deoxyadenosine-3'-phosphoric acid. Each nucleotide was then cut out and eluted from the paper with water, a fraction of the eluant being pipetted onto a planchette and evaporated down evenly to dryness under an infrared lamp. The activity of the sample was then determined using a windowless scintillation counting assembly with an anthracene crystal. A background count was obtained by eluting a strip of paper on which no ultraviolet absorption was observed, a fraction of the eluant evaporated to dryness on a planchette and its activity determined.

Two more polymerisations with analyses of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid were carried out in the same manner, except that in one case, analysis was carried out on a fraction without prior removal of mononucleotides and lower oligonucleotides, and the polymeric products were separated from by chromatography on Whatman No. 1 paper using Solvent G, and, after enzymatic degradation, 3'-nucleotides were separated on Whatman No. 1 paper using Solvent H.

The Polymerisation of thymidine-5'-phosphoric acid with deoxyadenosine-5'-phosphoric acid-P-32. - Sodium thymidine-5'-phosphate (19.5 mg.) was converted to its pyridinium salt by passage through an Amberlite IR 120

(pyridinium form) ion-exchange column. The eluant was evaporated to dryness under reduced pressure, the resulting gum dissolved in dry pyridine, and the solution added to the solution of deoxyadenosine-5'-phosphoric acid-P-32 (17 mg., 2964 x 10^3 c.p.s.) prepared as above (some carrier nucleotide having been added). The total solution was evaporated to dryness under reduced pressure, the residue being suspended in dry pyridine (10 mls.) and evaporated to dryness. This process was repeated using 10, 10 and 5 ml. aliquots of dry pyridine, the residue finally being dissolved in dry dimethylformamide (5 mls.), the solution being concentrated to 1 ml. Dicyclohexylcarbodiimide (56 mg.) was added followed by dry pyridinium Amberlite IR 120 ion-exchange resin (567 mg.). After 24 hours, in a tightly stoppered flask in the dark, dry dimethylformamide (1 ml.) and dicyclohexylcarbodiimide (53 mg.) was added to the solution.

After five days in the dark with exclusion of moisture at room temperature and with frequent shaking, water (10 mls.) was added, and the suspension left at room temperature for three hours, when it was extracted with ether (three 10 ml. aliquots). The aqueous layer was evaporated to dryness under reduced pressure, water (10 mls.) added to the residue, the solution adjusted to pH 7 using saturated aqueous lithium hydroxide solution, and evaporated to dryness. The residue was dissolved in water (10 mls.) and the pH adjustment and evaporation to dryness repeated to ensure complete removal of pyridine. The residue was finally extracted with water (10 mls.) and the suspension filtered, and the filtrate divided in half, the mononucleotides and oligonucleotides being

removed from each half as below.

(i) One half of the solution was diluted to c. 15 mls. and dialysed against water for 48 hours, the resulting solution being evaporated to dryness under reduced pressure.

(ii) The other half was concentrated to c. 1 ml. by evaporation under reduced pressure, and this solution chromatographed on Whatman Chromedia DE 81 paper using Solvent E. Polynucleotides and lower oligonucleotides were held near the origin, and this band was cut out and eluted from the paper with water, the eluant being evaporated to dryness.

Nearest neighbour base sequence analysis. - This was carried out essentially as described previously.

One more polymerisation of thymidine-5'-phosphoric acid and deoxyadenosine-5'-phosphoric acid-P-32 was carried out. Details were as above.

The polymerisation of thymidine-5'-phosphoric acid-P-32 with N-benzoyl deoxyadenosine-5'-phosphoric acid. - Sodium thymidine-5'-phosphate (17.9mg.) and diammonium thymidine-5'-phosphate (4.8 mg. of total activity 29,125 x 10^3 c.p.s.) were converted to the pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion exchange column, the eluant being evaporated to dryness under reduced pressure. The residue was dissolved in a solution of pyridinium N-benzoyl deoxyadenosine-5'-phosphate (36 mg. in 36 mls dry pyridine), the resulting solution

being evaporated to dryness. The residue was dissolved in dry pyridine (10 mls.) and the solution evaporated to dryness. The process was repeated using 10, 10 and 5 mls. of dry pyridine. The residue was finally dissolved in dry pyridine (1 ml.) and dicyclohexylcarbodiimide (50 mg.) and the solution left in a tightly stoppered flask in the dark for 24 hours. Dicyclohexylcarbodiimide (58 mg.) was again added.

After the flask had been shaken in the dark at room temperature with exclusion of moisture for five days, water (10 mls.) was added, and the solution left at room temperature for two hours. The aqueous suspension was extracted with ether (six x 30 ml.); the aqueous layer filtered and the filtrate passed through an Amberlite IR 120 (ammonium form) ion-exchange column, the eluant being evaporated to dryness under reduced pressure. The residue was dissolved in ammonia ($\overset{d}{o}$.880) (5 mls.) and kept at room temperature for 48 hours to remove benzoyl groups. The solution was evaporated to dryness, water (10 mls.) added and the solution divided into two portions. Mononucleotides and lower oligonucleotides were removed by two methods and analysis was performed as described previously.

Polymerisation of thymidine-5'-phosphoric acid with N-benzoyl deoxyadenosine-5'-phosphoric acid-P-32. - Operations as described above.

Results of the nearest neighbour base sequence analyses

Polymerisations of thymidine-5'-phosphoric acid-P-32
with deoxyadenosine-5'-phosphoric acid

Nucleotide	Separation of Mononucleotides by Dialysis			Separation of Mononucleotides by Paper Chromatography	
	Counts sec^{-1}		Ratio	Counts sec^{-1}	Ratio
	Expt.				
Deoxyadenosine-3'-phosphoric acid	1	286×10^2	1	7435	1
Thymidine-3'-phosphoric acid		585.3×10^2	2.1	30,110	4
Deoxyadenosine-3'-phosphoric acid	2	75	1	48	1
Thymidine-3'-phosphoric acid		827	11	310	6.5
Deoxyadenosine-3'-phosphoric acid	3	3,830	1	50	1
Thymidine-3'-phosphoric acid		18,000	4.1	275	5.5

Experiment in which mononucleotides and lower oligonucleotides were not separated from polynucleotides

Nucleotide	Counts sec^{-1}	Ratio
Deoxyadenosine-3'-phosphoric acid	992.2×10^2	1
Thymidine-3'-phosphoric acid	4843×10^2	4.87

Thymidine-5'-phosphoric acid initially labelled.

Polymerisations of thymidine-5'-phosphoric acid with deoxyadenosine-5'-phosphoric acid-P-32

Nucleotide	Separation of Mononucleotides by Dialysis			Separation of Mononucleotides by Paper Chromatography	
	Counts sec^{-1}		Ratio	Counts sec^{-1}	Ratio
	Expt.				
Deoxyadenosine-3'-phosphoric acid	1	225	1	13.5	1
Thymidine-3'-phosphoric acid		1415	6.3	88.5	5.9
Deoxyadenosine-3'-phosphoric acid	2	78	1	19	1
Thymidine-3'-phosphoric acid		260	3.3	91	4.7

Polymerisation of thymidine-5'-phosphoric acid-P-32 with N-benzoyl deoxyadenosine-5'-phosphoric acid

Nucleotide	Separation of Mononucleotides by Dialysis		Separation of Mononucleotides by Paper Chromatography	
	Counts sec^{-1}	Ratio	Counts sec^{-1}	Ratio
Deoxyadenosine-3'-phosphoric acid	92	1	20	1
Thymidine-3'-phosphoric acid	6130	67	129	6.4

Polymerisation of thymidine-5'-phosphoric acid with

N-benzoyldeoxyadenosine-5'-phosphoric acid-P-32

Nucleotide	Separation of Mononucleotides by Dialysis		Separation of Mononucleotides by Paper Chromatography	
	Counts sec^{-1}	Ratio	Counts sec^{-1}	Ratio
Deoxyadenosine-3'-phosphoric acid	1947	65	105	1
Thymidine-3'-phosphoric acid	30	1	122	1.15

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