STUDIES ON NUCLEOTIDES

AND

RELATED COMPOUNDS

by

Abdul Hameed Rehan, B.Sc. (Hons.), M.Sc., A.R.I.C.

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Department of Chemistry
University of Keele

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SUMMARY

The chemistry of polynucleotides is reviewed. Polydeoxyribonucleotides containing thymidylate and deoxyadenylate residues were synthesised using aqueous solutions and either 1-cyclohexyl-3 (2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate (water-soluble carbodiimide) or ethoxyacetylene as a polymerising agent. In each polymerisation, one of the mononucleotides was labelled with ^{32}P so that nearest neighbour base-sequence analysis could be carried out on the polymeric products. The results of these analyses show that the base-sequence is essentially random, but that thymidylic acid is incorporated some 39 to 44 times more efficiently than deoxyadenylic acid. The reasons for this predominance have been suggested.

The polydeoxyribonucleotides were also synthesised in the presence of a primer (polyuridylic acid) under the same conditions as those employed for the above-mentioned syntheses. The results showed a significant increase in the total amount of polymer and in the relative incorporation of deoxyadenylic acid into the polymer; the latter increasing by a factor of 8 to 9.

On the basis of these findings it is therefore, suggested that in the absence of primer, the copolymerisation of a mixture of nucleotides is controlled by kinetic factors whereas, in the presence of a suitable primer, the base-pairing due to hydrogen bonding can

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INTRODUCTION

INTRODUCTION

(α) Studies on Polynucleotides

(A) Nucleic Acids

Miescher's discovery of nucleic acid in 1869 and the theory of Deoxyribonucleic acid structure by Watson and Crick in 1953 provided the basis of a new outlook on cellular mechanism and have attracted the attention of scientists from many disciplines such as biochemistry, microbiology, genetics, virology and the like. The reasons for this keen interest lie with the importance of the nucleic acids in life where they occupy a position parallel with that of proteins themselves in providing the essential "bricks" of living organism, though they have nucleotides (rather than amino acid) as a repeating unit.

A century ago, the epoch of nucleic acid was ushered in by a Swiss chemist, Friedrich Miescher, who is now recognised as the founder of the chemistry of cell nucleus. He was working in Hoppe-Seyler's laboratory in Tübingen when he digested pus cells, from discarded surgical bandages, with pepsin in the presence of hydrochloric acid and shook the mixture with ether to yield pure nuclear material which settled at the bottom of the aqueous layer. He isolated, from this nuclear material, an acidic substance appropriately named "nuclein" which was shown to be readily soluble in dilute alkali but insoluble in dilute acid. It contained about

10 per cent of phosphorus which was higher even than the phosphorus content of lecithin, until then the only known phosphorus-containing compound in animal tissues.

These results were sufficiently startling to cause Hoppe-Seyler to refrain from publishing in his journal until he had repeated the work. So although the work was completed in 1869, the paper was not published until 1871.[1]

Since then scientists from many disciplines have been interested in this remarkable molecule - the first chemical to be able to reproduce itself. In 1889 Altman introduced the term nucleic acid (Nucleinsaüre). By 1930, identification and confirmation of components and the isolation of nucleic acid from almost all kinds of cell tissues was known and it was established as a common component of living systems. The structural model of Deoxyribonucleic acid proposed by Watson-Crick in 1953, and in recent years, the development of more sophisticated techniques used in the study of nucleic acid and related polynucleotides, have led to the elucidation of the "code" that determines the synthesis of specific gene-dependent proteins.[2]

(a) <u>The Structure of Nucleic Acids</u>

The work of Miescher which pointed to a polymeric structure for nucleic acid, was unfortunately rather overlooked by later workers, who, for investigations using the

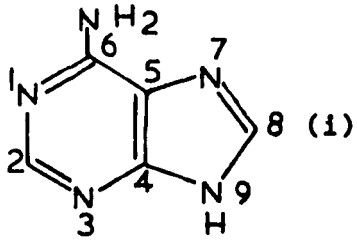
degradative methods of organic chemistry, made use of material which had been extracted from tissue by methods involving heat treatment and the use of acid and alkali. Thus many assumptions based on investigations making use of degraded material led to erroneous conclusions, some of which may have seriously retarded the development of ideas on the structure and function of nucleic acids. For example, the concept that the nucleic acid molecule was a tetranucleotide, which was accepted for some ten to fifteen years, grew up through the discovery of four nucleotides in approximately equimolecular proportions in the hydrolysate of yeast nucleic acid and a determination of molecular weight which was found to be 1.3×10^3 for ribonucleic acid. [3, 4]

However, by the evolution of new techniques for the isolation and separation of bases or nucleotides on columns of ion exchangers or on paper, it is now well established that there are two families of nucleic acids. One of these, deoxyribonucleic acid (DNA) is present in cell nucleus and has the molecular weight ~ 6 to $\sim 16 \times 10^6$ while the other, ribonucleic acid (RNA), is found largely in the cytoplasm and in viruses. It has molecular weight $\sim$90,000 to $\sim$150,000, but some RNAs with considerably lower weights have also been reported (see page 11). DNA and RNA have many similar structural features and these are summarised in Table 1 below.

TABLE 1

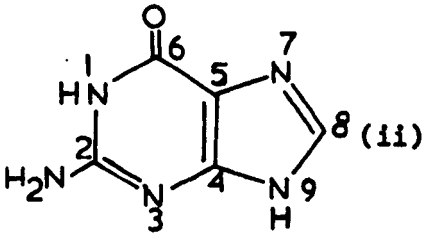
	DNA	RNA
Purines	Adenine and Guanine	Adenine and Guanine
Pyrimidines	Cytosine and Thymine	Cytosine and Uracil
Sugar	D-2-deoxyribose	D-ribose
Phosphate	One per sugar	One per sugar
Source	Plant and animal nuclei	Plant and animal cytoplasm and nuclei
Former Identification	Thymus, animal or deoxypentose nucleic acid	Yeast, plant or pentose nucleic acid

The structures of the purines and pyrimidines are shown in (i, ii, iii, iv and v), the carbon numbering system being shown.



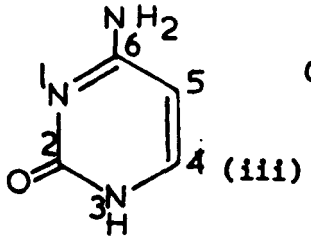
(i)

Adenine=6-amino-purine



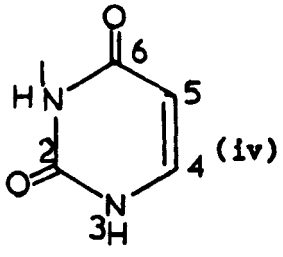
(ii)

Guanine=2-amino-1, 6-dihydro-6-oxopurine

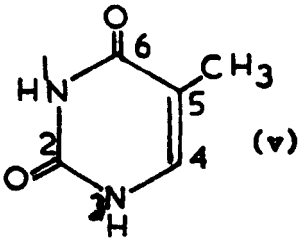


(iii)

Cytosine



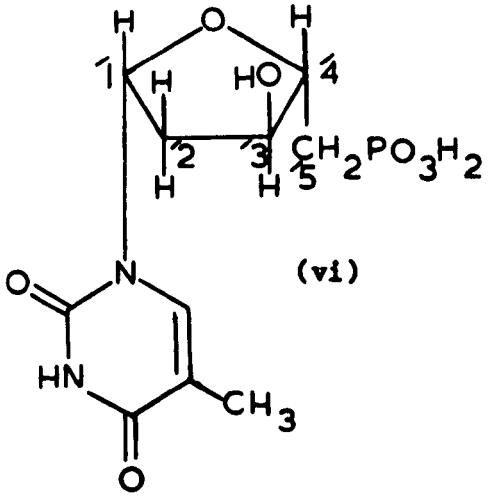
(iv)



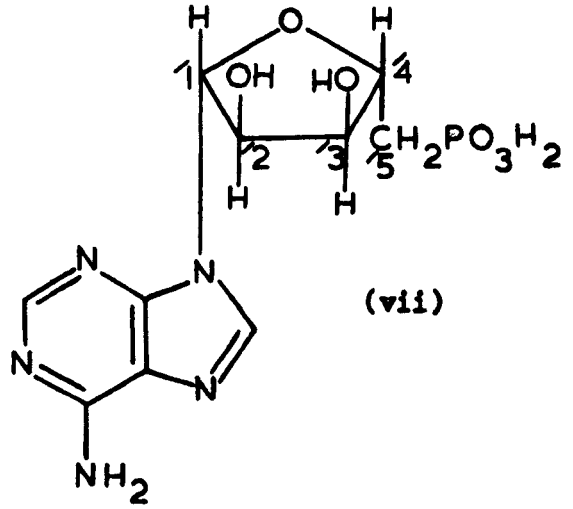
(v)

Other bases are also found in trace quantities in nucleic acids, and 5-hydroxymethylcytosine replaces cytosine completely in the T-even bacteriophages. The sugars are furanose and are β-linked at the 9-position of purines or 3-position of pyrimidines, giving the units known as nucleosides (ribonucleoside) or deoxynucleoside (deoxyribonucleoside). Nucleosides phosphorylated in the sugar moiety are called nucleotides. However, this term nucleoside has been extended to glycosides of many heterocyclic bases which are of biological interest and even to compounds such as riboflavin which has no glycosidic linkage but is a derivative of ribitol.

Infrared and ultraviolet data of derivatives as well as X-ray crystallography indicates that the hydroxyl groups exist predominantly in the keto-form and the amines exist predominantly in the amino form, as opposed to imino form.[5] (vi and vii) show the structure of two typical nucleotides.

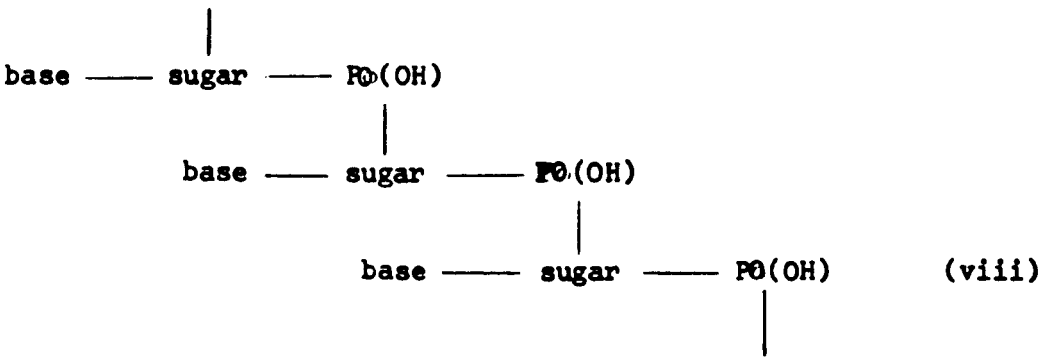


(vi)



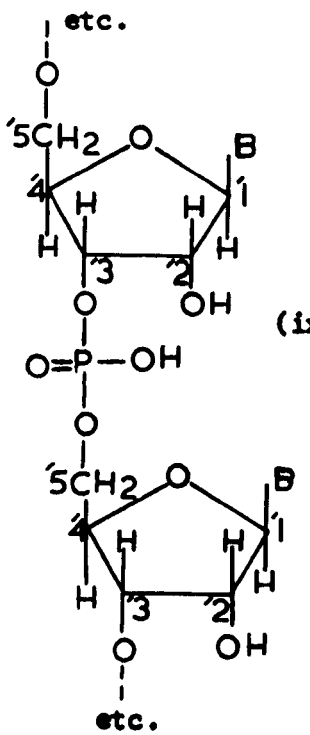
(vii)

Electrometric titration of monoribonucleotides of RNA and DNA led Leven and Simms[6] to the concept of nucleic acids as polynucleotides in which nucleoside residues were linked by phosphodiester bridges, as shown in (viii).



(viii)

Abundant chemical evidence indicates that nucleic acids are long chains of nucleotide units joined together by phosphodiester bridges between the primary hydroxyl at (C-5') of one nucleotide unit and the secondary hydroxyl at (C-3') of another and this has been well reviewed by Jordan[5] and by Brown.[7] A typical sequence for RNA is that (ix) shown below.



(ix) B = purine or pyrimidine base

The structure for the backbone of DNA is similar except that all sugar residues lack the C-2' hydroxyl group.

Interest in the macrostructure of DNA stems largely from the work of Astbury and Bell[8] who as a result of X-ray analysis explained the observed 3.4 Å spacing in terms of the thickness of the nucleotide bases by assuming the planar bases to be projecting perpendicularly to the long axis of the molecule. This work was followed by Furberg[9] who proposed two alternative structures for nucleic acid. In these structures, the plane of the purine and pyrimidine rings was at right angles to the direction of the long axis of the molecule, but the sugar residues and the $P-O_3$ bonds were in planes approximately parallel to the long axis.

Subsequently Pauling and Corey[10] put forward the concept of a helical structure for the nucleic acid molecule. Combining their own X-ray analysis data with that of Astbury, they suggested a structure which involved three interwined helical polynucleotide chains with the phosphate group on the inside and the bases on the outside. However, this arrangement does not accord with the chemical behaviour of DNA whose phosphate groups can be titrated but whose amino and -NH-CO- groups cannot, as was found by Gulland, Jordan and Taylor.[11] During the early 1950's Chargaff and co-workers[12] showed that the samples of DNA from a variety of sources contained equimolar amounts of adenine and thymine, and similarly equimolar

amounts of cytosine and guanine. Thus

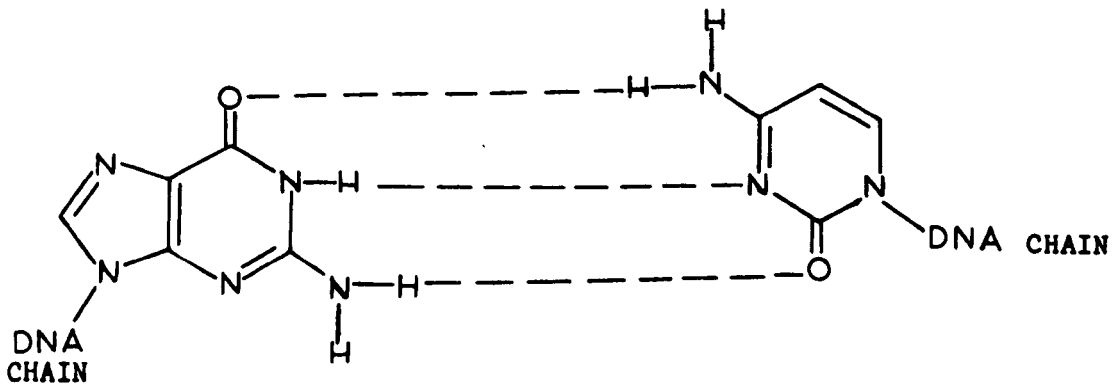
$$[\text{adenine}] = [\text{thymine}]$$
$$[\text{guanine}] = [\text{cytosine}]$$
$$[\text{adenine}] + [\text{cytosine}] = [\text{guanine}] + [\text{thymine}] \qquad (1)$$

This most valuable work by Chargaff et al. together with X-ray analysis data led Watson and Crick[13] to propose the double right handed helical structure for DNA. This structure with one or two modifications is still accepted today.

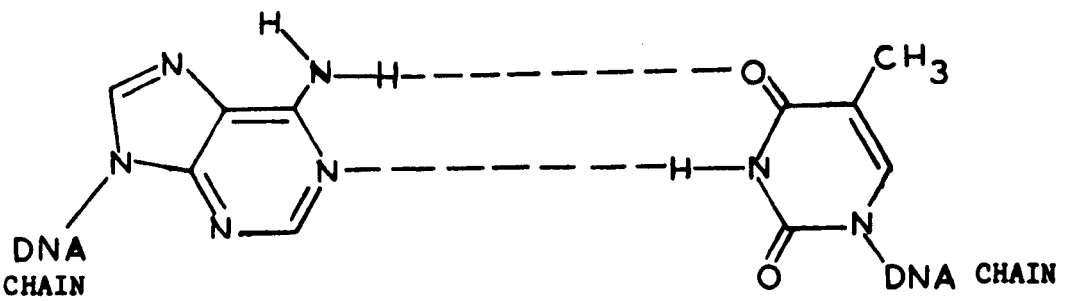
According to this structure, the DNA molecule consists of two polydeoxyribonucleotide chains winding around a common axis with their components arranged linearly but in opposite directions; i.e. the sequence C(3')-C(4')-C(5')-O-P-O runs up in one strand and down in the other. The pyrimidines and purines were located on the inside of the helix and the phosphates on the outside. The space between one pair and the next is 3.4 $\mathring{A}$, and the helix makes one complete turn every 34 $\mathring{A}$, i.e. after 10 pairs of bases. The orientation angle between adjacent nucleotides in the same chain was assumed to be 36°.

The heart of this revolutionary proposal for DNA structure was the inter-base hydrogen bonding which led to complete agreement with the analytical data of Chargaff et al. and Wyatt[14] mentioned above. It was postulated that an adenine residue on one strand could form a pair with a thymine on the second strand, and a

guanine residue on one strand could form a pair with a cytosine on the second strand. Thus each purine was opposite a pyrimidine in every pairing; two pyrimidines could not bridge the gap while two purines would be unable to enter the available space.



(x) Hydrogen bonding system for guanine-cytosine



(xi) Hydrogen bonding system for adenine-thymine

This postulated structure was confirmed and refined by Franklin and Gosling,[15] and Wilkins and his co-workers.[16] It has also been reported that while most naturally occurring molecules of DNA are

double stranded, a single stranded DNA molecule occurs in a bacteriophage that attacks Escherichia coli-bacteriophage ΦX174 discovered by Sinsheimer.[17] The single stranded DNA is believed to exist in the form of a circle with no free ends, and so the regularities in [purine] = [pyrimidine] do not obtain. Thus, ratios for adenine/thymine and guanine/cytosine of 0.75 and 1.3 are found respectively. As will be described later, the single stranded DNA has been of particular value in studies on the replication of DNA and on the synthesis of RNA. Other single stranded DNA molecules have also been reported.

Until recently, all attempts to obtain some insight into the secondary structure of RNA by means of X-ray diffraction have failed, probably because of the low degree of organisation of the secondary structure of RNA. However, a detailed study of cytoplasmic RNA became possible as a result of the introduction of modern cell fractionation techniques, and the biochemical studies have led to the identification of four types of RNA[18] - viral RNA, soluble RNA, ribosomal RNA and messenger RNA.

Viral RNA seems to replace DNA in some viruses, e.g. Tobacco-mosaic virus. Soluble RNA (s-RNA; since it was first observed in the soluble cytoplasm), or transfer RNA (t-RNA, because of its function in protein synthesis) constitutes from 10 - 15% of cellular RNA. There are many t-RNA's, at least one corresponding to each of the

amino acids that occur in proteins. These RNA's are quite small molecules with molecular weights of about 25,000 and consist of one polynucleotide chain made up of about 80 nucleotide residues with stretches of the polynucleotide folded back to make helical portions. These sequences of nucleotides therefore, run in opposite directions (as in DNA), making possible the pairing of complementary bases. Holley and his co-workers[19] were the first to determine the complete nucleotide sequence of a specific t-RNA (alanine t-RNA from Bakers' yeast). They reported that numerous bases of the t-RNA molecule are hydrogen-bonded to each other and thus form helical structures of the Watson-Crick type. On the basis of these studies they suggested the cloverleaf model for t-RNA in which three arms are folded up tightly together while the fourth arm is extended in the opposite direction. Many other scientists have now determined the sequences of different t-RNA's and this work has been well reviewed by Madison.[20]

Ribosomal RNA (r-RNA) constitutes about 80 - 90% of the RNA in most cells and was found in small particles "ribosomes" which have a diameter of 100 - 200 Å and are not resolved by the optical microscope.[21] Ribosomal RNA occasionally shows evidence of instability e.g. to heat[22,23] and this RNA can be divided into two types depending upon its size.

The first (23s r-RNA) derived from the larger subunit of the

ribosome has a molecular weight of between 1 and 2 x 10^6 depending upon the source of the ribosome. The second type (16s r-RNA), found in the smaller ribosomal subunit, has a molecular weight in the range of 5 x 10^5 to 1 x 10^6, again depending on the origin of the ribosomes. The two types (termed 16s and 23s according to the sedimentation coefficients of their respective units) have base sequences that appear to be complementary to regions of the DNA, along which they are presumably synthesised. It has also been reported that the molar base ratios of r-RNA differ in different species. However, a close correlation between (G + C): (A + U) ratios of the two r-RNA in various organisms has been found by Amaldi.[24]

Cotter and Gratzer[25] have studied the infrared difference spectra which indicated that the secondary structure of E. coli RNA involves 60% of the total bases in pairing and this agreed reasonably well with the estimates from the other methods. In E. coli, the rate of r-RNA formation is one nucleotide per second per gene (and each RNA molecule is over 1000 nucleotides long). The r-RNA then somehow combines with proteins to form 30s and 50s subunits. Once combined with protein, r-RNA is stable and is not easily degraded in vivo. In mamalian cells synthesis of ribosomes appears to be a more complex process, in which high molecular weight (45s) RNA is a precursor of r-RNA. The reason for the complex structure and RNA content of ribosomes is not yet clear.

Ribosomal function requires an additional RNA, termed messenger RNA (m-RNA) which serves as a template in protein synthesis and produces the correct amino acid sequence. M-RNA is not a homogeneous fraction and sedimentation constants ranging from 6 - 8s up to a maximum of 23 - 30s have been reported for m-RNA from E. Coli.[26] Because the molecular weights of the proteins vary, and m-RNA serves as a template for protein synthesis, this variation in the molecular weights of m-RNA is not surprising. However, in some cases the molecular weight has been found to be of the order of 5×10^5.

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

In addition to naturally occurring nucleic acids, synthetic polynucleotides may be prepared enzymatically and Arnott et. al.[27] have examined in detail the double helical complex formed by

mixtures of synthetic polynucleotides (poly-A plus poly-U; poly-I plus poly-C; poly-C plus poly-G). From the X-ray diffraction pattern they observed the same structures as with native RNA's though, in some cases, the RNA double helix existed in three forms = 11 fold helices; 12 fold helices and a non integral form similar to that observed with r-RNA fragments. Transitions from one form to another were related to changes in salt concentration. All these three conformations resembled the A form of DNA, but no structure like the DNA B form was seen.[28]

(b) Biological Role of Nucleic Acids

The cliché that "DNA makes RNA and RNA makes protein" was first stated several years ago when DNA was recognised as the primary genetic substance. There is now abundance of evidence pointing to DNA in this role. The reasons for believing that DNA is the ultimate source of genetic information include the localisation of DNA in the chromosomes, the constancy in the average amount of DNA per cell, the metabolic stability of DNA, the ability of mutagens (agents which cause alterations in inherited characteristics of an organism) to react with DNA, and the ability of DNA to cause transformations in the inherited characteristics of bacterial cells.[29]

The first convincing evidence for the biological role of DNA came from the work of Avery and his co-workers[30] in 1944; using pure DNA they showed that it was possible to transfer the capacity to

form capsules from one pneumococcal strain (S = smooth) to another (R = rough) and this property acquired by "Transformation" was transmitted to the daughter cells. Numerous virus studies further substantiated the role of DNA. Thus Herriot[31] in 1951 reported that bacteriophages multiply in host cells only if the former contained DNA. When DNA was removed from the phages, they were still capable of inducing an abortive infection, but no new phages arose. Later on Hershey and Chase[32] in 1952 observed when E. Coli is infected with bacteriophage T-2, only the phage DNA enters the cell, while protein remains outside. From these studies it appears that nucleic acids (both DNA and RNA) play a critical role in the control of biosynthetic processes.

Shortly after their suggestion of a double helical structure for DNA, Watson and Crick[33] discussed the fact that the structure could account for self-replication and that the sequence of bases along a DNA strand could, by self-replication, constitute a genetic message to subsequent generations. Several possible mechanisms for the replication of DNA have been considered, but experimental findings indicate that the replication of DNA takes place by a "semi-conservative" mechanism in which each of the daughter cells formed during mitosis receives one strand of DNA from the DNA of the parent cell, and the second strand in each daughter cell is formed by synthesis from small molecular precursors. This was

most clearly demonstrated by Meselson and Stahl[34] in 1958 and was confirmed by Kornberg and his co-workers.[35] The latter showed that the four monodeoxyribonucleotides under the influence of a polymerase enzyme undergo polycondensation in the presence of a separated strand of DNA primer to replicate the given DNA.

On the basis of these and numerous other studies, it is now generally accepted that DNA molecules are the repository of genetic information specifying the characteristics of all living cells. It is also believed that genetic information stored in the genes (physical units of heredity), as a linear sequence of the bases (A, C, G, and T) in DNA, is transcribed into a complementary base sequence (U, G, C, and A, respectively) in the m-RNA, as a linear sequence. This 4-letter "language" is "translated" in the process of protein biosynthesis into a linear sequence of the 20 amino acids within the protein polypeptide chain synethesised. Each nucleotide triplet or code word "codon" consisting of one of the 64 possible triplet combinations (of U, G, C, and A nucleotides) in a m-RNA molecule may specify one particular amino acid for incorporation into the polypeptide chain. It appears that certain amino acids may be specified by more than one of the 64 nucleotide triplets; in this respect, the genetic code is said to be "degenerate". A few particular triplet "words" may have special functions, such as to signal polypeptide-chain initiation, or

chain termination. The first identification of a particular triplet as the code word for a particular amino acid was the discovery that the sequence UUU (in the form of polyuridylate) appears to be the "code word" specifying incorporation of phenylalanine into polypeptide, in a cell-free, in vitro system containing ribosomes and other required components.[36]

Evidence that a nucleotide triplet (and not some smaller or larger run of nucleotides) is the "code word" for incorporation of a specific amino acid has come from studies of the fine structure of genes or DNA of a bacteriophage (virus).[37] Many tentative formulations of a "code" dictionary of m-RNA triplets, with the corresponding amino acid specified by each triplet, have been proposed on the basis of experimental results by the Nirenberg group[38] and the Ochoa group.[39] The exact determination of the genetic code, or pattern of correspondance between each possible nucleotide triplet of m-RNA and the amino acid specified has been an active field since about 1961. This has recently been quite firmly established, largely by the work of Nirenberg,[40] and confirmed and extended by Khorana and their respective co-workers.[41]

(c) Degradation of Nucleic Acids

(1) Chemical Hydrolysis of Nucleic Acids

The chemical hydrolysis of RNA and DNA has been extensively reviewed[5,42-44] and the products of hydrolysis by acid and alkali are well established. Generally speaking, the hydrolysis of DNA and RNA yields a variety of products. These include purines and pyrimidines, ribose and deoxyribose, according to the type of nucleic acids, the corresponding phosphorylated sugars, nucleosides, nucleotides, and oligonucleotides (i.e. polynucleotides of low molecular weight) as well as various degradation products formed by the further breakdown of these substances.

The type of nucleic acid, as well as the nature of the hydrolytic reagent, is very important in determining the course of the degradation. The two types of nucleic acids show different behaviour on alkaline hydrolysis; the ribonucleic acids are readily hydrolysed to mononucleotides by treatment with 1M alkali at room temperature; whereas DNA undergoes only slight hydrolysis. However, in both types of nucleic acids the purine-carbohydrate linkage is more labile to acid hydrolysis than the pyrimidine-carbohydrate linkage. The purines, adenine and guanine are thus readily formed during acid hydrolysis of both nucleic acids, while the pyrimidine bases remain for the most part as mononucleotides

in the case of RNA or as a nucleoside—diphosphates in the case of DNA. Nucleic acids can only be hydrolysed to give the free pyrimidines-cytosine, uracil, or thymine by drastic conditions (e.g. treatment with dilute sulphuric acid at 175° for several hours).[42]

(i) Chemical hydrolysis of RNA

It has been shown that when ribonucleic acids are treated with mild alkaline reagents under a variety of conditions, they are rapidly converted to a mixture of their component (2'- and 3'-) mononucleotides.[45-50] In the same way, mild acid hydrolysis also yields mononucleotides, although further degradation of purine nucleotides complicates the picture. The early observations that the final products of alkaline hydrolysis were mononucleotides has been confirmed by studies using paper chromatography,[42,51-54] electrophoretic separation[55] and ion-exchange chromatography.[56] By means of these techniques, it was shown that ribonucleic acid was converted to a mixture of nucleoside monophosphates, each of which occurred in two isomeric forms corresponding to the nucleoside-2' and 3'-monophosphates.[57-60]

The mechanism of alkaline degradation remained incompletely understood until 1952 when Brown and Todd[61] discussed these observations on the basis of their findings with the esters of mononucleotides. They concluded that 2'- and 3'- isomers were

readily interconvertible under acid conditions, each pure isomer being converted to a mixture containing both isomers; in alkali the isomers were stable.

Early studies of Bailly and Gaume[62] showed that while glycerol--phosphate was unaffected by alkali, its methyl ester was readily hydrolysed under alkaline conditions to methanol and to a mixture of glycerol-α- and β-phosphates. This and other investigations[63] showed that an exception to the general stability of alkyl and dialkyl phosphates to alkali occurred when a hydroxyl group was adjacent to the phosphoryl group. These findings were confirmed by Brown and Todd[61] by an examination of the hydrolytic behaviour of the adenosine benzyl hydrogen phosphates. Adenosine-2' and 3'-benzyl hydrogen phosphates, both of which contained a hydroxyl group adjacent to the phosphoric acid group, were readily hydrolysed by weak alkali at 30°C. Under the same conditions, however, adenosine-5'-benzyl hydrogen phosphate, which contained no hydroxyl group adjacent to the phosphoric acid group, was not affected.

These results led Brown and Todd to propose a mechanism for the alkaline hydrolysis of ribonucleic acids. According to this mechanism, the first step in the hydrolysis of RNA is the formation of a cyclic triester. As the triesters of phosphoric acid are extremely labile, being readily hydrolysed in both acid and alkaline media,[64,65] the intermediate triester formed during the hydrolysis

of ribonucleic acid will thus be rapidly hydrolysed. This mechanism is illustrated by (Fig. 1). For clarity only the 2' and 3' carbon atoms are shown. The final product is a mixture of 2'- and 3'-phosphates of the ribonucleosides.

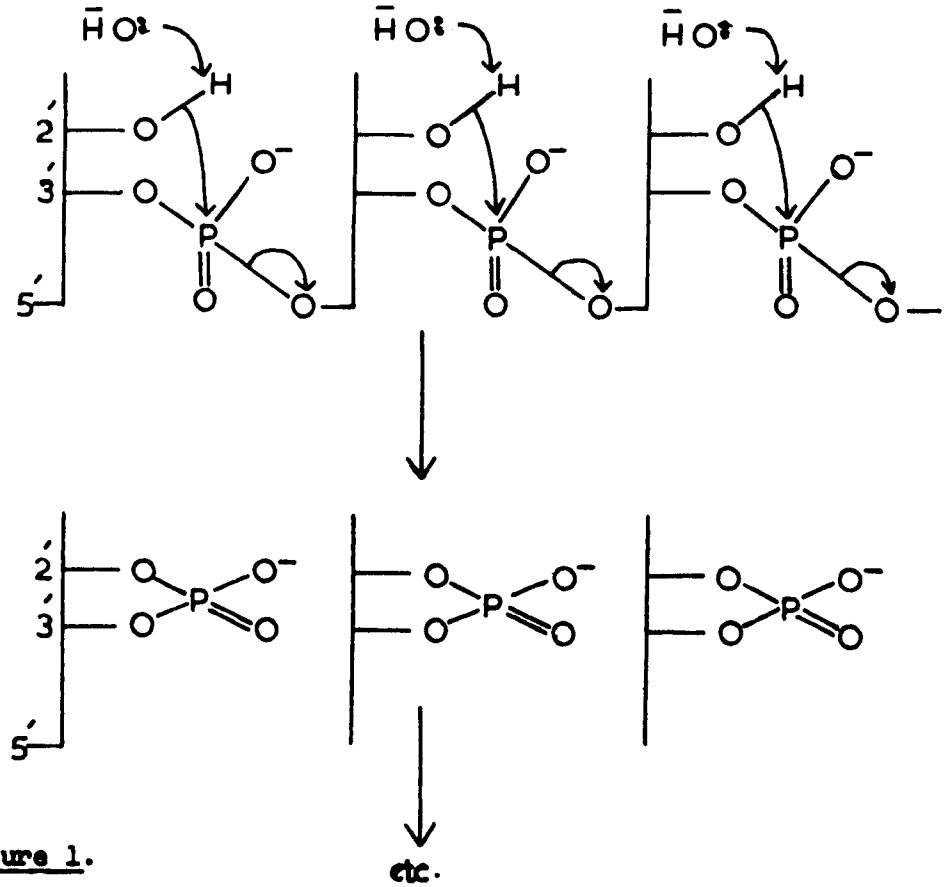


Figure 1.

Further support of this mechanism was furnished by the preparation of the cyclic esters by Brown et al.,[66] by Bockstahler and Kaesberg[67] and by the identification of the two adenylic acids, previously designated a and b, by Carter and Cohn.[57,58] Conclusive

proof came from Markham and Smith[68] who isolated experimentally all the 2'- and 3'-cyclic nucleotides. The pyrimidine cyclic mononucleotides were present in greater quantities than the purine cyclic nucleotides.

RNA is stable to mild acid but vigorous hydrolysis (e.g. with 1N sulphuric acid at 100° for several hours) first liberates the purine bases. Continued treatment yields initially pyrimidine oligonucleotides and finally cytidylic and uridylic acid. Hydrolytic cleavage of the pyrimidine glycosidic linkage is very difficult, requiring treatment with dilute sulphuric acid at 175° for several hours.[42]

(ii) Chemical hydrolysis of DNA

The mechanism discussed above for the alkaline hydrolysis of RNA is not applicable to DNA because of the absence of a hydroxyl group on position 2 of the deoxyribose sugar. The absence of a cis-1, 2-glycol system in the sugar moiety thus prevents the formation of the cyclic phosphotriester, an essential prerequisite for the facile alkaline hydrolysis of nucleic acids. For this reason, DNA is resistant to alkaline hydrolysis.

On the other hand treatment with strong mineral acids for a short time or prolonged exposure even to mild acid causes hydrolytic cleavage, not of the phosphate ester bonds but of the N-glycosidic linkage between deoxyribose and the purines, leaving a polymer

from which the purine bases have all been removed. This polymer is usually termed an apurinic acid or apurine DNA.[69,70,107,113,114] It has also been reported that more extensive hydrolysis of apurine DNA resulted in the isolation of pyrimidine oligonucleotides of the type pTpTpCp, i.e. oligonucleotides having a phosphoryl group at each end.[71-75] Although major amounts of the pyrimidine deoxyribonucleoside-3', 5'-diphosphates of thymidine and cytosine could be isolated from acidic digests of DNA,[76,77] every attempt to find a satisfactory hydrolytic preparation of the monodeoxyribonucleotides from DNA has, so far, been unsuccessful. The information thus obtained by the chemical degradation of DNA have been reviewed recently by Burton.[108]

(2) Enzymatic Degradation of Nucleic Acids

A number of enzymes have been discovered which catalyse the degradation of both deoxyribonucleic acid and ribonucleic acid and this has been reviewed by many investigators.[78-83,110] Generally speaking, enzymes which hydrolyse polynucleotide chains can be regarded as phosphodiesterases because they break the phosphodiester internucleotide linkage. These enzymes can be divided into two main groups;

(i) Endonucleases and (ii) Exonucleases.

(i) Endonucleases: The endonucleases attack linkages in the interior of the nucleic acid chain and break it into fragments

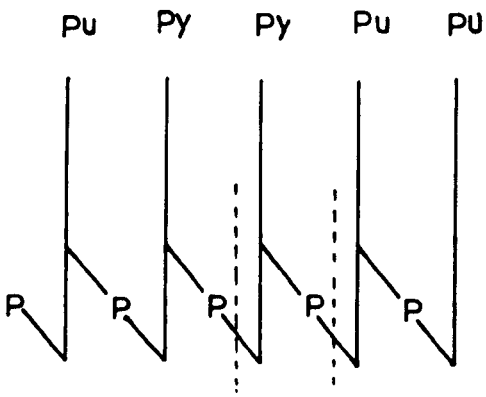
which may vary in size from mononucleotides up to acid precipitable polynucleotides. The endonucleases are further subdivided into two classes, the ribonucleases (RNase) which attack RNA and the deoxyribonucleases which attack DNA.

(a) Ribonucleases: Various forms of ribonuclease (RNase) have been isolated from different sources. The best known is that derived from pancreas. Pancreatic ribonuclease, which was discovered by Jones,[84] purified by Dubos and Thompson[85] and crystallised by Kunitz[86] and by McDonald,[87] is a highly specific phosphodiesterase which will hydrolyse only secondary phosphate esters of pyrimidine nucleoside-3' phosphates. It will also hydrolyse cyclic 2':3'-secondary phosphates of the pyrimidine

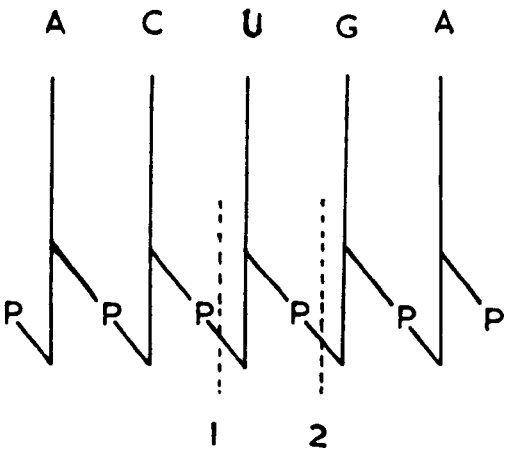
$$\text{Cytidine-2':3'-cyclic phosphate} + H_2O \xrightarrow{\text{RNase}} \text{cytidine-3'-phosphate} \qquad (2)$$

nucleosides[68,88-90] giving pyrimidine-3' phosphates either as a free nucleotides or as the terminal nucleotide residue in an oligonucleotide.

The action of ribonuclease may be illustrated by (xii and xiii).



(xii)



(xiii)

The pentanucleotide shown in (xii) in which pu and py represent purine and pyrimidine residues respectively, will be hydrolysed at the points shown by the dotted lines while the ribopolynucleotide chain shown in (xiii), which may also be expressed as pApCpUpGpAp (see also page 82,83), will be broken at position 1 and 2, thus pApCp/Up/GpAp to yield pApCp + Up + GpAp.

The ultimate action of ribonuclease on RNA is to produce pyrimidine mononucleotides together with oligonucleotides. The oligonucleotides consist of purine nucleotide units but terminate in a pyrimidine nucleotide as shown in the examples cited above. Furthermore, because its main role is to catalyze the migration of

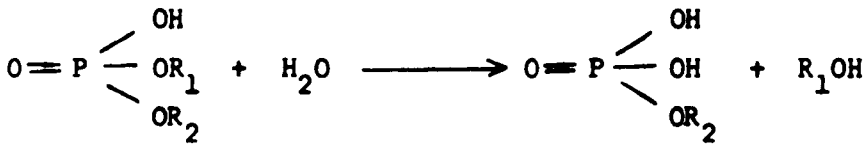
3'-5' phosphoryl RNA bond with transient formation of 2'-3' cyclic ester, this enzyme is also called polynucleotide-2'-oligonucleotide transferase (cyclising). The detailed discussion of these enzymes is outside the scope of this thesis. For a complete review, however, the reader is referred to the review by Schmidt,[78] Scherage and Rupley,[91] Stein,[92] and by Lehman.[93]

(b) Deoxyribonuclease: These enzymes hydrolyse polynucleotides of the deoxyribonucleic acid type with formation of mono- and oligonucleotides with either terminal 3'-phosphate or 5'-phosphate groups. Two main types of deoxyribonuclease (DNase) have been well characterised; both are endonucleases. The first type (DNase I) which was found in pancreas and in streptococci,[94,95] degrades DNA to yield 5'-phosphomonoesters. The second type (DNase II) which was found in spleen and thymus, degrades DNA to yield 3'-phosphomonoesters.[93,96]

Micrococcal deoxyribonuclease found in culture of staphylococcus degrades DNA to mixture of nucleoside-3'-monophosphates and oligonucleotides with 3'-phosphate termini. It preferentially attacks heat-denatured DNA and also RNA. It requires Ca^{2+} for maximum activity. Arnone et al.[109] have, recently, found that the molecular weight of this enzyme is 16807.

(ii) Exonucleases or phosphodiesterases: These are enzymes that attack the chain repeatedly giving a stepwise degradation of a

single mononucleotide at a time. All phosphodiesterases catalyse overall reaction of the type



(3)

where R_1 and R_2 represent either alkyl or phenyl or nucleoside residues. They catalyse this type of reaction as opposed to the phosphomonoesterases or phosphatases which hydrolyse phosphomonoesters with the production of alcohol or phenol and free inorganic phosphate.

These enzymes are, in fact, the enzymes that have been known as phosphodiesterases which degrade oligonucleotides to mononucleotides. The mononucleotides obtained are sometimes 3'-phosphates and sometimes 5'-phosphates, depending upon the specificity of the enzyme.[80-82] These enzymes can be subdivided into two classes.

(a) Phosphodiesterase-exonucleases, unspecific towards the nature of the residue esterifying the phosphoryl group, and whose action extends to all phosphodiesters. Example: snake venom phosphodiesterase.

(b) Phosphodiesterase-exonucleases, specific towards the nature of the residue esterifying the phosphoryl group, and whose action is limited to polynucleotide phosphodiesters. Example: the exonuclease I from E. coli.

Venom phosphodiesterase: The venom of several species of snakes contains a phosphodiesterase which is commonly employed in the preparation of nucleoside-5'-phosphates. The enzyme occurs naturally in association with a high concentration of phosphomonoesterase from which it can be separated by chromatography and acetone fractionation.[97,98]

Venom phosphodiesterase hydrolyses RNA to nucleoside-5' monophosphates starting at the 3'-hydroxyl end of the chain and it is also active in hydrolysing the oligonucleotides produced by the action of deoxyribonuclease I on DNA to deoxyribonucleoside-5' phosphates. The presence of a 3'-phosphoryl terminal group confers resistance on the substrate.

Bovine Spleen phosphodiesterase: Spleen contains several phosphodiesterases, one of these enzymes has been particularly studied, and it is usually this one that is referred as spleen phosphodiesterase.[99,100,101] This enzyme hydrolyses RNA to nucleoside 5'-monophosphates starting at the 5'-hydroxyl end and also acts on the mixture of oligonucleotides produced from DNA by spleen deoxyribonuclease II to yield deoxyribonucleoside 3'-phosphates.

It is inactive with oligonucleotides carrying a 5'-phosphomonoester end group.

Fig. 2, 3 and 4 illustrate the degradation of RNA and DNA using endo- and exonuclease enzymes.

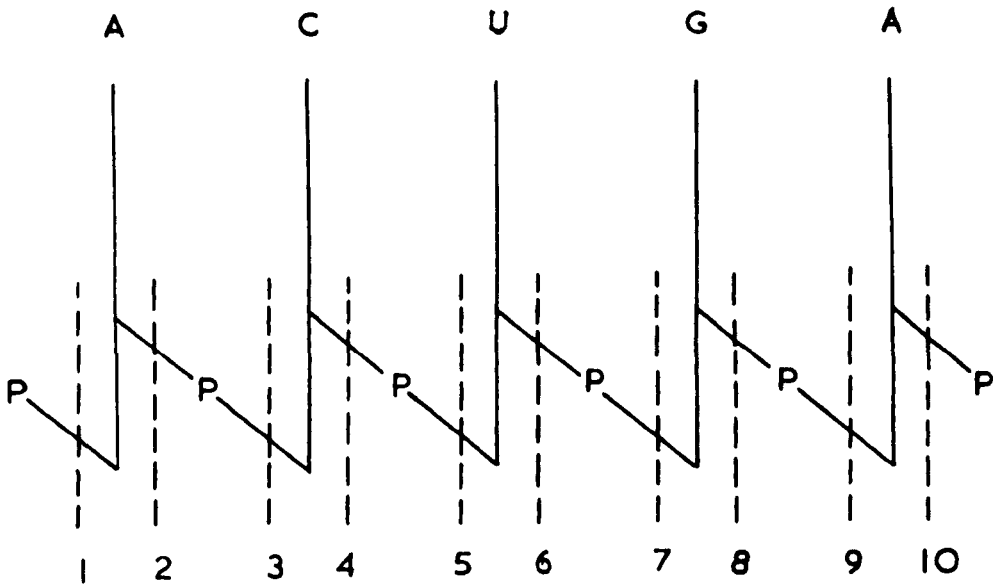


Figure 2.

A pentanucleotide containing 2 adenine nucleotide residues and one residue each of cytosine, uracil and guanine nucleotides with monoesterified phosphate residue at each end is split by ribonuclease at positions 5 and 7, by 5'-monoesterase at 1, by 3'-monoesterase at 10, by venom phosphodiesterase at 2, 4, 6 and 8, and by spleen phosphodiesterase at 3, 5, 7 and 9.

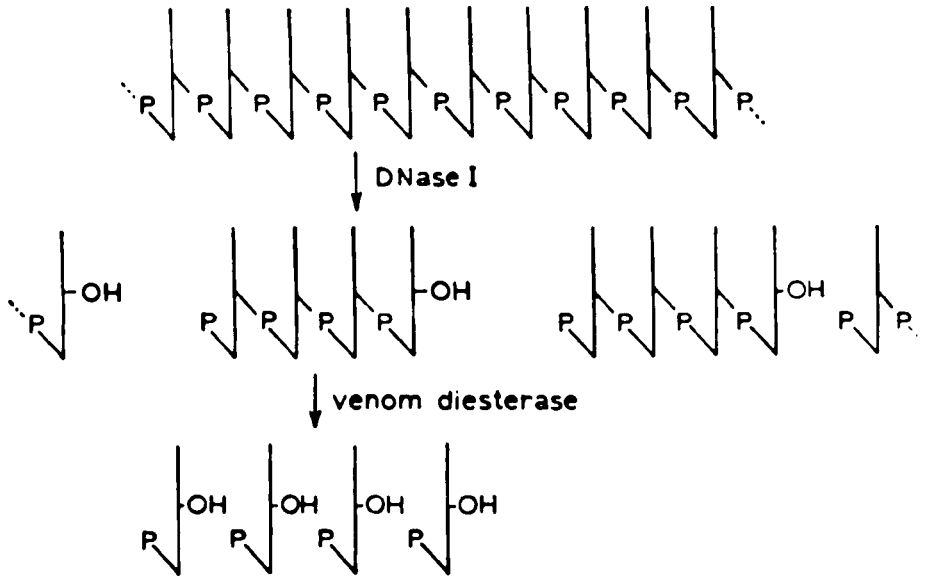


Fig.3. *The digestion of DNA by DNase I followed by venom diesterase to yield deoxyribonucleoside 5'-monophosphates*

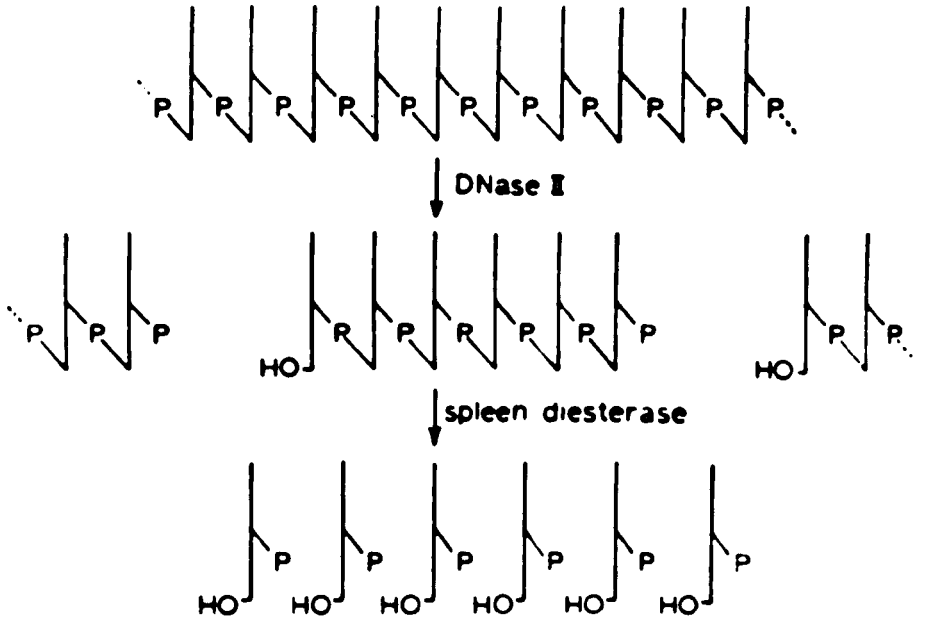


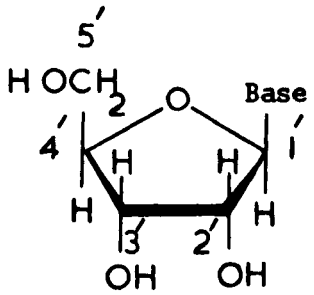
Fig.4. *The digestion of DNA by DNase II followed by spleen diesterase to yield deoxyribonucleoside 3'-monophosphates*

Thus, depending upon the method of degradation, one can prepare nucleoside 5'- or 3'- monophosphates. The problem of separation of oligonucleotides produced by chemical or enzymatic action has been solved mainly by the use of an ion-exchange media such as DEAE-cellulose[102] and especially by the use of urea[103] and Poly-N-vinyl pyrrolidine (polycar AT powder)[111] in column chromatography. Woods and Weitzman have recently reported the isolation of cyclic 3'-5'-adenosine monophosphate on a neutral silicic acid-glass matrix.[112] Two dimensional mapping techniques have been employed successfully by a number of workers[104,105] and Sanger et al.[53] have recently described a very useful technique for separation and identification of oligonucleotides based on their electrophoretic mobility.

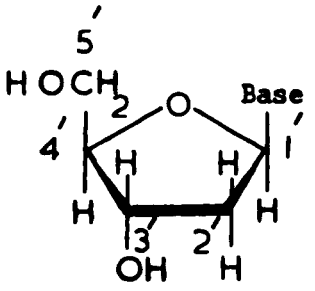
Exonucleases and some base specific ribonucleases have been widely used for structural analysis of small oligonucleotides; the stepwise degradation of oligonucleotides by venom phosphodiesterase and spleen phosphodiesterase has been elegantly exploited by Holley et al.[106] and by Sanger et al.[53]

(B) Nucleotides

Nucleotide, a term introduced by Levene and Mandel,[115] denotes the phosphate ester of nucleoside. In general, the word nucleotide is used to mean mononucleotide, while di, tri, and polynucleotides are so specified. A mononucleotide consists of one nucleoside (base and sugar) and one or more phosphates, which may carry additional substituents (as in the case of coenzymes). The structures (xiv) and (xv) show the two types of nucleosides with carbon numbering system.



(xiv) Ribonucleoside



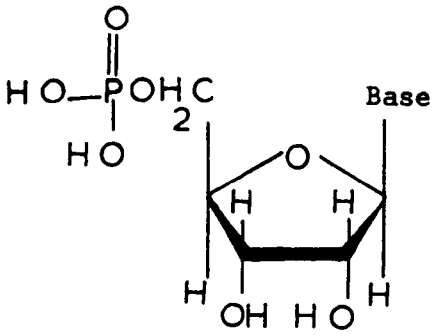
(xv) Deoxyribonucleoside

Base = Uracil / adenine, guanine, cytosine / thymine

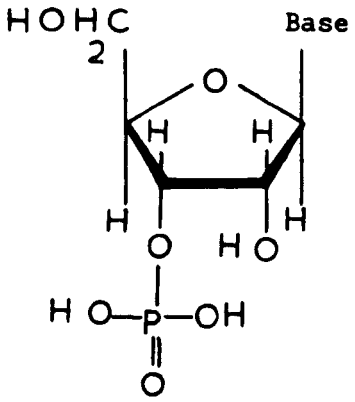
An examination of ribonucleoside structure (xiv) reveals that there are three hydroxyl groups available for esterification with phosphate while deoxyribonucleoside (xv) has only two positions. Depending upon the position of the phosphoryl group on the sugar, it is clear therefore, that a single base and single sugar will furnish two different isomeric nucleotides in the case of deoxyribose, according to whether the phosphoryl group is attached to C-2', C-3', or C-5'. Furthermore, 3'-5' cyclic phosphates (because of intramolecular cyclisation) and 2'-3' cyclic phosphates (because of 2 vicinal hydroxyl groups in ribose) can also be produced.

These isomeric mononucleotides have all been obtained by enzymatic hydrolysis of nucleic acids (described previously), or by chemical synthesis, or they have been found in the free state in nature. Therefore, it is necessary to specify the position of the phosphate (as 2'-, 3'-, 5'-, etc.) when a mononucleotide is mentioned. This is illustrated by Fig. 5. The numbers in parentheses refer to the carbon atoms of the sugar molecule to which the phosphoryl group is attached.

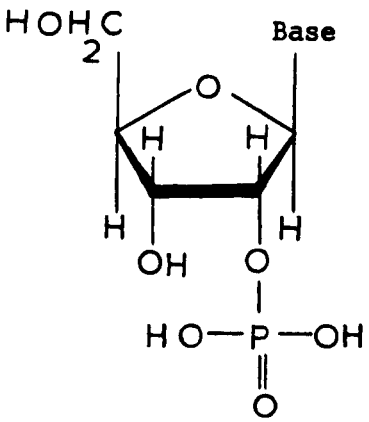
Until recently the phosphate esters of nucleosides were often characterised by naming the source material as in the use of the terms "yeast adenylic acid" and "muscle adenylic acid" to describe two distinct isomers of the nucleotide. Such terminology is now almost never used and has been superseded by defining the position



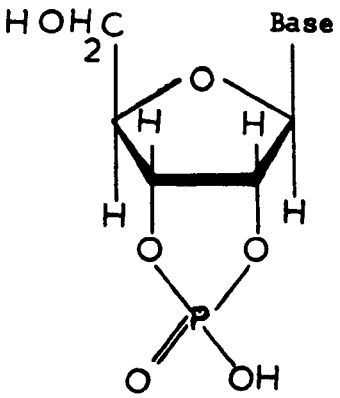
(5') Phosphate



(3') Phosphate

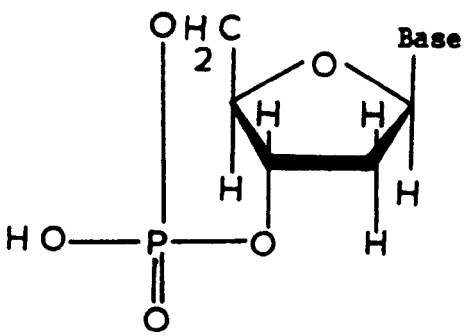


(2') Phosphate



(2',3') Cyclic Phosphate

Base = A, C, G, U, (T)



(3',5') Cyclic Phosphate

Figure 5

of the phosphate as in "adenosine-2'-phosphate" which is also known as adenylic-2'-acid. Table 2, essentially the same as that given by Potter,[116] summarises the nomenclature of the nucleotides.

Table 2

NAMES OF NUCLEOTIDES

Common Ribonucleotides		
Corresponding base	Nucleotide Named as Acid	Nucleotide Named as phosphate
Adenine	Adenylic-2'-acid, or Adenylic acid a	Adenosine-2'-monophosphate
Adenine	Adenylic-3'-acid, or Adenylic acid b	Adenosine-3'-monophosphate
Adenine	Adenylic-5'-acid	Adenosine-5'-monophosphate*
Adenine	Cyclic adenylic acid	Adenosine-2'-3'-monophosphate (cyclic)
Guanine	Guanylic-2'-acid etc.**	Guanosine-2'-monophosphate etc.
Uracil	Uridylic-2'-acid etc.	Uridine-2'-monophosphate etc.
Cytosine	Cytidylic-2'-acid etc.	Cytidine-2'-monophosphate etc.
Hypoxanthine	Ionosinic-2'-acid etc.	Ionosine-2'-monophosphate etc.

(Continued overleaf)

Table 2 (continued)

Common Deoxyribonucleotides		
Corresponding base	Nucleotide Named as Acid	Nucleotide Named as phosphate
Adenine	Deoxyadenylic-5'-acid	Deoxyadenosine-5'-monophosphate*
Adenine	Deoxyadenylic-3'-acid	Deoxyadenosine-3'-monophosphate*
Guanine	Deoxyguanylic-5'-acid etc.***	Deoxyguanosine-5'-monophosphate etc.*
Thymine	Thymidylic-5'-acid etc.	Thymidine-5'-monophosphate etc.*
Cytosine	Deoxycytidylic-5'-acid etc.	Deoxycytidine-5'-monophosphate etc.*

* Each of the 5'-monophosphates also occurs as the diphosphate and the triphosphate.

** Etc. indicates that the nucleotide occurs in other forms corresponding to the series shown for adenine.

*** Etc. indicates that each nucleotide occurs in a 3' form corresponding to that shown for Deoxyadenylic acid.

Note: 3'-5' cyclic phosphates are also known.

Synthesis of Nucleotides. The nucleotides can be prepared by the chemical or enzymatic hydrolysis of nucleic acids provided that the condition chosen do not affect hydrolysis of phosphomonoester groups and this route has already been discussed. (See page 18).

In recent years, numerous synthetic procedures have been developed which permit, in principle, the synthesis of any desired

nucleoside mono-, di-, or tri- phosphate. A complete discussion of the many synthetic methods is beyond the scope of this dissertation; the interested reader is referred to the detailed reviews of Baddiley,[117] Khorana,[118] Michelson,[69] and Brown[119]. To illustrate the steps in the synthesis of nucleotides, the following summary is, however given.

In principle, the synthesis of a nucleotide can take place either by combining a base with a phosphorylated sugar, or, more commonly, by the phosphorylation of a nucleoside. As mentioned above there are three hydroxyl groups available for esterification in ribonucleosides while two positions are available in deoxyribonucleosides. Therefore, the conversion of a nucleoside into a nucleotide generally requires three reactions. First, all hydroxyl and labile amino groups which are not to be phosphorylated must be blocked by treatment with suitable reagents (i.e. requirement of suitable protective groups), selected so as to be easily removed after phosphorylation. The next step is phosphorylation with a sufficiently active phosphorylating agent (i.e. requirement of suitable phosphorylating agent). The final step involves the removal of the protective groups.

(a) Suitable protective groups. Because nucleotides are sensitive towards strong alkali as well as acids, the protective groups should be of such a nature that they can be applied under mild conditions

to obtain the desired protected derivatives and can easily be removed after the phosphorylation of nucleosides. The most commonly used protective groups are summarised in Table 3.

Table 3

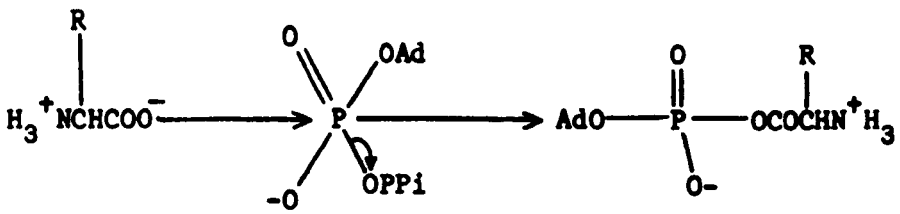
Common Protective Groups

	Protective groups	Position of nucleoside to be protected	Nature of the groups to be protected
1	Trityl (triphenyl-methyl) and its derivatives (monomethoxy, dimethoxy)	5'-position	primary alcoholic (OH)
2	Acetyl or Benzyl	2'- or 3'-position	secondary alcoholic (OH)
3	Isopropylidene or Benzylidene	2'- and 3'-positions (simultaneous blocking)	vicinal hydroxyls (in ribose)
4	Benzyl	6 position (Adenosine)	Amino group
5	Isobutyryl	2 position (Guanosine)	Amino group
6	Anisoyl	6 position (Cytosine)	Amino group

(b) Phosphorylating agents.

Phosphorylating agents, apart from a few unusual types; for example N-substituted phosphoramidates, have been phosphoric acid itself or mono-, and di-esters of ortho-, pyro- and poly-phosphoric acids, or their derivatives.[118-121,130] The chemistry of phosphate esters and anhydrides forms a large field of study in phosphorus chemistry, because of the great importance of phosphorylation in biological processes. In particular, the recognition of the significance of pyrophosphate and triphosphate esters in nucleic acid structure and hence in the genetic code and their importance in enzyme action have led to extensive investigations into all aspects of chemistry and biochemical action of phosphates.

These investigations have shown that the reaction of ATP, ADP, Conenzyme A, and similar compounds involved in the synthesis and action of nucleic acids, are basically phosphate transfers involving nucleophilic displacement at the highly electrophilic phosphorus atom; for example the phosphorylation of amino acid by ATP during protein synthesis.

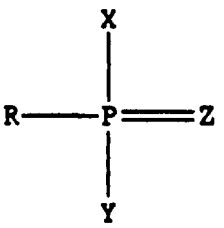


(4)

Thus phosphorylation may be defined as the transfer to a nucleophilic centre of a phosphoryl group $(RO)_2P\bar{O}$, which may or may not be substituted (one or both R = H, or an esterifying group).

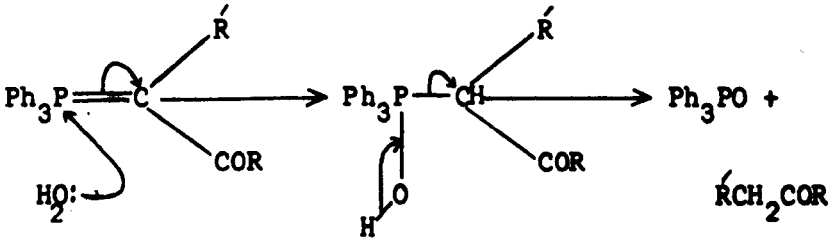
$$\bar{Y} + (RO)_2P(O)X \longrightarrow (RO)_2P(O)Y + X^- \qquad (5)$$

In other words, the mechanism of phosphorylation step is a nucleophilic displacement at a tetrahedral phosphorus. It is now known that the nucleophilic attack at the phosphorus atom in the compounds of the general structure (xvi).



(xvi)

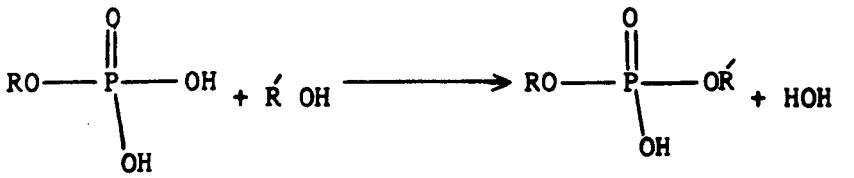
occurs when Z is C, N, O, S, or Se. Thus phosphoranes are hydrolysed by water.[122]



(6)

Chemical phosphorylation may be considered as analogous to the esterification of carboxylic acids.

(7)

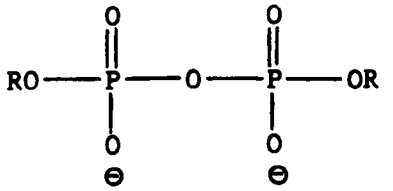


(8)

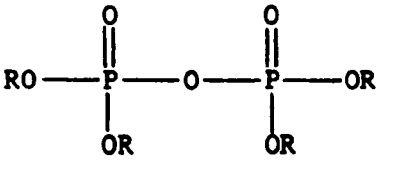
In parallel with acylating agents for the preparation of carboxylic esters, which are either carboxylic acid halides or anhydrides, phosphorylating agents have commonly been of two types, phosphorohalidates and anhydrides of the pyrophosphate or metaphosphate type. In broad terms, of course, the phosphorohalidates may also be considered mixed acid anhydrides. However, before discussing these phosphorylating agents, the problems which arise because of the polyfunctional nature of phosphoric acid, and the solutions to these problems, should be outlined.

Simple derivatives of phosphoric acid can themselves act as nucleophiles, especially if ionised. In activating a phosphoric acid molecule to produce a sufficiently powerful phosphorylating agent, one has to bear in mind the negative charges 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, the dialkyl pyrophosphates

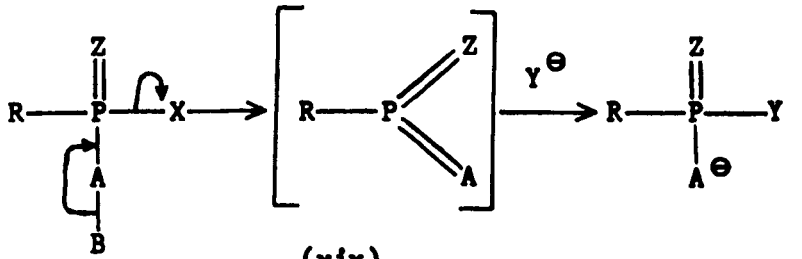


(xvii)

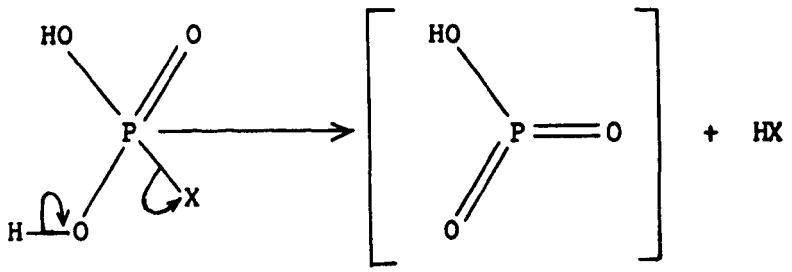


(xviii)

of the type (xvii) are not phosphorylating agents, but the corresponding tetraalkyl pyrophosphates (xviii) acquire the character of phosphorylating agents. One of the major problems in the development of efficient phosphorylating agents has therefore, been to confer on the phosphorus atom of the phosphoric acid molecule, an electropositive character. On the other hand, unsubstituted derivatives of phosphoric acid cannot bear the most effective leaving groups because of the possibility of internal elimination processes.[123]

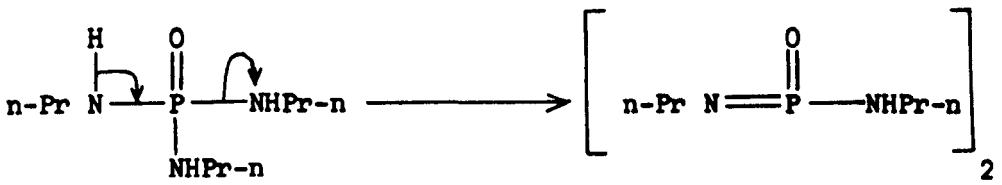


(xix) (9)



(10)

Although compelling evidence for the existence of (xix) or similar intermediates, has so far not been obtained, their presence has been recognised. Attempts to prepare them led to dimers or polymers which are now well characterised.[123,124]



(11)

Furthermore, attempts to prepare unsubstituted phosphorylating agents with good leaving groups such as phosphorochloridic acid $(HO)_2P(O)Cl$, lead to polymeric structures containing the P——O——P linkage.[125,126].

However, two approaches have been used successfully to overcome these problems: either using the fully protected phosphorylating agents $(RO)_2P(O)X$, or by generating the reagent

in situ, i.e. in the presence of the nucleophile, under such conditions that this can compete favourably with the nucleophilic group on the reagent itself.

Protected phosphorylating agents include the reagents of the phosphorohalidate type $(RO)_2P(O)X$, which can be considered as the mixed anhydride of the dialkyl or diaryl phosphoric acid and HX. The phosphorylation of a nucleophile is here a simple S_N2 (or more specifically S_N2P)[127] process as the reaction proceeds with complete inversion of configuration.

$$(PhO)_2POCl + ROH \xrightarrow{Base} (PhO)_2PO(OR) + Base\ HCl \qquad (12)$$

A variety of procedures is available to remove different protecting groups under mild conditions and these are discussed in the reviews mentioned above.

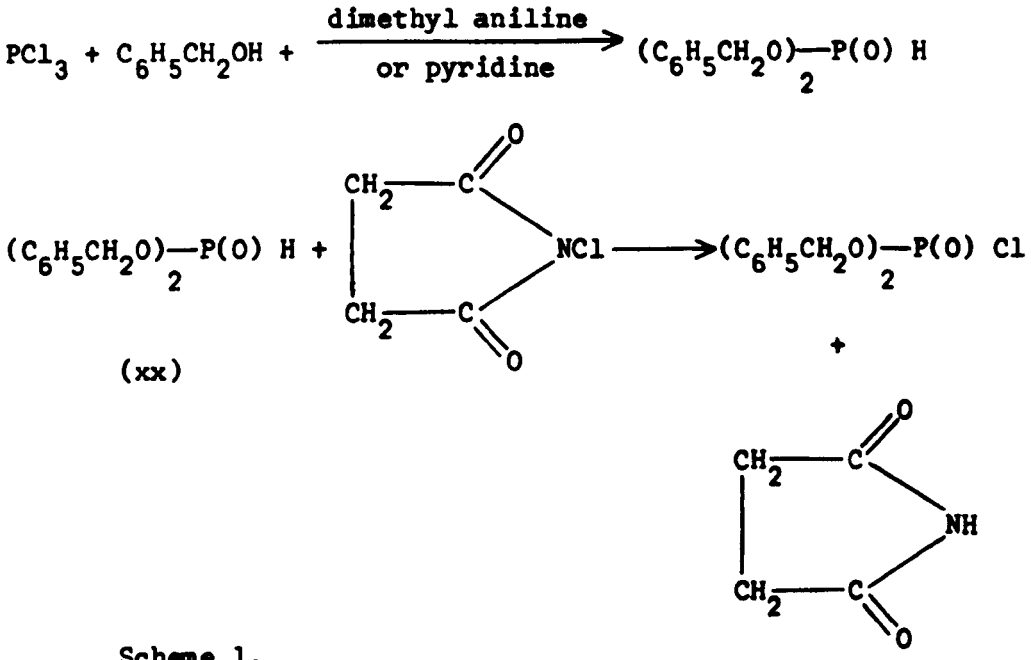
The direct displacement of a labile group from phosphorus by an anionic reagent sometimes leads to undesirable side reactions, and also subsequent exchange of the product residues particularly in the preparation of pyrophosphates. Milder methods of phosphorylation have been introduced by preparing the phosphorylating agent, in situ, which then reacts further to give the phosphorylated product.

In principle, a compound will require the activation of one substituent so that it can be converted into a good leaving group. The result will be increased electron-withdrawl from phosphorus, and this involves making the atom adjacent to phosphorus electron deficient. This can be achieved by electrophilic attack or by complete removal of electrons from the substituent by oxidation (i.e. oxidative phosphorylation).

Although a large number of phosphorylating agents have been introduced from time to time, only a few of them have been used widely, because of the limitations in the synthesis of nucleotides where such conditions as acid lability of purine glycosidic bonds, alkaline lability of the 6-amino group of cytosine,[128] and the catalytic reduction of pyrimidine rings[129] must be taken into consideration. However, today it is possible to convert all the ribonucleosides and deoxyribonucleosides into the corresponding ribonucleotides or deoxyribonucleotides. A list of these phosphorylating agents with a detailed discussion of their mode of action is included in the reviews of Khorana,[118,121] Brown,[119] and Clark.[120] Only one example from each of the two large groups of phosphorylating agents - the phosphorohalidates and the pyrophosphates - will be discussed here.

Among the phosphorohalidates, one reagent which has been used extensively in the nucleotide field is dibenzyl phosphorochloridate

developed by Todd and his co-workers.[119] Its preparation from dibenzyl hydrogen phosphate and phosphorus pentachloride was recorded by Zervas[131] who considered it too unstable for use, and from potassium dibenzyl phosphate and thionyl chloride by Deutsch and Fernö [132] who reported that it could be used successfully as a phosphorylating agent. The method which was developed by Todd and his co-workers[133] for the preparation of dibenzyl phosphorochloridate and is the one commonly used[134] is shown by (scheme 1).



(xx)

Scheme 1.

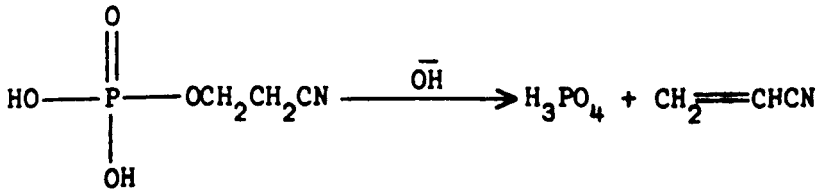
The reagent readily phosphorylates most primary alcoholic functions and the benzyl groups (protecting groups) can be removed from the intermediate phosphotriesters by mild catalytic

hydrogenolysis using a palladium catalyst and by a variety of other techniques. The reagent is, however, unstable and very sensitive to traces of water, and must be prepared from dibenzyl phosphite (xx) immediately before use. During the prolonged reaction time required to phosphorylate secondary alcohlic functions, the reaction solvent (pyridine) causes some debenzylation[135] of the intermediates thus giving lower yields of the desired products. Furthermore, dibenzyl phosphorochloridate is not extremely powerful as shown by its inability to phosphorylate guanosine nucleosides.[136] However, numerous nucleotides have been prepared using this reagent.

The second group of phosphorylating agents is the pyrophosphates. The best example of this group is, 2-cyanoethyl phosphate in combination with dicyclohexyl carbodiimide, the so called Tener's reagent.[137,138]

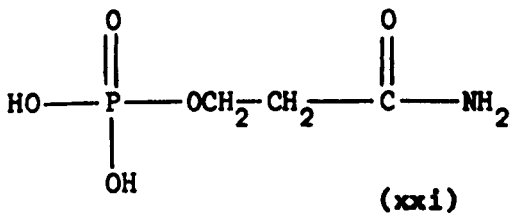
Any phosphorylating agent for use in the nucleotide field should satisfy the following requirements; it should be very powerful, and it should act as a monofunctional reagent from which the protecting group can be removed by very mild and specific methods. In addition, it is desirable to have a simple method for both the preparation of the reagent and its use. Since deoxyribonucleotides are unaffected by mild alkali, Tener[137] considered that a phosphorylating agent with protecting groups sensitive to mild alkali would prove useful. The reagent which satisfies this requirement

is 2-cyanoethyl phosphate. It was chosen because it had been shown by Cherbuliez and Rabinowitz[139] to break down under very mild alkaline treatment and to liberate orthophosphate, presumably by the reaction



(13)

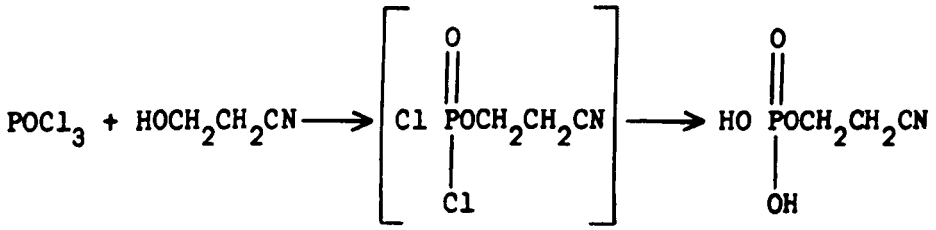
Cherbuliez and Rabinowitz reported its synthesis by heating a mixture of hydracrylonitrile and polyphosphoric acid at 100° for four hours. Following the same procedure Tener obtained the desired product in a very low yield together with a compound which he identified as phosphate ester of 3-hydroxypropionamide (xxi).



(xxi)

Therefore, a new method was developed to get pure 2-cyanoethyl phosphate in good yield. In this method an equimolar mixture of hydracrylonitrile and pyridine was added slowly to a cold solution of phosphorus oxychloride in ether. The reaction intermediate

presumably



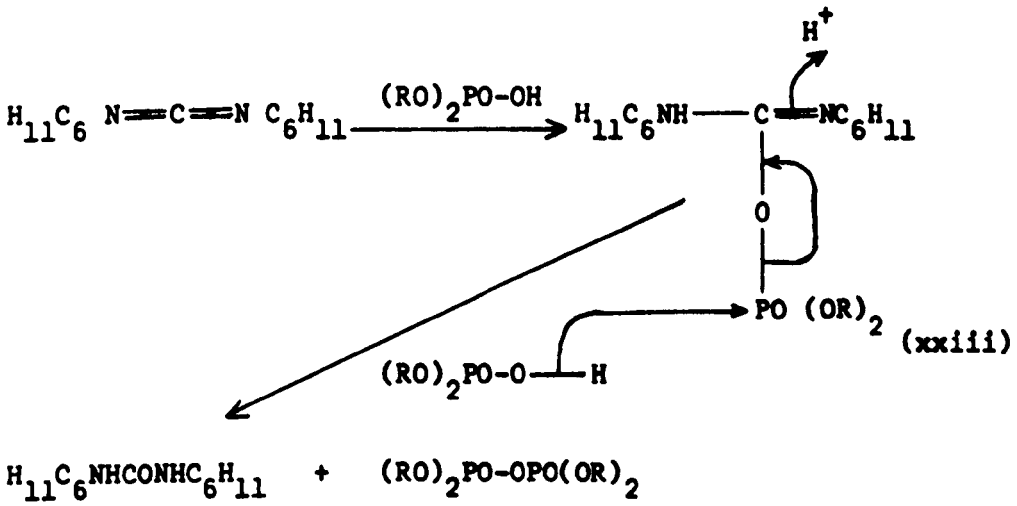
xxii (14)

(xxii) was decomposed by pouring the ethereal solution into a mixture of aqueous pyridine and ice. The product was collected as its barium salt in 60% yield.

Many examples of its use in the synthesis of nucleotides have been provided. Because 2-cyanoethyl phosphate can be easily prepared in a P^{32}- labelled form, the Tener reagent is also suitable for the synthesis of P^{32}- containing nucleotides. The reagent supersedes phosphorus oxychloride which is sensitive to traces of water and presents a hazard because of its volatility,[140] and labelled polyphosphoric acid which is suitable for the synthesis of pyrimidine nucleotides[141] but its use is limited because of its strong acidity and, further, with it the radioactive yields are very low, which necessitates the use of large amounts of radioactivity when preparing products of high specific activity.

The mechanism of the reaction of carbodiimides with acid has been very well discussed by Khorana,[118,121,142] who working in

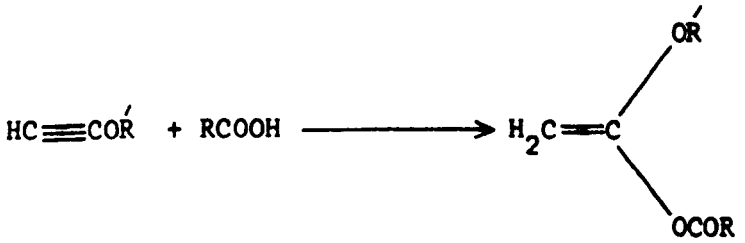
Todd's[143] laboratory at Cambridge found that under anhydrous conditions dicyclohexyl carbodiimide (DCC) with dibenzyl-, diphenyl-, and di- p- nitrophenyl phosphate gave corresponding pyrophosphates, whilst monophenyl phosphate gave P^1,P^2- diphenyl pyrophosphate. They put forward the hypothesis that the initial step of the reaction is the formation of the adduct (xxiii), and that the protonated form of this is attacked by another phosphate anion to give the pyrophosphate and the substituted urea (scheme 2). No success has attended efforts to isolate the adduct (xxiii).



Scheme 2

It should be added that the reaction of carboxylic acids with ethoxyacetylenes, where the 1, 2 adduct has been isolated, has been shown to involve after the formation of the 1, 2 adduct,

the addition of a second carboxylic anion to give the same type of intermediate (mentioned above) which "disproportionate" to give ethyl acetate and the acid anhydride.[144] Using different carboxylic acids, Wasserman[145] synthesised a variety of 1-methoxyvinyl esters (Scheme 3),

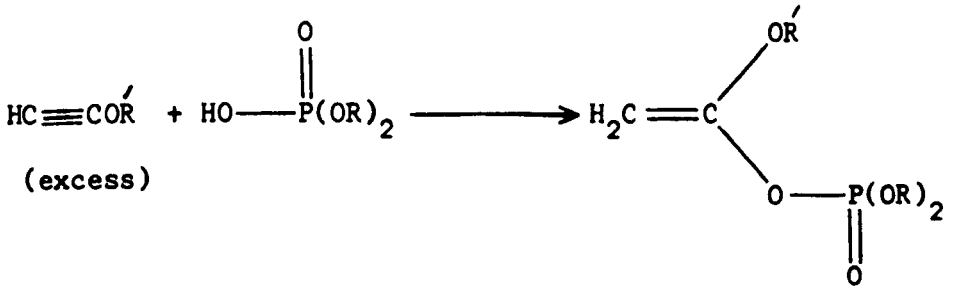


(xxiv)

$\acute{R}$ = CH_3; R = e.g. CH_3, CF_3, $CCl(Ph)_2$, C_6H_5, $PNO_2C_6H_4$

Scheme 3

and Cohen and Wasserman[146,147] extended this work to prepare 1-alkoxyvinyl phosphates by reacting ethoxyacetylene with phosphodiesters. The formation of the 1-alkoxyvinyl phosphates (xxiv, R = C_2H_5; R = C_6H_5, $C_6H_5CH_2$, P-$NO_2C_6H_4$) was demonstrated by spectral analysis and by chemical reaction, and in two cases (R = C_2H_5; R = C_6H_5, $C_6H_5CH_2$) analytical samples were obtained (Scheme 4).

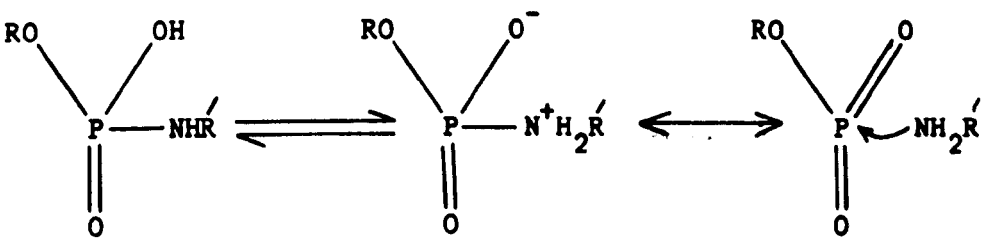


$\acute{R}$ = C_2H_5; R = C_6H_5, $C_6H_5CH_2$, $PNO_2C_6H_4$

Scheme 4

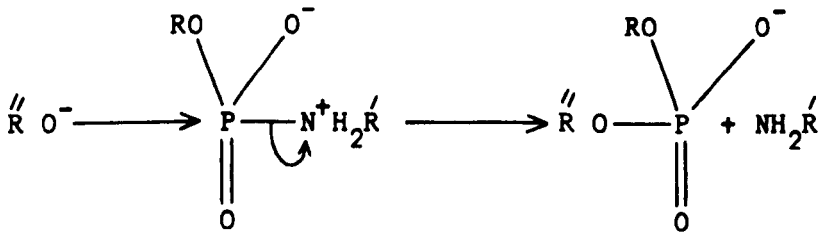
These 1-alkoxyvinyl phosphates have been shown to be successful phosphorylating agents for both acid anions and in some cases alcohols.

Earlier another type of phosphorylating agent had been developed;[148] the mono-alkyl phosphoroamidates which appear to act in the tautomeric form as base-metaphosphate complexes.



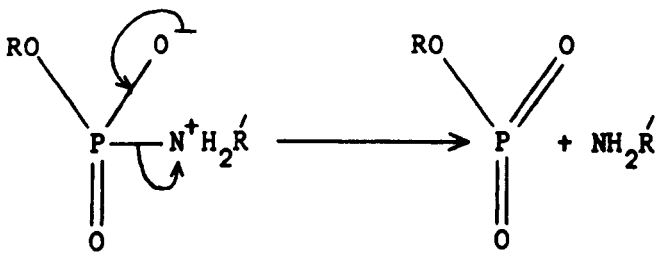
(15)

These reagents phosphorylate a potential or actual $R\ O^-$ anion by undergoing nucleophilic attack on the phosphorus atom with expulsion of a neutral amine molecule.



(16)

The expulsion of the amine molecule may precede the R O^- attack:

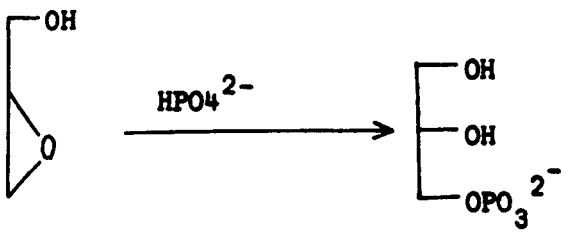


(17)

in which case the active phosphorylating intermediate would be the alkyl metaphosphate. These reagents readily phosphorylate phosphate diester anions ($R'' = (R''O)_2PO$) and have proved very useful in coenzyme synthesis.[149]

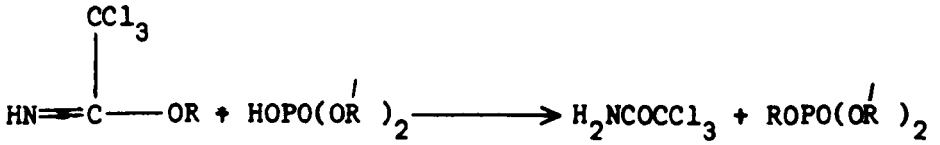
Furthermore, the activation of alcohol has also been achieved either using epoxides, iminoethers or diazomethane.

Thus Bailly showed that glycidol reacted with disodium hydrogen phosphate in water at room temperature to give disodium glycerol-1-phosphate.[150]



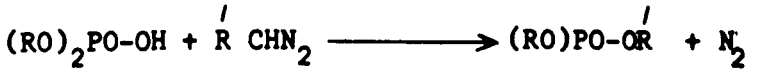
(18)

Cramer has prepared phosphate esters using the reaction of iminoethers with phosphoric acid.[178]



(19)

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



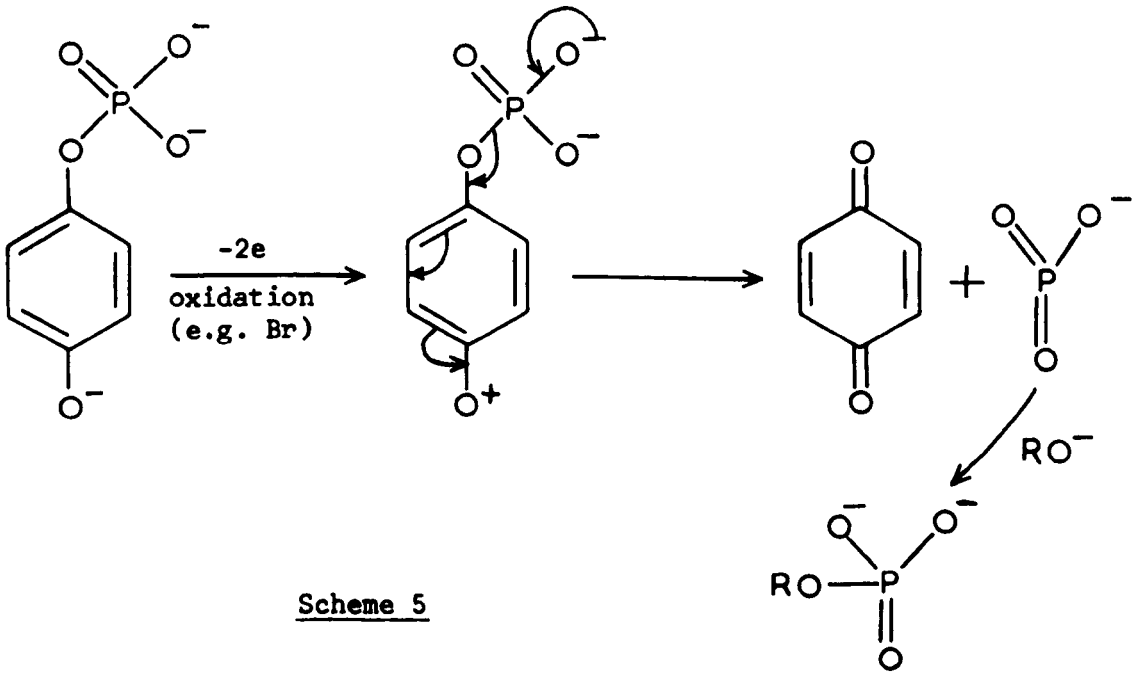
(20)

Thus Atherton et al.[179] reported that benzyl and benzhydryl esters could be prepared using phenyldiazomethane and diphenyldiazomethane. Brown et al.[62] converted uridine-3'-phosphate to the extremely unstable dimethyl and dibenzyl esters by titration in methanol with the corresponding diazoalkane. With other nucleotides, solubility problems became important but adenosine-2'(and -3') phosphate and cytidine-2'(and -3') phosphate were converted to their monobenzyl esters by phenyl diazomethane in dimethylformamide; the neutral triester was probably formed but converted to the diester during working up.

While excellent for the preparation of simple esters of phosphates, it must be concluded that the method is very limited on solubility grounds and by the inaccessibility of many substituted diazomethanes.

(c) Oxidative phosphorylation. At present an aspect of biochemistry that is receiving a great deal of attention is that of oxidative phosphorylation. This in general terms, is the generation of ATP from ADP coupled to the oxidation of carbon-containing substrate molecules.

From the chemical point of view, Clark et al.[151] have pointed out that the leaving group can be lost as a cation, or, more usually, as a proton and a neutral molecule, if the bond to phosphorus is activated by the oxidative removal of a pair of electrons. A pertinent example of this process studied in the laboratory is the oxidation of hydroquinone phosphates to the corresponding quinones, with the production of a phosphorylating agent, [152,153] possibly a metaphosphate, which will phosphorylate an alcohlic hydroxyl group, ROH or alkoxide anion RO^- (Scheme 5).

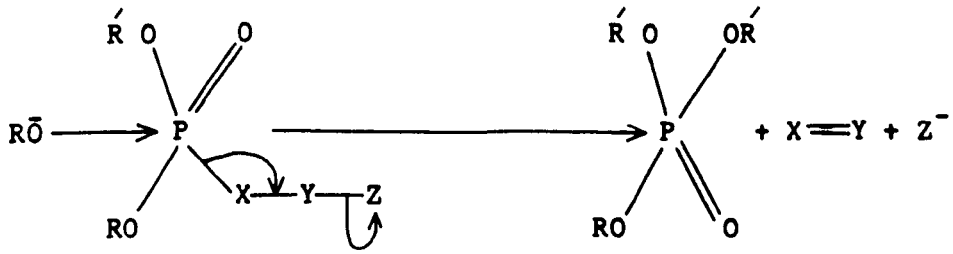


Scheme 5

Biochemical evidence implicates quinones as catalyst in coupling oxidation to phosphorylation in many cases, and it has been suggested that oxidation of a quinol phosphate is an essential step.[154,155] Lord Todd[153] proposed that all processes which involve activation of a mono-alkyl phosphate proceed through a metaphosphate intermediate, whereas Khorana[126] has produced evidence for a trimetaphosphate as the phosphorylating entity.

(d) The P-XYZ system. Clark et al.[120,156] have reported that any molecule of the general formula $(RO)_2P(O)$-X-Y-Z, where X, Y and Z are commonly atoms of H, C, N, O, S and Hal., is a potential phosphorylating agent if the electrons of the P-X bond can formally

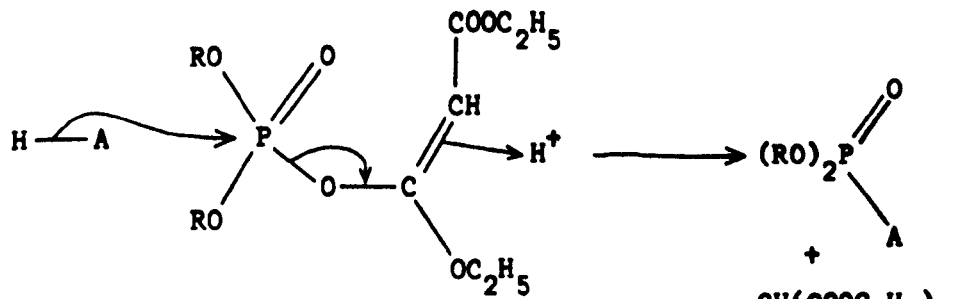
be accommodated on Z. On phosphorylation there is an increase of one in the bond order between X and Y, and a corresponding decrease between Y and Z.



(21)

Since Z acts as a primary electron-acceptor, the requirements of such a P-XYZ system are that the P-X bond must be weak and that Z must be a strong electron acceptor. 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. A few examples of P-XYZ systems (from phosphorylating agents) are quoted below.

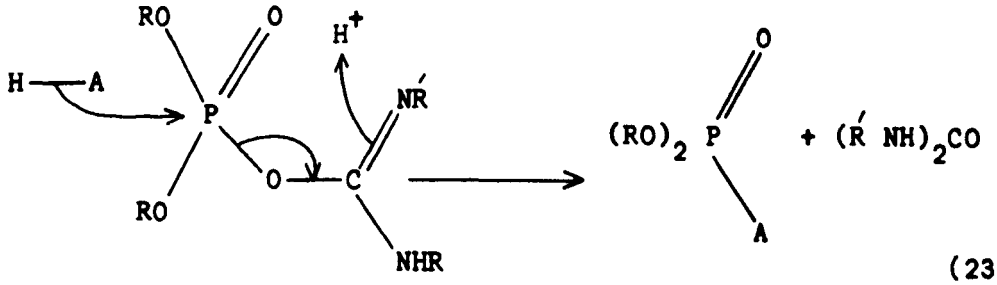
Enol phosphates under conditions leading to electron withdrawl from the carbon-carbon, for example, are capable of phosphorylating:



X = O, Y = Z = C.

(22)

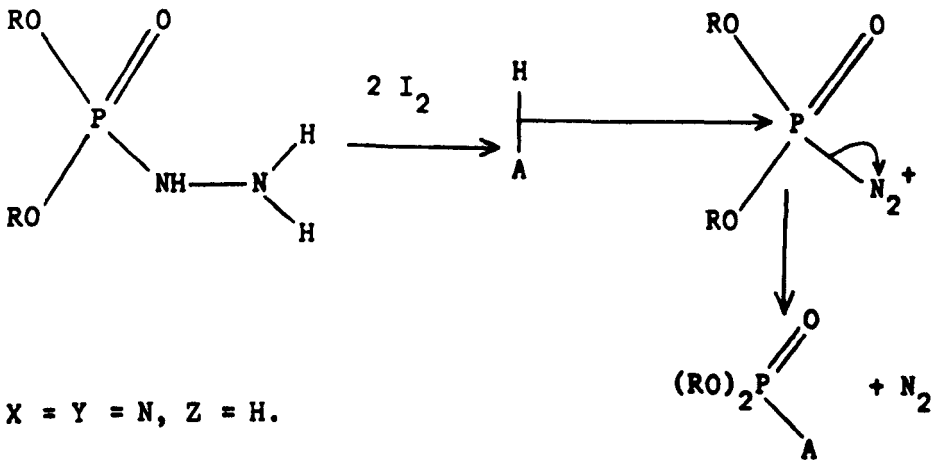
Imidoyl phosphates:



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

(23)

Phosphorohydrazidates:



X = Y = N, Z = H.

(24)

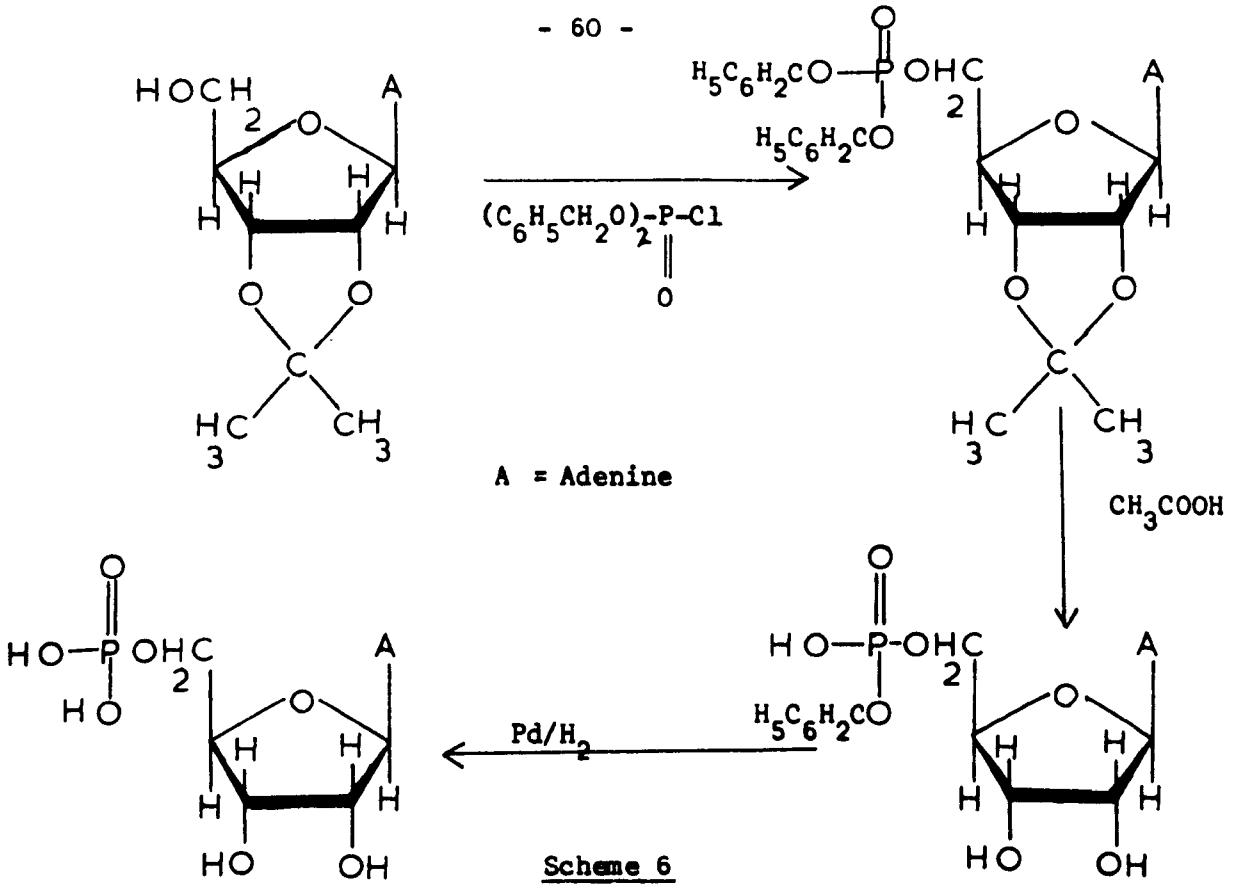
(e) Synthesis of Ribonucleotides.

Ribonucleoside-5'-phosphates.

It has already been mentioned that for the selective phosphorylation of a 5'-hydroxyl group, the 2'- and 3'-positions

in ribonucleosides can both be blocked simultaneously either using isopropylidene or benzylidene as protective groups. Thus using these protective groups and phosphoryl chloride or diphenyl phosphorochloridate as phosphorylating agents, synthesis of ribonucleoside-5'-phosphates has been recorded by many workers,[69,117] but the first satisfactory synthesis was not achieved until 1947 when Baddiley and Todd[157] phosphorylating 2':3'-O-isopropylidene-adenosine with dibenzyl phosphorochloridate obtained 2',3'-O-isopropylideneadenosine-5'-dibenzyl phosphate from which the protecting group (isopropylidene) and one of the benzyl groups were removed by acetic acid treatment[50] while the second benzyl group was lost by palladium-catalysed hydrogenolysis to obtain adenosine-5'-monophosphate in a good yield. The overall steps are shown by (Scheme 6).

Phosphorylation of the 2', 3'-O-isopropylidene derivatives of uridine, cytidine[136,159] and 9-β-D-ribofuranosyl purine[160] with dibenzyl phosphorochloridate gave the corresponding-5' phosphates. A difficulty arose when guanosine derivative was found to resist this type of reagent. However, Tener's reagent (2-cyanoethyl phosphate with dicyclohexyl carbodiimide)[137] was found to be useful for this purpose as was phosphoryl chloride.



Scheme 6

Direct phosphorylation of unprotected nucleosides with phosphoryl chloride ($POCl_3$) in pyridine[162,163,164] gives all three monophosphates (2', 3', or 5'), the molar proportions depending, to some extent, on the presence or absence of water,[165] but the yields are very poor. Recently Yoshikawa and his co-workers[166] have found that the treatment of 2', 3'-O-isopropylidene nucleoside with an excess of phosphoryl chloride in the absence of pyridine reduces the amount of by-products and improves greatly the yields of the corresponding 5'-phosphorochloridates which are readily hydrolysed by simple treatment with water to give 5'-nucleotides in good yields. During the course of their investigations on

accelerators for phosphorylation, they found that the use of trialkyl phosphate highly facilitated the phosphorylation with phosphoryl chloride. In addition, 2', 3'-O-isopropylidene nucleosides were moderately soluble in anhydrous trialkyl phosphates, such as trimethyl and triethyl phosphates.

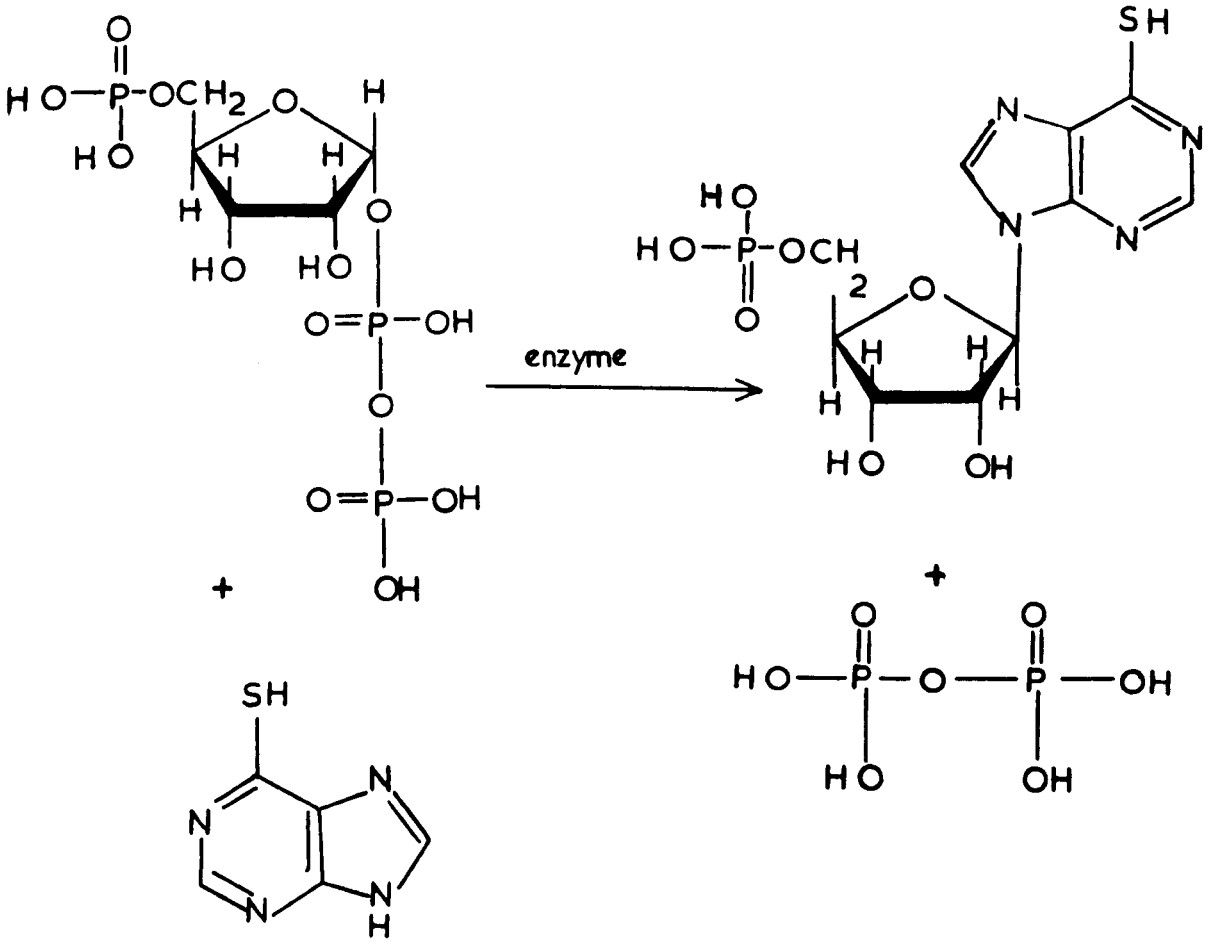
Taking advantage of the potentiality of these esters as useful solvents for phosphorylation, they developed a technique to synthesise nucleoside-5'-phosphates in one step. Thus when a 2', 3'-O-isopropylidene nucleoside was added to a cold mixture of trialkyl phosphate and phosphoryl chloride with stirring, it was converted smoothly into the corresponding 5'-phosphorochloridate in nearly quantitative yield. The nucleoside-5' monophosphate (in 92 - 98% yield) was obtained by rapid hydrolysis of the chloridate group followed by removal of the isopropylidene group at 70°C.

When unprotected nucleosides were treated directly with phosphoryl chloride in trimethyl or triethyl phosphate, the nucleosides were easily phosphorylated in good yields. The products were mainly 5'-phosphates along with 2' (or 3'), 5'-diphosphates. Furthermore, these studies showed that the formation of the diesters could be greatly reduced by the use of the phosphorylating agent previously treated with a small amount of water in trialkyl phosphate.

Very recently Yoshikawa et al.,[167] using trimethyl phosphate as an accelerator for phosphorylation, have made use of phosphorus trihalide to convert nucleosides into nucleotides in good yields. Thus the treatment of a 2', 3'-O-isopropylidene nucleoside with phosphorus trichloride in the presence of trimethyl phosphate and subsequent hydrolysis gave a nucleoside-5' phosphite in an excellent yield. They have reported that triaryl phosphates, trialkyl phosphine oxides, and trialkyl phosphites were also found to be potential accelerators of the reaction. Their effects were comparable to that of trimethyl phosphate. The similar treatment of 5'-O-acetyl nucleosides gave mixed nucleoside-2' and 3' phosphites in an approximate ratio of 4:6. The reaction intermediate, nucleoside phosphorochloridate was subjected to partial hydrolysis, giving the corresponding product which was readily oxidised to give the 5'-nucleotide. Chlorine was generally suitable as an oxidising agent, but for guanosine derivatives iodine gave better results than did chlorine or bromine. The treatment of an unprotected nucleoside with phosphorus trichloride in a trialkyl phosphate, prior to the reaction with phosphorus trichloride, could be used for the synthesis of a nucleoside 2' (or 3'), 5'-diphosphate in a good yield.

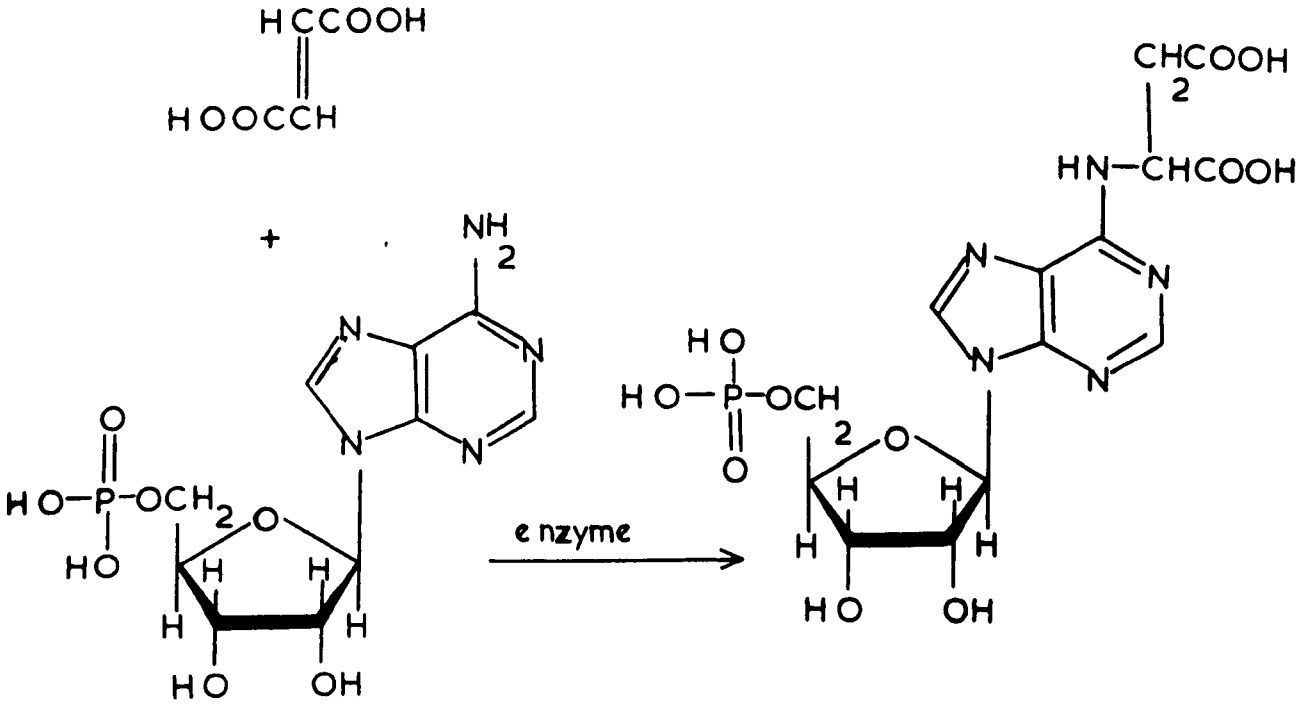
The chemical synthesis of nucleotides from the free base and a sugar phosphate has not been extensively explored. However, the

biochemical synthesis of nucleoside-5' phosphates by this method is occasionally convenient, particularly in the case of nucleotides derived from "unnatural" bases which in the general chemical route would require prior synthesis of the nucleoside. This has been well reviewed by Michelson.[69] Only two examples are recorded below to illustrate the necessary steps in this method. Preparation of 9-β-D-ribofuranosyl-6-mercaptopurine-5'-phosphate[168] by the interaction of 6-mercaptopurine with 5-phosphoribosyl-1 pyrophosphate (α-D-ribofuranose-5 -phosphate-1 pyrophosphate)[169] in the presence of a pyrophosphorylase from beef liver. (Scheme 7)



Scheme 7

The use of adenylosuccinase in the preparation of adenylosuccinic acid by the condensation of adenosine-5'-phosphate and fumaric acid.[170] (Scheme 8)

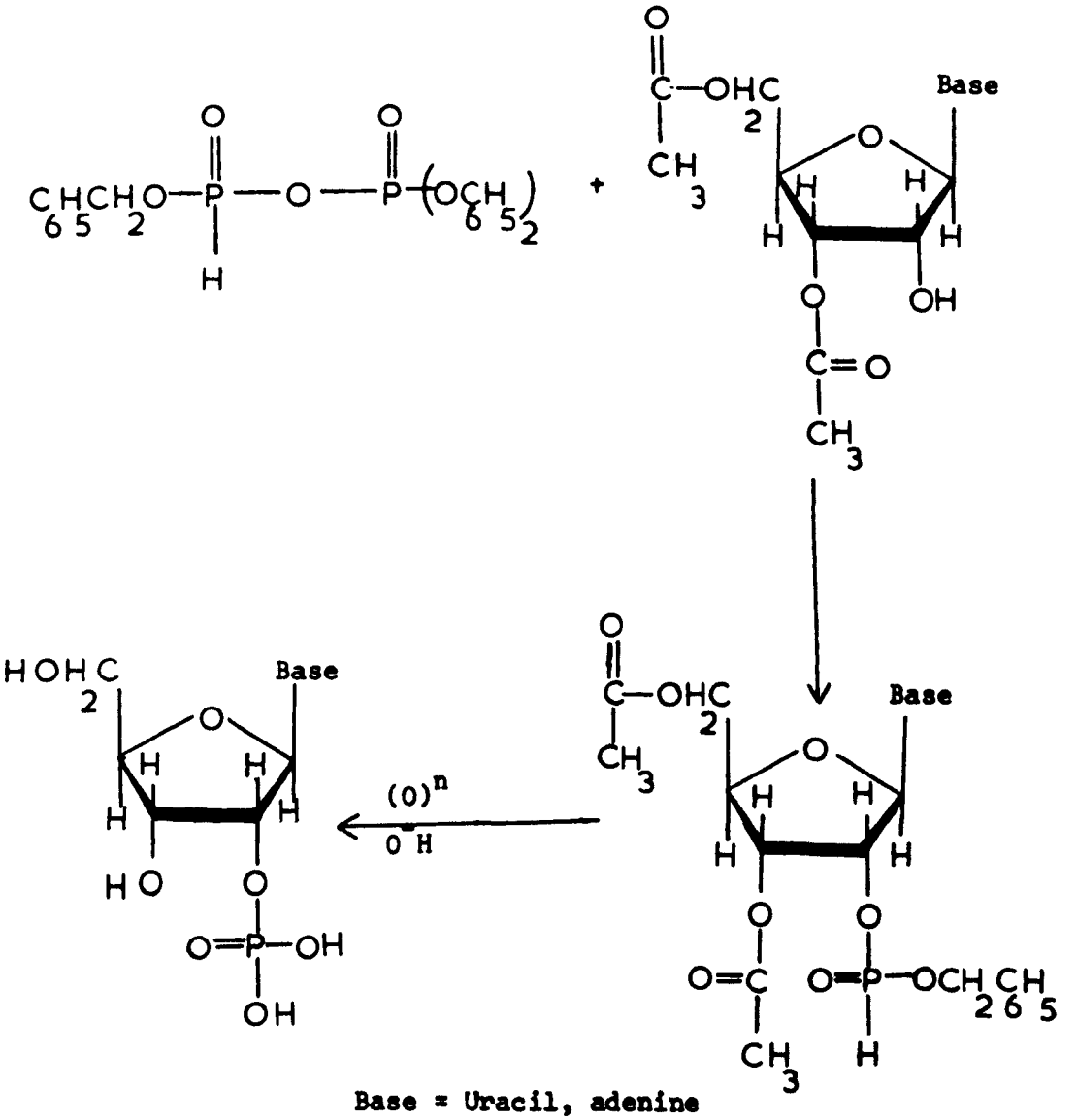


Scheme 8

Ribonucleoside-2' and 3'-phosphates.

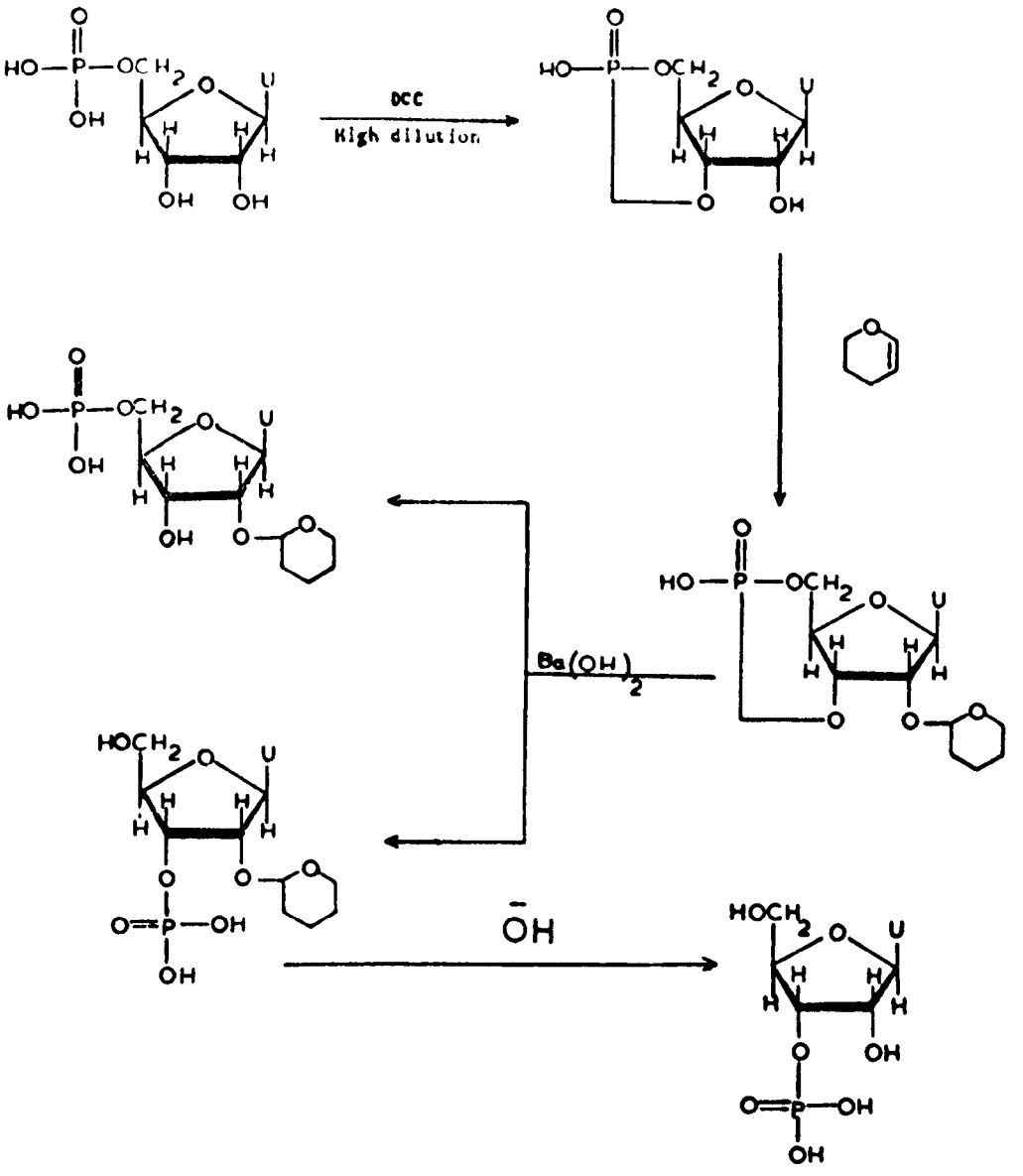
Because of the facile group migration via cyclic phosphate intermediates (page 87) between the 2'- and 3'-hydroxyl groups, unambiguous synthesis of either 2' or 3' phosphate esters of ribonucleosides requires derivatives containing 2'- and 5'- or 3'- and 5' hydroxyl groups appropriately blocked. In this way

adenosine-2' phosphate and uridine-2' phosphate have both been synthesised by unambiguous routes involving phosphorylation of the 3', 5'-di-O-acetyl nucleoside with O-benzylphosphorous-O,O-diphenyl phosphoric anhydride to give the phosphite, followed by oxidation and hydrolysis.[158,171] (Scheme 9)



Scheme 9

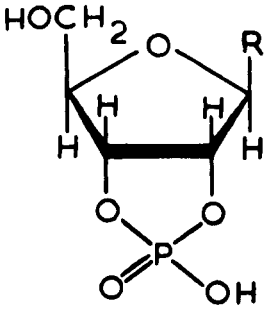
An unambiguous synthesis of -3' phosphates proceeds through the reaction of dihydropyran with, for example, uridine-3', 5' cyclic phosphate to give the 2'-O-tetrahydropyranyl derivative.[172] (Scheme 10)



Scheme 10

The chemical synthesis of a mixture of ribonucleoside-2' and 3' phosphates requires only the protection of the 5'-hydroxyl group. Thus Brown and Todd[66] prepared a mixture of adenosine-2' and 3' phosphates by phosphorylation of 5'-O-trityladenosine with dibenzyl phosphorochloridate. Syntheses of corresponding nucleotides from other nucleosides should be analogous. The separation of 2'- and 3'- phosphate isomers of all ribonucleotides can be readily effected by the ion exchange techniques.[56]

The 2', 3'-cyclic nucleotides (xxv) of adenosine, cytidine and uridine have been synthesised by Brown, Magrath and Todd,[65] and identified with the products isolated from ribonucleic acid.



(xxv)

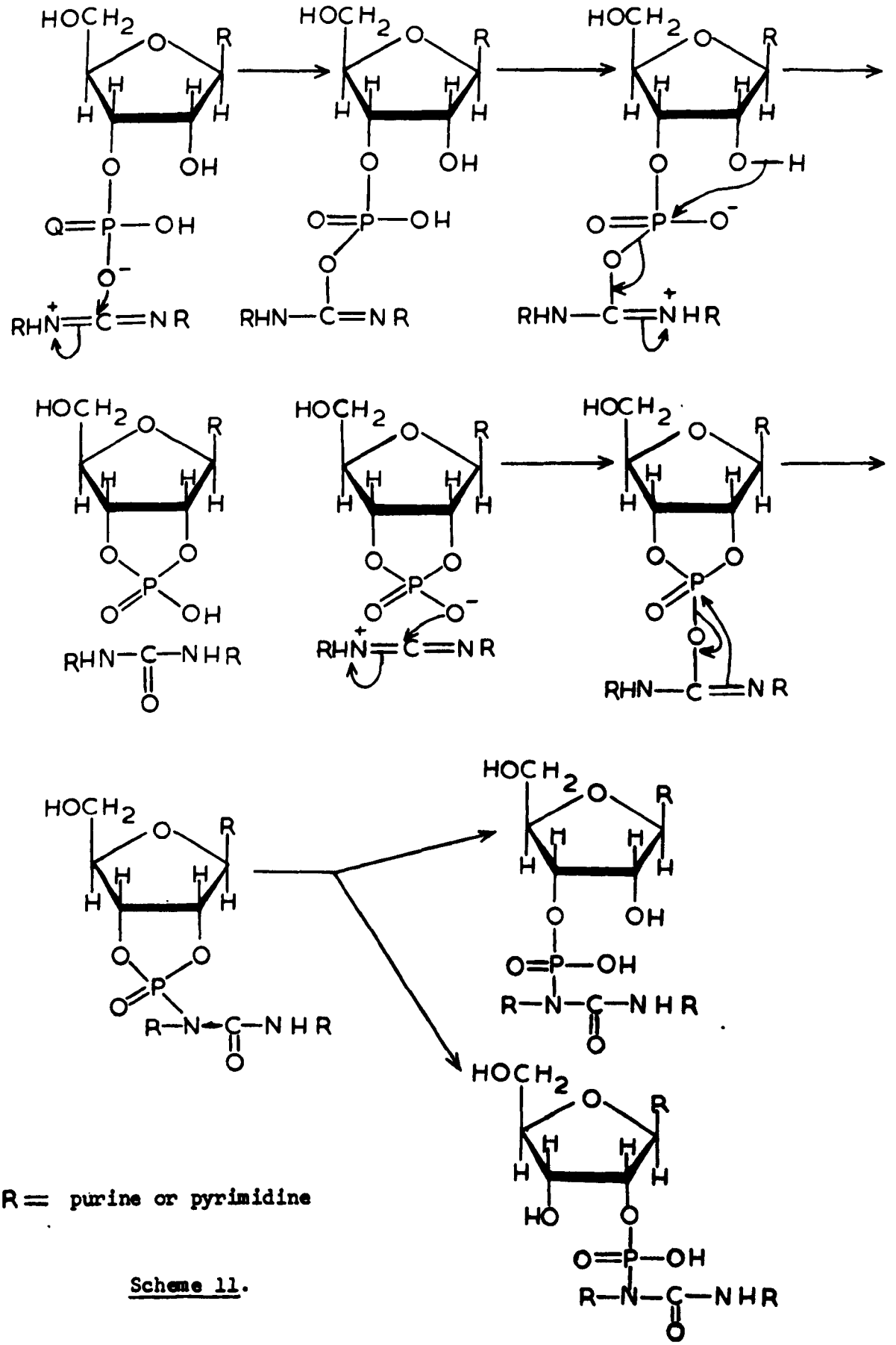
R= purine or pyrimidine

The pyrimidine cyclic nucleotides are readily converted into the 3'-phosphate by the further action of ribonuclease[65,60] and into the 2'-phosphate isomers by the action of acid. The cyclic phosphates of adenosine and guanosine, however, while being readily hydrolysed to the 2' and 3' isomers by acid, are not attacked by ribonuclease.[60]

Nucleoside-2', 3' cyclic phosphates have also been obtained by the action of carbodiimides on nucleoside-2' (or -3') phosphates.[173] (Scheme 11) In wet pyridine a mixture of the cyclic phosphate and the 2' and 3' phosphoryl ureas was obtained. The phosphoryl ureas resulted from the further reaction of the cyclic phosphate monoanion with dicyclohexyl carbodiimide. This can be avoided by the use of ammonium or trialkyl ammonium salts[142] of the nucleotides in anhydrous solution. In the presence of the stronger bases, addition of a proton to the carbodiimide is greatly reduced so that reaction with the cyclic phosphate, which is also considerably less nucleophilic than the nucleotide di-anion, is negligible. Similar results can be obtained by using a less basic carbodiimide.

Nucleoside-3' (or 2'), 5' Diphosphates.

Direct phosphorylation of adenosine with dibenzyl phosphorochloridate followed by alkaline hydrolysis and hydrogenolysis of



R = purine or pyrimidine

Scheme 11.

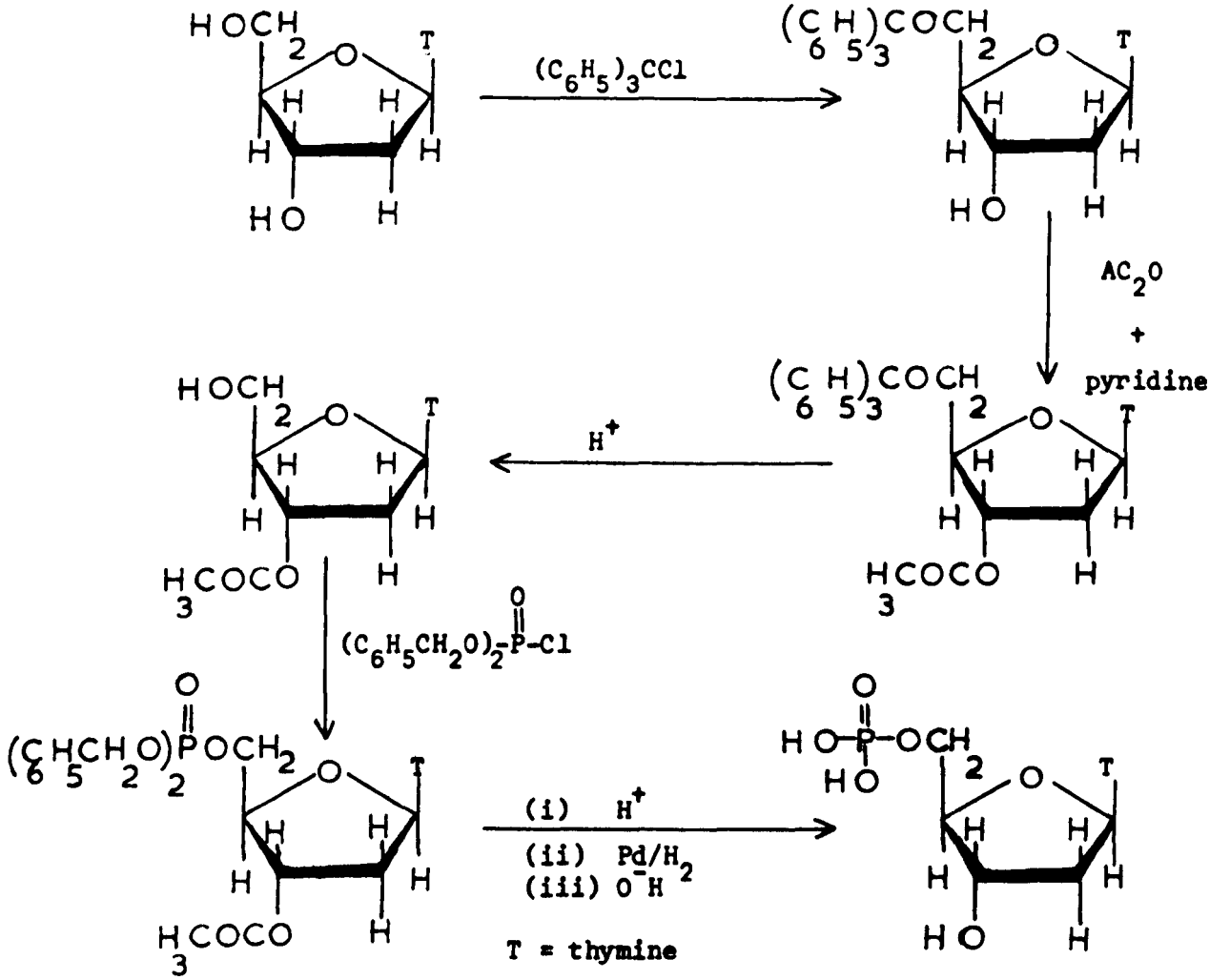
the benzyl groups gave a mixture of the nucleoside-2', 5' and 3',5'-diphosphates,[174] which can be separated by ion exchange chromatography and characterised by chemical or enzymatic hydrolysis.[175] The mixture also contained adenosine-2',3',5' triphosphate which upon deamination gave inosine-2',3',5' triphosphate.[175] Fogarty and Rees[176] have reported the phosphorylation of adenosine with 2-cyanoethyl phosphate and dicyclohexyl carbodiimide.

Adenosine-2' (3'), 5' diphosphate has also been obtained by phosphorylation of adenosine-2',3' cyclic phosphate with O-benzyl phosphorous O,O-diphenyl phosphoric anhydride (followed by oxidation with chlorine, mild alkaline treatment and hydrogenolysis),[177] with dibenzyl phosphorochloridate (followed by hydrogenolysis), or with 2-cyanoethyl phosphoric acid and dicyclohexyl carbodiimide (followed by treatment with alkali).[69] The last method has also been applied for guanosine derivatives. The pyrimidine ribonucleoside-2',5' and 3',5' diphosphates were prepared by the direct treatment of nucleoside with polyphosphoric acid.[69]

(f) Synthesis of Deoxyribonucleotides.

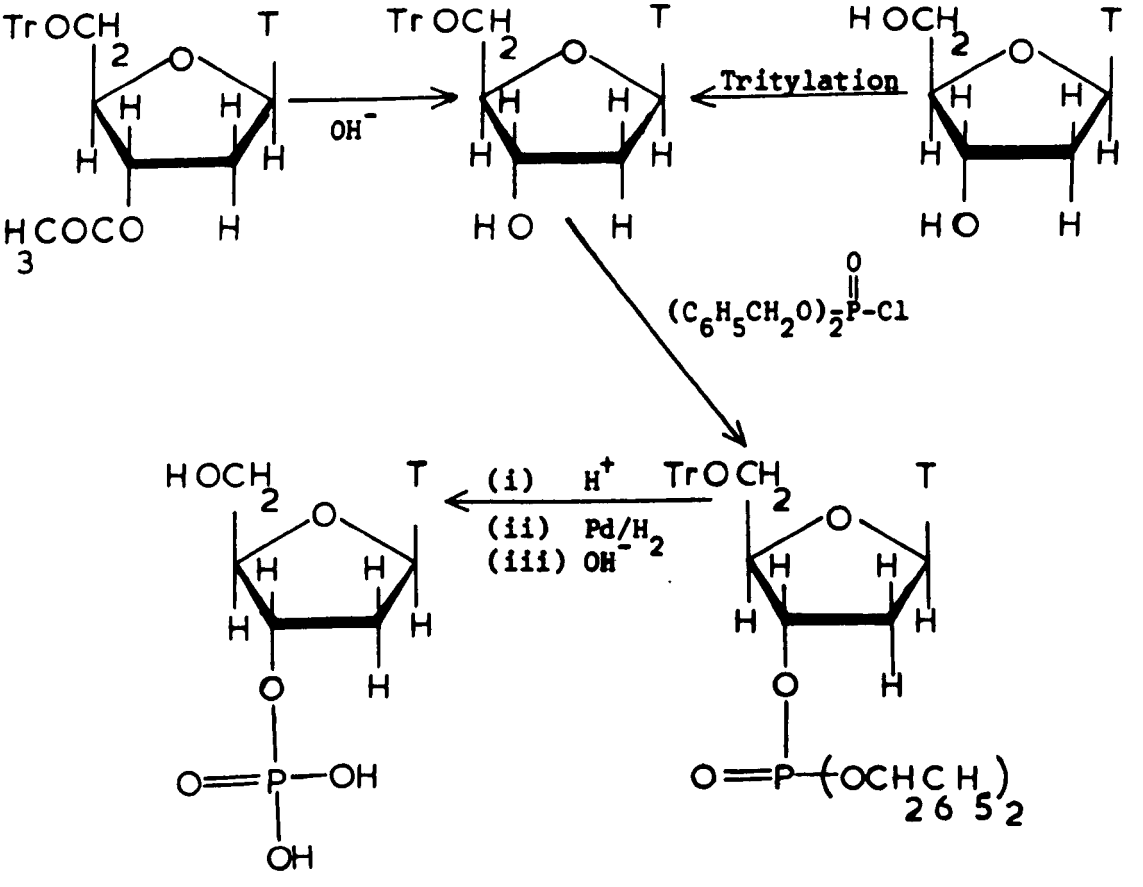
Deoxyribonucleoside-3' and 5' phosphates.

Thymidine-5' phosphate has been prepared by phosphorylation of 3'-O-acetyl thymidine with dibenzyl phosphorochloridate followed by the removal of protective groups.[161,180] (Scheme 12)



Scheme 12

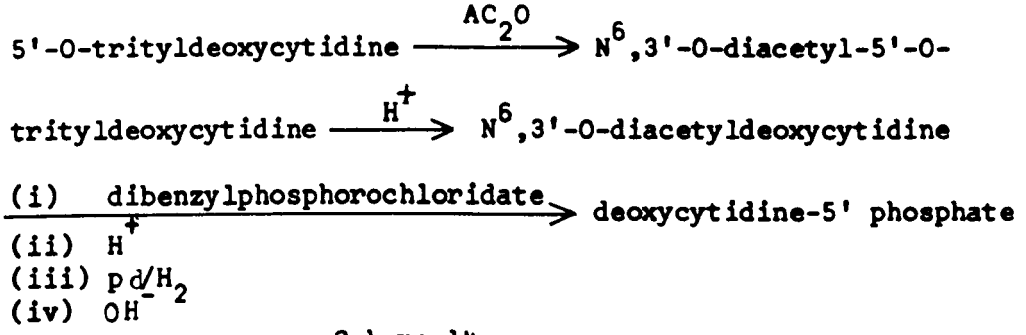
In a similar way thymidine-3' phosphate was prepared by treating 5'-O-trityl thymidine with dibenzyl phorochloridate and then removing the protective groups from the product. (Scheme 13)



T = thymine, Tr = trityl

Scheme 13

For the synthesis of deoxycytidine-5' phosphate, the same technique was used except that the starting material was N^6, 3'-O-diacetyl-5'-O-trityl deoxycytidine.[181] (Scheme 14)



Scheme 14.

The same method (described above), however, could not be applied for the synthesis of the purine deoxyribonucleoside-5' phosphates, because of the high lability of the glycosyl bond under the acidic conditions necessary for the removal of 5'-trityl group. The possibility of removing the 5'-trityl group by hydrogenolysis also met with little success.[182] Alternative procedures were, therefore, developed for the preparation of 5'- and 3'-O-monoacetyldeoxyadenosine and the corresponding guanosine compounds.[183] These involved complete acetylation of the nucleosides followed by partial deacetylation. Thus deoxyadenosine was acetylated with acetic anhydride to yield 3',5'-O-diacetyldeoxyadenosine which was partially hydrolysed with methanolic ammonia, and 3'-O-acetyldeoxyadenosine and 5'-O-acetyldeoxyadenosine were separated from the mixture by counter-current distribution. When these monoacetylated deoxyadenosine products were phosphorylated with dibenzyl phosphorochloridate and the protective groups were removed, the corresponding deoxyadenosine phosphates were obtained.

For the synthesis of the corresponding deoxyguanosine phosphate isomers, monoacetates were likewise prepared by partial deacetylation of 3',5'-O-diacetyl-deoxyguanosine or by partial acetylation of deoxyguanosine. Each monoacetyl (3' or 5') deoxyguanosine was then phosphorylated with O-benzyl phosphorous O,O-diphenyl phosphoric anhydride and the product converted into the corresponding nucleotides. The yield was poor.

Although deoxyribonucleoside-3' or 5' phosphates have been prepared using many other phosphorylating agents, perhaps the most useful reagent is 2-cyanoethyl phosphate with carbodiimide. Thus using this reagent all the ribo and deoxyribonucleotides have been synthesised in very good yields.[69,121,137] In addition the reagent is very useful to prepare ^{32}p-labelled nucleotides and Tener has used this reagent for the synthesis of ^{32}p nucleotides by the direct phosphorylation of deoxyribonucleosides. This will be discussed in detail in the experimental section.

(C) Polymerisation Methods

The last decade has seen remarkable progress in the techniques for the synthesis of polynucleotides. The object of these syntheses has been the preparation of oligo- and polynucleotides of known base sequence or random polymerisation in an attempt to prepare polynucleotides containing bases in known ratios. The interest in the synthesis of polynucleotides has arisen from the discoveries of (1) internucleotidic linkage in nucleic acid, (2) the macromolecular organisation of DNA, (3) the discovery of DNA polymerase with which a particular DNA could be replicated to produce more DNA in a well characterised enzymatic reaction, (4) the discovery of DNA-dependent RNA polymerase which clarified the manner in which information from DNA may be transcribed to a ribonucleic acid (messenger RNA) and (5) the development of amino acid polymerisation which brings about in vitro the formation of polypeptide material in response to certain varieties of RNA.

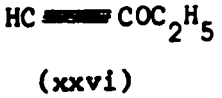
Polynucleotides can be synthesised by chemical synthesis by enzymatic synthesis or by a combination of both methods.

It is difficult to deal with all aspects of this rich and interesting field and this thesis will be restricted to the more chemical and synthetic aspect.

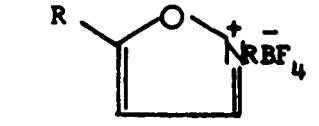
Before discussing the chemical synthesis, the nomenclature of polynucleotides and condensing reagents which are used in their preparations, are described.

(a) Condensing Reagents.

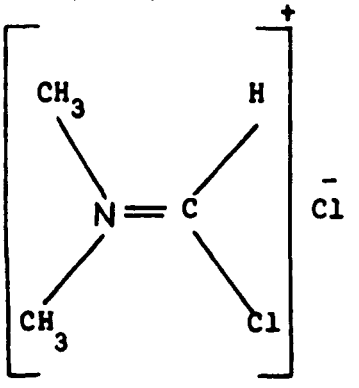
During the past few years a number of reagents have been proposed for the activation of carboxylic and phosphoric acid groups.[184] The reagents proposed by many workers from time to time include ethoxyacetylene (xxvi), substituted isoxazolium salts (xxvii) the product (xxviii) from the reaction of phosgene with dimethylformamide, ethyl metaphosphoric acid prepared by the reaction of ether with phosphorus pentoxide, trichloroacetonitrile (xxix) carbonylbis (imidazole) (xxx) carbodiimides (xxxi) and aryl sulphonyl chloride (xxxii).



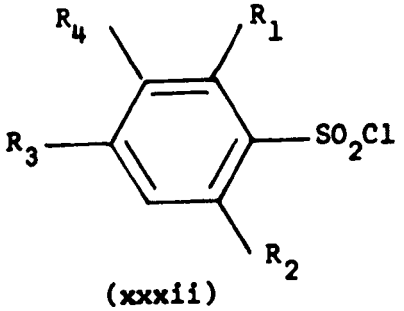
(xxvi)



(xxvii) $R = C_2H_5$, $R = C_6H_5$



(xxviii)



(xxxii)

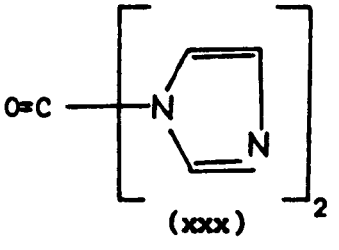
(a) $R_1, R_2, R_3, R_4 = H$

(b) $R_1, R_2, R_4 = H$; $R_3 = CH_3$

(c) $R_1, R_3 = H$; $R_2, R_4 = CH_3$

(d) $R_4 = H$; $R_1, R_2, R_3 = CH_3$

(e) $R_4 = H$; $R_1, R_2, R_3 = CH(CH_3)_2$



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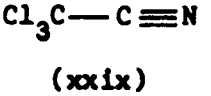
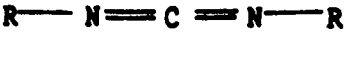


(xxix)



(xxi)

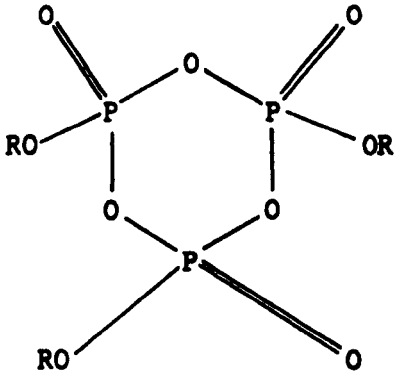
$R = C_6H_{11}$, $CH(CH_3)_2$

Jacob and Khorana[184] made a comparative study of these reagents to observe their efficiency in the synthesis of a $C_{3'}$-$C_{5'}$ internucleotidic linkage using a nucleotide and a nucleoside derivative. Thus the formation of thymidylyl (3'-5') thymidine by the condensation of (a) pyridinium 5'-O-acetylthymidine-5' phosphate with 5'-O-tritylthymidine, and of (b) pyridinium 5'-O-acetylthymidine-3' phosphate with 3'-O-acetylthymidine was studied under identical conditions using the several reagents mentioned above. Among all these reagents dicyclohexylcarbodiimide and aryl sulphonyl chlorides were found to be the most efficient reagents as they gave 90% yields (or better, using stoichiometric amounts of the protected nucleotide and the nucleoside) of the desired product. The rate of internucleotide bond synthesis using the aromatic sulphonyl chlorides was much higher than that obtained with dicyclohexyl carbodiimide. On the basis of their findings they were also able to discuss the mechanism of internucleotide bond synthesis using the aryl sulphonyl chloride in relation to the findings with dicyclohexyl carbodiimide.

Weimann and Khorana,[126] in their mechanistic studies of the formation of an internucleotide bond by the condensation of protected mononucleotide with the hydroxyl group of a suitably protected nucleoside in the presence of dicyclohexyl carbodiimide, carried out the following two sets of experiments:

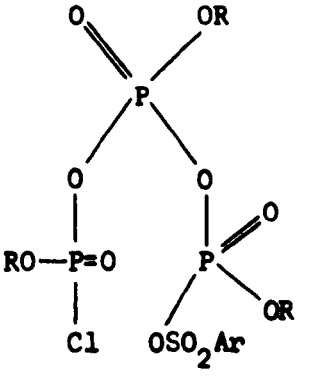
1, A study of rates of phosphorylation of 3'-O-acetylthymidine, 5'-O-tritylthymidine by a mixture of 2-cyanoethyl phosphate (stoichiometric amount) and DCC (excess).

2, A study of the rate of formation and the final yield of TpT in condensation of 3'-O-acetylthymidine with different 5'-O-substituted thymidine-3' phosphates (5'-O-trityl, 5'-O-mesitoyl, 5'-O-trimethylacetyl, and 5'-O acetylthymidine-3' phosphate). Their results showed that the size of the organic groups in the nucleoside (the hydroxyl component) did not have very significant effect but that the size of the organic groups in the phosphomonoester component (the nucleotide) has a profound influence on the rate and the yields of the phosphodiester. Furthermore, on the basis of their findings, they were able to postulate that the initial phosphorylating species was probably an alkyl trimetaphosphate (xxxiii).

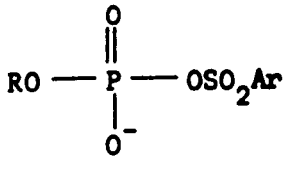


(xxxiii)

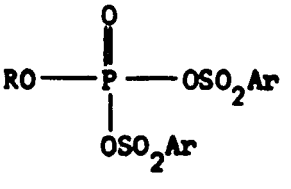
Since the rate of synthesis of the internucleotide bond using the arylsulphonyl chlorides was found to be 5 - 10 times higher than that using DCC, it was thought that a species other (and more reactive) than trimetaphosphate was the phosphorylating species in the reaction mediated by arylsulphonyl chlorides, and Khorana has suggested the species (xxxiv - xxxix) might be formed in the presence of an excess of the sulphonyl chloride.



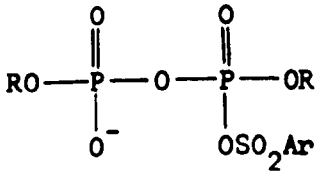
(xxxiv)



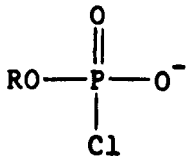
(xxxv)



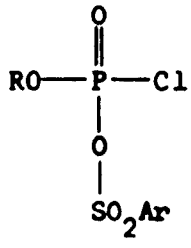
(xxxvi)



(xxxvii)



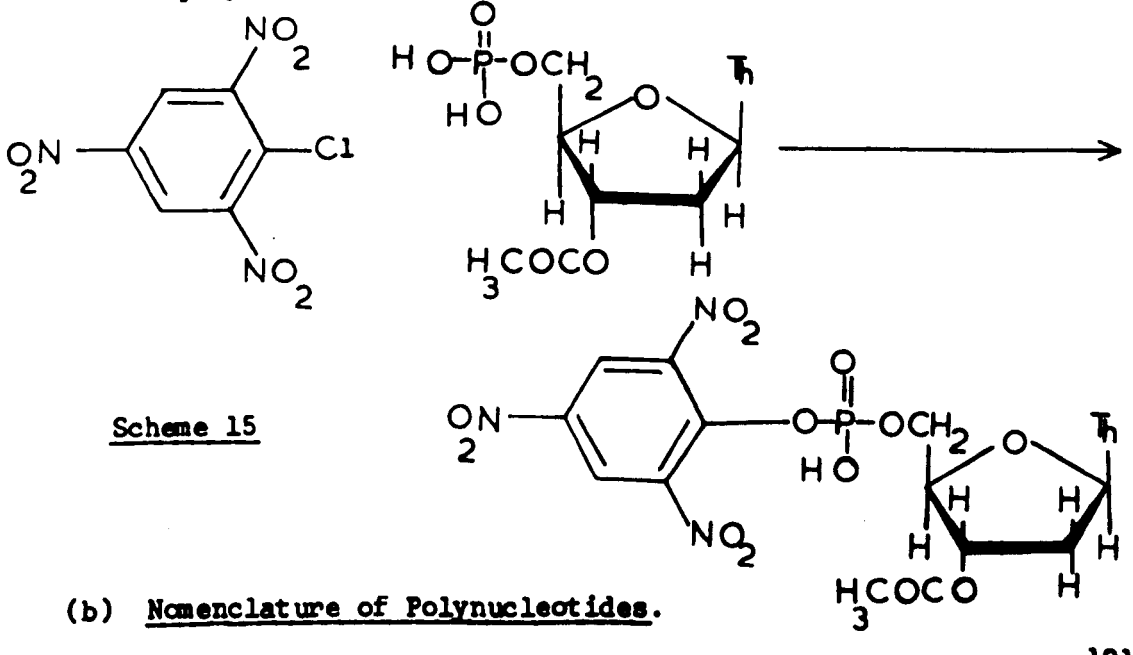
(xxxviii)



(xxxix)

whilst all these would be expected to be reactive phosphorylating entities, lack of information at the moment prevents a definitive mechanism to be deduced.

Recently, Cramer[185] has reported that picrylchloride is a preferable condensing reagent for the condensation of mononucleotides to homopolymers. (Scheme 15)



Scheme 15

(b) Nomenclature of Polynucleotides.

For the nomenclature of oligo- and polynucleotides Khorana[121] proposed a system which is now widely accepted. In this system a

polynucleotide chain is regarded as a chain in which each nucleotide esterifies the hydroxyl groups of the succeeding one rather than as a number of nucleoside joined together by phosphodiester bonds. For the mononucleotides, the trivial names, for example, thymidylic-(5') acid and adenylic-(3') acid are used; the number in parentheses refers to the carbon atom attached to the phosphoryl group (see page 34). In naming the oligo- or polynucleotides, each nucleotide residue is followed by two numbers, separated by an arrow, in paranetheses, inserted into the names by hyphen. The first number refers to the carbon atom by which phosphodiester is linked to the preceeding nucleoside. The second number denotes the point of linkage to the succeeding nucleoside. It should be mentioned that the system allows one to name the compound equally well from either terminus. In this presentation, however, the polynucleotide chains are named in the direction of C_3,-C_5, so that it can be related to the abbreviations system which will be described later.

The second system, known as diagramatic representation or shorthand formulation, was introduced by Brown and Todd[60] when they discussed the chemical hydrolysis of nucleic acid in 1952. The method is very useful to illustrate many chemical and enzymatic reactions. In this system the letters A, C, G (also T or U) represent the initial letters of purines and pyrimidines while sugar residues are represented by vertical lines. The diagonal

line broken by the letter "P" joining the midpoint ($C_{3'}$-position) of one vertical line with the bottom ($C_{5'}$-position) of the other vertical line, represent the phosphodiester bridge. The conventions regarding the terminal phosphomonoester groups shown by letter "P" are the same as in the abbreviated formulation (see later). The 2'-hydroxyl groups in polyribonucleotides as well as any terminal hydroxyl groups in any polynucleotide may be shown by horizontal lines attached to the vertical lines as shown in Fig. 6.

The third system, commonly known as the abbreviations or shorthand formulation, was first used by Markham and his co-workers.[186] It is a very convenient and commonly used system. In this system the letters A,C, G (also T and U) represent the nucleosides. The letter "P" to the left of the nucleoside initial indicates a 5'-phosphomonoester group whereas the same letter on the right denotes a 3'-phosphate. The deoxyribose series can be distinguished from their ribo-counterparts by prefixing the initial letters with the letter "d". It should be mentioned that in this system the polynucleotide chain is specified in the direction $C_{3'}$-$C_{5'}$. The reverse method in which the polynucleotide chain is $C_{5'}$-$C_{3'}$ has also been used by Chargaff et al.,[187] but the former method is the most commonly used and has been adopted by the Editors of the Journal of Biological Chemistry.

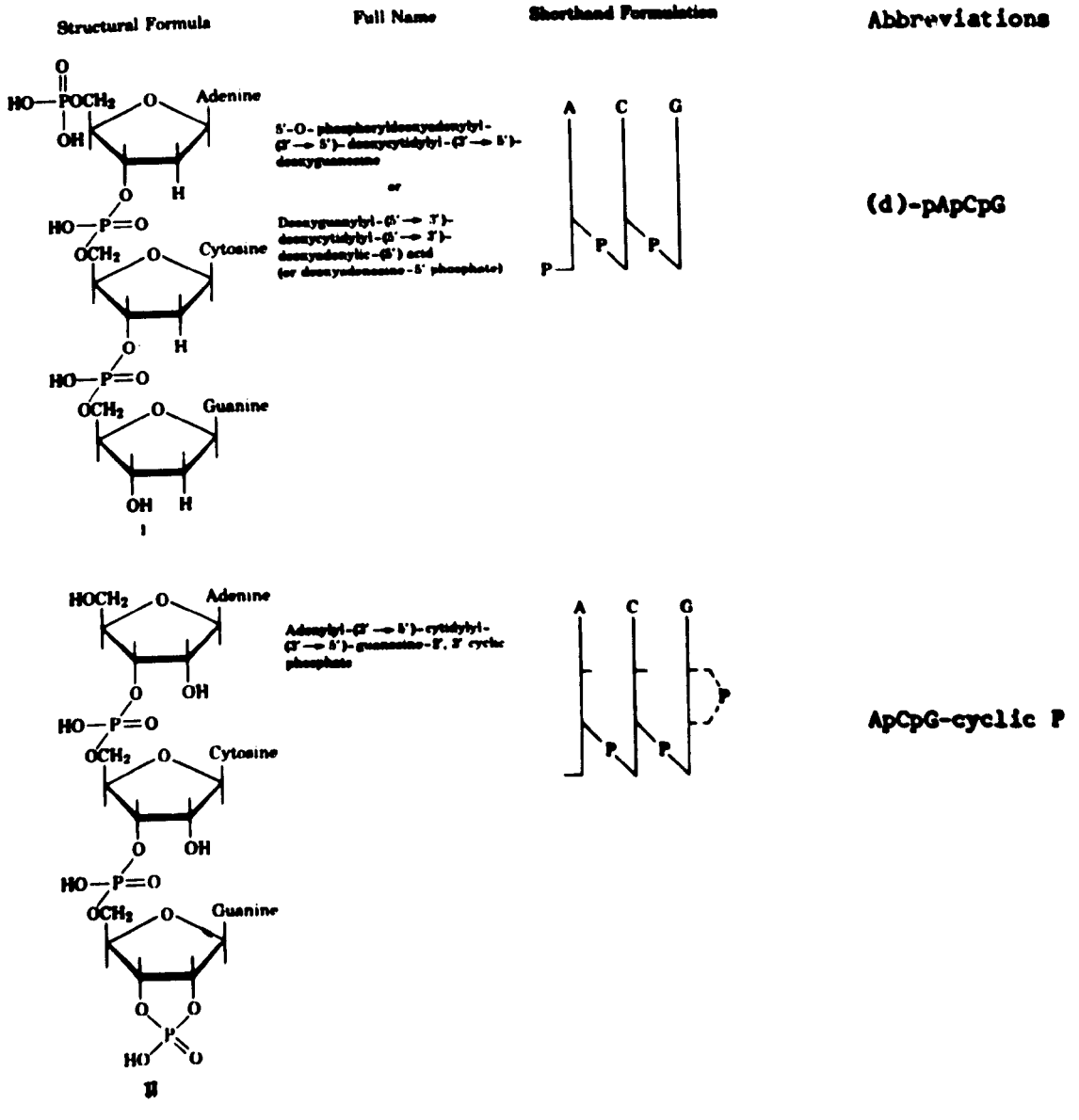


Figure 6. Nomenclature of Polynucleotides

Recently Khorana[188] has developed and modified the system in which the chain runs in the 3'-5' direction. He has designated the protecting groups on the purine or pyrimidine rings by two-letter abbreviations added as superscripts after the nucleoside initial: thus d-A^{Bz} for N-benzoyldeoxyadenosine, C^{An} for N-anisoyl-cytidine, G^{Ac} for N-acetylguanosine. The acetyl group at the 3'-hydroxyl group of a nucleoside was shown by -OAC added after the nucleoside initial. Thus, pT-OAC was the abbreviation of 3'-O-acetylthymidine-5' phosphate. CE was the abbreviation for β-cyanoethyl: thus, d-CE-pG^{Ac} stands for N-acetyldeoxyguanosine-5' β-cyanoethyl phosphate. Thus using this system, the abbreviation of d-$pC^{An}pG^{Ac}pA^{Bz}$-OAC stands for the protected trinucleotide, 5'-O-phosphoryl-N-anisoyldeoxycytidylyl-(3'-5')-N-acetyldeoxyguanylyl-(3'-5')-3'-O-acetyl-N-benzoyl-deoxyadenosine.

The use of all three systems is demonstrated in this thesis.

(c) Synthesis of Polynucleotides.

Polyribo- and polydeoxyribonucleotides (including lower oligonucleotides) have been synthesised either by stepwise synthesis or by random polymerisation. Stepwise synthesis involves the addition of mononucleotides one after the other in a strictly sequential fashion, with isolation of the product at each step; whilst in the random polymerisation mixtures of mono-, di-, or trinucleotides are used as starting materials, a whole range of products resulting

which vary in their degree of polymerisation. It should be obvious that the success of both methods depends upon the method of separation of the products.

(i) Stepwise Synthesis of Polynucleotides.

In principle, organic chemists could attempt to put together nucleotides and nucleosides to form short chains of polynucleotides which would be identical in every way with naturally occurring nucleic acids. But this becomes very complicated because of the different functional groups present in nucleosides and nucleotides, e.g. the hydroxyl groups in ribose or deoxyribose sugars. Therefore, to get the required polynucleotide chain ($C_{3'}$-$C_{5'}$) one has to protect the appropriate groups (as has already been described) to prepare suitable nucleosides and nucleotides. These protected nucleosides and nucleotides having been made, the next step will be their condensation to get higher nucleotides. This can be achieved in two ways; in one method mononucleotides are added one by one to a growing polynucleotide chain and the second approach, which is more attractive, consists in the preparation of oligonucleotide blocks and their subsequent condensation to form successively longer chains. Both of these techniques have been developed and investigated systematically over the last few years.[189, 190,191].

(1) Stepwise Synthesis of Polyribonucleotides.

In the RNA field, things are more complicated. The 2'-hydroxyl group in the ribose ring creates an additional formidable problem. Consequently methods had to be developed for the specific linkage of the 3'-hydroxylic group of one nucleoside to the 5'-hydroxylic group of the next. In its simplest form, the problem of specific synthesis of an inter-ribonucleotidic linkage may be approached in two ways as shown in Figure 7.

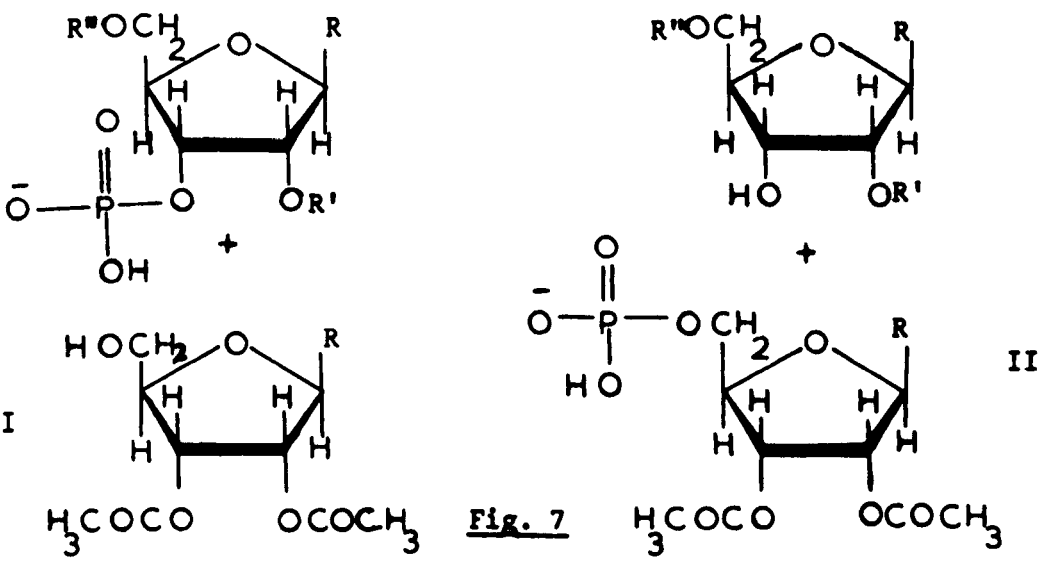


Fig. 7

In the first, a suitably protected ribonucleoside-3' phosphate and a suitably protected ribonucleoside bearing a free C5'-hydroxyl group may be used as the two components in a condensation reaction. In the second approach, a suitably protected ribonucleoside-5' phosphate and a second component, a protected ribonucleoside bearing a free C3'-hydroxyl group, may be used in the condensation reaction.

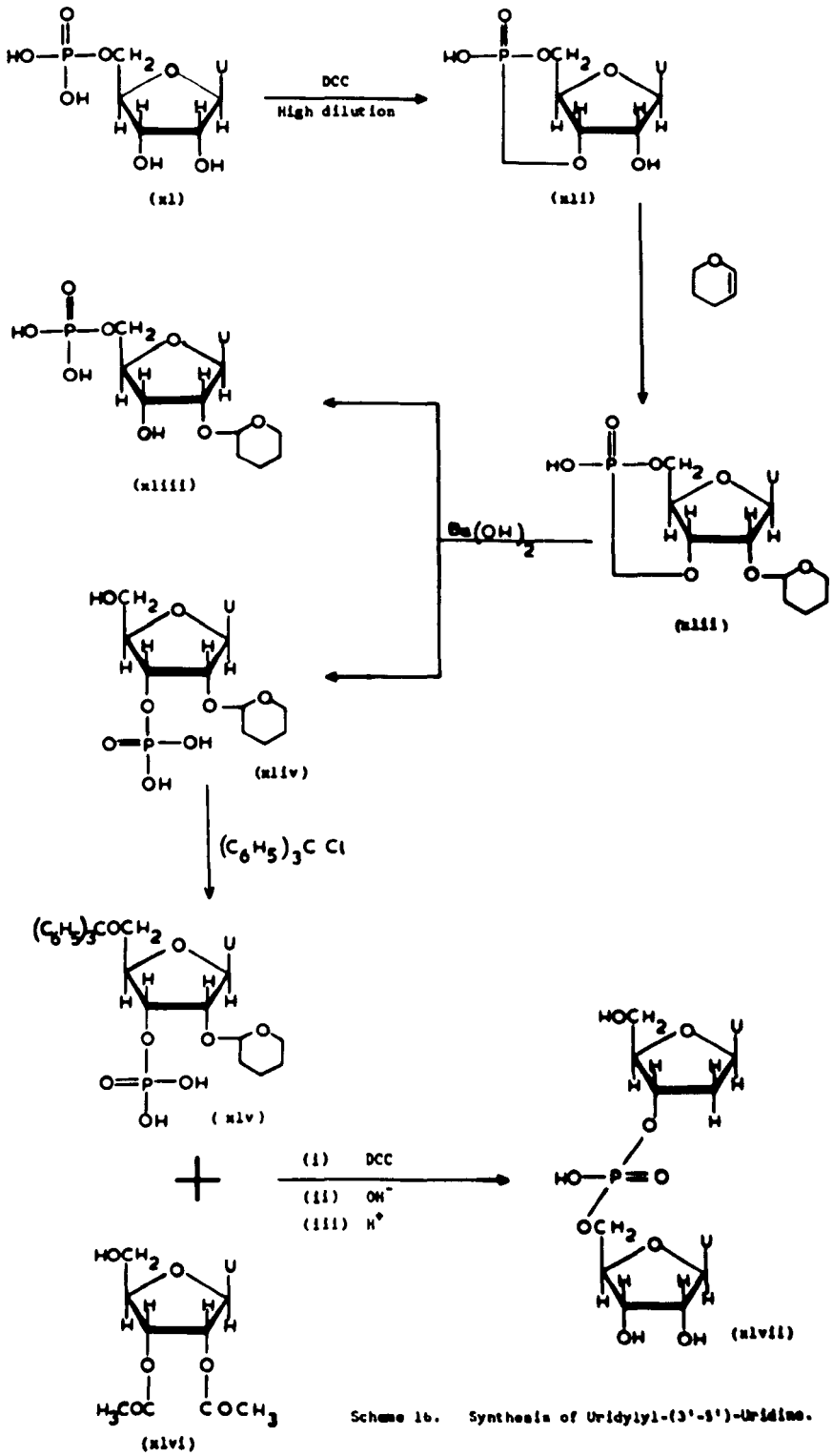
A systematic investigation of both approaches has led to the conclusion that the second approach is unsuitable in the polyribonucleotide field[192,193] because of the tendency of the acyl group (the normal protective group) to migrate from the $O^{2'}$- to the $O^{3'}$- positions in ribonucleotides.[192] Thus $N^6,O^{3'},O^{5'}$-tribenzoylcytidine (prepared by controlled debenzylation of $N^6,O^{2'},O^{3'},O^{5'}$-tetrabenzoylcytidine), after phosphorylation with 2-cyanoethyl phosphate and DCC followed by the removal of protective groups, gave pure cytidine-2' phosphate. In contrast, $N^6,O^{2'},O^{5'}$-tribenzoylcytidine gave, after similar treatments, a mixture of cytidine-3' and cytidine-2' phosphates.

These results, namely the production of the two isomeric nucleotides as a result of the phosphorylation of $N^6,O^{2'},O^{5'}$-tribenzoylcytidine and the formation of cytidine-2' phosphate alone by phosphorylation of $N^6,O^{3'},O^{5'}$ tribenzoylcytidine, indicate that there is a tendency for an acyl group to migrate from the $C_{2'}$-hydroxyl to $C_{3'}$-hydroxyl group in the ribonucleosides. Apparently, this migration may be acid or base catalysed. Thus during the partial debenzoylation of $N^6,O^{2'},O^{3'},O^{5'}$-tetrabenzoylcytidine the only tribenzoylcytidine obtained was the $N^6,O^{3'},O^{5'}$-tribenzoylcytidine isomer whereas the rates of debenzoylation of the groups on the $C_{2'}$- and $C_{3'}$-hydroxyl groups would be expected to be not dissimilar. It is believed that under the basic conditions used rapid migration

of the 2'-O-benzoyl group to the $C_{3'}$-hydroxyl group occurred. This interpretation also agrees with the experimental results of Todd and his co-workers[194] who recorded the formation in good yield of 3',5'-di-O-acetyladenosine by fusion of an equimolar mixture of 5'-O-acetyladenosine and 2',3',5'-tri-O-acetyladenosine. None of the 2',5'-di-O-acetyladenosine was detected and it would appear that, presumably, the basic catalysis provided by glass resulted in the migration of the 2'-O-acetyl group to form 3',5'-di-O-acetyladenosine.

From the standpoint of the synthesis, of the $C_{3'}$-$C_{5'}$ internucleotidic linkage, the acyl group on the $C_{2'}$-hydroxyl group and a free $C_{3'}$-hydroxyl group was thus found to be unsuitable. The only possibility deserving of further investigation was that of using the easily accessible 3',5'-di-O-acetylribonucleosides as the starting materials and introducing on the $C_{2'}$-hydroxyl group a protecting group, such as the tetrahydropyranyl group, which would not migrate to the adjoining position. Subsequent deacetylation would then give the potentially useful 2'-O-substituted ribonucleoside.

Following these experiments, Khorana and his co-workers[172,195] developed the first approach for the synthesis of a $C_{3'}$-$C_{5'}$ internucleotidic linkage in the polyribonucleotide field. Thus they synthesised,for example, uridylyl-(3'-5')-uridine and uridylyl-(3'-5')-adenosine. (See Scheme 16). Uridine-5'-phosphate (xl)



Scheme 16. Synthesis of Uridylyl-(3'-5')-Uridine.

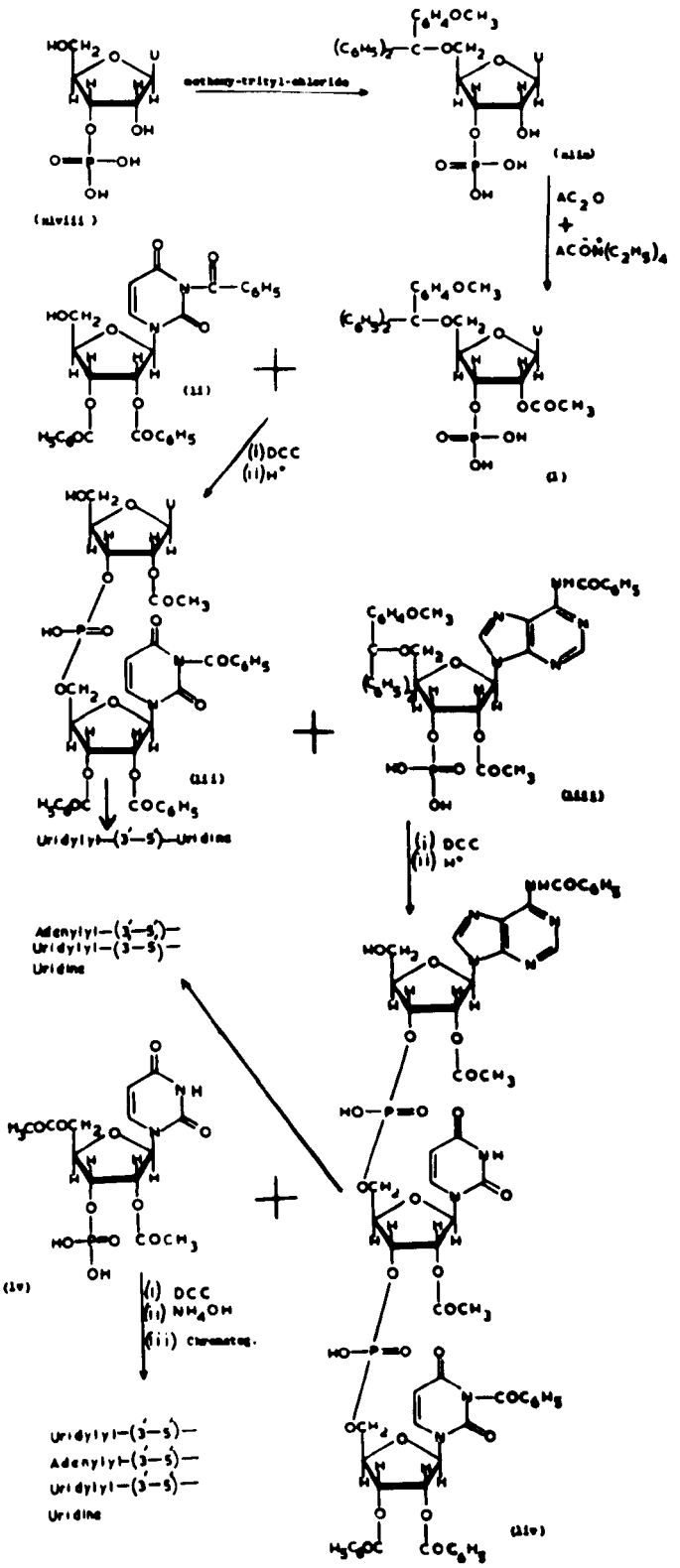
was converted in high yield to uridine-(3'-5') cyclic phosphate (xli) by reaction with DCC under high-dilution conditions. Reaction of (xli) as the free acid with dihydropyran gave the 2'-O-tetrahydropyranyl derivative (xlii) quantitatively. The cyclic phosphate ring was then opened by barium hydroxide-catalysed hydrolysis, and the resulting mixture (xliii) and (xliv) containing the two substances in the ratio 1 : 5 respectively, was treated with triphenylmethyl chloride in pyridine. Only (xliv) reacted, and its trityl derivative (xlv) was separated by partition chromatography. Subsequent condensation with 2',3'-O-diacetyluridine (xlvi), followed by mild alkaline and acidic treatments, yielded uridylyl-(3'-5')-uridine (xlvii).

The stepwise synthesis of the next higher nucleotide requires the selective removal of trityl group from (xlvii). The acid-catalysed selective removal of this group, however, without affecting the acid labile tetrahydropyranyl group did not prove possible. However, during their work they observed that the rate of removal of the tetrahydropyranyl group varied considerably in mononucleotide derivatives.

For example, the tetrahydropyranyl group in 2'-O-tetrahydropyranyluridine-3' phosphate was twice as labile as the corresponding group in the 2'-O-tetrahydropyranyluridine-5' phosphate,[195] and this group was consistently more stable in the products containing the

phosphodiester linkage than in the mononucleotides.[196] Furthermore, the acidic conditions necessary for the complete removal of the tetrahydropyranyl group from the synthesised dinucleotides caused detectable isomerisation of the inter-ribonucleotidic linkage ($C_{3'}—C_{5'} \rightleftharpoons C_{2'}—C_{5'}$) and, therefore, caution was necessary in the duration of the acidic treatment. No such isomerisation was, however, detectable under alkaline conditions. Thus the lack of migration in the inter-ribonucleotidic linkage on ammonical and sodium hydroxide treatment was confirmed. These results led Khorana et al.[196] to search for a new protective group which should be alkali labile. 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',N,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine gave UpA in 84% yield after removal of protecting groups.

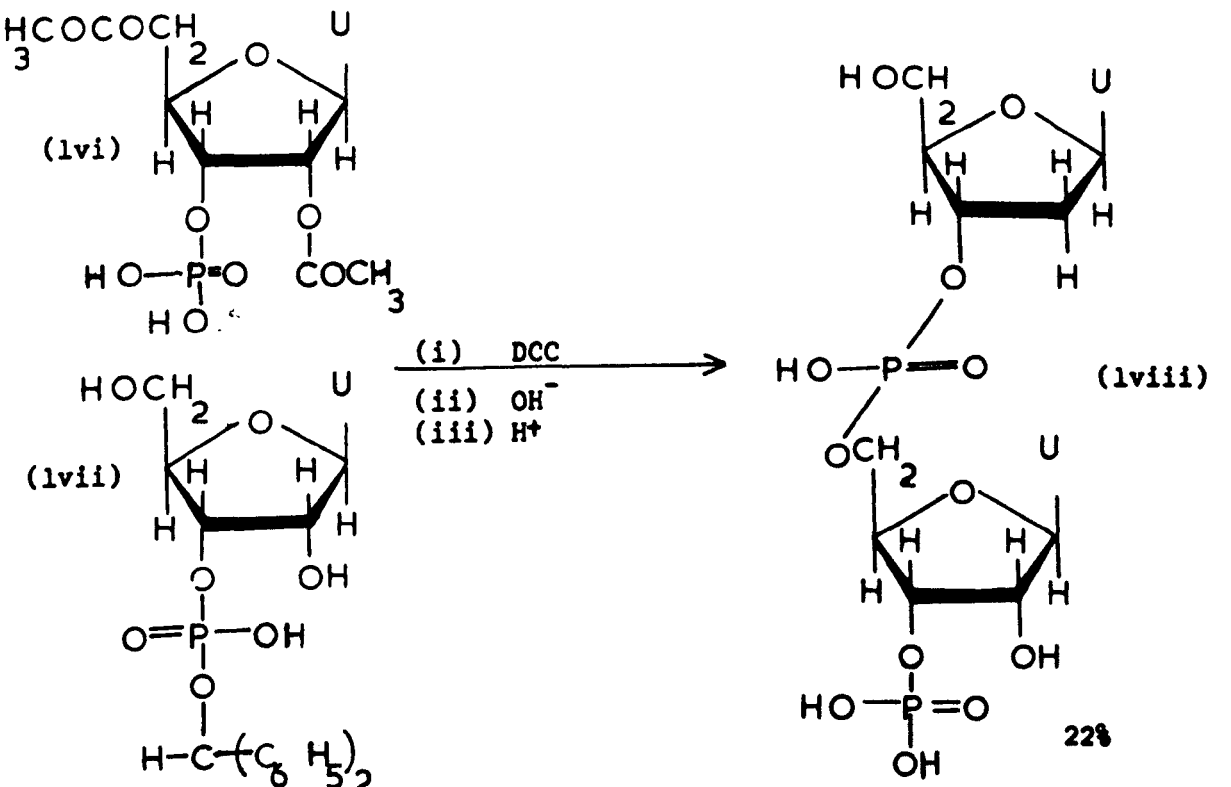
Thus the observation that ribonucleoside-3' phosphates could be directly reacted with trityl or methoxytrityl halides to form the 5'-O-tritylribonucleoside-3' phosphates and the crucial finding that the 2'-OH group in these products could be quantitatively acetylated, provided carefully defined conditions were used, played a very important role in the practical development of the polyribonucleotide field. Thus the tetranucleotide uridylyl-(3'-5')-



Scheme 17 Synthesis of UpApUpU

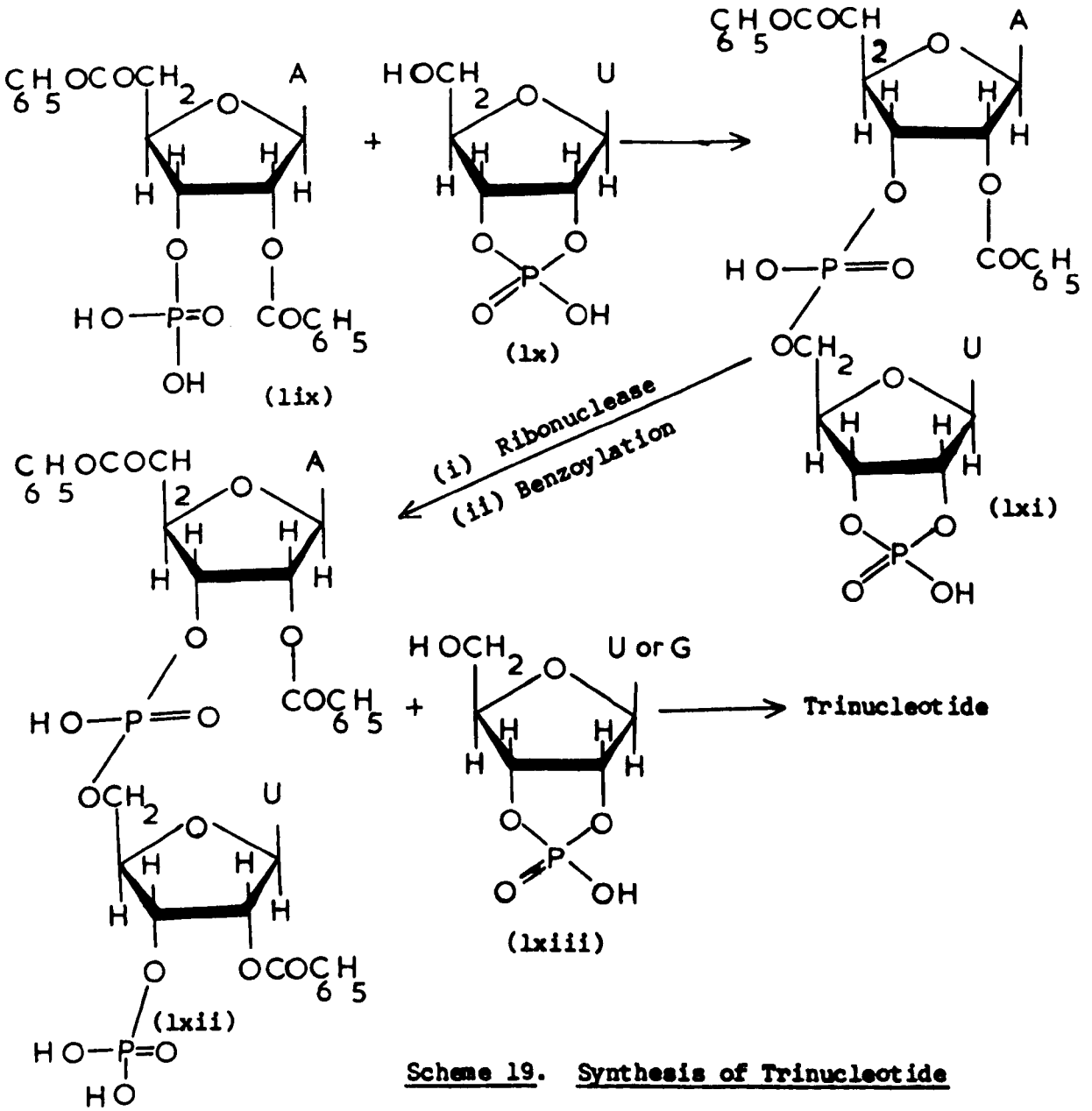
-adenylyl-(3'-5')-uridylyl-(3'-5')-uridine was synthesised[193] as shown in (Scheme 17).

Using the benzhydryl ester method, Cramer et al.[197,198] were able to synthesise dinucleoside phosphates of the type UpUp, UpAp, ApUp, ApAp, CpAp, and CpUp. The benzhydryl group can be removed with acid. The yields were poor ranging from 29 to 11%. This is exemplified in Scheme 18. (Synthesis of UpUp).



Scheme 18

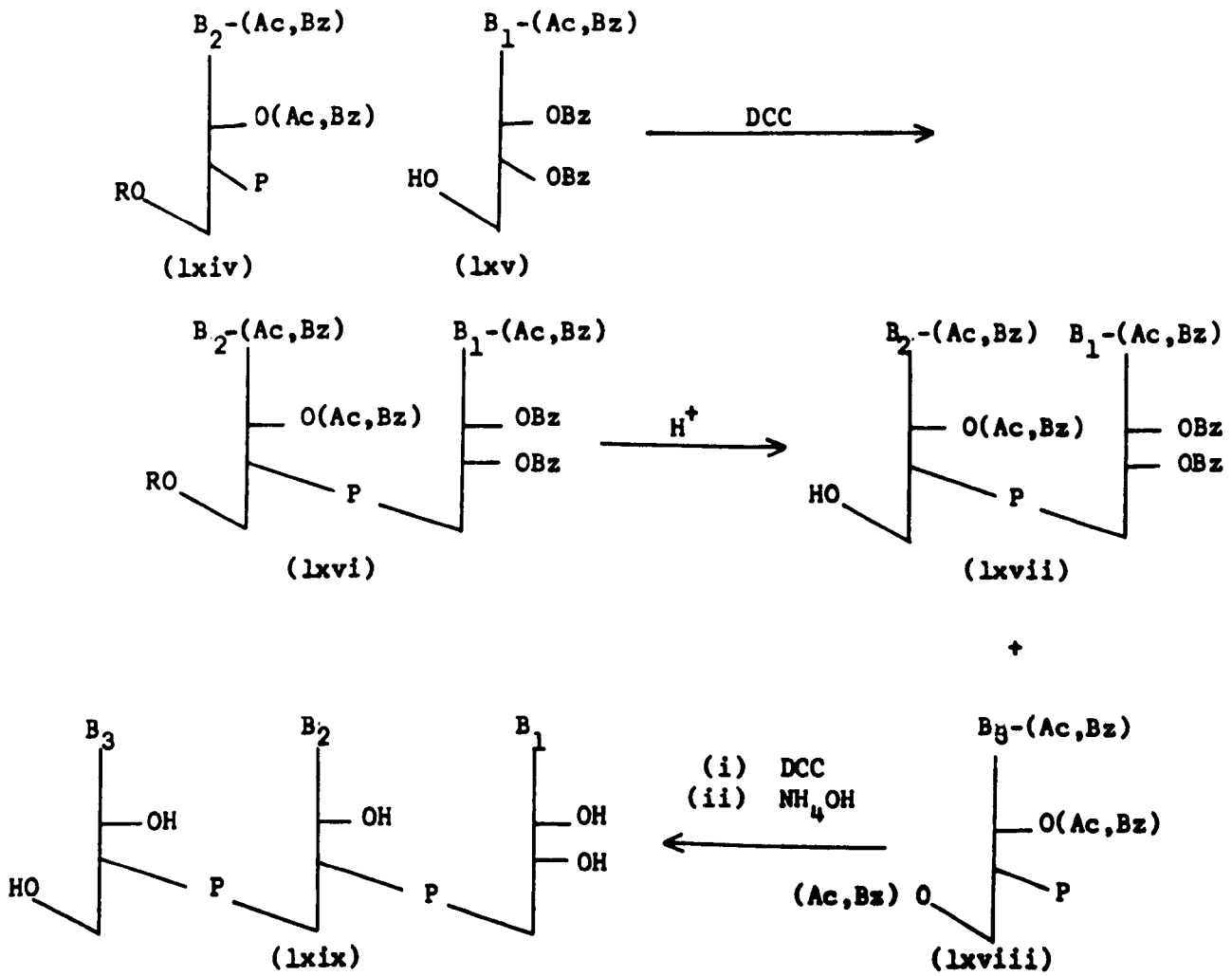
Another possibility is to use the cyclic phosphate as protecting units[199] (Scheme 19). This method can be used to give trinucleotides with a 3'-terminal phosphate in good yield.



Scheme 19. Synthesis of Trinucleotide

Using these techniques, Khorana has developed satisfactory methods for the preparation of three sets of compounds; (i) a set of the four protected ribonucleosides with free 5'-OH groups, (ii) a set of the four protected (5'-O-methoxytrityl,N,2'-O-acetyl) ribonucleoside-3'-phosphates; and (iii) a set of the four protected (N,2',5'-O-acyl) ribonucleoside-3' phosphates. Using these protected

derivatives, unambiguous syntheses of all the possible ribonucleotides derivable from the four common mononucleotides have recently been realised.[190,191] Khorana[189] generalises his synthetic approach as shown in Scheme 20.

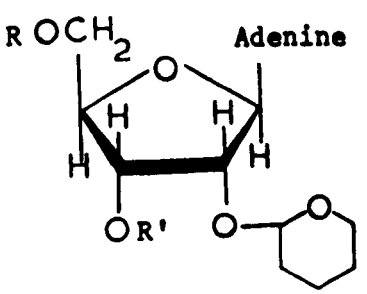


B_1, B_2, B_3 = purines or pyrimidines
R = trity, methoxytrityl
Ac = acetyl; Bz = benzyl

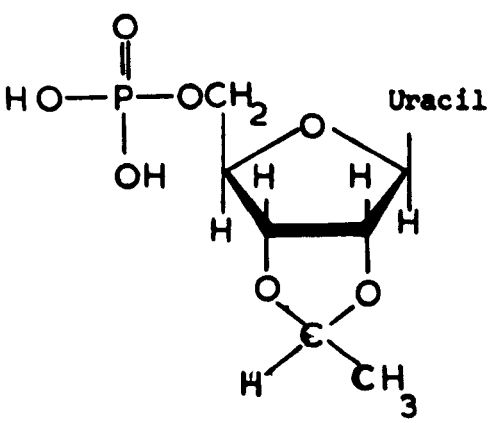
Generalised scheme for the synthesis of oligoribonucleotides

Scheme 20

It should be mentioned that in Reese's laboratory in Cambridge, the second approach continues to be investigated and some success has been achieved in the synthesis of oligoribonucleotides, for example, when 2'-O-tetrahydro-5'-O-pivalyl-adenosine (lxx; 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 methoxymethylidene group was found to be suitable (lxxi). Condensation of such a protected 3'-nucleotide did, in fact, produce uridylyl-(3'-5')-adenylyl-(3'-5')-uridine in 30% yield after removal of protecting groups.[200]



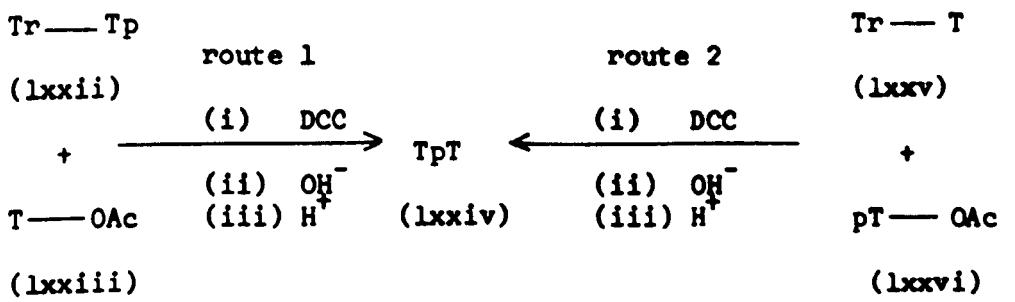
(lxx)



(lxxi)

(2) Stepwise Synthesis of Polydeoxyribonucleotides.

The stepwise synthesis of polydeoxyribonucleotides has been carried out by both approaches described above. For example, (Scheme 21) thymidylyl-(3'-5')-thymidine (lxxiv) can be prepared

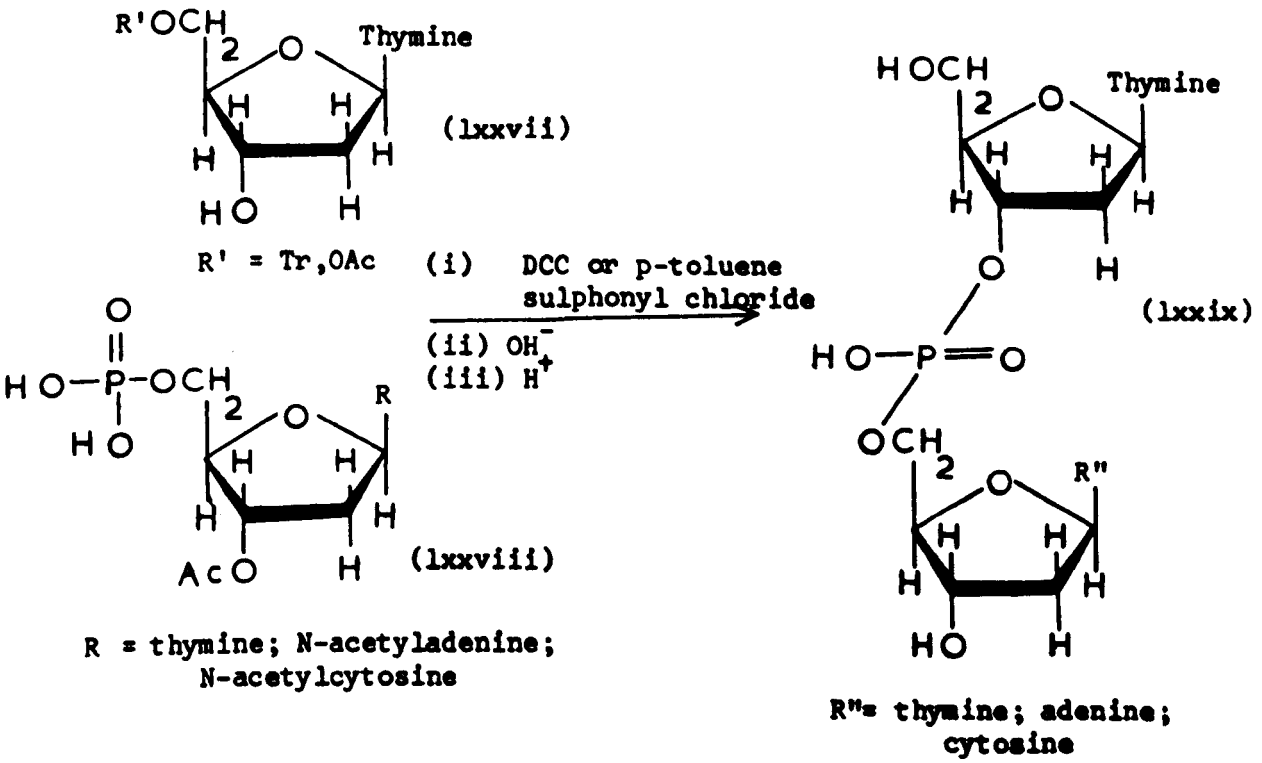


Scheme 21

either by condensing 5'-O-tritylthymidine-3'-phosphate (lxxii) with 3'-O-acetylthymidine (lxxiii), route 1, or by the condensation of 5'-O-tritylthymidine (lxxv) and 3'-O-acetylthymidine-5'-phosphate (lxxvi), route 2.

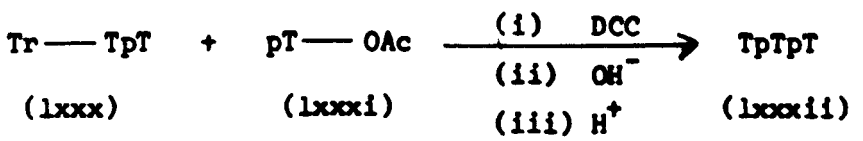
A systematic study by Khorana and his co-workers[189] has shown that the (route 2) approach which involves the condensation of a 5'-phosphoryl group of nucleotide with the 3'-hydroxyl group of a second component (nucleoside) is more suitable. This is rationalised in terms of (1) the lack of complete stability of the compounds bearing a 5'-hydroxyl group, for example, cyanoethyl thymidine-3' phosphate under the conditions of the reaction to prepare the phosphodiester linkage, (2) the ready availability of deoxyribonucleoside-5' phosphates compared with the relative inaccessibility of the

-3' phosphates, (3) the lower yield obtained when bulky substituents were present in the nucleotide component,[126] as, for example, in the condensation of 5'-O-tritylthymidine-3' phosphate with 3'-O-acetylthymidine which gave poor yield compared with that obtained by the condensation of 3'-O-acetylthymidine-5'-phosphate with 5'-O-tritylthymidine, and (4) the fact that a component bearing a 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-acetylthymidine reacted with DCC in presence of pyridine giving by-products.[201] Thus using the second approach Khorana et al.[180] synthesised TpT, TpA, TpC in good yield as shown in (Scheme 22).



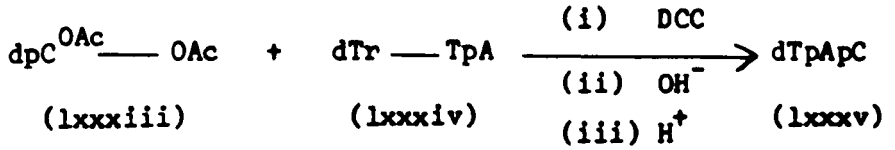
Scheme 22

The synthesis of larger oligonucleotides would entail the formation of a phosphodiester linkage between a molecule bearing a phosphomoester group and a second fragment bearing the appropriate hydroxyl group, one or both of these molecules containing preformed diester bonds. Thus when 5'-O-tritylthymidylyl-(3'-5')-thymidine (lxxx) was reacted with an excess of DCC in pyridine for one hour and then with two molar equivalents of 3'-O-acetylthymidylic-(5') acid (lxxxi) for two days at room temperature, the product obtained after successive alkaline treatment and catalytic hydrogenolysis, was found to be TpTpT[202] (lxxxii, Scheme 23) which was chromatographically identical with the product obtained by the dephosphorylation by prostatic phosphomonoesterase of TpTpTp.[102]



Scheme 23

In a similar fashion, mixed oligonucleotides have been synthesised. Thus the N,O$^{3'}$-diacetyl deoxycytidylic-(5') acid (lxxxiii) was treated with 5'-O-tritylthymidylyl-(3'-5')-deoxyadenosine (lxxxiv) and the product afforded, after successive alkaline and acidic treatments, thymidylyl-(3'-5')-deoxyadenylyl-(3'-5') deoxycytidine (lxxxv) in 31% overall yield. (Scheme 24).



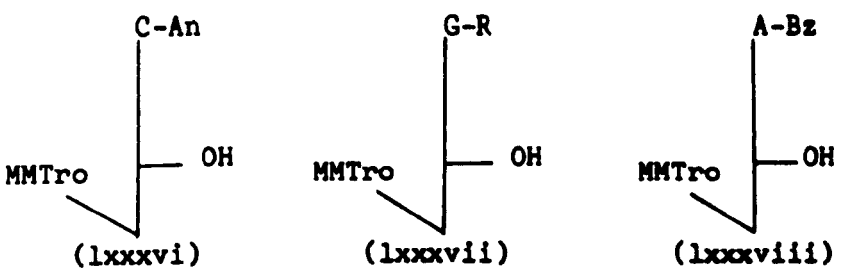
Scheme 24

During their studies on the stepwise synthesis of polydeoxyribonucleotides, Khorana and his co-workers observed that the protection of the 5'-hydroxyl group by an unmodified trityl group was quite satisfactory as long as pyrimidine nucleosides were used in oligonucleotides. If purines were used, the glycosyl bonds in the purine deoxyribonucleotides were too sensitive for the prolonged acetic acid treatment required for the removal of the trityl group. For this reason, a new group similar to the trityl group for the protection of primary alcohlic function was sought. They observed that if the trityl group was mono-, or di-methoxy substituted at the p-position of the phenyl rings then the modified trityl group was more labile to acid. The acid lability of the trityl group was increased in the order trityl group <monomethoxyl trityl <di-methoxytrityl. Thus mono- and di-p,p',-methoxytrityl groups for the protection of 5'-hydroxyl groups were selected for subsequent syntheses.[203]

It was also found that the protection of the amino groups on purines or pyrimidines, was necessary because (a) the activated phosphates reacted with amino groups; for example, when thymidine-5' phosphate was condensed with 5'-O-tritydeoxycytidine, the major

product, phosphoramidate, was obtained.[204] Similarly the amino groups in adenine and guanine were found to possess a similar character although the reactivity of the amino functions varied a great deal, (b) if the amino groups were unprotected then the nucleosides and nucleotides were highly insoluble under anhydrous conditions. Therefore, a new class of protecting groups for the amino functions was required to overcome these problems. The protected derivatives used currently for different deoxynucleoside and deoxynucleotides are shown in Figure 8. (See also Table 3)[189]

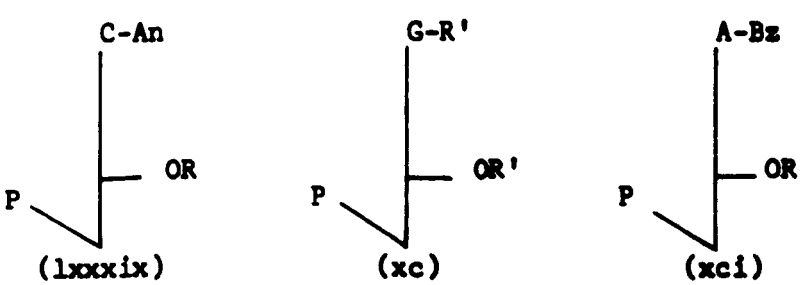
Deoxynucleoside.



(lxxxvi) (lxxxvii) (lxxxviii)

An = anisoyl, R = isobutyryl, Bz = benzoyl,
MMTr = monomethoxytrityl or acetyl.

Deoxynucleotides.

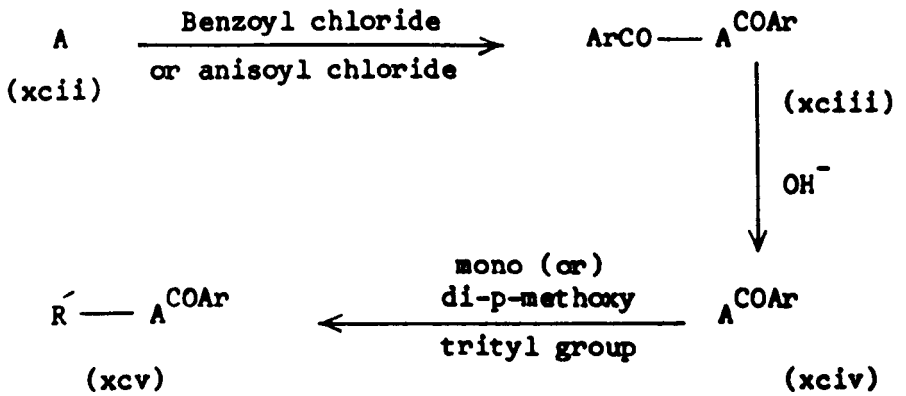


(lxxxix) (xc) (xci)

R = H or OAc, R' = isobutryl or acetyl

Figure 8: Protected derivatives of deoxyribonucleosides and deoxyribonucleotides.

The general method currently available to synthesise deoxyribonucleosides with blocked amino and 5'-hydroxyl groups is exemplified by adenosine shown in Scheme 25.



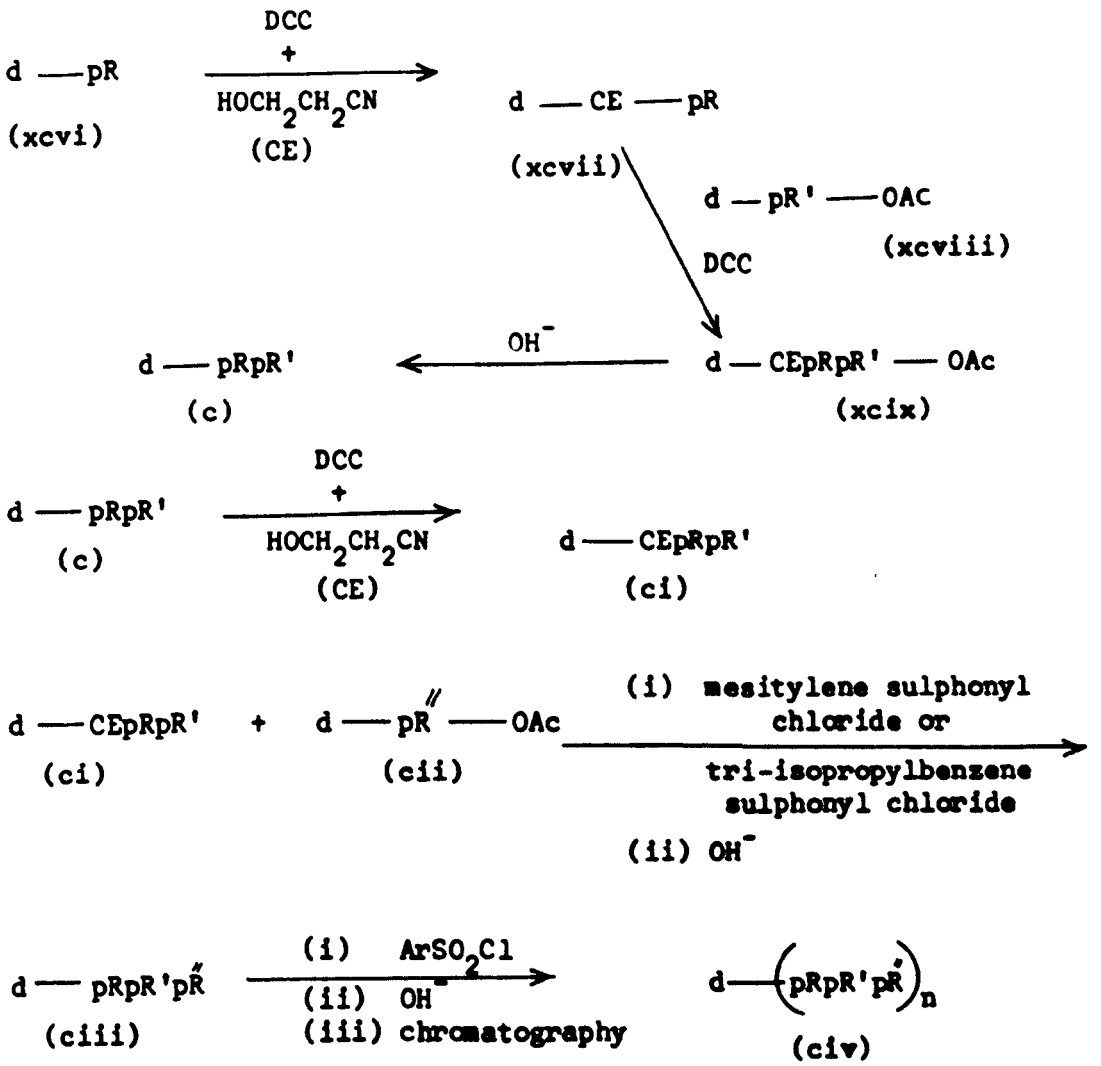
Ar = phenyl or p-methoxyphenyl

R' = mono (or) di-p-methoxytrityl

Scheme 25. General method for the preparation of protected deoxyribonucleosides.

In this way, all the deoxyribonucleosides and nucleotides can be prepared in the N-protected form. The succeeding step to protect the 5'-hydroxyl function is a standard one.

One other protecting group which has also been used for the protection of 5' phosphomonoester group is cyanoethyl group (CE) which is readily lost by mildly alkaline treatment.[188] The general method for the synthesis of dinucleotides (c), trinucleotides (ciii), and polynucleotides (civ) of this type is shown in Scheme 26.



R,R',R'' = thymine, or N-acetyl (or N-isobutyryl) guanine, or N-benzoyladenine, or N-anisoylcytosine.

$ArSO_2Cl$ = mesitylene or tri-isopropyl benzene sulphonyl chloride.

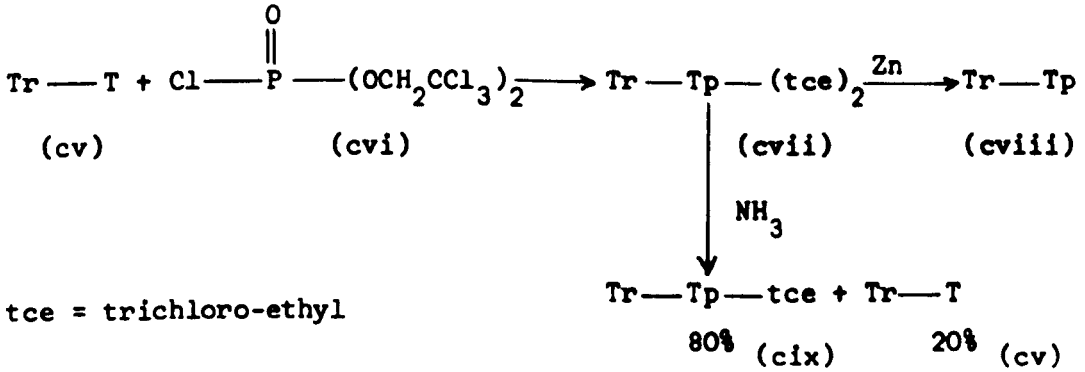
Scheme 26

By treatment of a deoxyribonucleoside-5' phosphate (xcvi) with acrylonitrile in the presence of dicyclohexylcarbodiimide, the cyanoethyl protected phosphoryl derivative (xcvii) can be obtained. The condensation of (xcvii) with 3'-O-acetyldeoxyribonucleoside-5' phosphate (xcviii) in the presence of DCC followed by alkaline treatment gives the dinucleotide (c).

For the synthesis of trinucleotide with a 5'-end group the dinucleotide as prepared above, may be converted quantitatively to the cyanoethyl derivative (ci) again and this may then be condensed with 3'-O-acetyl-mononucleotide (cii). Alkaline treatment of the product will give the trinucleotide (ciii). Repeating the same procedure higher members (civ) can be synthesised.

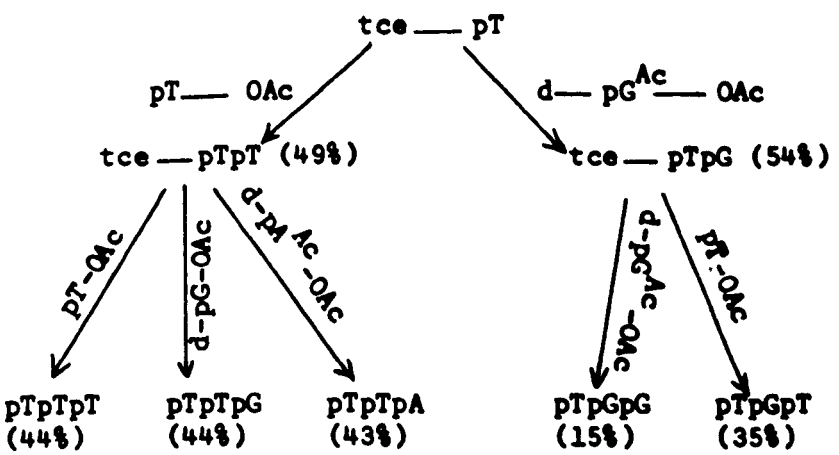
Using this method all the different sets of di-, tri-, and tetranucleotides have been prepared in a good yield.[188]

More recently an alternative group, the 2,2,2-trichloroethyl group, originally introduced by Woodward and his co-workers,[205] has been investigated by Eckstein[206] for work in the nucleotide field. The trichloroethyl group can be removed by reduction with zinc or copper-zinc.[207] These esters can be prepared as shown in Scheme 27.[198]



Scheme 27: Synthesis of trichloro-ethyl esters of nucleotides

When 5'-O-tritylthymidine (cv) was allowed to react with di (trichloroethyl)-phosphorochloridate (cvi), 5'-O-tritylthymidine-di (trichloroethyl)-3' phosphate (cvii) was obtained. The latter, if reduced with zinc, gave 5'-O-tritylthymidine-3' phosphate (cviii), whilst treatment with ammonia yielded 5'-O-tritylthymidine-trichloro-ethyl-3' phosphate in 80% yield (cix) and 5'-O-tritylthymidine (cv) in 20% yield. Using these esters Cramer et al.[198] have synthesised the trinucleotides shown in Scheme 28.



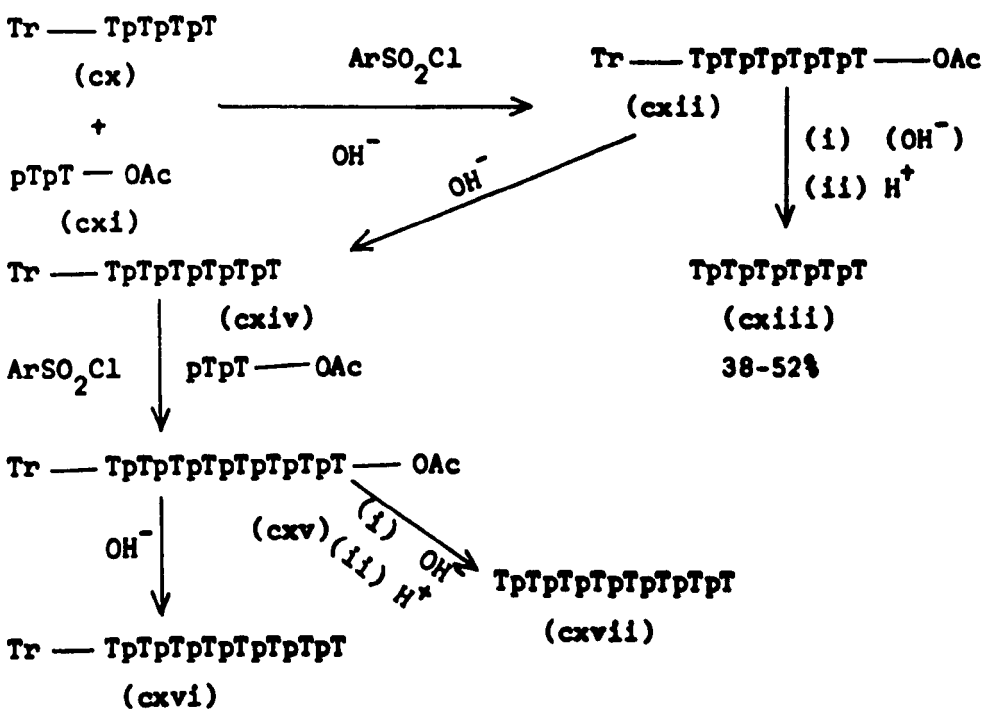
tce = trichloroethyl, AC = OAc = acetyl

Scheme 28

(ii) Block Condensation

Although a polynucleotide chain can be built by successive addition of mononucleotide units to the 3'-hydroxyl end of a growing oligonucleotide chain as has been demonstrated above, the alternative approach, which theoretically is more attractive, involves the use of preformed di- and higher oligonucleotide blocks in repetitive condensation steps.[208]

Using this technique Khorana et al.[209] prepared hexa- and octanucleotides of thymidine. The steps involved are shown in Scheme 29.



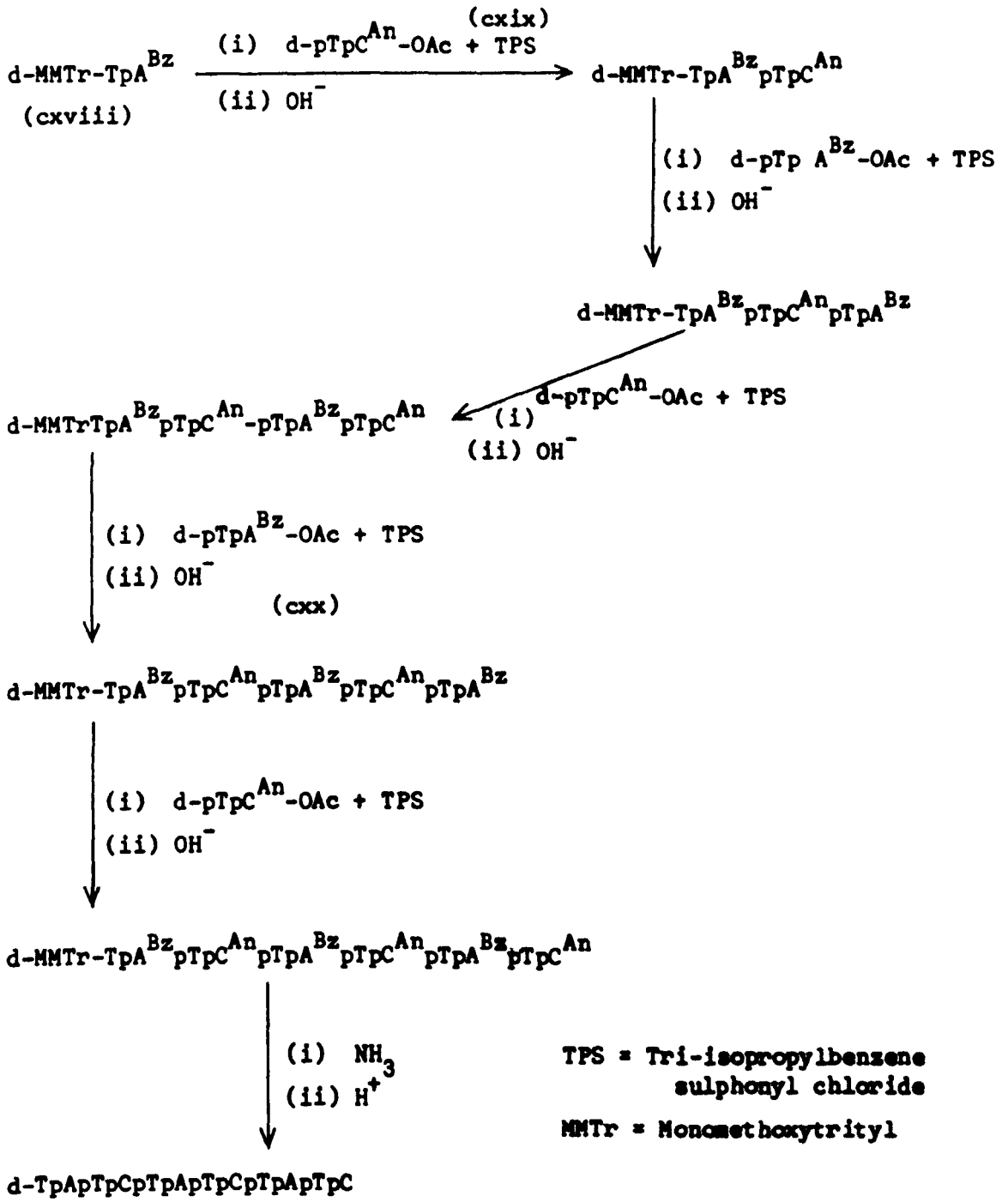
$ArSO_2Cl$ = mesityl or tri isopropyl benzene sulphonyl chloride

Scheme 29

When 5'-O-trityl [thymidylyl-(3'-5')]$_3$-thymidine (cx) was condensed with 2 equivalents of 3'-O-acetylthymidylyl-(3'-5')-thymidylyl-(3'-5')-thymidine-5'-phosphate (cxi) using mesitylene sulphonyl chloride as a condensing reagent, followed by the removal of protective groups, the hexanucleotide, [thymidylyl-(3'-5')]$_5$ thymidine (cxiii) was obtained in 52% yield. In a similar way when the hexanucleotide (cxiv) was condensed with the dinucleotide (cxi), the product after the removal of protecting groups was the octanucleotide (cxvii), [thymidylyl-(3'-5')]$_7$-thymidine in 38% yield.

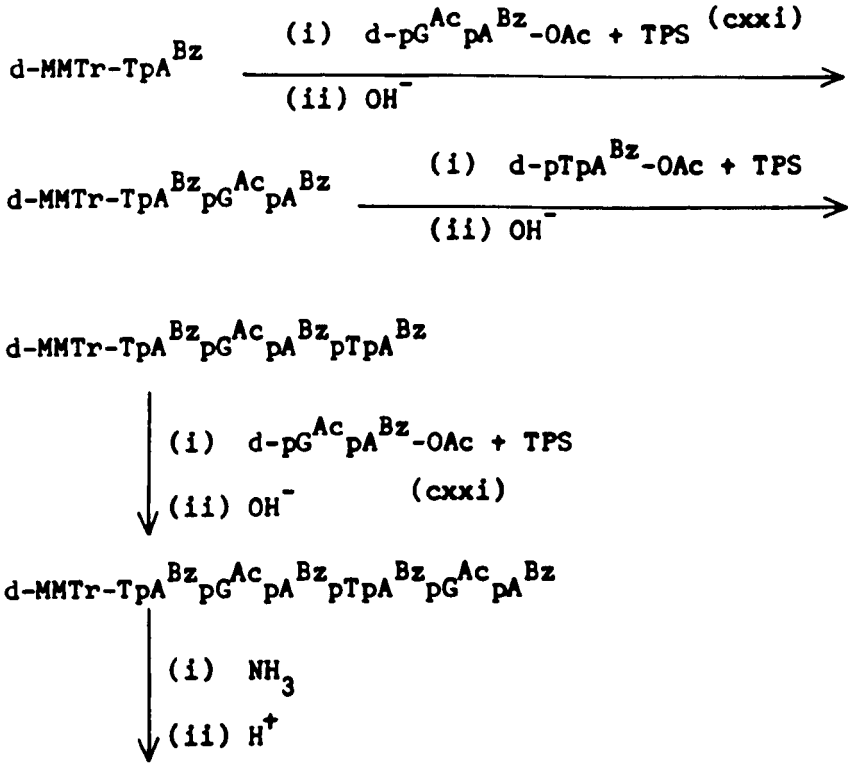
Kössel, Büchi and Khorana[210] extended this work by preparing (1) a dodecanucleotide containing the repeating sequence thymidylyl-deoxyadenylylthymidylyldeoxycytidylyl (Scheme 30), (2) an octanucleotide containing the repeating sequence thymidylyldeoxyadenylyl-deoxyguanylyldeoxyadenylyl (Scheme 31) and (3) another octanucleotide containing the repeating sequence thymidylyldeoxyadenylyldeoxyadenylyl-deoxyguanylyl (Scheme 32).

The general procedure used involved the stepwise condensation of preformed protected dinucleotides with the 3'-hydroxyl end of the growing deoxyoligonucleotide chains. The starting blocks containing 3'-hydroxyl end groups were 5'-O-monomethoxytrityl-thymidylyl-(3'-5') N-benzoyldeoxyadenosine (cxviii) or 5'-O-dimethoxytritylthymidylyl-(3'-5') N-benzoyldeoxyadenosine (cxxii). The protected dinucleotide blocks containing 5'-phosphomonoester groups were:



Synthesis of Dodecanucleotide

Scheme 30

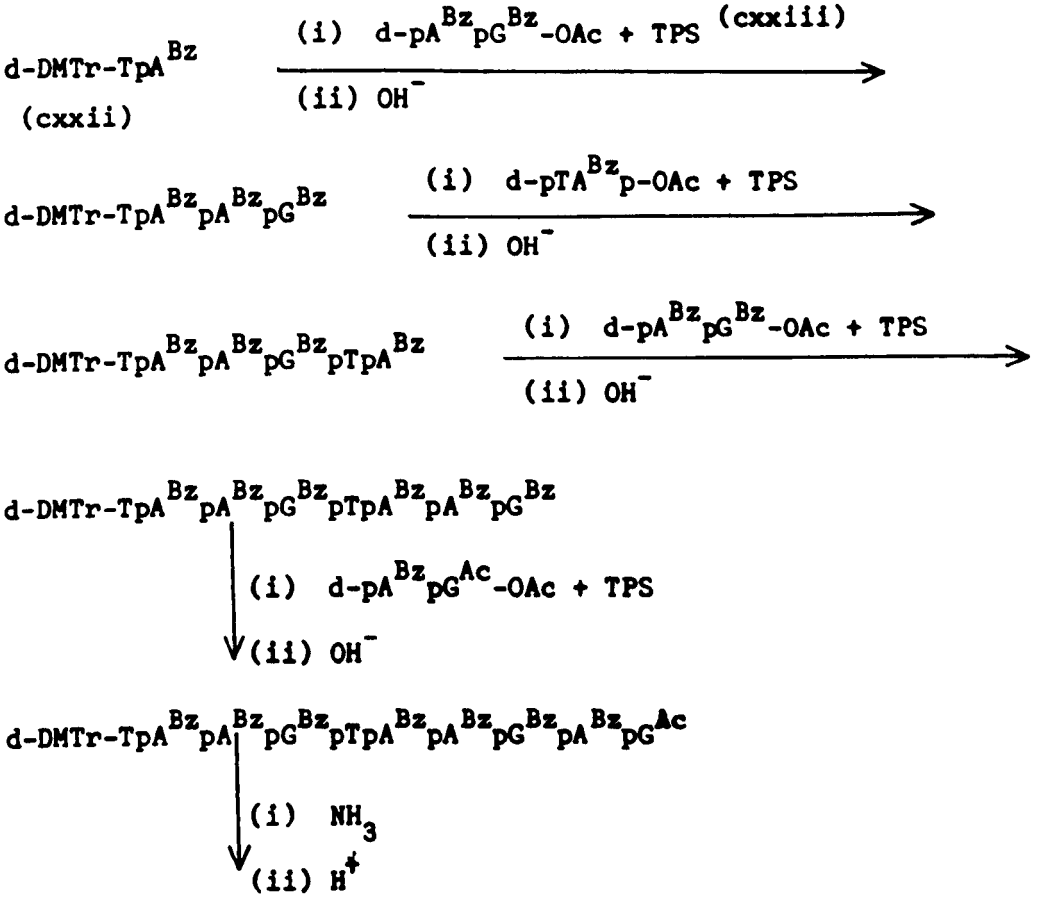


TPS = Tri-isopropylbenzene sulphonyl chloride

MMTr = Monomethoxytrityl

Scheme 31.

Synthesis of Octanucleotide d-TpApGpApTpApGpA



DMTr = dimethoxytrityl

TPS = tri-isopropylbenzene sulphonyl chloride

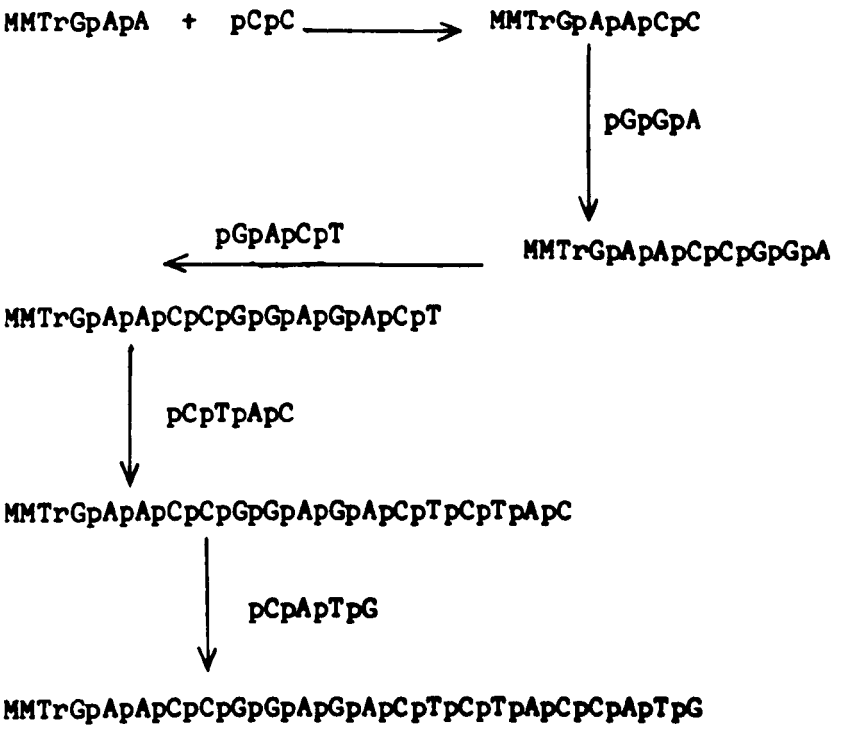
Scheme 32.

Synthesis of Octanucleotide d-TpApApGpTpApApG

5'-O-phosphorylthymidylyl-(3'-5') N-anisoyl-3'-O-acetyldeoxycytidine (cxix), 5'-O-phosphorylthymidylyl-(3'-5') N-benzoyl-3'-O-acetyldeoxyadenosine (cxx), 5'-O-phosphoryl-N-acetyldeoxyguanylyl-(3'-5') N benzoyl-3'-O-acetyldeoxyadenosine (cxxi), and 5'-O-phosphoryl-N-benzoyldeoxyadenylyl-(3'-5') N-benzoyl-3'-O-acetyldeoxyguanosine (cxxiii).

With each increase in the chain length of the deoxyribopolynucleotides, an increasing excess of the protected dinucleotide was used and the condensations were carried out in the presence of 2,4,6-triisopropyl-benzene sulphonyl chloride. After each condensation, the terminal 3'-O-acetyl group was selectively removed from the protected oligo- or polynucleotides by mild alkaline treatment, and the products were purified by chromatography on DEAE-cellulose anion-exchange columns. In a similar way polynucleotides with repeating dinucleotide,[211] trinucleotide,[188,212] and tetranucleotide[210,213] sequences have been prepared.

Very recently the method just described (block condensation of an appropriately protected oligonucleotide with a free 3'-hydroxyl group and 5'-phosphates of tri- and tetra-nucleotides) have led to the synthesis of the eicosadeoxynucleotide[214] as shown in Scheme 33.



Synthesis of eicosadeoxyribonucleotide

Scheme 33

Khorana considers this to be the maximum chain length that current methods of chemical synthesis and purification allow for deoxyribonucleotides.[215]

(iii) Random Polymerisation.

As described above, a random polymerisation is one in which mixtures of mono-, di-, or trinucleotides (depending on the system) are used as a starting materials leading to a whole range of products which vary in their degree of polymerisation. Random polymerisation has been achieved; (a) either with a primer (a pre-existing polymer) or without a primer, and (b) either enzymatically or by purely chemical means.

Polyribonucleotides.

(1) Chemical synthesis of polyribonucleotides.

Since the use of unprotected nucleotides may produce C_3'-C_5', C_2'-C_5' and C_5'-C_5' internucleotidic linkages, here again the use of protecting groups play an important role in obtaining the desired internucleotidic linkages in a polymer. Thus using protected nucleotides, all types of polyribonucleotides (homopolymer, complex polymer and even "ribonucleic acid") have been prepared. This subject has been extensively reviewed by Michelson,[69,216] Banks,[217] Steiner and Beers,[4] and Cramer.[198] Here, only the recent examples of primed chemical synthesis of polyribonucleotides in aqueous medium are described.

Complementary polynucleotides are known to react to form a variety of helical structures and in some cases oligonucleotides have been shown to be capable of undergoing base-pair interactions.[218]

Howard et al.[219] showed that a mononucleotide, guanosine-5'-phosphate, is capable of forming both two- and three-stranded helices with poly C. Their later investigations showed that the phenomenon of helix formation by complementary monomer polymer pairs is a general one when the monomeric component is a purine.[220] Thus a number of monomeric nucleic acid components (purine, purine nucleoside, or purine nucleotide; but no pyrimidine derivative) interacted with polynucleotides to form well defined complexes having helical structures similar to those formed between corresponding pairs of high polymers. They demonstrated that 2-aminoadenosine and adenosine form regular ordered structures with poly U or poly UC. The extent of the interaction was dependent upon temperature and upon the concentration of the reactants. Gilham et al.[221] used water-soluble carbodiimides as reagents for the activation of the terminal phosphate group of oligonucleotides in aqueous solution. In model experiments these reagents were shown to be capable of effecting the rapid cyclisation of nucleoside 2'(3')-phosphates at pH6 and to be capable of converting adenosine-5'-phosphate to its ethyl ester and to adenylyl-5'-5'-adenosine on reaction with the appropriate hydroxylic component.

These preliminary results provided a basis for further understanding of how life may have evolved.[222] The study of the biological role (described earlier) has shown that in contemporary

life forms information is carried from one generation to the next by nucleic acids - DNA and RNA - and by proteins; DNA is usually the repository of genetic information. It is probable that, when natural selection began to operate, either DNA or RNA molecules were present. It is also possible that these molecules arose in the first place by spontaneous chemical synthesis.

To investigate the conditions which might be required for spontaneous nucleic acid synthesis, Sulston et al.[223] have attempted to build a polyadenylic acid molecule using a template of poly uridylic acid; the complex of poly A and poly U is, in fact, a triple helix of two poly U chains and one poly A chain as has been described.. Sulston et al. used conditions under which two poly U chains and 5'-adenylic acid could form a triple helix in which the adenylic acid monomers were simply held in place by the two poly U chains. Under similar conditions a double poly U: adenosine helix can also be formed. Thus they carried out the polymerisation using equimolar amounts of adenosine and adenosine-5'-phosphate as substrate, water soluble carbodiimide, 1-ethyl-3 (3-dimethylamino-propyl)-carbodiimide hydrochloride, as a condensing reagent and poly uridylic acid as a primer. The reaction was followed by paper chromatography and the products were shown to be polymers. Their control experiments lacked poly U. They found that the poly U does indeed "catalyse" the formation of oligoadenylic acids; the presence

of poly U accelerated the rate of formation of oligomers by a factor of about ten.

On the basis of their base specificity experiments[224] they found that; (1) the presence of poly U under conditions permitting formation of triple helices with A or pA enhances greatly the yields of dinucleoside monophosphates from pA and A but has little effect on the reaction of pA with other nucleosides, (2) the presence of other nucleosides does not affect the condensation of pA with A on a poly U template, and (3) poly C does not enhance the formation of dinucleoside monophosphate from pA and A, but poly C incorporates only G into oligonucleotides. Thus the Watson-Crick-base pairing rules apply to nonenzymatic syntheses using carbodiimide as condensing agent. This observation has been confirmed during the course of the present work (see experimental discussion).

(2) Enzymatic synthesis of polyribonucleotides.

Polynucleotide phosphorylase was first isolated from the microorganism Azotobacter Vinelandii by Grunberg-Manago, Ortiz and Ochoa in 1955.[225,227] This enzyme catalyses the synthesis of high molecular weight polyribonucleotides from ribonucleoside-di-phosphates in the presence of magnesium ions with the release of inorganic phosphate. The reaction can be represented by the general equation (25) where X = base, PP = pyrophosphate, P = orthophosphate, and R = ribose.

$$nx\text{——}R\text{——}PP \underset{Mg^{++}}{\overset{\text{enzyme}}{\rightleftharpoons}} (X\text{——}R\text{——}P)_n + np \qquad (25)$$

The base may be A, G, C, and U; later it was discovered that thymine riboside-di-phosphate could also be used.

During subsequent investigations Mii and Ochoa[226] observed that polynucleotide phosphorylase catalyses the synthesis not only of RNA from the mixture of 4 naturally occurring ribonucleoside-diphosphates but also polyribonucleotides containing one, two, or three different kinds of nucleotides in their chain from non-naturally occurring ribonucleoside-diphosphates. The nature of the product depends upon the substrate used for the synthesis.[227] The main types of polyribonucleotides which have been prepared with polynucleotide phosphorylase are shown in Table 4.

Table 4.

Substrate	Synthetic polyribonucleotides
ADP	Poly A
GDP	Poly G
UDP	Poly U
CDP	Poly C
IDP	Poly I
Ribothymidine-di-phosphate	Polyribothymidylic acid
ADP + UDP	Poly AU
GDP + CDP	Poly GC
ADP + GDP + CDP + UDP	Poly AGUC

This enzyme has now been isolated from many other sources. Scheit and Gaertner[228] have recently reported the copolymerisation of UDP and 4-thiouridine-5'-diphosphate (S^4UDP) by polynucleotide phosphorylase from micrococcus lysodeikticus. This enzyme did not catalyse the polymerisation of S^4UDP to measurable amounts of poly 4-thiouridylic acid (poly S^4U). But when 4-thiouridine-5'-diphosphate was used as substrate for polynucleotide phosphorylase from E. Coli, poly S^4U was obtained. The formation of a complex of the latter with poly A has also been reported.

Beers[4] observed that polynucleotide phosphorylase extracted from micro-organisms was in fact impure and contained oligonucleotides which served as primers in the synthesis of polyribonucleotides. Thus when the enzyme was used in its highly pure state, a long lag-time phase was observed before the reaction eventually started. Mii and Ochoa[226] showed that this lag phase could be shortened by adding a small amount of certain high molecular weight polynucleotides which appear to act as primers. The length of these primers could be quite short and even a dinucleotide (e.g. ApA) was shown to possess priming ability. It was found that priming by oligonucleotides was not specific; priming by polynucleotides was, however, shown to possess a certain degree of specificity. Some primers, in fact, inhibited polymer synthesis as shown in Table 5.

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	+	-	+	0	+	+
Poly U	-	+	+	0	+	+
Poly C	-	-	+			-
Poly G	0	0	+			
Poly I	-	0	+	+		
Poly AU					+	
Poly AGUC	0	0	+			+

Key: + denotes priming; - denoted inhibition;
0 denotes no effect; blank spaces - no information.

The chain length can be increased by increasing the ratio of substrate to primer. Recently, Crothers and Ludlum[229] have synthesised polyadenylic acid of high molecular weight (about 5×10^6) by increasing the ratio of ADP to primer and using polynucleotide phosphorylase from micrococcus lysodeikticus.

Apart from polynucleotide phosphorylase some other enzymes (e.g. polynucleotide kinase, polynucleotide ligase) have also been used in polyribonucleotide synthesis. Perhaps the best example of

the use of these enzymes is the recent total synthesis of the gene for an alanine t-RNA from yeast by Khorana and his co-workers.[230] In summary, they synthesised 15 single-stranded oligonucleotides (by stepwise synthesis) which were 5 to 20 residues long and had free 3' and 5'-OH termini. The segments destined to comprise the complementary strands of the final duplex had an overlap of some 4 to 5 bases, so that the segments taken together corresponded to the length of the gene. After phosphorylating the 5'-termini of the segments with ATP by using polynucleotide kinase (from phage T_4) the segments aligned head to tail by their ability to base pair in the overlapping regions and the linkage was made covalent by use of the T_4-polynucleotide ligase.

Three large duplex fragments, each with a single stranded end, were made to correspond to the first 20 nucleotides of the gene (A); to residues 17-50 (B); and to the residues 46-77 (C), at the end of the gene. These were then joined to give the complete gene in each of two ways. In one scheme, A was joined to B, taking advantage of the overlap in residues 17-20, and C was then added in the presence of a small segment to assist the formation of the correct overlap. In another scheme, B was added to C in the presence of this small segment, and A was subsequently added. Analysis of the overall product showed that in each case it comprised a length of duplex RNA of predicted sequence.

Polydeoxyribonucleotides.

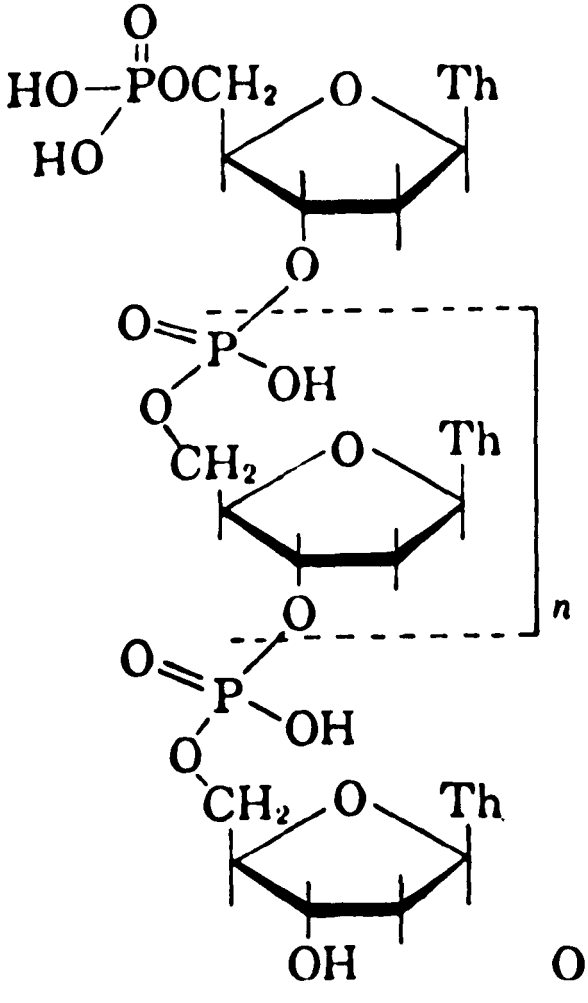
This very rich subject has been reviewed many times[1,41,198,214,217,231,232] so that this chapter will merely attempt to summarise and classify the major developments.

(3) Chemical synthesis of polydeoxyribonucleotides.

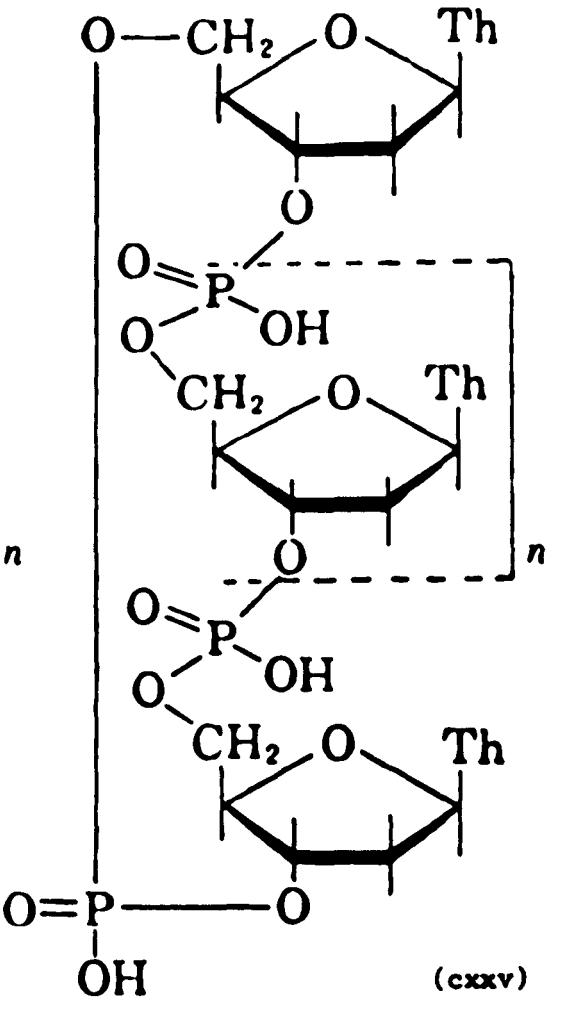
Thymidine-5'-phosphate was the first mononucleotide to be polymerised using either dicyclohexylcarbodiimide or p-toluene-sulphonate in anhydrous pyridine by Khorana and co-workers.[102,233] The separation and preparation of pure components from the mixture was achieved by chromatography on cellulose anion exchangers often followed by paper chromatography. Two series of homologous oligonucleotides were characterised; (a) the linear oligonucleotide (general structure, cxxiv),and (b) the cyclic oligonucleotides (general structure, cxxv) which represented about 15 - 20% of the product and arose by intramolecular cyclisation.

In addition to these major series of compounds, a minor byproduct was also observed; the monomeric thymidine-3'-5'-cyclic phosphate (which could be regarded as the first member of the cyclic series),(cxxvi).

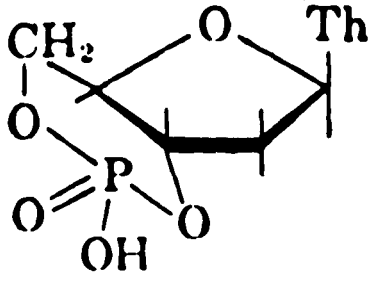
The linear oligonucleotides were characterised up to pentanucleotide but a further 15% of the total product remained on the column and was thought to consist of high polymers. Later Khorana et al.[234] reported that the formation of cyclic products could be reduced either by increasing the mononucleotide concentrations or



(cxxiv)



(cxxv)



(cxxvi)

Th = Thymine

by using some terminating residues. The former technique was not applicable because of the low solubility of mononucleotides in anhydrous pyridine. However, when 3'-O-acetylthymidine (20 - 25%), which acted as a terminating residue, was added to thymidine-5'-phosphate before polymerisation, no cyclic products were found.

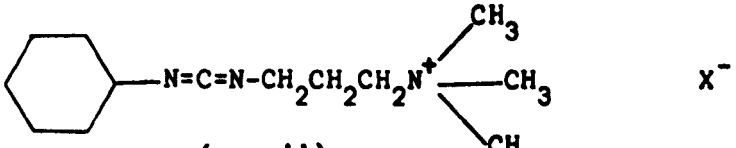
Since it was considered that phosphoramidates would result from the reaction of activated phosphate residues with purine or pyrimidine ring amino groups, N-benzoyldeoxyadenosine-,N^6-benzoyldeoxycytidine-, and N-acyldeoxyguanosine-5'-phosphates were used as starting materials to yield the corresponding polymeric products.[235]

A useful outcome of these studies was the observation that these polymerisations could be effected equally successfully in dry dimethylformamide with a little cationic exchange resin (pyridinium form) present, thus solving many solubility problems. Using the same technique Cohen and Banks[217,236,237] polymerised mixtures of thymidine- and deoxyadenosine-5'-phosphates using DCC. In this case, however, the base sequences of the resulting polymers were analysed by the nearest neighbour base frequency method described later.

Short oligomers of known base sequence have also been used as starting material for unprimed polymerisation. Polynucleotides containing thymidine and deoxyadenosine in alternating sequence were the first of this type to be synthesised.[203] Thus the dinucleotide

($pTpA^{Bz}$) 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 an alternating thymine-deoxyadenine base sequence. In a similar way oligonucleotides containing deoxyguanosine and deoxycytidine units were obtained.[235c]

Random polymerisations of deoxyribonucleotides have also been performed on a polymer support. Thus the reaction of a mixture of a thymine hexanucleotide and polyadenylic acid (primer) with water soluble carbodiimide (cxxvii) at -3° in 1M sodium chloride gave a



(cxxvii)

dodecanucleotide in 5% yield.[221] The product was identified by comparison with an authentic sample of the dodecanucleotide obtained by the polymerisation of thymidine-5'-phosphate in anhydrous solution. The reaction appeared to be catalysed by the complex formed by the two polynucleotides and appeared also to be a consequence of the activation of the terminal phosphate of the hexanucleotide since no dodecanucleotide was produced when either poly A or the reagent was omitted from the reaction mixture. Under the

same conditions, thymidine pentanucleotide, poly A, and the activating reagent gave a small yield of thymidinedecanucleotide. Water-soluble carbodiimide as condensing reagent has also been used during the course of the present work and is described later. (See Experimental section).

4. Enzymatic Synthesis of Polydeoxyribonucleotides.

The first enzymatic synthesis of a deoxyribonucleic acid was achieved by Kornberg et al.[231] from the four common deoxyribonucleoside-triphosphates. DNA Polymerase (which has now been obtained from many sources[232]), from E. coli[238] was used to catalyze the reaction. The requirements for the net enzymatic synthesis of DNA can be generalised by equation (26) where TP = triphosphates, MP = monophosphates and PP = pyrophosphate.

$$\left.\begin{matrix}\text{nTTP}\\ \text{ndGTP}\\ \text{ndATP}\\ \text{ndCTP}\end{matrix}\right\} + \text{DNA} \underset{\text{Mg}^{++}}{\overset{\text{Mn}^{++}\ \text{or}}{\rightleftharpoons}} \text{DNA}\left[\begin{matrix}\text{nTMP}\\ \text{ndGMP}\\ \text{ndAMP}\\ \text{ndCMP}\end{matrix}\right] + 4\,(n)\,\text{PP} \qquad (26)$$

Thus it was found that the enzymatic synthesis of DNA requires (a) all the four deoxyribonucleoside-triphosphates, and (b) a high molecular weight DNA which acts as primer. It was further observed that the omission of any deoxyribonucleoside-triphosphate reduces the reaction to below 1%, and that triphosphates could not be replaced by the corresponding diphosphates.

The analysis of enzymatically synthesised DNA revealed that it had essentially the same physical properties as DNA isolated from natural sources.[231] Thus the molecular weight was found to be in the neighbourhood of $5 - 6 \times 10^6$. Its behaviour on heating at 100°C, namely the collapse of macromolecular structure, was again similar to that of calf thymus DNA.

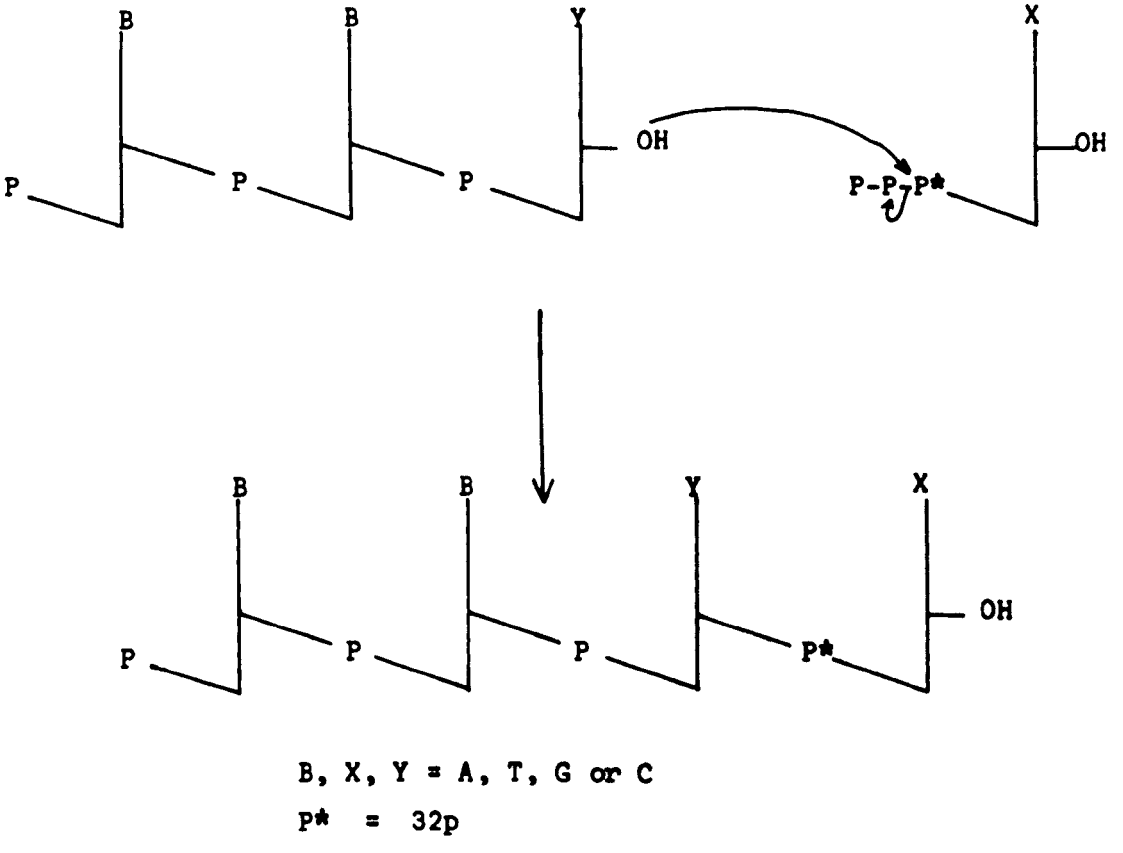
Chemical analysis of the base composition of the enzymatic product disclosed the fact that $[A] = [T]$ and $[G] = [C]$ as was the case in the primer DNA. Moreover, the ratio $[A] + [T] / [G] + [C]$ was equal to the ratio that was characteristic of the primer DNA. Thus the nucleotide sequence of the priming DNA appeared to be reproduced in the synthetic DNA product.

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 his co-workers.[239] This is the nearest approach yet obtained to a complete base sequence determination of high molecular weight nucleic acid and has also been used during the course of the present work. (See Experimental).

The technique as used by Kornberg involved several steps as summarised below.

(1) DNA was enzymatically synthesised by using one nucleoside triphosphate labelled with ^{32}P at α position, the other three were

unlabelled. The radioactive phosphorus attached to the 5'-carbon of the deoxyribose then became the bridge between the substrate molecule (X) and the nucleotide at the growing end of the chain (Y) with which the substrate reacted. (See Figure 9).



B, X, Y = A, T, G or C

P* = 32p

Figure 9. DNA Synthesis (by polymerase)

(2) At the end of the synthetic reaction, the DNA was isolated and digested enzymatically to yield the 3'-deoxynucleotides quantitatively. The phosphorus atom formerly attached to the 5'-carbon of the deoxyribonucleoside triphosphate substrate (X) was then attached to

the 3'-carbon of the nucleotide (Y) with which the substrate reacted during the synthesis of the DNA chain. (See Figure 10).

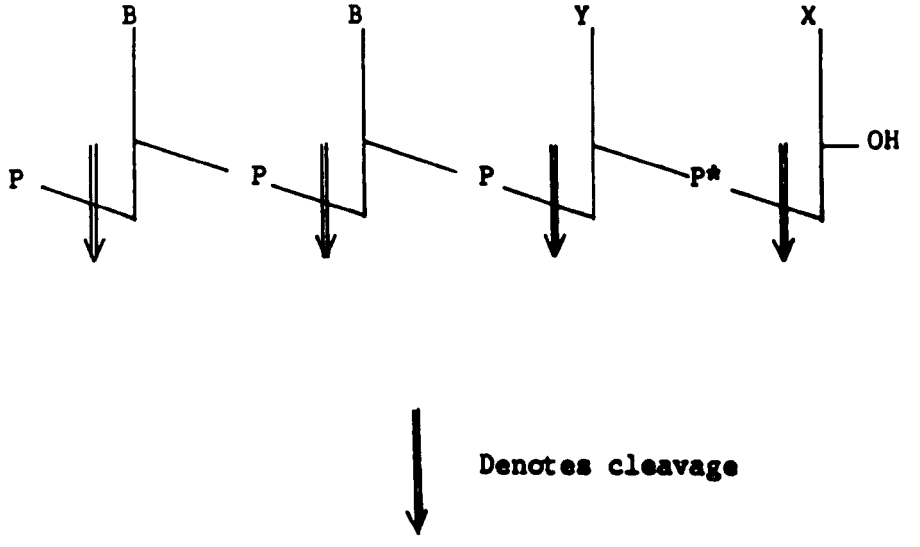


Figure 10. Degradation (by micrococcal DNase and splenic diesterase)

(3) The phosphorus-32 content of each of the 3'-deoxyribonucleotides, isolated by paper electrophoresis, was a measure of the relative frequency with which the labelled substrate reacted with each of the four nucleotides in the synthesis of the DNA. This procedure, carried out four times with a different labelled substrate (see Figure 11) in each case, yielded the relative frequencies of all

the 16 possible kinds of dinucleotide (nearest-neighbour) sequences.

Reaction 1.	dATP-32,	TTP ,	dGTP ,	dCTP
2.	dATP ,	TTP-32,	dGTP ,	dCTP
3.	dATP ,	TTP ,	dGTP-32,	dCTP
4.	dATP ,	TTP ,	dGTP ,	dCTP-32

Figure 11

Reaction pattern for each DNA primer

Thus using this technique Kornberg et al.[231,239] confirmed that the enzymatically synthesised DNA was double stranded like natural DNA. It has the base-pairing of the Watson-Crick model and the opposite polarity of the two strands. It has a nonrandom set of nearest-neighbour frequencies; the incorporation of thymidylate is equal to that of deoxyadenylate and the incorporation of deoxycytidylate is equal to that of deoxyguanylate. The ratio of incorporation of purine nucleotides to pyrimidine nucleotides is exactly that of the chemical composition of the primer DNA as isolated from nature. These results indicated that, in each of these experiments, a faithful replication of the overall composition of the primer DNA had been achieved.

Later Kornberg et al.[240] reported further very interesting observations. Some polymerisations were observed only after lag periods of several hours and occurred in the absence of DNA primer. The products of the reactions were double stranded rod shaped

macromolecules like DNA. These could, in turn, serve as primers and thus remove the time lag in the synthesis of identical polymers. From the mixture of deoxyadenosine- and thymidine-5'-triphosphates, in the DNA polymerase system, the product was found to be a copolymer in which deoxyadenylate and thymidylate residues were in perfectly alternating sequence (nonrandom copolymer, poly dAT).[241] From a mixture of deoxyguanosine- and deoxycytidine-5'-triphosphates, in the DNA polymerase system, the product was a mixture of the two homopolymers poly dG and poly dC.[242]

The formation of these two types of polymers under the same conditions, using two pairs of nucleoside-5'-triphosphates, raised the question of the factors causing the interesting specificity in the structure of the two polymers. These factors have been investigated in this department at Keele and are further discussed in this thesis (see Experimental Discussion). It should be added that the enzyme, DNA polymerase, which for several years had been thought to be responsible for making DNA molecules (as described above) in vivo is, instead, normally used to repair defects in the structure of DNA molecules. Kornberg and his colleagues at Stanford University[243] have recently shown how their versatile enzyme will work in the other direction, taking DNA molecules apart as well as assembling them. These new experiments have shown that it will also select parts of a DNA molecule that are not properly matched with a complementary molecule and remove these altogether.

While the Kornberg enzyme, DNA-dependent DNA polymerase, has won an importance for itself in molecular biology, another enzyme RNA-dependent DNA polymerase has been discovered very recently by two independent groups of investigators.[244,245]

For the past twenty years or so the cardinal tenet of molecular biology has been that the flow or transcription of genetic information is from DNA to messenger-RNA and then its translation to protein is strictly one way. But Baltimore,[244] and Mizutani et al.[245] claim independently that RNA tumour viruses contain an enzyme which uses viral RNA as a template for the synthesis of DNA and thus reverses the direction of genetic transcription. This discovery if upheld, will have important implications not only for carcinogenesis by RNA viruses but also for the general understanding of genetic transcription; apparently the classical process of information transfer from DNA to RNA can be inverted.

(β) Keto-esters of Phosphoric acid

Stability of Phosphoric acid Esters

Phosphoric acid esters include the fully substituted derivatives (triesters of phosphoric acid), the disubstituted derivatives which possess one acidic hydroxyl group, and the monosubstituted which possess two ionisable hydroxyl functions. Furthermore, both cyclic and acyclic phosphoric acid esters are known. As this subject has been reviewed by many workers,[121,249-253] this chapter will merely summarise the data related to the stabilities of phosphoric acid esters.

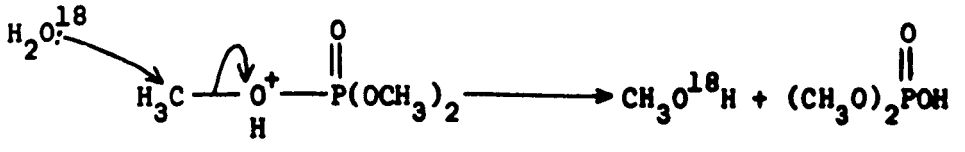
(A) Acyclic esters of phosphoric acid.

(a) Triesters of phosphoric acid.

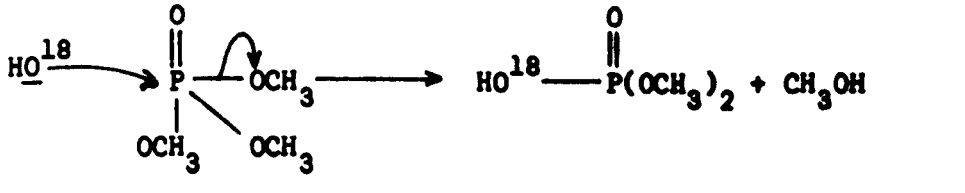
Triesters of phosphoric acid are unstable to alkali. Alkaline hydrolysis produces salts of disubstituted phosphoric acid derivatives and this has been used preparatively.[62] Perhaps the behaviour of trimethyl phosphate and triphenyl phosphate, recently examined by Barnard et al.[254] may be taken as characteristic of the reactions of the triesters of primary alcohols, phenols, and thiols with hydroxide ion. The hydrolysis of trimethyl phosphate in aqueous base is first order with respect to hydroxide ion and first order with respect to the ester. Isotopic tracer experiments show that the phosphorus-oxygen bond is broken exclusively; furthermore, within the limit of experimental error of the isotopic analysis, no isotopic exchange occurs prior to hydrolysis between the phosphoryl oxygen and oxygen atom of the solvent. A small depression of rate is

observed on changing the solvent from water to 75% dioxane-25% water. The hydrolysis of triphenyl phosphate in 75% dioxane-25% water was found also to be first order with respect both to hydroxide ion and to the ester.

The hydrolysis of trimethyl phosphate in acidic solution is slow, and is not acid catalysed.[254,255] It is interesting to note that for trimethyl phosphate, the position of bond fission changes as the nucleophilic reagent is changed from hydroxide ion to water molecule. Thus, whereas phosphorus-oxygen bond cleavage occurs in alkaline solution, acidic hydrolysis cleaves the C-O bond. Isotopic labelling experiments have shown that there is no O^{18} incorporated in the dimethyl phosphate produced by the hydrolysis of trimethyl phosphate in acidic H_2O^{18} but that one atom of O^{18} is incorporated when the hydrolysis is conducted in alkali. In acidic solution, the water molecule attacks at carbon but in alkali the hydroxide ion attacks at phosphorus.[254,256]



(27)



(28)

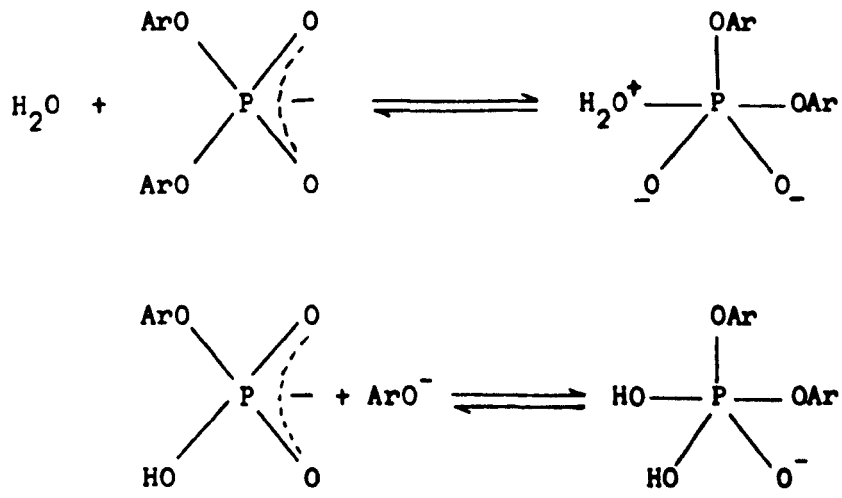
The effect of substituents on the reaction rate is very large. Thus, for example, alkoxy groups withdraw electrons inductively but can act as π-donors, increasing the electron density at phosphorus and making bond formation with the nucleophile more difficult. The order of inhibition by alkoxy substituents is the order of their electron releasing power.[257] On the other hand electrophilic substituents increase the instability. For example, tri-p-nitrophenyl phosphate is converted into di-p-nitrophenyl phosphoric acid simply by being heated under reflux in 2% aqueous methyl ethyl ketone.[258] This is a useful preparative method and has been used during the present course of work (see Experimental).

(b) Diesters of phosphoric acid.

With the exception of esters in which the phosphorus is included in a five-membered ring, or esters with 2-hydroxyalkyl substituents and related derivatives in which conversion to a five membered ring diester can occur by intramolecular transesterification (discussed later), diesters of phosphoric acids are in general unreactive. These are normally quite stable in alkaline solution although again their reactivity towards hydrolysis depends strongly on the basicity of the leaving group. Acid hydrolysis is not so rapid as the initial hydrolysis stage of tertiary esters.[62] As yet, of the group of normal diesters, only dimethyl phosphate and dibenzyl phosphate have been studied in detail. Westheimer and his co-workers[259] found the hydrolysis of the monoanion of dimethyl phosphate to be extremely

slow. In strongly alkaline solution the reaction was first order with respect to hydroxide ion and first order with respect to the ester; only about 10% of this reaction resulted in cleavage of the phosphorus-oxygen bond, the remainder involving nucleophilic attack at carbon.[260]

When a good leaving group is present in a disubstituted phosphate, more rapid attack at phosphorus is possible. Thus the dihalo-acids and their salts, $HOPOX_2$ which presumably are intermediates in the hydrolysis of the phosphoryl halides, are not known as isolatable substances.[261] Many modern phosphorylation techniques involve "activating" phosphoric acid or one of its monoesters so as to incorporate a good leaving group in a disubstituted derivative.[121] A wide variety of reagents is available for this purpose and this has already been described in an earlier section (see phosphorylating agents). Recently Kirby and Younas,[262] and Bunton and Farber[263] have studied the hydrolytic behaviour of a number of diesters of phosphoric acid. As a result, they reported that the difference in reactivity between diester and monoester monoanions decreases rapidly as the basicity of the leaving group decreases, and with very good leaving groups the reactivity of the two classes of compounds is comparable. The mechanism of hydrolysis of phosphate diester anions appeared to involve bimolecular nucleophilic attack of solvent on phosphorus as shown in Scheme 34.



Scheme 34

(c) Monoesters of phosphoric acid.

These are normally stable to alkali but readily hydrolysed by acid.[264] Bunton et al,[265] and Butcher and Westheimer[266] have reported that the hydrolyses of alkyl phosphates have a rate maximum at pH4. The pH rate profile for such esters is the now familiar bell-shaped curve which is observed for monoalkyl phosphates,[252,267] monoaryl phosphates[268,269,270] and monobenzyl phosphate.[271] The rate maximum at pH4 corresponds to a maximum in concentration of the anion[266] (cxxviii) or of the kinetically equivalent ion pair (cxxix), and these are, indeed, the only species which can show what is,

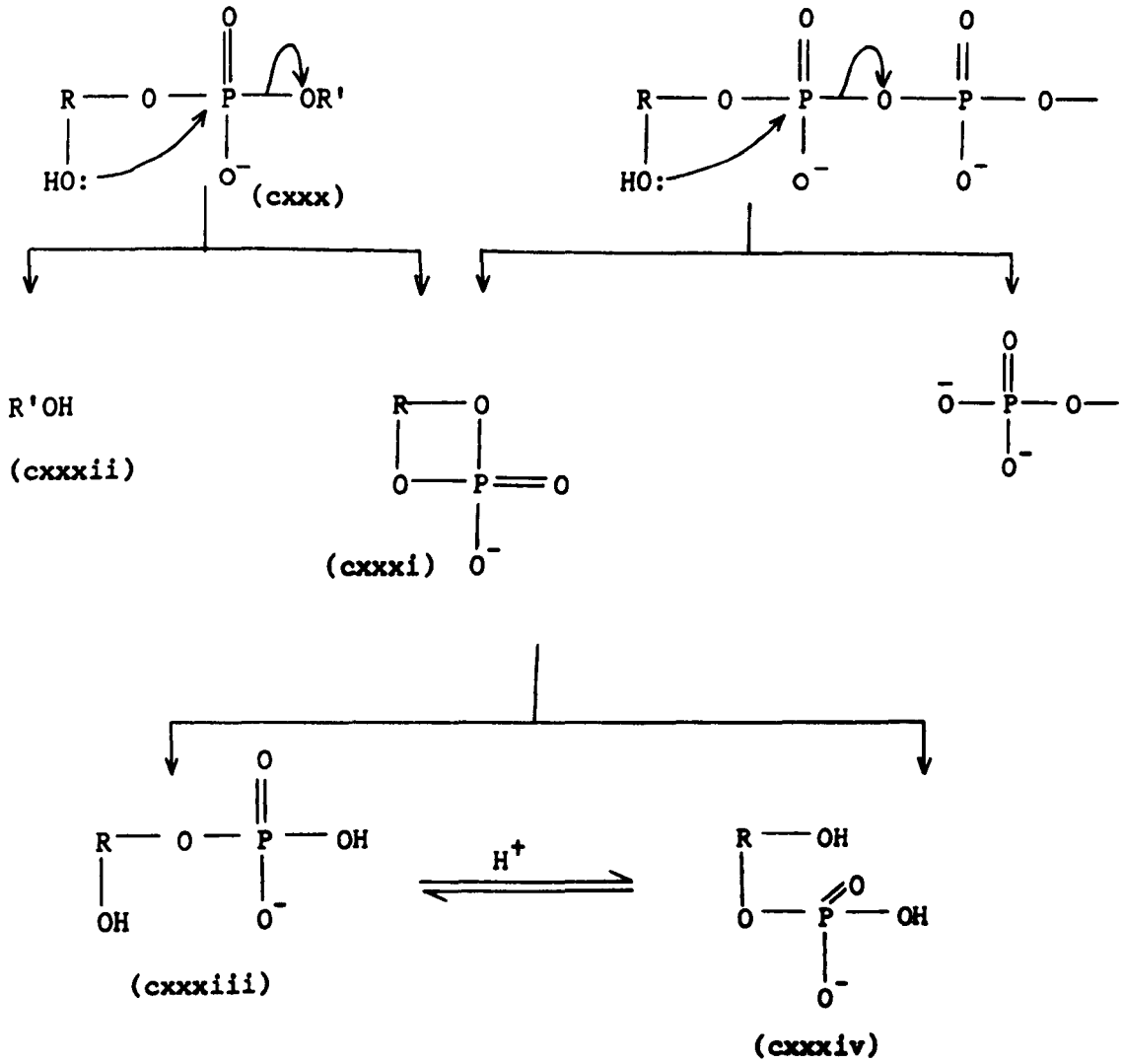
(cxxviii) (cxxix)

apparently, required for such rate acceleration; i.e. a negatively charged oxygen atom and a hydroxyl group, or two negative charges and a proton for the leaving group.[272] It should also be added that when electrophilic substituents are present, the monoesters are often very alkali labile (see under phosphorylating agents).

(B) Cyclic esters of phosphoric acid.

Cyclic esters of phosphoric acid have been recognised as very important compounds, because of their biological role. Two examples should be enough to demonstrate this fact: (a) the hydrolysis of ribonucleic acids involves the formation of 2',3' cyclic phosphates (see hydrolysis of RNA), (b) Adenosine-3',5' cyclic phosphate stimulated the conversion of inactive glycogen phosphorylase to the active form in tissue preparations.[273] Furthermore phosphodiesterases hydrolysing adenosine-3',5' and other ribonucleoside-3',5' cyclic phosphates to the corresponding ribonucleoside-5' phosphates appear to be present in different tissues.[274,275]

Cyclic phosphates can be obtained by the intramolecular cyclisation of phosphate esters provided part of the molecule bears a hydroxyl function suitably placed for an internal displacement on phosphorus as shown by the Scheme:

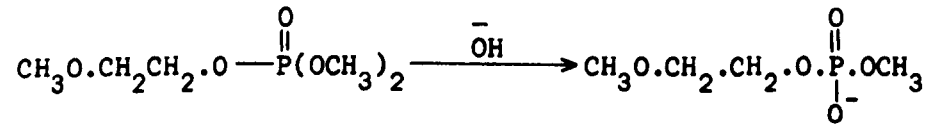


Scheme 35

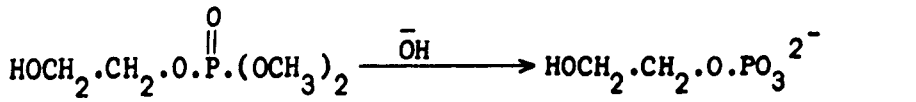
The displacement results in the formation of the cyclic phosphate (cxxxi) and the release of a second ester group as the free hydroxylic compound (cxxxii). The cyclic phosphate thus formed may hydrolyse in either of two ways to form two isomeric monoesters (cxxxiii, cxxxiv).

In sharp contrast to the behaviour of the usual diesters of phosphoric acid, the reactivity of diesters in which a hydroxyl group

is present on a carbon adjacent to the esterified hydroxyl group is high. Thus, for instance, while methyl 2-methoxyethyl phosphate is nearly inert to mild basic hydrolysis, methyl 2-hydroxyethyl phosphate is rapidly hydrolysed under the same conditions to hydroxyethyl phosphate.[62] (equations 29, 30).



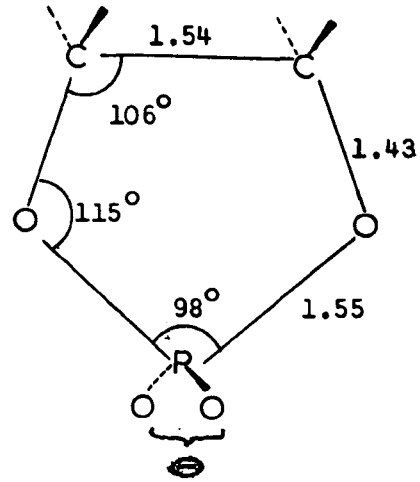
(29)



(30)

Proceeding on the assumption that this structural feature makes available a reaction pathway of low activation energy which is not possessed by ordinary diesters, Brown and Todd[61] interpreted these reactions as involving five-membered ring cyclic esters of phosphoric acid as intermediates (see hydrolysis of nucleic acids for further detail).

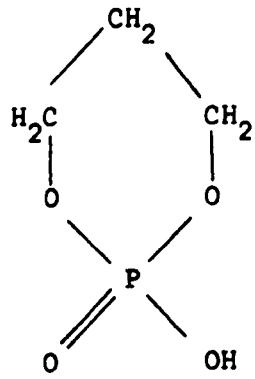
Westheimer and his co-workers[259] reported the synthesis of ethylene phosphate and determined its rate of hydrolysis to be about 10^7 times faster than that of its acyclic analoge dimethyl phosphate. Westheimer explained this high reactivity of five-membered cyclic phosphate esters in terms of strain on the ring.[276,277]



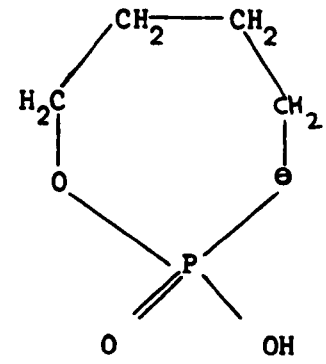
(cxxxv)

He suggested that the total angular strain in this system (cxxxv) would be in the neighbourhood of 3 kcal/mole. Since the ratio of the rate of hydrolysis of ethylene phosphate to that of dimethyl phosphate is around 10^6 - 10^7, the difference in free energy of activation is of the order of 10 kcal/mole. Westheimer proposed that the bond-bond interaction of cyclopentane increases its energy by ca. 7 kcal/mole. There are, however, insufficient bond interactions in structure (cxxxv) to account for this, but he suggested the possibility of analogous interactions involving the unpaired electrons on the oxygen atoms. Recent work by Westheimer and his colleagues[278] has shown that the opening of the 5-membered ring does in fact release about 5.5 kcal/mol. in excess of the heat of hydrolysis of a simple triester.

It should also be added here, while five-membered cyclic phosphate hydrolyses some 10^6 - 10^7 times as fast (half life of 50 minutes only in 0.5 N sodium hydroxide at 25° [259]) as dimethyl phosphate, the six-membered analogue (structure cxxxvi) is much more stable than the five-membered and hydrolyses only less than a power



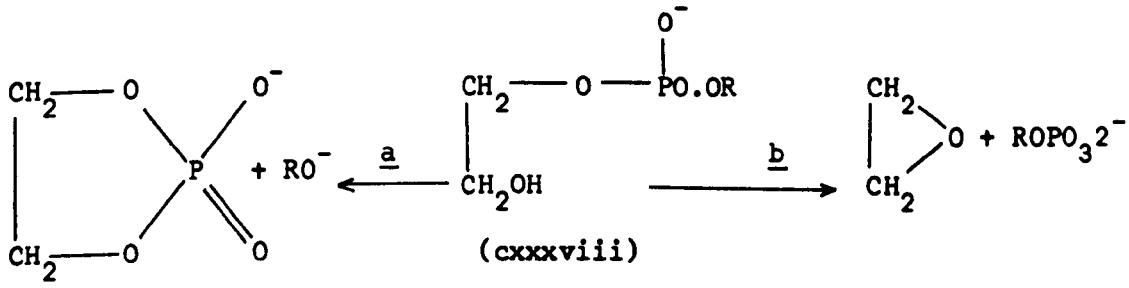
(cxxxvi)



(cxxxvii)

of ten more rapidly[279] than dimethyl phosphate. The seven-member cyclic phosphate (cxxxvii) is even more stable than the six-membered ring.

As described above, the neighbouring hydroxyl group has a marked labilising effect. This effect has usually been explained in terms of displacement (route a). Brown and Usher[280] reported an alternative base catalysed decomposition (route b, epoxide formation). They found that the ester (cxxxviii) (R = cyclohexyl) gave on alkaline hydrolysis 67% of cyclohexyl dihydrogen phosphate and 33% of the normal products. (Scheme 36).

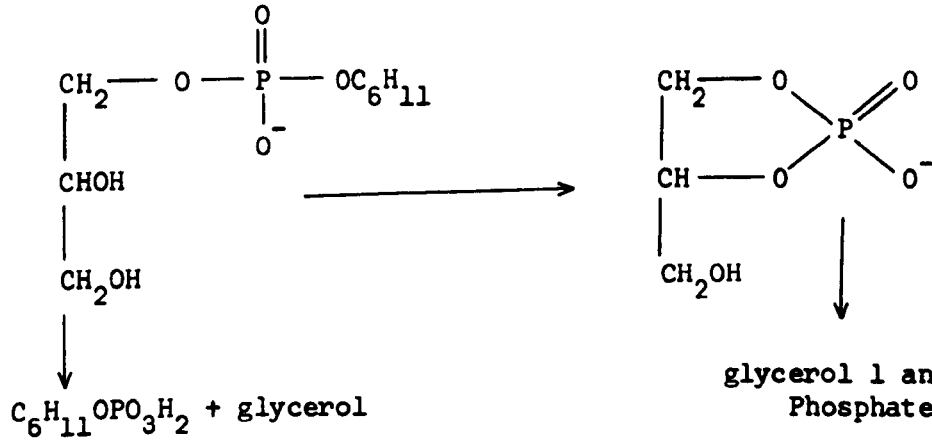


(cxxxviii)

Scheme 36

On the basis of their isotopic labelling experiments they concluded that the reaction is one of epoxide formation. However, dibenzyl trans-2-hydroxycyclohexyl phosphate was decomposed by base in three ways:[281] by competitive attack of the neighbouring hydroxyl group (a) on phosphorus with cyclic phosphate formation and (b) on carbon with formation of cyclohexene oxide and dibenzyl phosphate (hydroxide and t-butoxide); and (c) by attack on phosphorus with expulsion of cyclohexane-1,2-diol (methoxide). Qualitatively similar behaviour was shown by dibenzyl-2-hydroxyethyl phosphate.

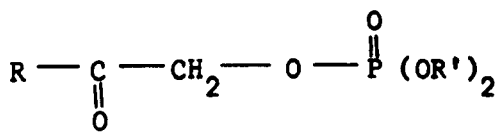
The alkaline hydrolysis of the cyclohexyl esters of a variety of 2-hydroxyalkyl phosphates was found to proceed by two mechanisms;[282] the normal one in which a cyclic phosphate is first formed, and one in which an epoxide is formed with displacement of cyclohexyl phosphate. (Scheme 37).



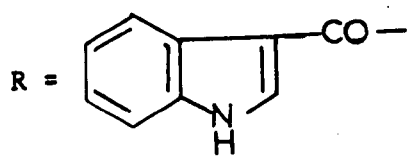
Scheme 37

Brown and Usher[283] have also studied the rates and products of alkaline hydrolysis of a series of esters of 2-hydroxypropyl phosphate. They observed that decomposition by the epoxide route was dominant in the cyclohexyl and absent in the phenyl ester in which hydrolysis occurred through the cyclic phosphate only.

The influence of the α-keto group in fully esterified phosphoric acid has been reported by Archer and Harley-Mason[289] who observed the unusual alkali lability of the phenyl groups in 3-indolylglyoxalylmethyl diphenyl phosphate (cxxxix).



(cxxxix)

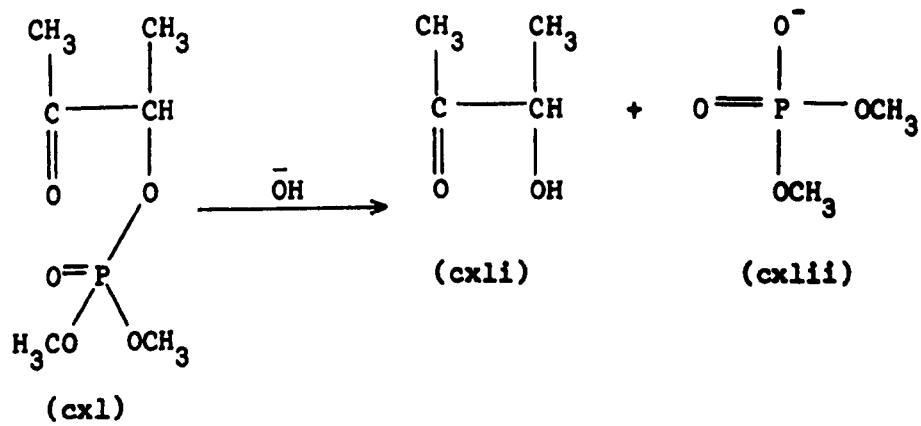


R' = Ph.

A nucleophilic attack on the carbon atom of the α-keto group was postulated leading to the formation of the cyclic intermediate

which would be attacked by hydroxyl ion at the phosphorus atom to give the monophenyl ester. Repetition of this process would lead to the mono-ester of phosphoric acid which was isolated. Cohen[284] has prepared simple members of the α-keto triesters of phosphoric acid and has studied their alkaline hydrolytic behaviour. On the basis of his findings, he concluded that the alkaline hydrolysis of these esters proceeded through a 5-membered cyclic phosphate formation (see page 170 for further detail). The author has also carried out alkaline hydrolysis of α-keto triesters of phosphoric acid with the same results as those reported by Cohen. The mass spectral data of the α-keto triesters of phosphoric acid have also indicated a similar cyclisation to that involved in the alkaline hydrolysis. This is discussed in detail elsewhere (see page 175).

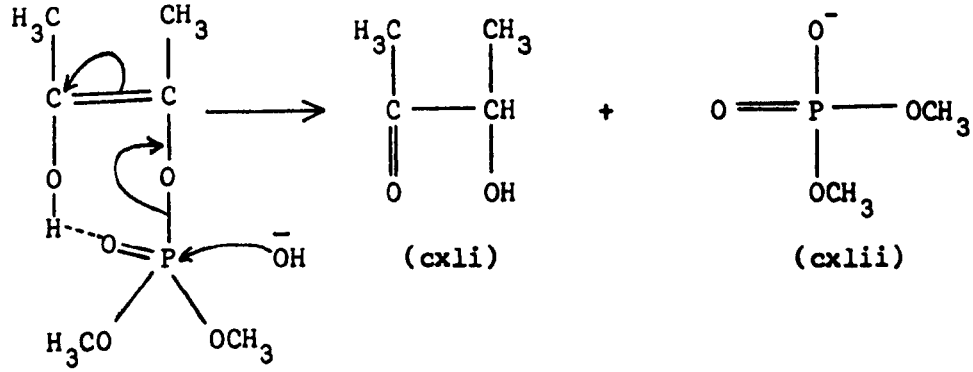
Ramirez and his co-workers[285] have examined the hydrolysis (in the pH range 7.7 - 8.3) of dimethyl phosphoacetoin (cxl). They reported that dimethyl phosphoacetoin, on alkaline hydrolysis, gave acetoin (cxli) and dimethyl phosphate (cxlii).



(cxl) (cxli) (cxlii)

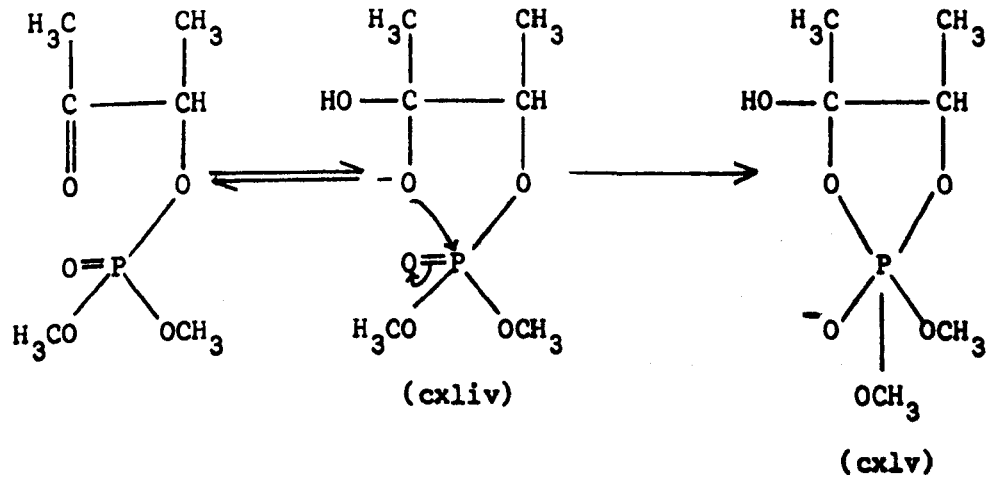
Scheme 38

The reaction was found to be first order with respect to hydroxide ion, and at least 2 x 10^6 times faster than that of trimethyl phosphate. They suggested that this enormous acceleration could be explained in two ways: (1) substitution at phosphorus involving the enediol-phosphate (cxliii) in which the rate enhancement would be due to the unsaturated nature of the leaving group and to hydrogen bonding assistance.

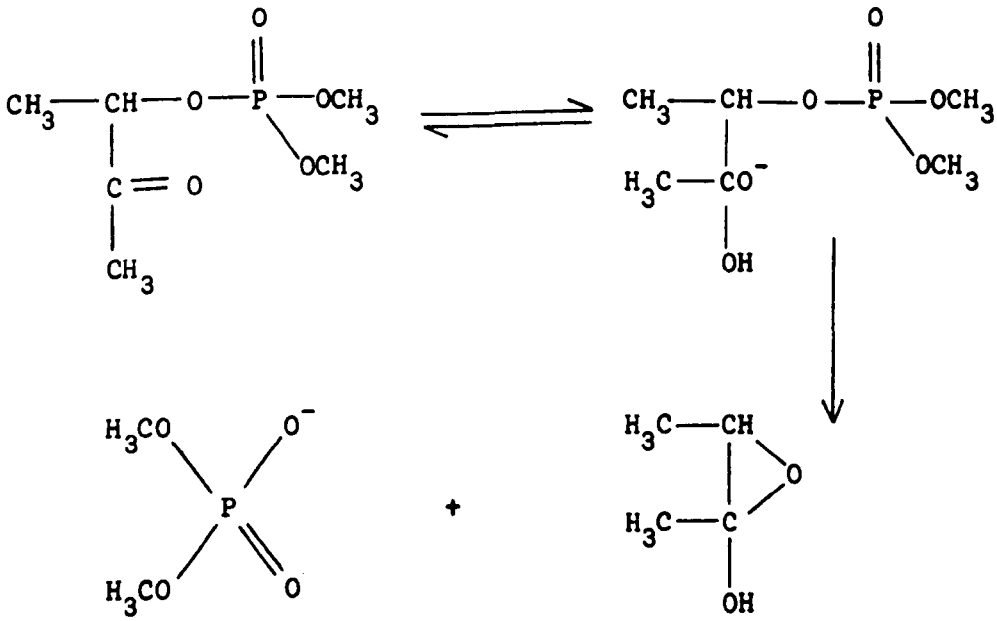


(cxliii) (cxli) (cxlii)

(2) Carbonyl participation (cxliv) and oxyphosphorane formation (cxlv) in the hydrolysis.



(cxliv) (cxlv)



Scheme 39

Recently Brown and Frearson[288] have discussed the possible mechanisms for the base catalysed hydrolysis of α-keto phosphate triesters and supported the view of Witzal et al. that an epoxide intermediate was involved in MeOH/MeO^- hydrolysis rather than the phosphorane proposed by Ramirez et al. Thus the fast methoxide-catalysed cleavage of $CH_3CO.CHCH_3.O.PO(OCH_3)_2$ and $CH_3CO.C(CH_3)_2.O.P.O.(OCH_3)_2$ yielding dimethyl phosphate, also gave products expected for a reaction involving a methoxyoxiran intermediate, i.e. $CH_3C(OCH_3)_2CH.CH_3.OH$ and $(CH_3)_3C.CO.CH_3$ respectively. It should also be added that Brown and Frearson noted that there was a competitive route which may be dominant in phenyl esters. This is discussed later (see page 172).

PART II

EXPERIMENTAL DISCUSSION

(α) Base Sequence specificity in chemically synthesised Polydeoxyribonucleotides.

The enzymatic synthesis of deoxyribonucleic acid has already been described in detail (see introduction). In summary, Kornberg et al.[238] isolated an enzyme, DNA polymerase*, from E.Coli (a micro-organism which reproduces itself 20 times a minute). This enzyme, DNA polymerase, has the ability to synthesise DNA from the four commonly deoxyribonucleoside triphosphates. The requirements for net synthesis are that (i) all the four deoxyribonucleoside-5'-triphosphates which form the adenine-thymine and guanine-cytosine base-pairs must be present, (ii) the substrate must be tri- and not di-phosphates and only the deoxyribose sugar compounds are active, and (iii) DNA of the high molecular weight must be present.

Later Kornberg et al.[240] found that if the DNA, which acts as a primer directing the synthesis of exact copies of itself, was omitted from the reaction mixture, there was a considerable delay before the commencement of rapid synthesis of a polymer which contained only deoxyadenylate and thymidylate residue. The synthesis was of importance because it did not require deoxycytidine and deoxy-guanosine-triphosphates and proceeded in the same fashion even in

---

* This enzyme is not now believed to be responsible for replication of DNA during reproduction. It is, instead, able to repair defects in the structure of DNA (Kornberg et al; 224, 495, (1969)).

See page 130.

their absence. The nearest neighbour analysis technique[239] (see also page 126) showed that the polymer was a copolymer of deoxyadenylate and thymidylate which had the physical size and properties of natural DNA and in which the adenine and thymine residues were in a perfectly alternating sequence (i.e. a nonrandom copolymer) as shown in Figure 11.

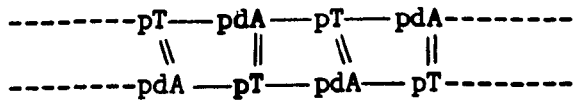


Figure 11

Nonrandom copolymer of pdA and pT

The discovery of poly dAT led Kornberg to search for naturally occurring poly dAT.

Sueoka[246] discovered in crab testes a separable component DNA with a buoyant density corresponding to poly dAT and representing 30% of the total DNA. In order to eliminate the possibility that adventitious materials, such as protein, might be responsible for the low buoyant density of this DNA band and with the thought that this component might be a "natural" dAT polymer, Kornberg et al.[247] tested this light DNA component from crab as a primer for replication. It supported synthesis at a rate comparable to that primed by authentic poly dAT but, in contrast with the latter, all four deoxyribonucleoside triphosphates were required, although synthesis could be achieved without using dGTP and dCTP, at a rate which was only

19% of that observed with the four triphosphates.

The most remarkable result, to support the view that light Crab DNA was poly dAT, came from nearest neighbour analysis which showed that alternating A and T residues comprised 93% of the sequences. Thus, on the basis of this close relation of the Crab testes light DNA to poly dAT, Kornberg suggested that the light DNA might be a natural form of this polymer.

It should also be added that when deoxyguanosine-5'-triphosphate and deoxycytidine-5'-triphosphate were used as substrate under the same conditions as those used for poly dAT, rapid polymerisation was observed after an initial lag period. The product was again found to be a rigid and double stranded macromolecule like deoxyribonucleic acid. However, this time the product was a mixture of the two homopolymers (poly dG and poly dC) which were not always present in equal amounts.[242] (Figure 12).

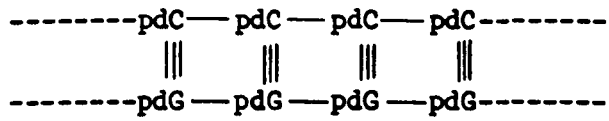


Figure 12

Homopolymers of pdG and pdC

The discovery of these two types of polymer (copolymer and homopolymer) using two pairs of deoxyribonucleoside-5'-triphosphates under the same conditions, led to an investigation of the factors

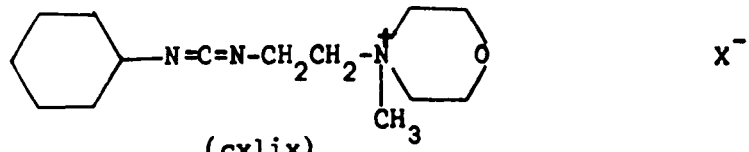
causing the specificity in the structures of the above two polymers.

Cohen and Banks[236] suggested that the reason for the specificity could be either that the molecular configuration of the nucleotides or the nature of the enzyme system determines the base order if no primer is used. To investigate which factor is operative it is desirable to eliminate one of the above factors and this can be done conveniently by eliminating the enzyme, i.e. by synthesising the polymer using chemical condensing reagents. Thus Cohen and Banks[236] synthesised poly dAT chemically using equimolar amounts of deoxyadenosine-5'-monophosphate and thymidine-5'-monophosphate in the presence of dicyclohexylcarbodiimide and anhydrous pyridine (as reaction medium). On the basis of their findings they concluded that it was the enzyme which determined the base order of the nucleotides in Kornberg's unprimed enzymatic synthesis of polydeoxyribonucleotides.

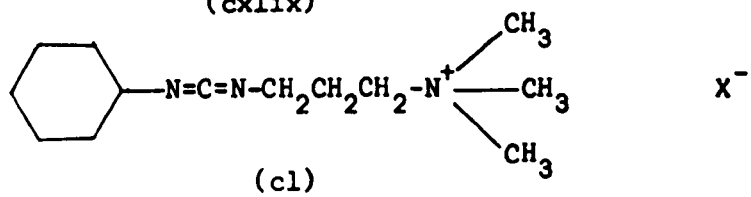
When Khorana et al.[102] polymerised thymidine-5'-phosphate in the presence of dicyclohexylcarbodiimide and pyridine, they found two types of products; (a) linear oligonucleotides and (b) 3',5'-cyclic oligonucleotides formed by intramolecular cyclisation. Later Khorana et al.[234] reported that increase in the concentration of the nucleotides favoured bimolecular condensations compared with the intramolecular reaction. Since the nucleotides are readily soluble in water, a chemical polymerisation in aqueous medium may be carried out in highly concentrated solutions. Hence a longer polymer,

compared with the polymer obtained by using anhydrous pyridine could be expected.

Gilham et al.[221] studied the use of diimides of the type I which by virtue of the quaternary ammonium groups are water soluble.



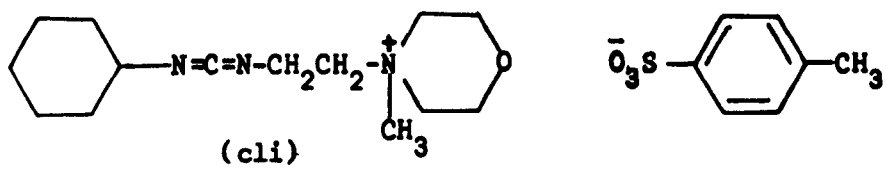
(cxlix)



(cl)

This has already been described (cf. page 124). It was found that these carbodiimides in aqueous solution like dicyclohexylcarbodiimide in anhydrous pyridine activate the terminal phosphate group. Since the basic life processes in cells operate in water rather than in pyridine and also since the use of an aqueous medium provides a better basis for comparison with the results of Kornberg et al.,[240] it was decided to carry out further chemical polymerisations in aqueous medium.

The condensing reagent 1-cyclohexyl-3-(2-morpholinoethyl) metho--p-toluenesulphonate (water soluble carbodiimide) was used in place

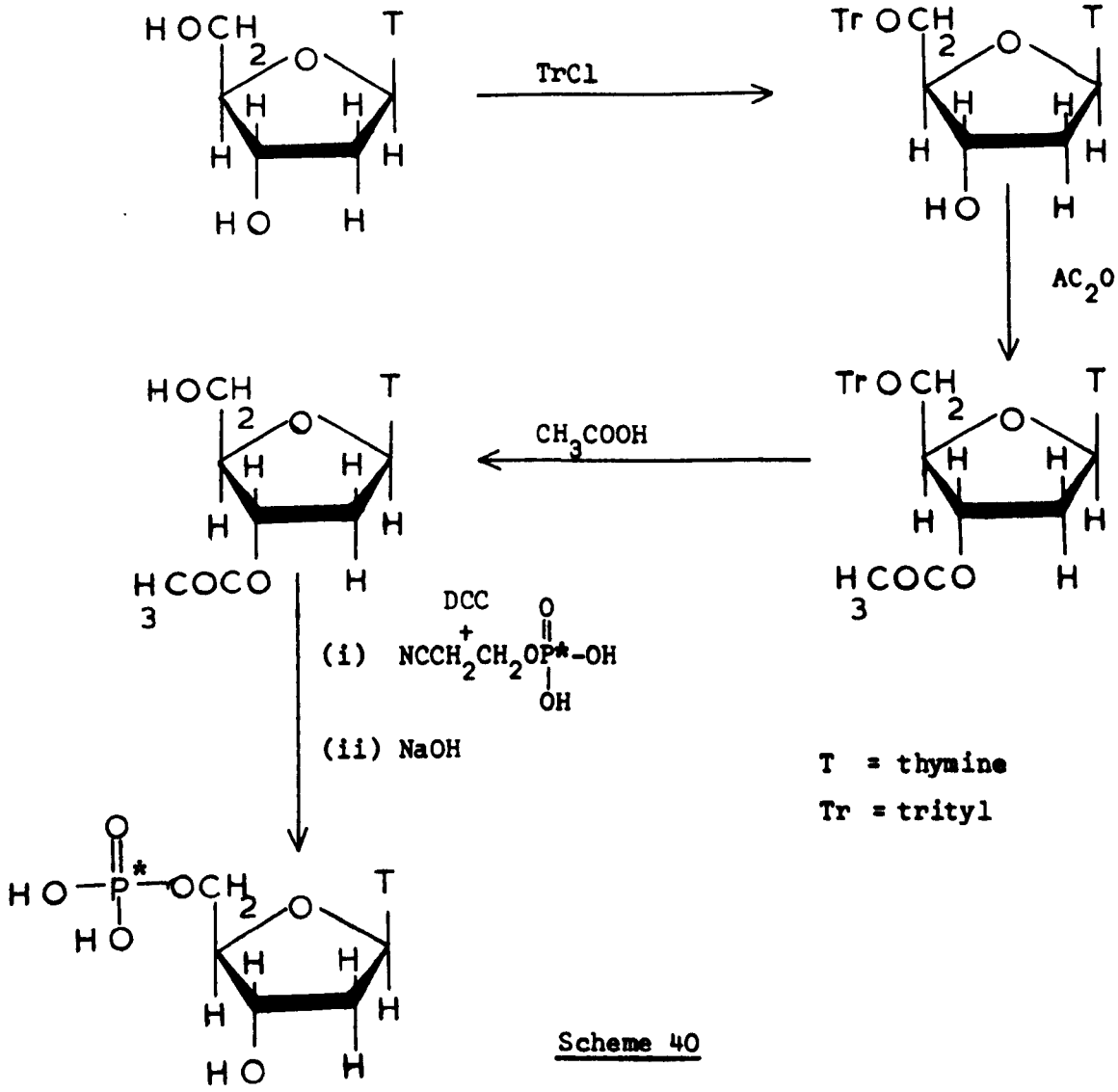


(cli)

of dicyclohexyl carbodiimide. The two deoxyribonucleotides chosen for polymerisation by water-soluble carbodiimide were thymidine-5'-monophosphate and deoxyadenosine-5'-monophosphate which, it will be remembered, were the components of the poly dAT copolymer with a complete alternating sequence of nucleotides in the unprimed enzymatic synthesis, and of the poly dAT copolymer with a random sequence of nucleotides in the unprimed chemical synthesis. The base sequence of the polymeric products were analysed by the nearest neighbour frequency technique which involved 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 provide the deoxyadenosine-5'-phosphate-P-32.

Thymidine-5'-phosphate-P-32 was synthesised by the method used by Michelson and Todd[77] and modified by Tener.[137] This involved (a) the blocking of the 5'-OH group of thymidine by tritylation, (b) acetylation at the 3'-position of the above protected nucleotide, (c) the removal of the trityl group to expose the 5'-primary hydroxyl group, (d) the phosphorylation of the exposed 5'-primary hydroxyl group using pyridinium 2-cyanoethyl phosphate-P-32 and dicyclohexylcarbodiimide (the former was synthesised by the Radiochemical Centre, Amersham, using the technique of Pfitzner and Moffat[248]), and (e) the removal of the acetyl and 2-cyanoethyl groups by alkaline

treatment to yield thymidine-5'-phosphate-P-32. (Scheme 40).



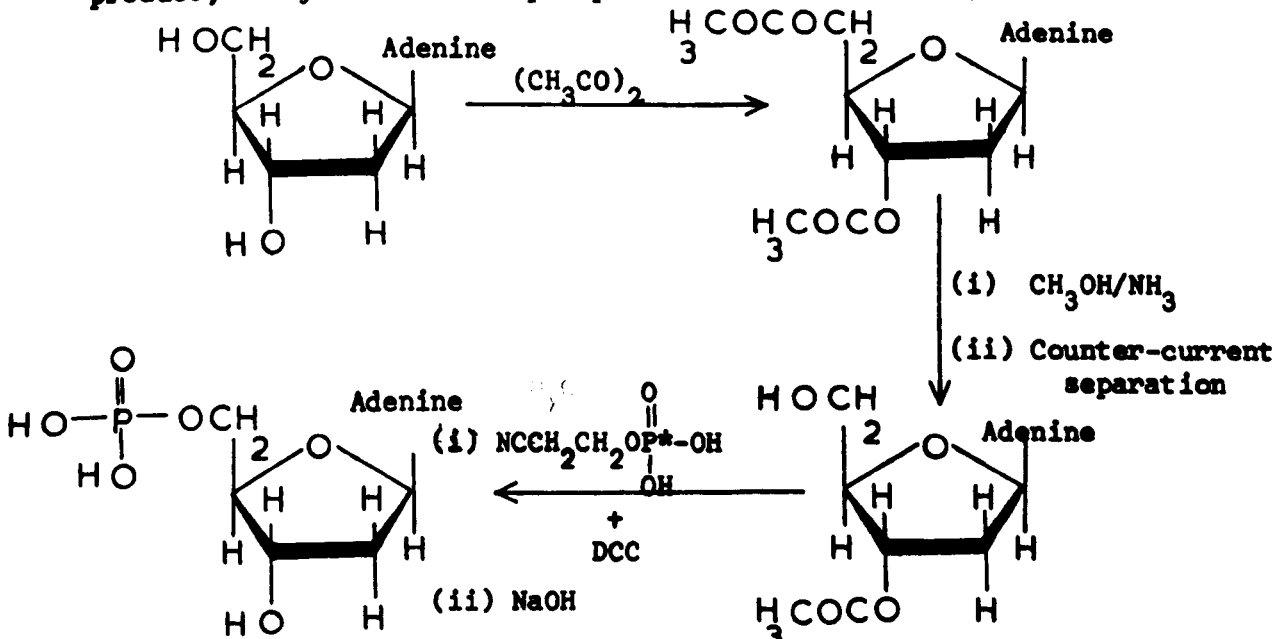
Scheme 40

The second method of synthesis of thymidine-5'-phosphate-P-32 involved the direct phosphorylation of anhydrous thymidine using 2-cyanoethyl phosphate-P-32 and dicyclohexylcarbodiimide. The required nucleotide was then isolated by paper chromatography (Whatman No. 1, solvent F).

The first method, however, could not be applied to the synthesis of deoxyadenosine-5'-phosphate-P-32, because of the acid lability of the glycosidic linkage in the purine nucleotides. Two methods were employed for the synthesis of this nucleotide.

The first was developed by Anderson, Hayes, Michelson and Todd,[182, 183] who treated deoxyadenosine with acetic anhydride to produce complete acetylation at 3'- and 5'- positions. Partial deacetylation of the product with saturated methanolic ammonia yielded a mixture of 5'-acetyldeoxyadenosine, 3'-acetyldeoxyadenosine, 3',5'-diacetyl-deoxyadenosine, and deoxyadenosine. These four products were then separated by counter-current distribution and the components identified by paper chromatography of the fractions.

Phosphorylation of the isolated 3'-acetyldeoxyadenosine with 2-cyanoethyl phosphate and dicyclohexylcarbodiimide followed by alkaline treatment to remove the protecting groups yielded the desired product, deoxyadenosine-5'-phosphate-P-32. (Scheme 41).



Scheme 41

The second method of synthesis of deoxyadenosine-5'-phosphate-P-32 involved direct phosphorylation of deoxyadenosine using 2-cyanoethylphosphate-P-32 and dicyclohexylcarbodiimide.[137] The required nucleotide was then isolated by ion-exchange (Dowex 1) using a linear concentration gradient of hydrochloric acid. The overall yield from deoxyadenosine using this method was better than that of the former method.

Each polymerisation experiment was performed in an identical manner; equimolar quantities of each deoxyribonucleotide were used in each case, only one of the deoxyribonucleotides being labelled with ^{32}P. The components were allowed to condense in the presence of water-soluble carbodiimide (50 fold excess with respect to the weights of nucleotides). After the reaction time, the product was dialysed exhaustively against distilled water to remove any mono- or lower oligonucleotides and then filtered. Paper chromatography showed a spot at the origin indicating the presence of polymer. The filtrate was evaporated under reduced pressure and at room temperature to c. 0.5 ml. This solution was employed for nearest neighbour base-sequence analysis which was accomplished by mixing the above solution with Tris buffer and calcium chloride and incubating the whole with Micrococcal DNase and Bovine spleen phosphodiesterase at 37°C; this treatment degraded the polymers into deoxyribonucleoside-3'-phosphates. These were separated by paper chromatography; each ultra-violet absorbing band was cut out and the nucleotide eluted from the paper with

water. An aliquot of the eluant was transferred to an aluminium planchette and evaporated under an infrared lamp. The activity of the sample was determined using a windowless scintillation counting technique, the scintillator being an anthracene crystal.

Since the initial radioactivities of the labelled substrates were known, it was then possible, by measuring the radioactivity in each of the two, 3'-monodeoxyribonucleotides from the enzymatic digest of the polymer (and making allowance for the loss of activity due to time-lapse), to obtain directly the amounts of these nucleotides and to calculate the progress of the reaction. Thus in the polymerisation of thymidine-5'-phosphoric acid-P-32 with deoxyadenosine-5'-phosphoric acid using water-soluble carbodiimide the ratio of active 3'-thymidylate to active deoxyadenylate derived from the polymer had a value of 6.7:1. In the polymerisation of deoxyadenosine-5'-phosphoric acid-P-32 with thymidine-5'-phosphoric acid, the ratio of active 3'-deoxyadenylate and thymidylate derived from the polymer had the value of 1 : 6.58.

If the polymer obtained were an alternating copolymer like Kornberg's poly dAT, the activity should transfer from the originally labelled deoxyribonucleotide to the second deoxyribonucleotide. (See Figure 13).

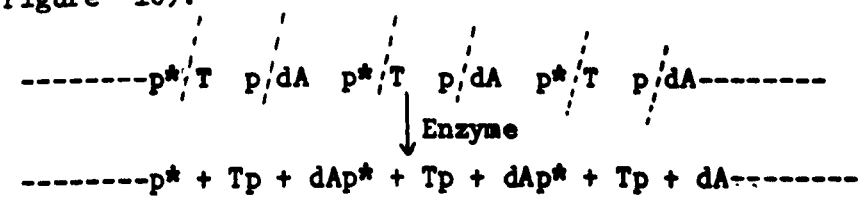


Figure 13 Dotted lines show the cleavage by the enzyme.

Thus if thymidine-5'-phosphoric acid was originally labelled with ^{32}p in the polymerisation of thymidine-5'-phosphate and deoxyadenosine-5'-phosphate, then after the micrococcal DNase and splenic phosphodiesterase digestion, the activity should transfer entirely to the second mononucleotide. In this case the 3'-deoxyadenylate should have 100% activity. If, however, the polymer was a homopolymer then the activity should remain on the originally labelled deoxyribonucleotide. (See Figure 14).

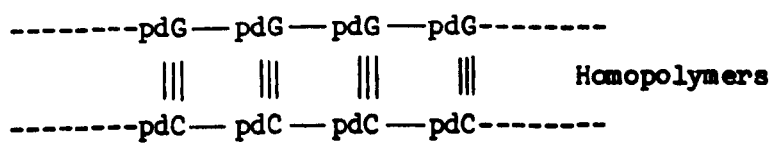


Figure 14

As can be seen from the result recorded above, both 3'-mononucleotides were active which clearly indicates that the polymer obtained was neither a copolymer with complete alternating sequence of the nucleotides nor a homopolymer but probably contained random sequences of nucleotides with the number of thymidylate residue predominating over deoxyadenylate in a ratio of about 45 : 1. (See Figure 15).

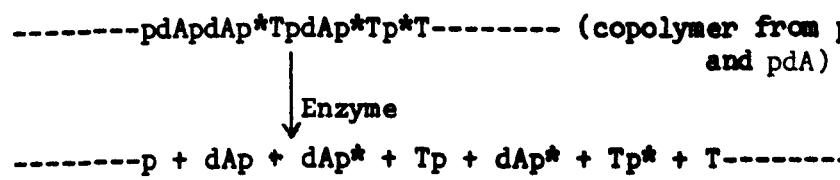
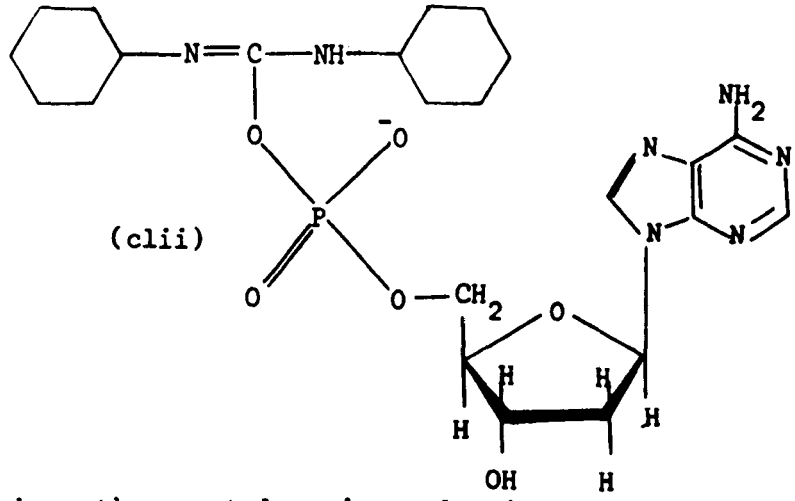


Figure 15

Thus in the products shown in the figure each dAp had been nearest neighbour to dA, each dAp* had been nearest neighbour to T, each Tp had been nearest neighbour to dA, and each Tp* had been nearest neighbour to T. With this technique, the polymer was found to be a copolymer with a random sequence of nucleotides and this is in general agreement with the findings by Cohen and Banks.[236,237] When they polymerised equimolar amounts of thymine and adenine deoxyribonucleoside-5'-phosphates, thymidylic acid being labelled with phosphorus-32, and analysed the product by using the nearest neighbour base sequence analysis technique, they found that the polymer contained TpT linkages and dApT linkages in a ratio, the estimate of which varied from 2 : 1 to 6 : 1; and when they used labelled deoxyadenylic acid in the polymerisation of thymidine-5'-phosphate and deoxyadenylic-5'-phosphate the polymer had dApdA linkages and TpdA linkages in a ratio of approximately 1 : 6. Thus they found that thymidylic residues predominated over deoxyadenylic acid in a ratio somewhere between 12 : 1 and 36 : 1 but they suggested that corrections for the finite lengths of the molecules would tend to raise these ratios slightly.

They suggested that the lack of incorporation of deoxyadenylic residue could be due either to the greater ease of phosphorylation of thymidine-3' hydroxyl group, or more probably, when carbodiimide is used as the condensing reagent, to the lower susceptibility to nucleophilic attack of the hypothetical active intermediate (clii)

analogous to the isolatable intermediates



(clii)

formed when ethoxyacetylene is used. (See Figure 16).

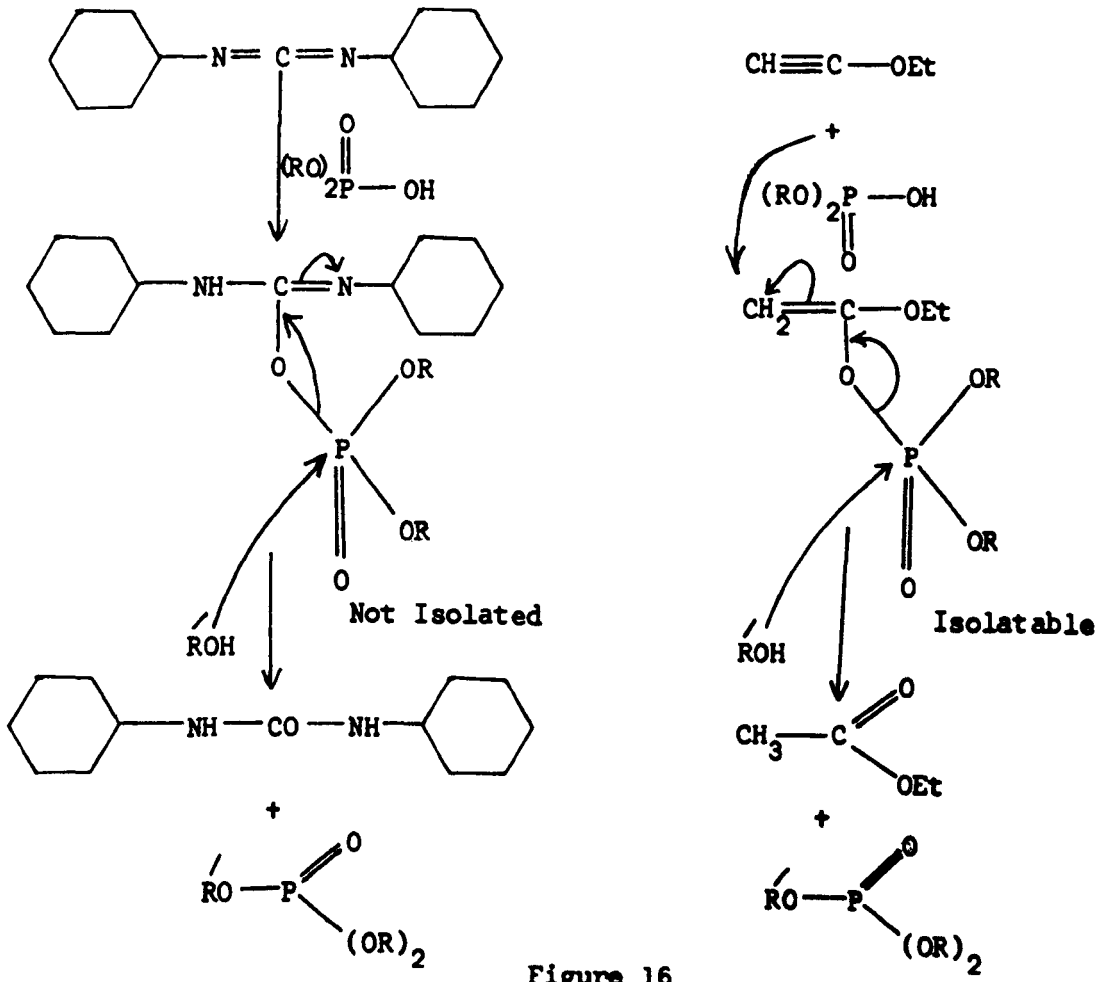


Figure 16

Evidence pointing to the former as the important factor comes from the work by Tener[137] who studied the rate of phosphorylation of the various functional groups, namely the 3'- and 5'-hydroxyl and the purine or pyrimidine ring amino groups. The technique used for this purpose was simple but effective. 2-cyanoethylphosphate plus dicyclohexylcarbodiimide in pyridine was allowed to compete for the groups in the presence of large excess(nine fold) of the nucleoside and then the amount of each phosphorylated product was determined. The results are summarised in Table 6.

Table 6

Ratio of Products from direct

Phosphorylation of Nucleosides

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

With nucleoside bearing ring amino groups, the mononucleotide fraction was heated at 100° for an hour to break the phosphoamide linkages. The hydrolysate was then rechromatographed and the ratio

of the 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. It can be seen that (a) even in the most unfavourable case, that of the cytosine nucleosides, the loss of phosphorylating agent due to phosphorylation of the amino group is less than 50%, (b) (of interest in the present case) no N-phosphate could be detected when deoxyadenosine was used, and (c) the 3'-hydroxyl group of deoxyadenosine is much less susceptible to phosphorylation (compared with the 5'-hydroxyl) than the corresponding group in either thymidine or deoxycytidine.

These experimental results are thus in agreement with the observed preferential incorporation of thymidylic residues in the synthetic copolymers.

Wasserman and Cohen[147] have demonstrated that an internucleotide linkage can be formed by using ethoxyacetylene as condensing reagent in pyridine solution. Thus they synthesised thymidylyl-3'-5'-thymidine by condensing the pyridinium salt of 3'-acetylthymidine-5'-phosphoric acid with 5'-tritylthymidine in the presence of ethoxyacetylene (6 moles excess) followed by the removal of protecting groups by successive treatment with alkali and acid. On the basis of their findings they concluded that ethoxyacetylene was a versatile reagent for the activation of mono- and dialkyl phosphates and undoubtedly the method is capable of extension in the field of mononucleotides and nucleotide coenzymes.

Khorana et al.[184] during their comparative study on several condensing reagents found that ethoxyacetylene was not as powerful a polymerising reagent as dicyclohexylcarbodiimide. In fact, when pyridinium thymidine-5'-phosphate was polymerised with ethoxyacetylene in anhydrous pyridine, the major product was P^1, P^2-dithymidine pyrophosphate. They suggested that whereas the activation of phosphomonoesters may occur with ethoxyacetylene, this results in the formation of pyrophosphates which lack the nucleophilicity to undergo further reaction with ethoxyacetylene, a step which is essential for the conversion of the initially formed symmetrical pyrophosphate into a phosphorylating agent.

However, if the formation of pyrophosphate can be reduced or the resulting pyrophosphate can be destroyed by some means, ethoxyacetylene can also be used in polymerisation reactions. Banks[217] in his chemical polymerisation of thymidine-5'-phosphate and deoxyadenosine-5'-phosphate with ethoxyacetylene found that pyrophosphates were destroyed by using water in the reaction mixture.

In view of these facts, we decided to use ethoxyacetylene in our chemical polymerisations in aqueous solution. Thus a model polymerisation of thymidine-5'-phosphate and deoxyadenosine-5'-phosphate was carried out using the same conditions as those employed for water-soluble carbodiimide (ethoxyacetylene was used in 50 molar excess). Although polymerisation was observed, the yield was very low compared with that obtained with water-soluble carbodiimide.

This poor yield could be the result of the low solubility of ethoxyacetylene in water. Ethoxyacetylene is partially soluble in water and forms a heterogeneous reaction mixture, thus reducing the efficiency. This difficulty can in principle be overcome either by using a large quantity of water or by using a larger amount of ethoxyacetylene. The former approach, of course, can not be followed because of the reasons mentioned above, i.e. dilution will encourage intramolecular cyclisation and increase the rate of conversion of ethoxyacetylene to ethyl acetate. Thus we decided to use an increased amount of ethoxyacetylene, and observed that when more ethoxyacetylene (200 molar instead of 50 molar excess) was used, the progress of polymerisation was equal to or in some cases more than that using water-soluble carbodiimide. On the basis of our findings, it seems that ethoxyacetylene is about 4 to 6 times less efficient than water-soluble carbodiimide in our system. If the solution were homogenised, it is probable that the efficiency of ethoxyacetylene could be increased considerably.

In view of these results, we decided to study also the base sequences in the polymerisation products obtained by using ethoxyacetylene. The polymerisations were carried out in the same fashion as described previously using water-soluble carbodiimide. When thymidine-5'-phosphate-P-32 and deoxyadenosine-5'-phosphate were allowed to polymerise using an excess of ethoxyacetylene (200 molar excess) and nearest neighbour analysis was employed, both 3'-mononucleotides were found to be active, thymidylate residue predominating

over deoxyadenylate residues in the ratio 6 : 1. Using deoxyadenosine-5'-phosphate-P-32 and thymidine-5'-phosphate in the polymerisation, the ratio had the value 6.53 : 1.

As discussed previously, these results indicate that the polymer was again a copolymer with a random sequence of the nucleotides. It had TpT, dApT, TpdA and dApdA linkages. Thymidylic residues predominating over deoxyadenylic acid in a ratio of about 40 : 1.

The structure of deoxyribonucleic acid has already been described in detail (see page 2). Very briefly, Watson and Crick[13] in their hypothesis of the structure of DNA, proposed that the 6-amino group of adenine is linked by hydrogen bonds to the 6-keto group of thymine and that in a similar manner guanine is hydrogen bonded to cytosine (see structure x and xi, page 9). Later workers have confirmed this proposal. For example, Kornberg et al.[231] by their enzymatic synthesis of deoxyribonucleic acid indicated that it is the capacity for base—pairing by hydrogen bonding between the pre-existing DNA and the nucleotides added as substrates that accounts for the requirement for a DNA primer.

In order to take advantage of such hydrogen bondings, it was, therefore, decided to use polyuridylic acid as a primer in our chemical polymerisations (note; this is a reverse of the normal natural sequence where DNA acts as a template for RNA). Since polyuridylic acid contains uracil whose 6-keto group is able to hydrogen bond with the 6-amino group of adenine (see Figure 17), the possibility

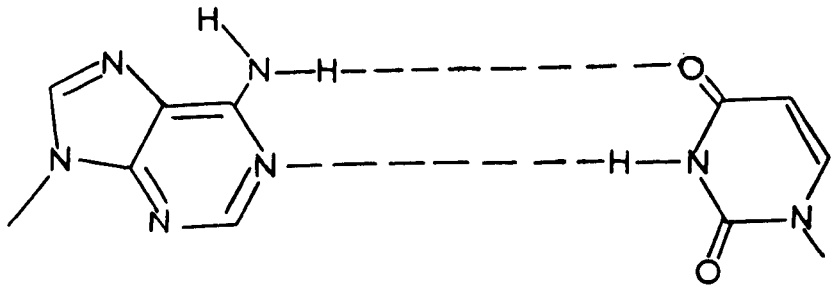


Figure 17

Uracil and Adenine Hydrogen Bondings

of increasing the incorporation of deoxyadenylate in the polymer was the motivation for this work.

Since water enhances hydrogen bonding while organic solvents do not have much positive affect (in fact, some such as dimethylformamide inhibit hydrogen bonding), it should be added here that this was also one of the reasons for conducting the chemical polymerisations in aqueous medium.

Polyuridylic acid, obtained commercially as the ammonium salt, was converted into its acid form (ion-exchange) and chemical polymerisations were carried out by using both chemical condensing reagents (water-soluble carbodiimide and ethoxyacetylene in excess, 50 fold and 200 fold respectively, equivalent to weights of deoxyribonucleotides) under the same conditions as those employed for the unprimed chemical polymerisations except that the excess of polyuridylic acid, at the end of the reaction, was destroyed by mild alkaline treatment (adjusting the pH to 12 for 24 hours at 37°C).

The results were as expected. Thus the use of polyuridylic acid increased the amount of polymerisation 4 - 6 times and nearest neighbour analysis revealed increased incorporation of deoxyadenylate compared with that in the unprimed chemical polymerisations.

When thymidine-5'-phosphate-P-32 was used in the polyuridylic acid primed chemical polymerisation of thymidine-5'-phosphate and deoxyadenosine-5'-phosphate using water-soluble carbodiimide, the ratio of active thymidylate to active deoxyadenylate (i.e. the ratio of TpT and dApT linkages in the polymer) residues had a mean value 2.14 : 1 with a standard deviation of 0.14. In the experiments where deoxyadenosine-5'-phosphate was labelled with ^{32}P, the ratio of active thymidylate to active deoxyadenylate (i.e. the ratio of TpdA and dApdA linkages in the polymer) residues had a mean value 1.62 : 1 with a standard deviation 0.33 in the first figure. Thus thymidylic residues predominating over deoxyadenylic residue in a ratio of about 5 : 1.

Using ethoxyacetylene, under similar conditions, when thymidine-5'-phosphate was labelled with 32p, the active thymidylate and deoxyadenylate residues were found to be in a ratio the mean value of which was 2 : 1 with a standard deviation of 0.2. When deoxyadenosine-5'-phosphate-P-32 was used the active residues had a ratio of the mean value of 2.1 : 1 with standard deviation of 0.13. Thus the polymer had random sequences of nucleotides with the number of

thymidylic residues predominating over deoxyadenylic acid in a ratio of about 5 : 1.

Results obtained in the present investigations on poly dAT compared with those obtained by Kornberg and by Cohen and Banks are summarised in Table 7.

These results show that poly U does catalyze the reaction and increases the relative incorporation of deoxyadenylic acid by a factor of 8 to 9. On the basis of these findings we suggest that, in the absence of enzyme and primer, the copolymerisation of the mixture of nucleotides is controlled by kinetic factors whereas, in the presence of a suitable primer, the base-pairing due to hydrogen bonding can influence the nature of the polymer even when the polymerisation is non-enzymatic.

Table 7

Synthesis of poly dAT		Ratio of 3'-Thymidylate to 3'-Deoxyadenylate residues	Nature of Polymer
A.Kornberg	Enzymatic (primer DNA)	1 : 1	Copolymer (alternating sequence)
	Enzymatic (No primer)	1 : 1	Copolymer (alternating sequence)
D.Cohen & G.R.Banks	Chemical with DCC in anhydrous Pyridine (No primer)	12 : 1 to 36 : 1	Copolymer - random sequence
	Chemical with DCC* in H_2O (No primer)	44 : 1 to 44.9 : 1	Copolymer - random sequence
	Chemical with DCC* in H_2O (primer poly U)	4.45 : 1 to 5.19 : 1	Copolymer - random sequence
	Chemical with E-A* in H_2O (No primer)	39 : 1 to 42.64 : 1	Copolymer - random sequence
	Chemical with E-A in H_2O (primer poly U)	4.9 : 1 to 4.97 : 1	Copolymer - random sequence

E-A* = Ethoxyacetylene

DCC* = water-soluble DCC

(β) α-Ketotriesters of phosphoric acid

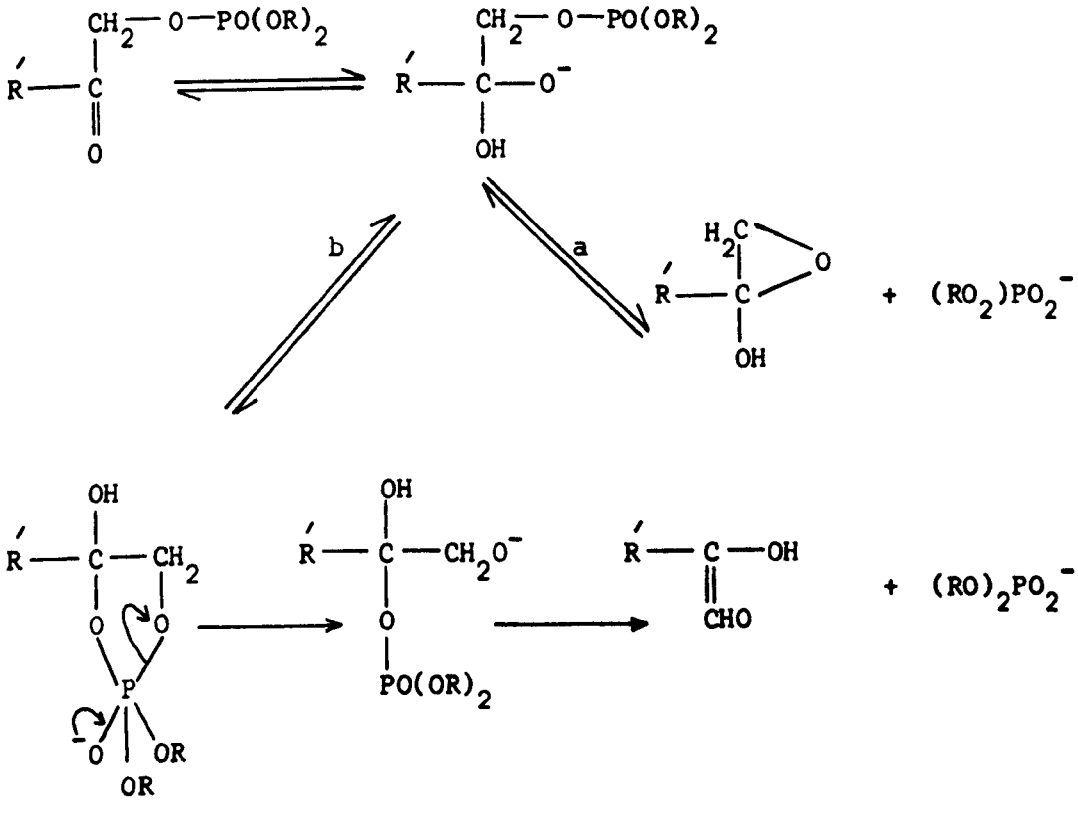
Cohen[284] prepared and studied the hydrolytic behaviour of a number of phosphate ketotriesters. During the alkaline hydrolysis of phenacyl di-p-nitrophenyl phosphate carried out titrimetrically, he observed that the solution became yellow at pH 6 and eventually two moles of p-nitrophenoxide ion were released with the uptake of four moles of alkali.

When he performed the alkaline hydrolysis at pH 9 using a pH-stat, he found that one mole of base was consumed at the same rate as one mole of p-nitrophenoxide ion was released ($t_{\frac{1}{2}}$ = 75 seconds). At pH 10 a further mole of base was consumed ($t_{\frac{1}{2}}$ = 310 seconds) without further liberation of p-nitrophenoxide. With the pH-stat set at pH 11, two moles of base were rapidly consumed ($t_{\frac{1}{2}}$ = >30 seconds) with one mole of p-nitrophenoxide being liberated.

On the basis of these findings Cohen concluded that the alkaline hydrolysis of α-ketophosphate triesters involved an intermediate, a 5-membered cyclic phosphate which was not isolated. In the proposed mechanism, hydroxide ion attacks the carbon atom of the carbonyl group with formation of a 5-membered cyclic phosphate and release of p-nitrophenoxide ion. The 5-membered cyclic phosphate intermediate reacts with another mole of alkali with opening of the ring (as shown in scheme 42).

Westheimer and his co-workers[276,277] have also studied the alkaline hydrolysis of 5-membered cyclic phosphates. They reported the high rate of hydrolysis of 5-membered cyclic phosphates compared with the corresponding open chain compounds. For example, the rate of alkaline hydrolysis of cyclic ethylene phosphate was found to be about 10^6-10^7 times faster than that of its acyclic analogue dimethyl phosphate. Cox, Wall and Westheimer[277] compared the heats of hydrolysis of the cyclic and acyclic phosphates and concluded that this enormous reactivity of 5-membered cyclic phosphate was associated with the thermodynamic strain of the five membered ring (see also page 139).

Recently Brown and Frearson[288] have discussed the possible mechanisms for the alkaline hydrolysis of α-ketotriesters of phosphoric acid and supported the view of Witzel, Botta and Dimroth [287] who suggested that an epoxide intermediate (path a) was involved in MeOH/MeO hydrolysis rather than the oxyphosphorane (path b) proposed by Ramirez, Hansen and Desai[285] although the latter penta co-ordinate compound was the major intermediate in water (scheme 43).



Scheme 43

However, Brown and Frearson[288] noted that there was a competitive route (also suggested by Cohen[284]) which may be dominant in case of phenyl esters (see scheme 42).

The work by Cohen[284] on the alkaline hydrolysis of α-ketotriesters of phosphoric acid was carried out in aqueous ethanol. Thus phenacyl di-p-nitrophenyl phosphate was dissolved in aqueous ethanol and titrated against sodium hydroxide at constant pH (automatic titrator). Since ethoxide ion is more reactive than hydroxide ion,

it was thought possible that ethoxide ion might have interfered with the alkaline hydrolysis. In view of this fact, it was therefore, decided to repeat the hydrolysis in the absence of ethanol, thus eliminating any influence of the ethoxide ion.

α-Ketotriesters of phosphoric acid (general formula; cliii) were prepared by the method used by Cohen.[284]

$$(RO)_2PO\cdot OH + R'\cdot COCHN_2 \longrightarrow (RO)_2PO\cdot OCH_2\cdot COR' + N_2$$

(cliii)

R' = C_6H_5, t-Bu, CH_3

R = C_6H_5, $C_6H_5CH_2$, C_6H_4-P-NO_2

α-Diazoketones were synthesised by the established methods i.e. reacting acid chlorides with excess of diazomethane. Disubstituted phosphoric acids were prepared by known methods and were also obtained from R.N. Emanuel or Sigma Chemicals U.S.A.

The ketophosphate triesters were obtained in their pure state. Phenacyl di-p-nitrophenyl, pivaloyl di-p-nitrophenyl, and phenacyl di-phenyl phosphates were obtained as solids while the other members of the series were obtained as liquids (see experimental).

The compounds were characterised by their physical properties, and their micro-analyses; the mass spectral data agreed with their emperical formulae. The u.v. and i.r. spectra showed a carbonyl group

adjacent to a methylene group (ν_{max} 1720-1730 cm^{-1} and 1450-1455 cm^{-1}; $CH_2-\overset{\overset{O}{\|}}{C}-$) and P=O (1290-1300 cm^{-1}). The n.m.r. spectra had multiplets between 1.5-2.8τ (corresponding to phenyl absorptions) and a doublet assigned to methylene proton absorptions, probably split by the long range coupling effect of phosphorus (P^{31} has a magnetic moment, $I = \frac{1}{2}$). The position of the doublet (4.3-4.6τ) indicated the methylene group to be a part of the open chain structure of the α-ketotriesters of phosphoric acid.

Phenacyl di-p-nitrophenyl phosphate was used for alkaline hydrolysis under the same conditions as those employed by Cohen[284] except that aqueous tetrahydrofuran was used in place of aqueous ethanol. The triester was dissolved in 50% aqueous tetrahydrofuran and titrated against N/5.191 sodium hydroxide both at pH 9 and at pH 11 (automatic titrator) in an atmosphere of CO_2-free nitrogen. The results were the same as those recorded by Cohen i.e. one mole of base was consumed at pH 9 while pH 11 was attained with the uptake of two moles of alkali. This result eliminated the possibility of ethoxide ion interference.

The mass spectra of α-ketotriesters of phosphoric acid were found to be interesting. In only one case ($R = C_6H_5$; $R' = (CH_3)_3C$) was the molecular ion observed at normal ionisation voltages, but in all cases where $R = C_6H_4$-p-NO_2, C_6H_5, $C_6H_5CH_2$, the major peak at high m/e corresponded to the loss of OR. It was thought that this easy loss of OR (which is not shown by e.g. triphenyl phosphate,[290]

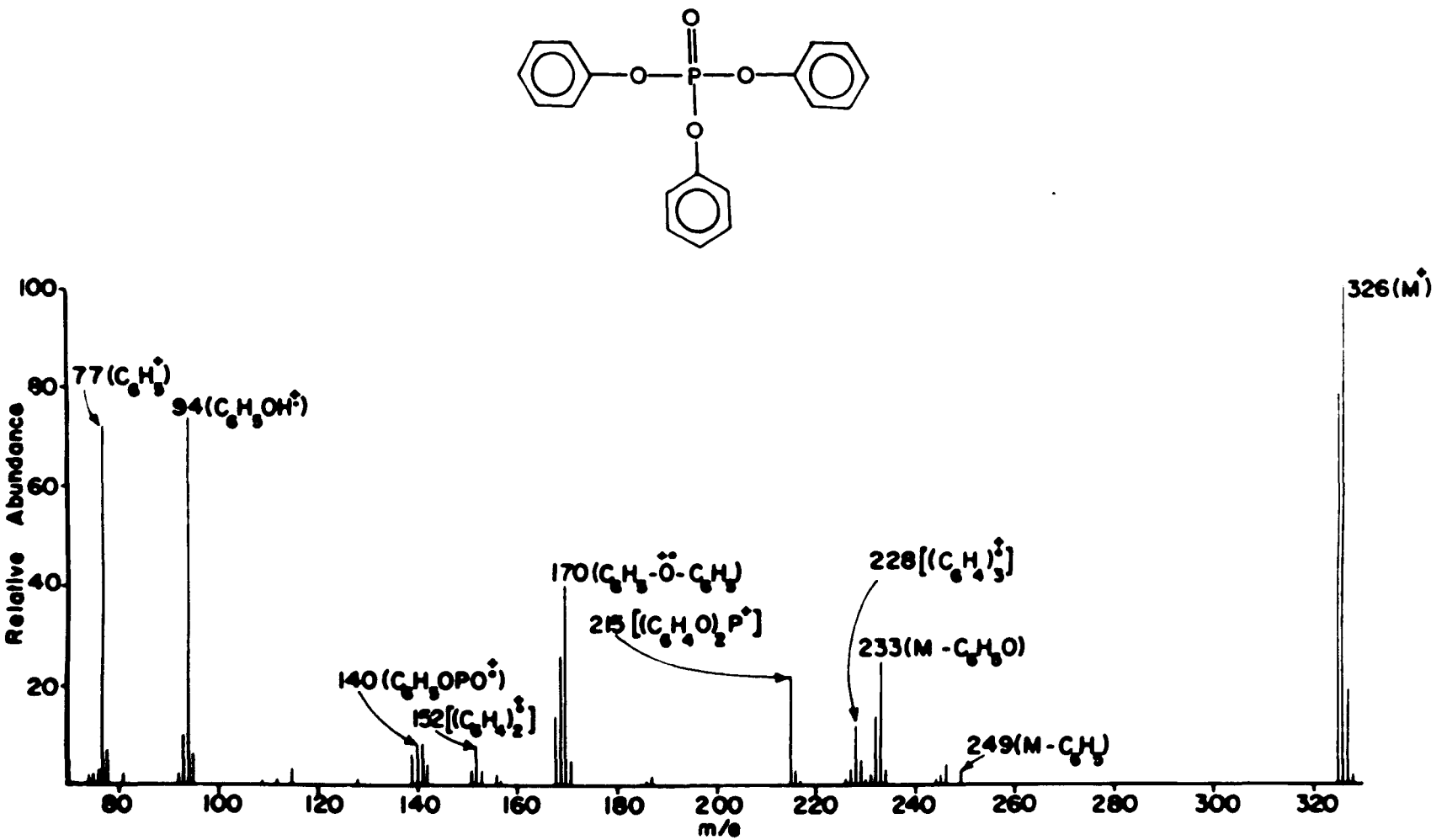
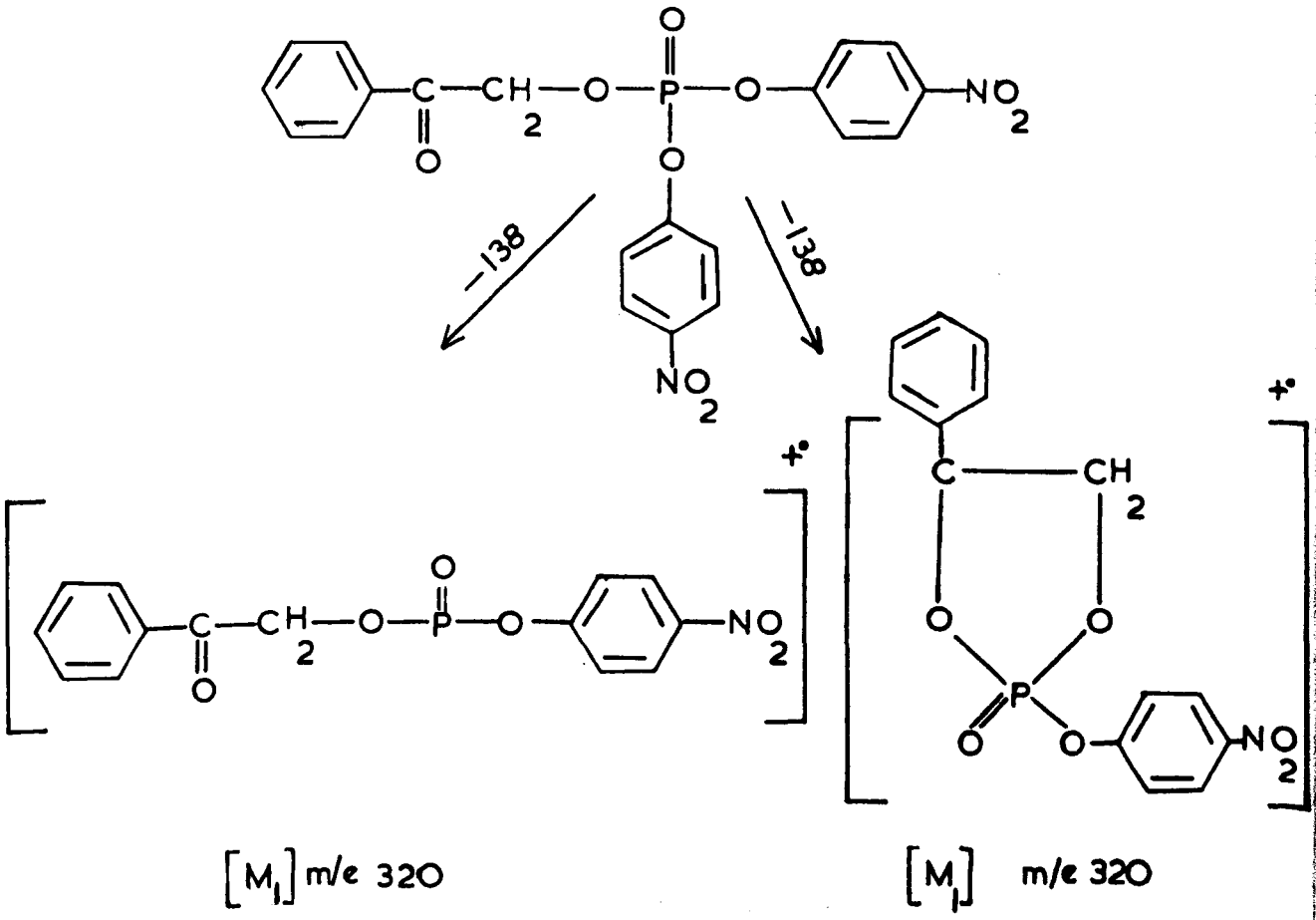

Relative Abundance
m/e
77($C_6H_5^+$)
94($C_6H_5OH^{+\cdot}$)
140($C_6H_5OPO^{+\cdot}$)
152[$(C_6H_4)_2^{+\cdot}$]
170(C_6H_5-$\ddot{O}$-C_6H_5)
215[$(C_6H_4O)_2P^+$]
228[$(C_6H_4)_3^{+\cdot}$]
233(M - C_6H_5O)
249(M - C_6H_5)
326($M^{+\cdot}$)

Figure 18

see Figure 18) might have resulted from a cyclisation similar to that involved in alkaline hydrolysis. The mechanism proposed for the fragmentation pattern of α-ketotriesters of phosphoric acid is applicable for every compound presented in this thesis. This is exemplified by the mass spectrum of phenacyl di-p-nitrophenyl phosphate.

The mass spectrum of phenacyl di-p-nitrophenyl phosphate (Fig. 19) showed the apparent molecular ion $[M_1]$ m/e 320 (relative abundance 64.28%), possibly due to the loss of a p-nitrophenoxide ion (loss of 138) which could occur in either of the following ways:



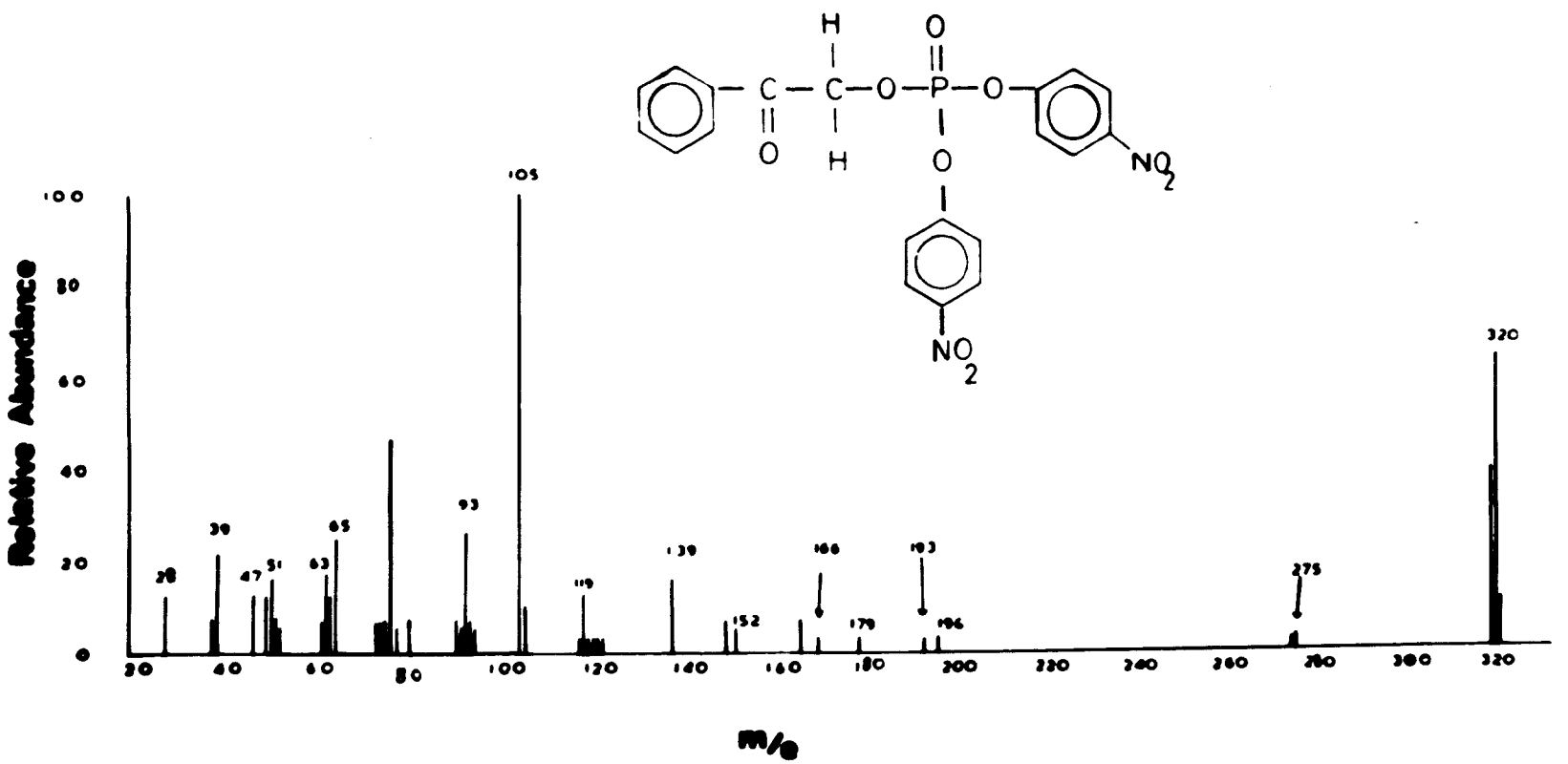
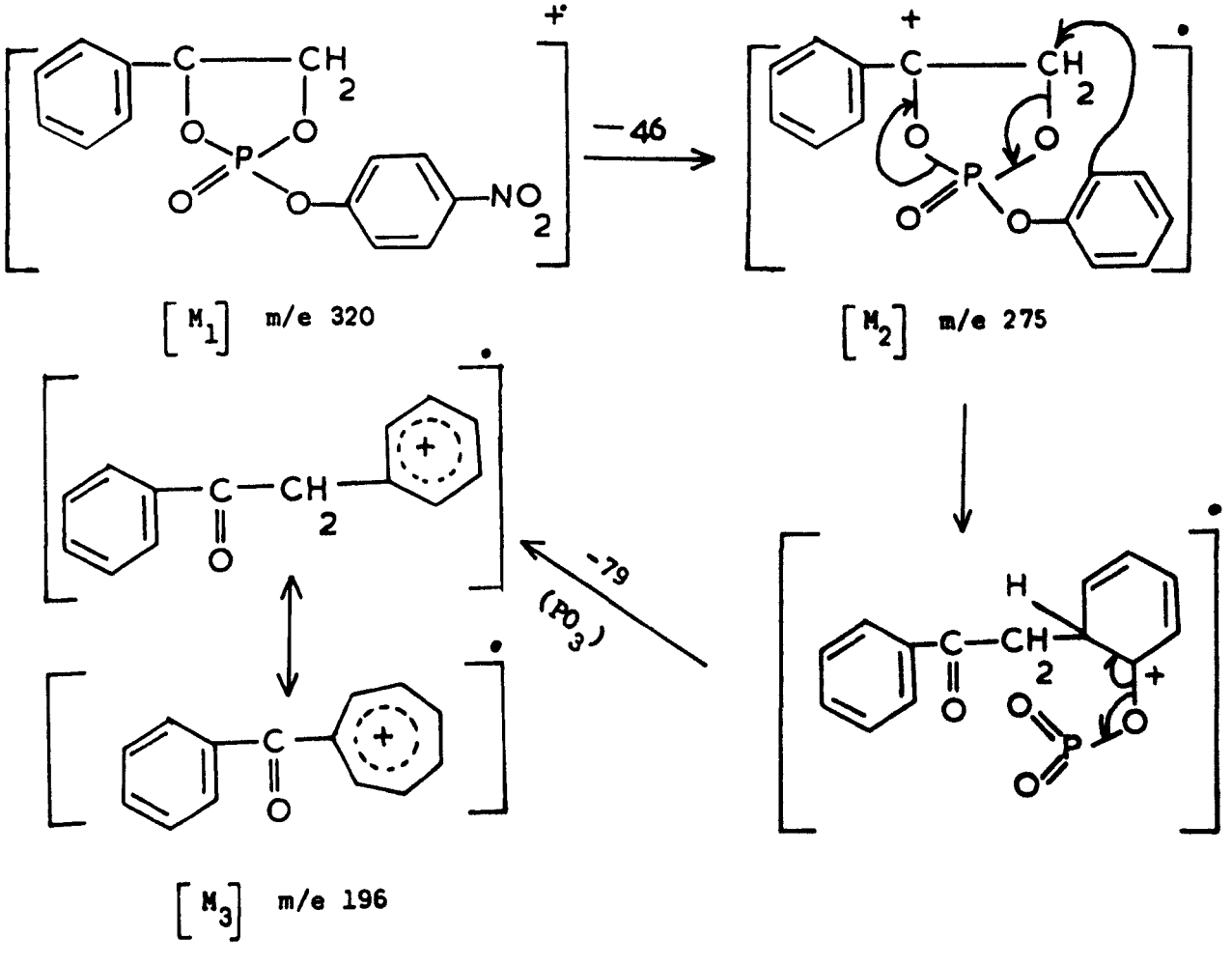

Relative Abundance
m/e
105
320
275
93
65
39
28
47
51
63
119
139
152
166
179
193
196
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320

Figure 19

The $[M_1]$ ion then lost NO_2 to give a peak m/e 275 (relative abundance 3%).

The next fragmentation gave rise to a peak m/e 196 (relative abundance 3-6%); a loss of 79 from the peak m/e 275 $[M_2]$. There was a metastable peak, m/e 138.5, corresponding to a transition of m/e 275 $[M_2]$ to m/e 196 $[M_2 - 79]$ (79 = metaphosphate).

Presumably the elimination of metaphosphate followed a rearrangement, supporting the postulate that the $[M_1]$ ion m/e 320 is a 5-membered cyclic ion;

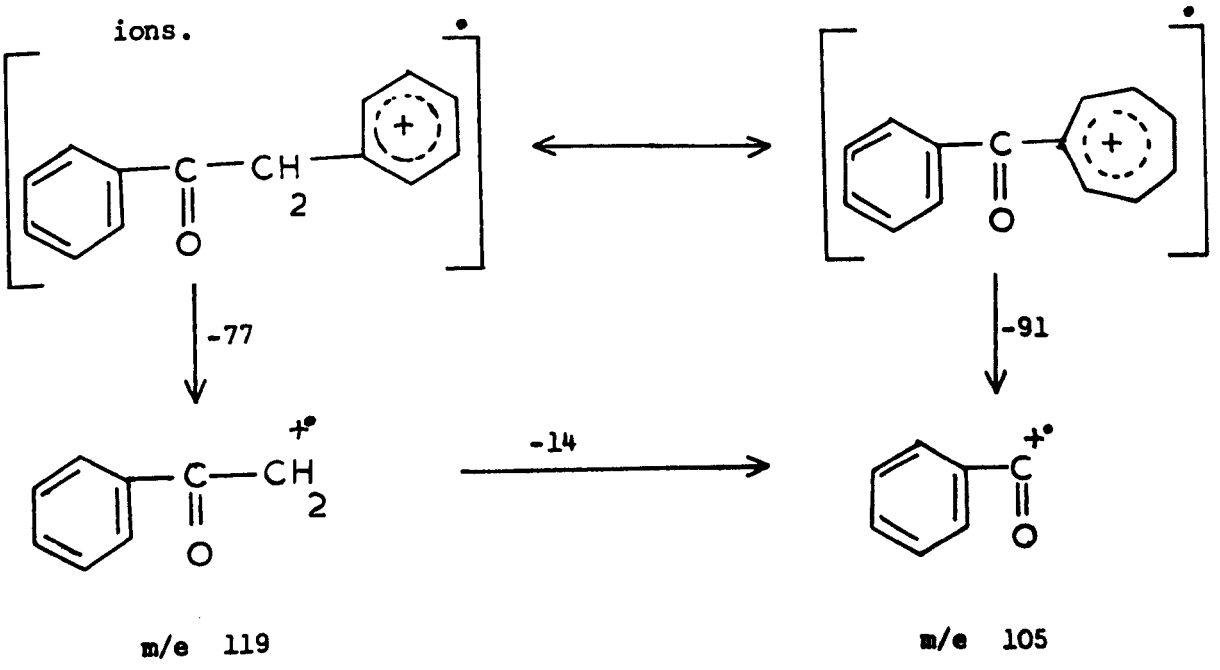


$[M_1]$ m/e 320

$[M_2]$ m/e 275

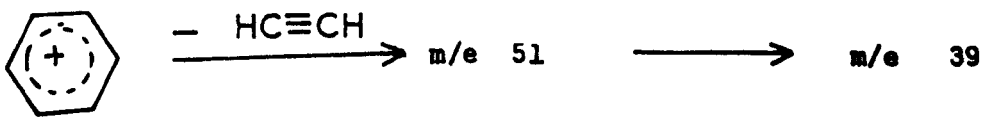
$[M_3]$ m/e 196

The $\left[M_3\right]$ ion could lose either 77 (phenyl ion) or 91 (tropylium ion). Since the tropylium ion is relatively stable, the peak m/e 105 (base peak) was stronger than the peak m/e 119 (relative abundance 12.5%). The fragments m/e 65 (relative abundance 25%), m/e 51 (relative abundance 5.35%) and m/e 39 (relative abundance 21.42%) arose from the breakdown of tropylium and phenyl ions.



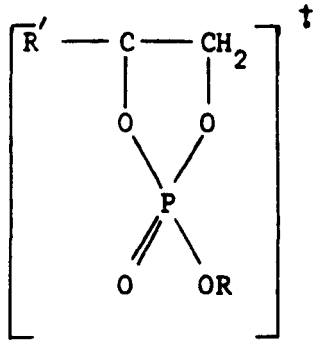
m/e 119 m/e 105

m/e 91 m/e 65 m/e 39



m/e 77 m/e 51 m/e 39

The mechanism discussed above is equally applicable to the mass spectra of other triesters i.e. an ion such as (cliv) is formed in each case.



(cliv)

The mass spectral data is summarised in Table 8. The mass spectra of the triesters are also shown by (Figure 20-23).

The fragmentation pattern of acetonyl benzyl phosphate (Figure 24) was complex. Although it exhibited the loss of OR giving a peak m/e 227 (relative abundance 0.26%), no satisfactory mechanism could be proposed to explain the fragmentation pattern. The possible structures for the fragments are, however, shown below;

TABLE 8

Mass Spectral Data

$(RO)_2PO\cdot O\cdot CH_2\cdot COR'$		M.wt.	Base peak	M - OR		Fragment after rearrangement and metaphosphate elimination	
R	R'		m/e	m/e	% of base peak	m/e	% of base peak
i $PNO_2\cdot C_6H_4$	C_6H_5	458	105	320	64.28%	196	3.57%
ii $PNO_2\cdot C_6H_4$	$(CH_3)_3C$	438	57	300	63.46%	176	1.15%
iii C_6H_5	C_6H_5	368	275	275	100.0%	196	2.08%
iv C_6H_5	$(CH_3)_3C$	348	57	255	48.27%	176	24.13%
v $C_6H_5\cdot CH_2$	C_6H_5	396	91	289	1.7%	196	57.62%
vi $C_6H_5\cdot CH_2$	CH_3	334	18	227	0.26%		

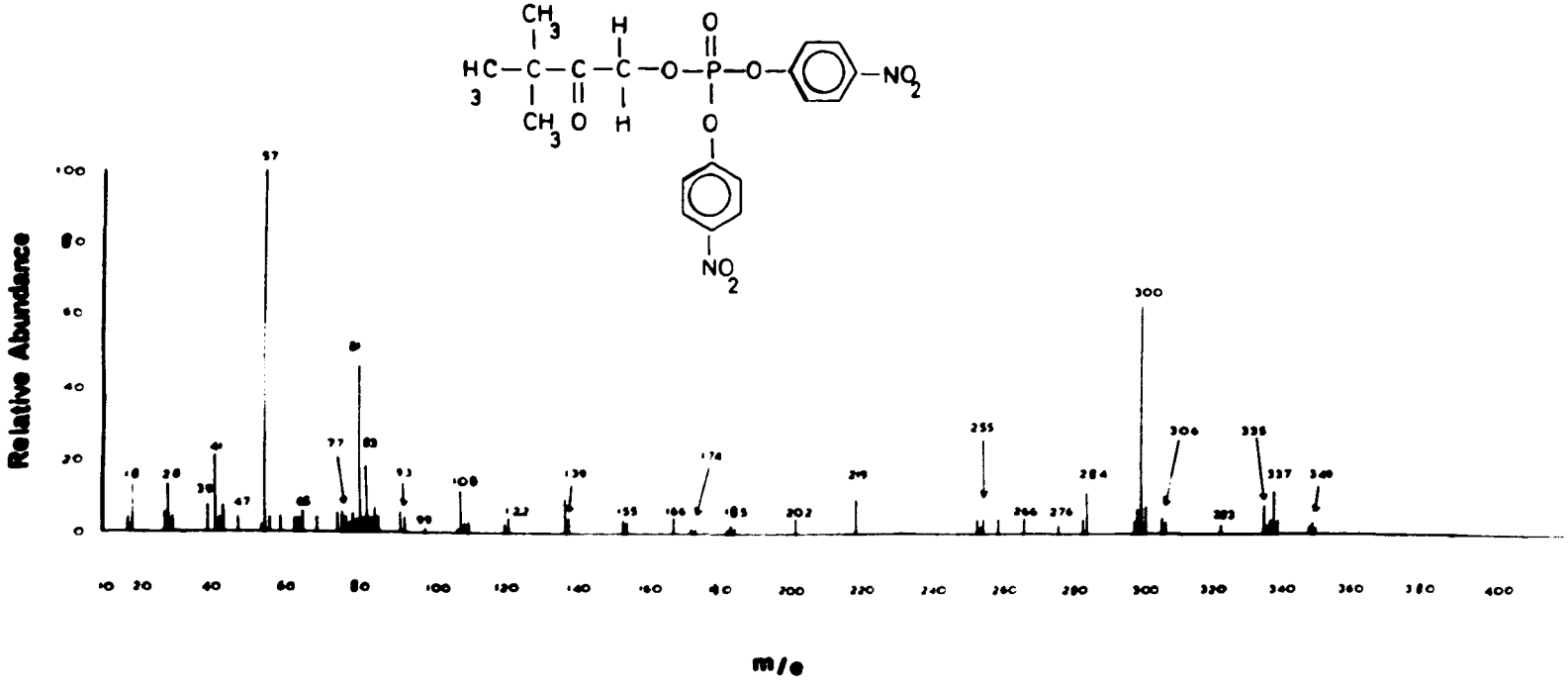

Relative Abundance
m/e
57
300
255
306
335
337
284
174
139
108
77
93
99
122
155
166
185
202
219
266
276
283
340
100
80
60
40
20
0
10
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400

Figure 20

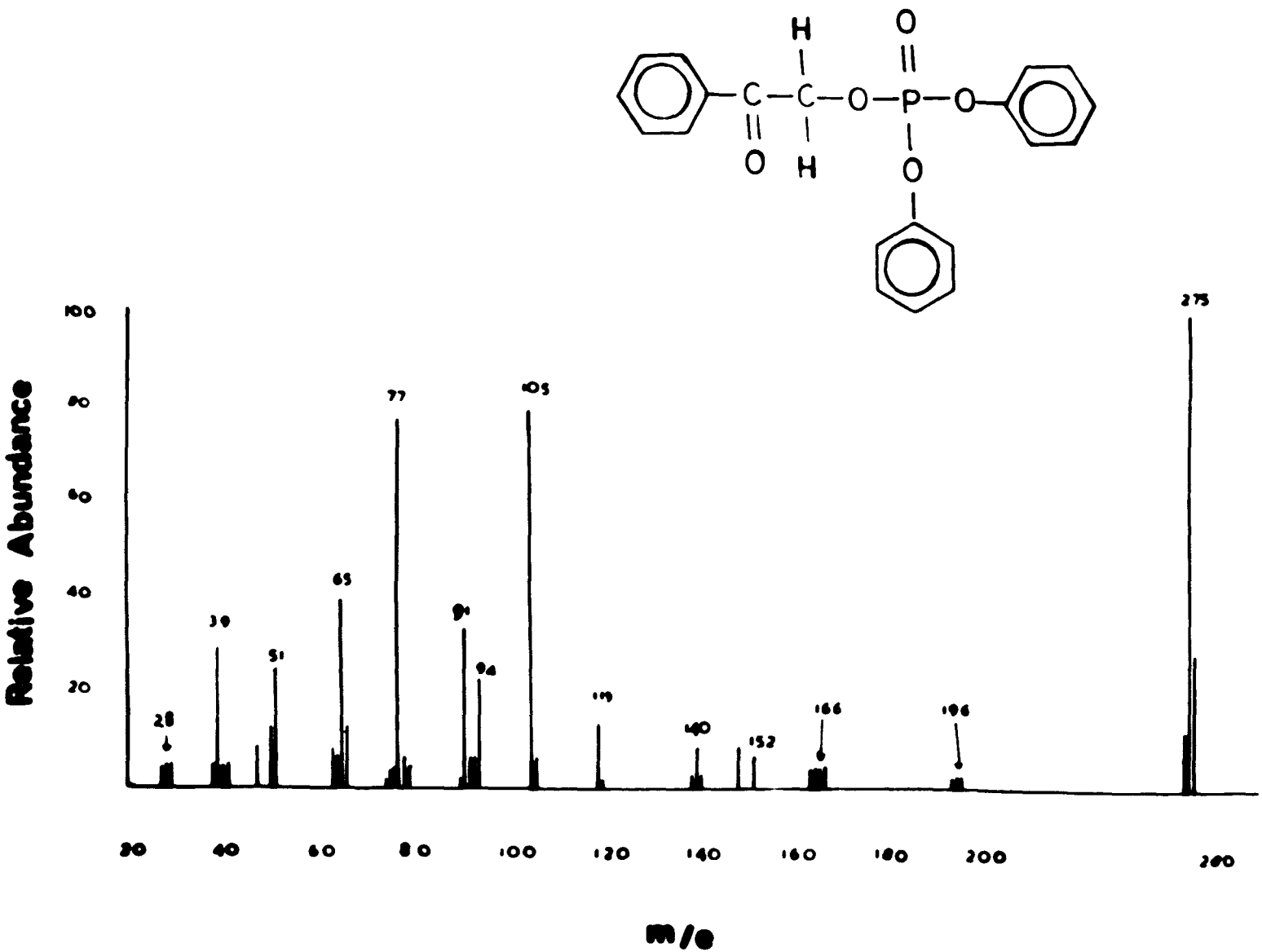

Relative Abundance
100
80
60
40
20
28
39
51
65
77
91
94
105
119
140
152
166
196
275
20
40
60
80
100
120
140
160
180
200
200
m/e

Figure 21

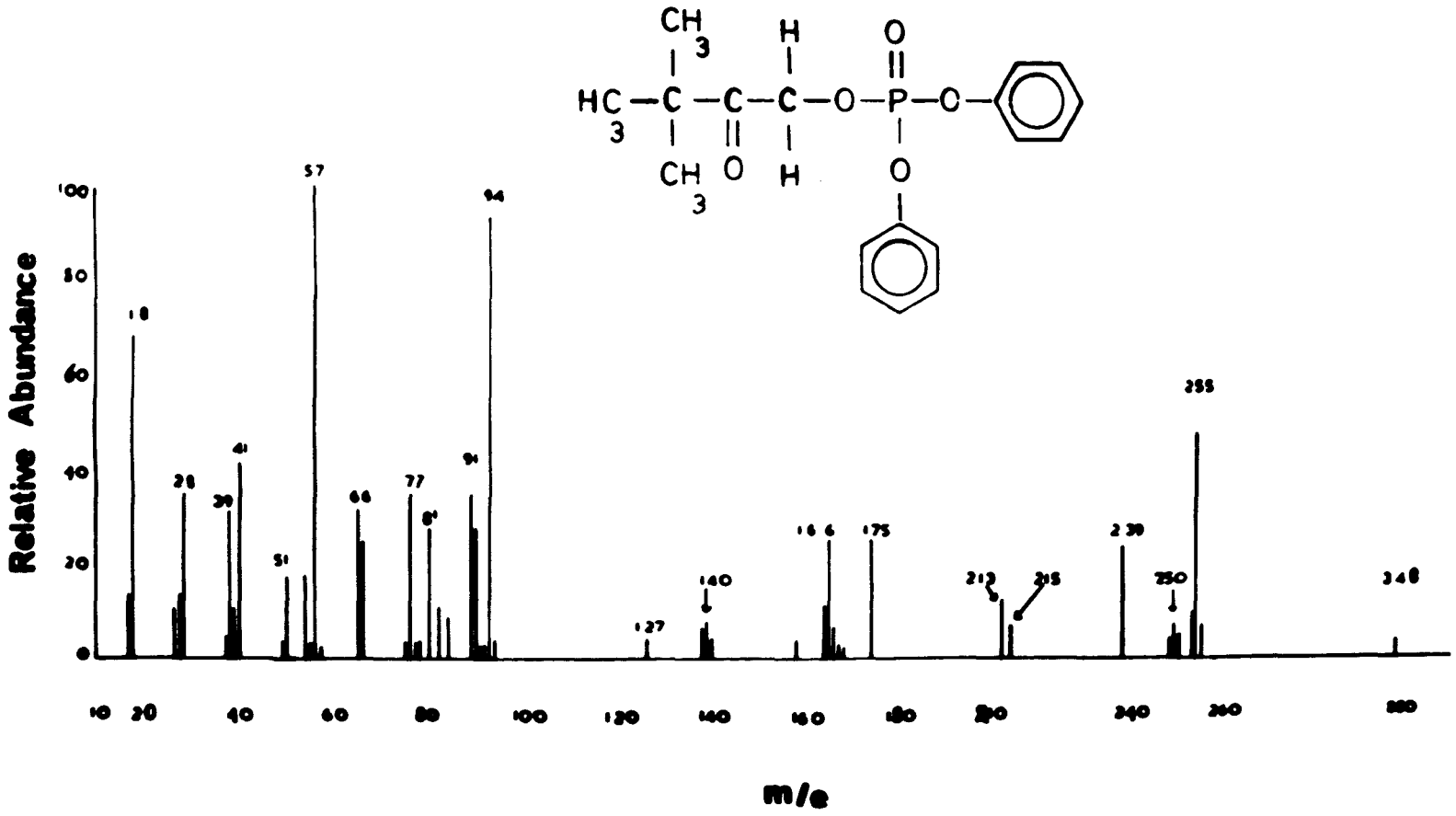


Figure 22

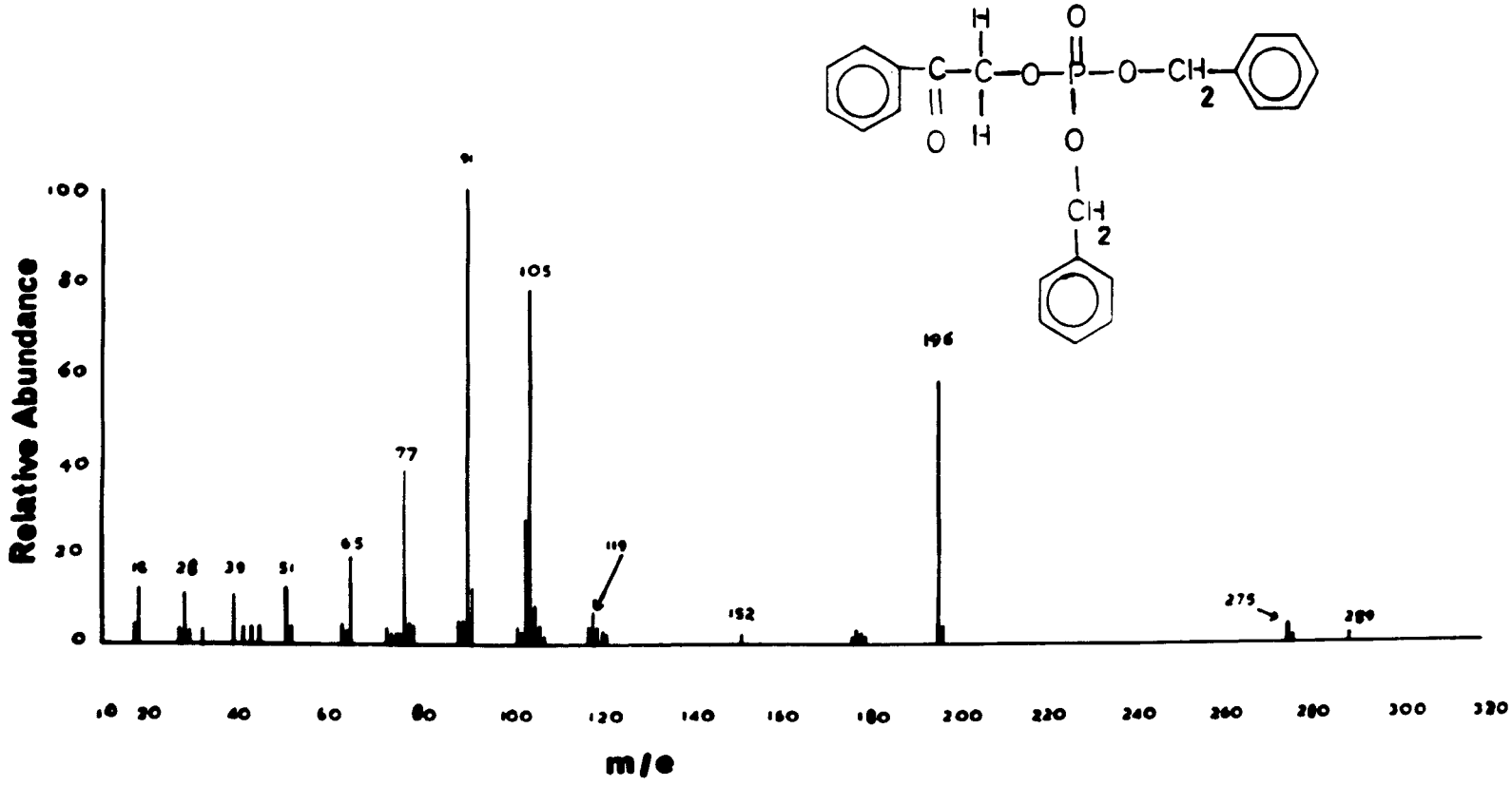

Relative Abundance
100
80
60
40
20
0
91
105
77
65
196
119
152
275
289
10 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320
m/e
H
O
C—C—O—P—O—CH
2
O H
O
CH
2

Figure 23

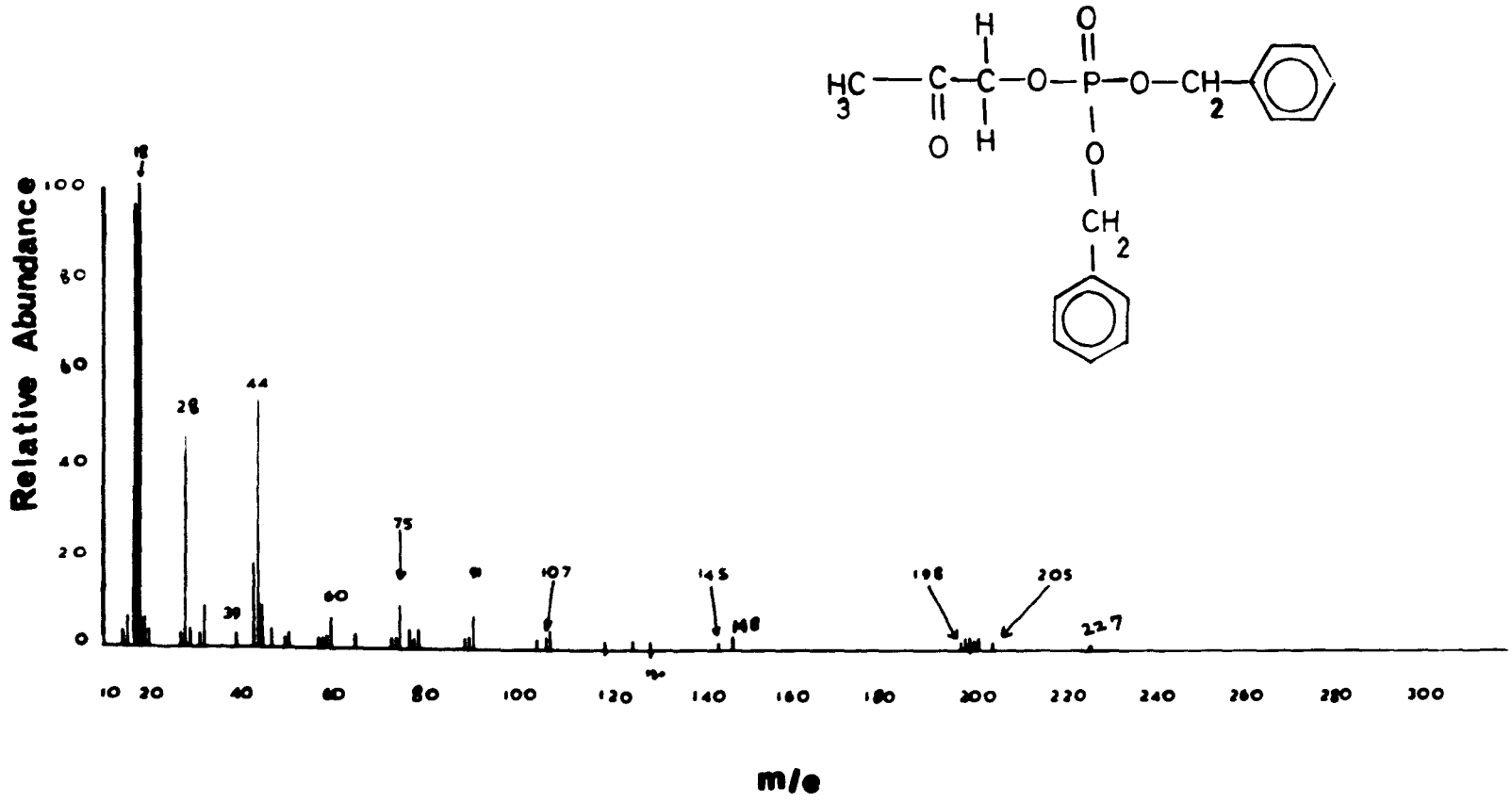
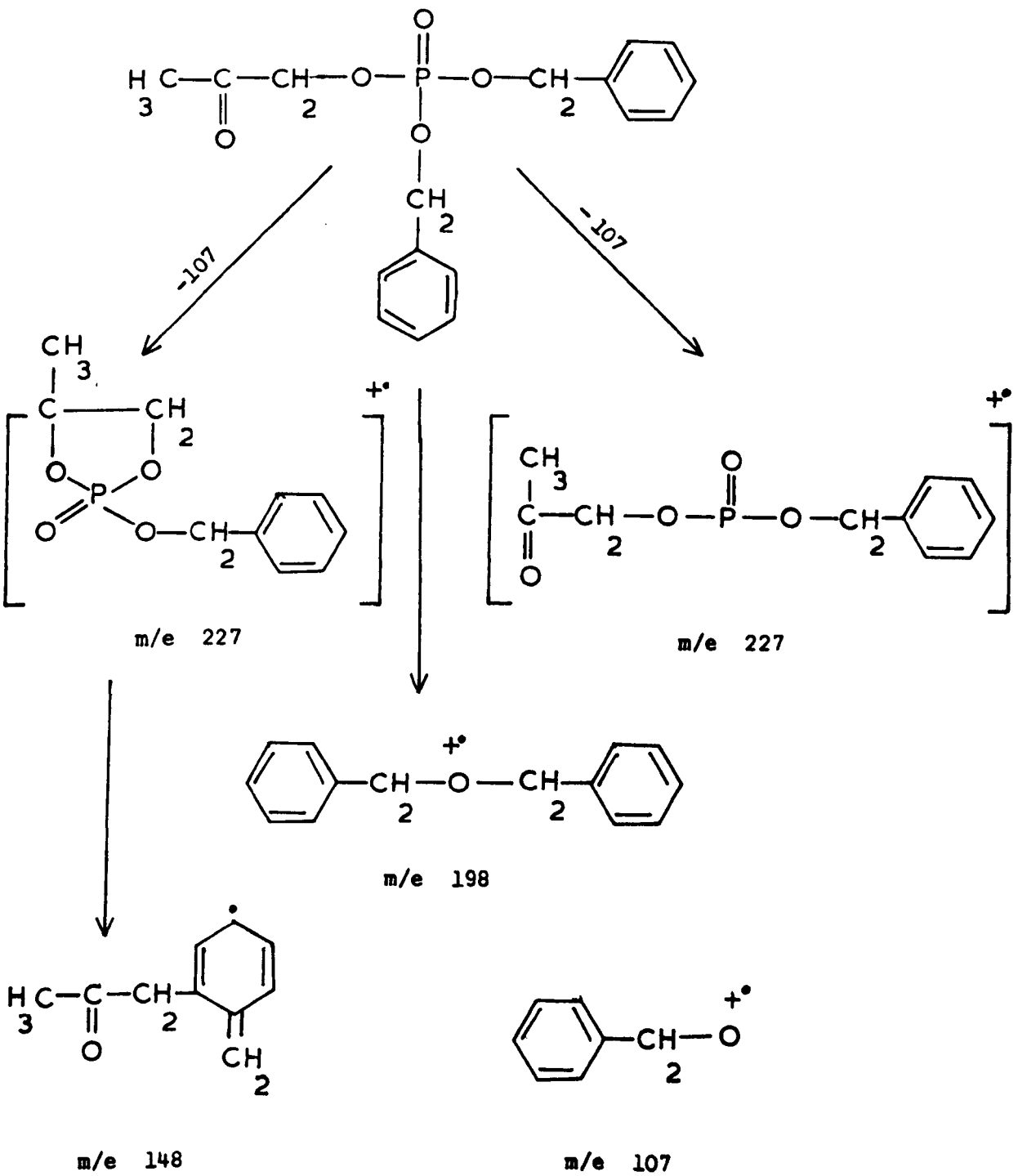

Relative Abundance
100
80
60
40
20
0
28
44
39
60
75
107
145
198
205
227
M+
10 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
m/e

Figure 24


-107
-107
m/e 227
m/e 227
m/e 198
m/e 148
m/e 107

Attempts were also made to isolate the 5-membered cyclic phosphate formed during hydrolysis (suggested intermediate). Although a variety of reactions was carried out, no success could be met. In one case only, where phenacyl di-p-nitrophenyl phosphate was homogenised with alkali (reaction of $LiOH \cdot H_2O$; in $CDCl_3$), the n.m.r. spectrum indicated the presence of a mixture of phenacyl di-p-nitrophenyl phosphate and the product with the loss of p-nitrophenoxide (75:25). No pure cyclic phosphate, however, could be isolated. The reason for this could well be the high instability of the 5-membered cyclic phosphate as described before.

PART III

EXPERIMENTAL

EXPERIMENTAL SECTION

Preliminary Notes

Melting points were determined on a Kofler block and are uncorrected. Infrared absorption (i.r.) spectra were measured on a Perkin-Elmer Infracord or a Perkin-Elmer 221 spectrophotometer. The spectra of solids were determined as potassium bromide discs (K Br Disc), Nujol mulls (Nujol) or in solution (e.g. CCl_4). The spectra of liquids were determined as liquid films (film) or in solution (e.g. CCl_4).

Ultraviolet (u.v.) spectra were recorded on either a Unicam SP 800 or occasionally on a Unicam SP 500 spectrophotometer, whilst nuclear magnetic resonance (n.m.r.) spectra were recorded on a Perkin-Elmer R10,60MHz, instrument and are quoted in units of "tau" (τ) using a tetramethyl silane (T.M.S.) standard. When spectra were recorded in deuterium-oxide, the T.M.S. standard was recorded externally in carbon tetrachloride solution. The following abbreviations are used; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, cs = complex splitting, and H = proton.

Accumulated n.m.r. spectra were obtained using a Jeol J.R.A.1 spectrum accumulator in conjunction with the Perkin-Elmer R10,60MHz instrument. Mass spectra were determined on a Hitachi-Perkin Elmer RMU-6 instrument.

Titrations were performed using a Radiometer automatic titrating machine, Titrigraph pH-stat. Elemental analyses were carried out using a F & M Carbon/Hydrogen/Nitrogen analyser (Model 180) or by Drs. Weiler and Strauss of Oxford. Total phosphorus was determined by the method described by Allen.[298] Phosphorus-32 (P-32) activity was determined using Panax modular equipment and a windowless scintillation counting technique.

Pyridine, methylene dichloride and dimethylformamide were all distilled before use, pyridine being stored over calcium chloride 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 30°C. Tetrahydrofuran was purified and dried by heating under reflux over sodium hydroxide pellets for 3 hours, distilled and heated under reflux over sodium wire for 6 hours, and finally distilled from the sodium.

Paper chromatography. Paper chromatography was carried out on Whatman chromatographic paper No. 1, 4, 3MM and diethylaminoethyl cellulose (DEAE) (Whatman chromedia DE81) ion exchange paper. Unless, otherwise mentioned, a descending technique was used at room temperature, except solvent A used for ascending technique. The solvent systems used were as follows:

Solvent System

A	0.3 ammonium formate
B	n-butanol saturated with water
C	n-propanol - 2 N hydrochloric acid (3 : 1 V/V)
D	ethyl alcohol - 1 M ammonium acetate PH 7.5 (7 : 3 V/V)
E	5% aquous disodium hydrogen phosphate - isopentyl alcohol (3 : 2 V/V)
F	isopropanol - ammonia - water (7 : 1 : 2 V/V)
G	n-butanol - acetic acid - water (4 : 1 : 5 V/V)
H	n-butanol - acetic acid - water (5 : 2 : 3 V/V)
I	isopropanol - ammonia - acetic acid - water (4 : 1 : 2 : 2 V/V)
J	n-propanol - conc. ammonia - water (55 : 10 : 35 V/V)

Nucleotides were located by inspection under ultraviolet light, phosphate esters were detected with the ammonium molybdate/perchloric acid spray.[209]

(α) Base Ratio and sequence specificity in synthetic Polydeoxyribonucleotides

1. Materials

Nucleosides and Nucleotides. Deoxyadenosine, thymidine, and di-sodium salt of thymidine-5'-monophosphate and deoxyadenosine-5'-monophosphate, were obtained either from Sigma chemicals, U.S.A. or from Koch-Light Laboratories. The ammonium salt of thymidine-5'-monophosphate-P-32 was made by the Radiochemical Centre, Amersham.

Phosphorylating Agents. Barium 2-cyanoethyl phosphate (as dihydrate) was obtained from B.D.H. Laboratories, and the pyridinium salt of 2-cyanoethyl phosphoric acid-P-32 was prepared by the Radiochemical Centre, Amersham.

Primer. Polyuridylic acid (as ammonium salt) was obtained from Sigma chemicals, U.S.A. Before use, it was dialysed against 0.5 M sodium chloride, then against distilled water, and finally lyophilised in order to yield the ammonium salt free of small molecules. The product was checked for oligomers in solvent system J, using Whatman 3MM paper.

Condensing reagents. Dicyclohexylcarbodiimide (DCC) and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide, metho-p-toluene sulphonate (water soluble carbodiimide) were obtained from Ralph N. Emanuel Ltd. Ethoxyacetylene was prepared by a modification of the method used by Nazarov and co-workers. It was stored at

-10°c and redistilled before use. The method used was as follows:

1,2-Dibromo-1-ethoxyethane. Ethyl vinyl ether (190 g; 250 mls.) was slowly added to stirred and cooled (-20° to -30°) bromine (422 g; 136 mls.). The mixture was allowed to reach room temperature and the resulting 1,2-dibromo-1-ethoxyethane was used without further purification.

2-Bromo-1-ethoxyethylene. N,N-Diethylaniline (928 g; 988 mls.) was stirred and heated to 105°C under a reduced pressure of 18 mm. The 1,2-dibromo-1-ethoxyethane was added dropwise over the course of three hours during which the 2-bromo-1-ethoxyethylene distilled over into an ice-cooled flask. On fractional distillation of the product, 2-bromo-1-ethoxyethylene (ca 260 g.) distilled b.p. 45 - 50°C at 18 mm. pressure.

Ethoxyacetylene. 2-Bromo-1-ethoxyethylene (260 g.) and powdered potassium hydroxide (520 g.) were stirred and heated to about 110°C, when ethoxyacetylene distilled rapidly into an ice-cold flask. The product was fractionally distilled to give ethoxyacetylene (95 g. ca. 50% from ethyl vinyl ether) b.p. 51 - 53°C.

Enzymes. Phosphodiesterase from Bovine spleen and micrococcal deoxyribonuclease, extracted from staphylococcus strain v.aureus, were obtained from Sigma Chemicals, U.S.A.

Stock solution of 2-Cyanoethyl phosphoric acid-P-32. Barium-2-cyanoethyl phosphoric acid (3.2 g.) and pyridinium-2-cyanoethyl phosphate-P-32 (19.5 mg.) were converted to the acid form by passage

through an Amberlite 1R-120, H^+ form, ion-exchange column. The eluant was evaporated to ca. 10 mls. under reduced pressure and the volume was made up to 50 mls. with pyridine in a graduated flask and this stock solution (1 mM ≡ 5 mls.) was used in the phosphorylation of deoxyribonucleotides.

2. Preparation of P-32 deoxyribonucleotides.

(a) Thymidine-5'-phosphoric acid-P-32.

(i) From Ammonium salt. The ammonium salt of thymidine-5'-phosphoric acid-P-32 (3.95 mg.) which had been prepared by the Radiochemical Centre, Amersham, was converted into its acid form by passage through an Amberlite 1R-120, H^+ form, ion-exchange column. The solution was concentrated to ca. 10 mls. and thymidine—5'-phosphoric acid (171 mg.) added. The volume was made up to 35 mls. to obtain a stock solution (1 ml. ≡ 5 mg.) which was stored under refrigeration and was used in the polymerisation experiments.

(ii) From 3'-acetylthymidine. The method of Michelson and Todd[161] followed by that of Tener[137] was used in this case.

5'-Tritylthymidine. Tritylation of anhydrous thymidine (2.5 g.) with triphenylmethyl chloride (3.5 g.) in anhydrous pyridine (60 mls.), and crystallisation of the product from acetone-benzene, gave pure 5'-tritylthymidine in 83% yield (4.12 g., m.p. 123°, lit., 125°).

3'-Acetyl-5'trityl thymidine. 5'-Tritylthymidine (4.12 g.) was acetylated with freshly distilled acetic anhydride (10 mls.) in

dry pyridine. The pale cream-coloured residue was recrystallised from benzene-light petroleum (40 - 60°) to give pure 3'-acetyl-5'-trityl thymidine in 84% yield (4.075 g., m.p. 103 - 104°, lit. 105°).

3'-Acetylthymidine. 3'-Acetyl-5'tritylthymidine (4.075 g.) was detritylated using 80% acetic acid (17.5 mls.). The white precipitate of triphenylmethanol was separated by filtration and the filtrate evaporated under reduced pressure. The residue was recrystallised from acetone-light petroleum (40 - 60°) to give 3'-acetylthymidine in 67% yield (1.96 g., m.p. 172 - 174°, lit. 176°).

Thymidine-5'-phosphoric acid-P-32. 3'-Acetyl thymidine (56.8 mg.) was phosphorylated using 2-cyanoethylphosphate-P-32 (2 mls. of stock solution) in anhydrous pyridine. After alkaline treatment (1N aqueous sodium hydroxide solution) of the resulting solution followed by filtration, the filtrate was passed through an Amberlite 1R - 120, H^+ form, ion-exchange column. The product in 73% yield (23.58 mg.) was identical in all respects with a commercial sample of thymidine-5'-phosphoric acid.

(iii) From Anhydrous Thymidine. In this case, anhydrous thymidine (242 mg.) was directly phosphorylated with 2-cyanoethyl phosphate-P-32 (2.5 mls. of the stock solution) using dicyclohexylcarbodiimide (310 mg.) in dry pyridine. The product was treated with ammonium hydroxide (5 mls.) and N,N-dicyclohexylurea was separated by filtration. The filtrate was concentrated to ca. 10 mls. and the

mixture was separated by paper chromatography (Whatman chromatographic paper No. 1, solvent F). Thymidine-5'-phosphoric acid-P-32 in 47% yield (150.84 mg.) was obtained.

(b) Deoxyadenosine-5'-phosphoric acid-P-32.[182,137]

3',5'-Diacetyldeoxyadenosine. Anhydrous deoxyadenosine (2 g.) dissolved in dry pyridine (10 mls.) was allowed to react with freshly distilled acetic anhydride (5 mls.) for 24 hours at room temperature. 3',5'-Diacetyldeoxyadenosine was crystallised from ethyl acetate-light petroleum (40 - 60°) in 79% yield (m.p. 135 - 136°, lit. 151 - 152°).

3'-Acetyl and 5'-acetyldeoxyadenosine. 3',5'-Diacetyldeoxyadenosine (21 g.) in ethanol (250 mls.) was partially deacetylated using saturated methanolic ammonia solution (70 mls.) for two hours at room temperature. The residue was separated into its components by counter-current distribution (200 tubes), using ethyl acetate-water as a solvent system, to give deoxyadenosine (0.472 g.), 5'-acetyldeoxyadenosine (0.75 g.), 3'-acetyldeoxyadenosine (0.429 g.) and unchanged 3',5'-diacetyldeoxyadenosine (1.115 g.).

Deoxyadenosine-5'-phosphoric acid-P-32. Phosphorylation of 3'-acetyldeoxyadenosine (34.7 mg.) with 2-cyanoethylphosphoric acid-P-32 (0.5 mls. of the stock solution) using DCC (175 mg.),and then alkaline hydrolysis to remove the acetyl group during work-up, yielded a product which was identified by paper chromatography (Whatman

chromedia DE81, solvent A), using a nucleotide marker. Deoxyadenosine-5'-phosphoric acid-P-32 was isolated in 85% yield (32.89 mg.)

(ii) Tener's method[137] was also used; deoxyadenosine (153 mg. as trihydrate) was allowed to react with 2-cyanoethyl phosphoric acid-P-32 (5 mls. of the stock solution) in the presence of DCC (325 mg.). The 2-cyanoethyl group was removed during work-up by treating with ammonia, and the components of the solution were separated by chromatography on Dowex 1-X8 (chloride 200 - 400 mesh) ion-exchange column. Elution was carried out using a linear gradient of one litre of 0.01N hydrochloric acid into one litre of water. Fractions of about 10 mls. were collected and the optical density of each determined at 260 mμ. Unreacted deoxyadenosine came off at the front followed by a second unidentified nucleotidic component. The deoxyadenosine-5'-phosphoric acid-P-32 came off the column in fractions no. 60 - 86 (48.5 mg.), 30% yield.

3. Polymerisation of deoxyribonucleotides.

(a) General Procedure.

(i) Polymerisation. Thymidine-5'-phosphoric acid and deoxyadenosine-5'-phosphoric acid (one being labelled with P-32) were polymerised either with or without a primer (polyuridylic acid) and using a condensing reagent (ethoxyacetylene or water-soluble DCC). Paper chromatography was performed at each stage and the activity of the sample was determined. In all the experiments, labelled substrates were allowed to react according to the following pattern:

$\overset{*}{p}T$, pdA, soluble DCC

pT, $\overset{*}{p}dA$, soluble DCC

$\overset{*}{p}T$, pdA, E.A.

pT, $\overset{*}{p}dA$, E.A.

where $\overset{*}{p}T$ = thymidine-5'-phosphoric acid-P-32, pT = thymidine-5'-phosphoric acid, $\overset{*}{p}dA$ = deoxyadenosine-5'-phosphoric acid-P-32, pdA = deoxyadenosine-5'-phosphoric acid, soluble DCC = 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate, and E.A. = ethoxyacetylene.

After an appropriate reaction time, polyuridylic acid (if used) was hydrolysed at pH 12 with 0.1N sodium hydroxide for 24 hours at 37°C, the whole was dialysed exhaustively against cold water to yield pure polymer (poly dAT).

(ii) Enzymatic digestion of poly dAT to 3'-nucleotides.

Poly dAT from each preparation was incubated with phosphodiesterase (from Bovine spleen) and micrococcal DNase (from strain v.aureus). (Phosphodiesterase is an exonuclease whilst micrococcal DNase is an endonuclease, and both are specific for 3' → 5' phosphodiester linkage). The 3'-nucleotides were separated by paper chromatography using the solvent system A or F. Each nucleotide was eluted with water, concentrated under reduced pressure, and its activity determined using a windowless scintillation counting assembly with an anthracene crystal. A background count was obtained by eluting a strip of paper where no ultraviolet absorption was observed.

(iii) Calculation. Because the specific activities of the labelled substrates were known, it was then possible by measuring the radioactivity in each of the two 3'-nucleotides from the enzymatic digest of the polymer, to obtain directly the amounts of these nucleotides. The activity lost by paper chromatography was calculated and when compensation was made for this loss, more than 92% of the activity applied on the paper was obtained in all experiments.

(b) Detailed Procedure.

(i) Polymerisation of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid using water soluble carbodiimide as a condensing reagent.

Thymidine-5'-phosphoric acid-P-32 (2.5 mg. of activity 8.0280×10^4 c.p.s.), deoxyadenosine-5'-phosphoric acid (2.5 mg.) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulphonate (water soluble carbodiimide; 300 mg. = 50 fold excess with respect to weight of nucleotides) were dissolved in distilled water (1 ml.) and the reaction flask allowed to stand at room temperature for 48 hours. The product was dialysed exhaustively (48 hours) against cold water and then filtered. The filtrate being concentrated to ca. 0.5 ml., showed only one spot (Rf = 0) when paper chromatograms (developed in solvent system A, B or F using Whatman chromatographic paper No. 1 or Whatman chromedia DE81) were inspected under an ultraviolet light.

A fraction of the solution was pipetted on to a planchette and evaporated to dryness under an infrared lamp. The activity of the sample (3.576×10^3 c.p.s.) was then determined using a windowless scintillation counting assembly with an anthracene crystal.

Nearest Neighbour Base Sequence Analysis. The above solution was shaken with Tris buffer solution (0.2 ml. = 2 μM = 12.2 mg. in 10 mls. water) and calcium chloride solution (0.2 ml. = 1 μM = 5.5 mg. in 10 mls. water). Micrococcal DNase (4.5 mg. = 1350 units) was added and the solution was allowed to digest at 37° for two hours, when the pH was adjusted to 7 using 0.1N hydrochloric acid. Phosphodiesterase (1 unit) was added to this solution which was then

incubated for one hour at 37°. Another unit of phosphodiesterase was added and the solution again incubated (1 hour). This process was repeated once more to make a total of three phosphodiesterase units for the complete degradation of the polymer.

Finally, the solution was concentrated by evaporating under reduced pressure to ca. 0.3 ml.. It was chromatographed on Whatman chromedia DE81 using solvent A to separate thymidine-3'-phosphoric acid from deoxyadenosine-3'-phosphoric acid. Each nucleotide was then cut out and eluted from the paper with water, the eluants being concentrated to 5 mls. A fraction of the solution was pipetted on to a planchette and evaporated to dryness under an infrared lamp. The activity of each sample was determined as mentioned above. (95.67% of radioactivity applied to the paper was recovered in the nucleotide bands).

A background count was obtained by eluting a strip of paper on which no ultraviolet absorption was observed. A fraction of the eluant was evaporated to dryness under an infrared lamp and its activity determined. The results are summarised on page 204.

(ii) Polymerisation of deoxyadenosine-5'-phosphoric acid-P-32 and thymidine-5'-phosphoric acid using water soluble carbodiimide as a condensing reagent.

Polymerisation of deoxyadenosine-5'-phorphoric acid-P-32 (2.5 mg. of activity 1.5893 x 10^4 c.p.s.) and thymidine-5'-phosphoric acid

(2.5 mg.) in the presence of water soluble carbodiimide (300 mg. = 50 fold excess) was achieved in the same manner and under the same conditions as described above.

The reactants were allowed to stand in a reaction flask at room temperature for 48 hours after which, the product was dialysed exhaustively (against cold water) to yield a polymer (Rf = 0; activity 1.427×10^3 c.p.s.). This polymer was then incubated with enzymes at 37^0 for complete degradation. The two components (deoxyadenosine-3'-phosphoric acid and thymidine-3'-phosphoric acid) were separated by paper chromatography and the activities of each nucleotide were determined using a windowless scintillation counting technique (97.68% of the radioactivity applied to the paper was recovered).

A background count was obtained by eluting a strip of paper on which no ultraviolet absorption was observed. The results obtained are tabulated on page 204.

(iii) Polymerisation of deoxyadenosine-5'-phosphoric acid-P-32 and thymidine-5'-phosphoric acid using ethoxyacetylene as a condensing reagent.

Deoxyadenosine-5'-phosphoric acid-P-32 (2.5 mg. of activity 1.8347×10^4 c.p.s.) and thymidine-5'-phosphoric acid (2.5 mg.) were dissolved in distilled water (1 ml.). Ethoxyacetylene (3 mls. = 200 fold excess with respect to weight of nucleotides) was added to the

solution and the whole was allowed to stand at room temperature for 48 hours. The product was dialysed exhaustively (48 hours) against cold water to remove mono- and lower oligonucleotides and then filtered. The filtrate being concentrated to ca. 0.5 ml., showed only one spot (Rf = 0) when paper chromatograms (developed in solvent system A, B or F using Whatman chromatographic paper No. 1 or Whatman chromedia DE81) were inspected under an ultraviolet light. A fraction of the solution was pipetted on to a planchette and evaporated evenly to dryness under an infrared lamp. The activity of the sample (2.829×10^3 c.p.s.) was then determined using a windowless scintillation counting assembly with an anthracene crystal. Nearest Neighbour Base Sequence Analysis. The same procedure was used as mentioned before. The results are shown on page 205.

(iv) Polymerisation of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid using ethoxyacetylene as a condensing reagent.

The polymerisation of thymidine-5'-phosphoric acid-P-32 (2.5 mg. of activity 7.8206×10^4 c.p.s.) and deoxyadenosine-5'-phosphoric acid (2.5 mg.) in the presence of ethoxyacetylene (3 mls. = 200 fold excess) was carried out in the same manner and under the same conditions as described above.

The polymer (Rf = 0 of activity 5.143×10^3 c.p.s.) was dialysed exhaustively against cold water (to remove mono- and lower oligo-

nucleotides) and was used for nearest neighbour base sequence analysis. 97.64% of the radioactivity applied to the paper was recovered in nucleotide bands.

The results are tabulated on page 205.

(v) Polymerisation of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid using water soluble carbodiimide as a condensing reagent and polyuridylic acid as a primer.

The ammonium salt of polyuridylic acid (5 mg.) was converted into its acid form by passage through an Amberlite IR - 120, H^+ form, ion-exchange column. The eluant was concentrated to ca. 0.5 ml., and thymidine-5'-phosphoric acid-P-32 (2.5 mg. of activity 6.3750×10^4 c.p.s.), deoxyadenosine-5'-phosphoric acid (2.5 mg.), and 1-cyclohexyl-3 (-2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate (300 mg. = 50 fold excess with respect to weight of nucleotides) were added one after the other. The material was dissolved and the reaction flask was allowed to stand at room temperature for 48 hours.

The resulting solution was adjusted to pH 12 with 0.1N sodium hydroxide and kept at this pH for 24 hours at 37°C. The alkaline solution was dialysed exhaustively (48 hours) against cold water, to remove mono- and lower oligonucleotides and then filtered. The filtrate after concentration to ca. 5 mls., showed only one spot

(Rf = 0) when paper chromatograms (developed in solvent system A, B or F using Whatman chromatographic paper No. 1 or Whatman chromedia DE81) were inspected under an ultraviolet light.

A fraction of the solution was pipetted on to a planchette and evaporated evenly to dryness under an infrared lamp. The activity of the sample (2.130×10^3 c.p.s.) was then determined using a windowless scintillation counting assembly with an anthracene crystal.

Nearest Neighbour Base Sequence Analysis. The same technique, as described before, was used.

The solution was shaken with Tris buffer solution (0.2 ml. = 2 μM = 12.2 mg. in 10 ml. water pH 8.5) and aqueous calcium chloride solution (0.2 ml. = 1 μM = 5.5 mg. in 10 ml. water). Micrococcal-DNase (4.5 mg. = 1350 units) was added and the solution incubated at 37°C for two hours after which the pH was adjusted to 7 using 0.1N hydrochloric acid. To this solution phosphodiesterase (1 unit) was added and the solution incubated for a further hour at 37°C. Another unit of phosphodiesterase was added, followed by one more 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 ca. 0.3 ml. by evaporation under reduced pressure. It was chromatographed on Whatman chromedia DE81 using solvent A to separate thymidine-3'-phosphoric acid from

deoxyadenosine-3'-phosphoric acid. Each nucleotide spot was then cut out and eluted from the paper with water. The eluants were concentrated to ca. 0.3 ml. Aliquots of the solutions were pipetted on to a planchette and evaporated down evenly to dryness under an infrared lamp. The activity of each was determined as mentioned above.

A background count was obtained by eluting a strip of paper on which no ultraviolet absorption was observed, a fraction of the eluant was evaporated to dryness and its activity determined.

One more polymerisation of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid was carried out in the same manner. Nearest neighbour base sequence analysis was performed as mentioned above except that 3'-nucleotides were separated on Whatman chromatographic paper No. 1 using solvent F, after enzymatic degradation. The results obtained are summarised on page 206.

(vi) Polymerisation of deoxyadenosine-5'-phosphoric acid-P-32 and thymidine-5'-phosphoric acid using water soluble carbodiimide as a condensing reagent and polyuridylic acid as a primer.

The same procedure as described above was used. Polymerisation was achieved successfully in the same fashion and under the same conditions except that in this case deoxyadenosine-5'-phosphoric acid was labelled with P-32 in place of thymidine-5'-phosphoric acid.

Thus polyuridylic acid obtained from its ammonium salt (5 mg.) by passing through an Amberlite I.R 1 120, H^+ form, ion-exchange

column was mixed with deoxyadenosine-5'-phosphoric acid P-32 (2.5 mg., of activity 1.7772 x 10^4 c.p.s.) and thymidine-5'-phosphoric acid (2.5 mg.). 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate (300 mg. = 50 fold excess) was added and the solution kept at room temperature for 48 hours, after which the pH was adjusted to 12 with 0.1N sodium hydroxide and kept at this pH for 24 hours at 37°c. The alkaline solution was then dialysed exhaustively and the polymeric product (total activity 7.025 x 10^3 c.p.s.) was used for the nearest neighbour base sequence analysis (as described previously). The 3'-mononucleotides resulting from the enzymatic degradation of the polymer were separated on Whatman chromedia DE81 using solvent A as well as on Whatman chromatographic paper No. 1 using solvent F. 98% of the radioactivity applied to the paper was recovered. One more polymerisation was carried out using the same procedure. The results are tabulated on page 207.

(vii) Polymerisation of thymidine-5'-phosphoric acid—P-32 and deoxyadenosine-5'-phosphoric acid using ethoxyacetylene as a condensing reagent and polyuridylic acid as a primer.

The ammonium salt of polyuridylic acid (5 mg.) was converted into its acid form by passage through an Amberlite IR - 120, H^+ form, ion-exchange column. The eluant was concentrated to ca. 0.5 ml. and shaken with thymidine-5'-phosphoric acid-P-32

(2.5 mg., of activity 1.2044 x 10^4 c.p.s.) and deoxyadenosine-5'-phosphoric acid (2.5 mg.). Ethoxyacetylene (3 mls. = 200 fold excess with respect to weight of nucleotides) was added and the solution allowed to stand at room temperature for two days.

The resulting solution was adjusted to pH 12 with 0.1N sodium hydroxide and kept at this pH for 24 hours at 37°C. The alkaline solution was dialysed exhaustively (two days) against cold water, to remove mono- and lower oligonucleotides and then filtered. The filtrate after being concentrated to ca. 5 mls. showed only one spot (Rf = 0) when paper chromatograms (developed in solvent system A, B or F using Whatman chromatographic paper No. 1 or Whatman chromedia DE81) were inspected under an ultraviolet light.

A fraction of the solution was pipetted on to a planchette and evaporated evenly to dryness under an infrared lamp. The activity of the sample (2.415 x 10^3 c.p.s.) was then determined using a windowless scintillation counting assembly with an anthracene crystal.

Nearest Neighbour Base Sequence Analysis. This was carried out essentially as described previously and 97.5% of the radioactivity applied to the paper was recovered.

One more polymerisation of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid was carried out. Details were as above. The results are tabulated on page 208.

(viii) Polymerisation of deoxyadenosine-5'-phosphoric acid-P-32 and thymidine-5'-phosphoric acid using ethoxyacetylene as a condensing reagent and polyuridylic acid as a primer.

Two experiments were carried out with the parameters as described above. Polymerisations were achieved successfully in the same fashion and under the same conditions except that in this case deoxyadenosine-5'-phosphoric acid was labelled with P-32 in place of thymidine-5'-phosphoric acid. The polymeric product was used for nearest neighbour base sequence analysis (described above). The 3'mononucleotides, resulting from enzymatic degradation of polymer, were separated by paper chromatography and 92 - 94% of the total activity applied to paper was recovered. The results are summarised on page 209.

RESULTS OF NEAREST NEIGHBOUR BASE SEQUENCE ANALYSIS

*Polymerisation of thymidine-5'-phosphoric acid, and deoxyadenosine-5'-phosphoric acid, in the absence of primer

Labelled Nucleotide	Initial Activity counts sec^{-1}	Activity after Dialysis counts sec^{-1}	Separation of Mononucleotides by paper chromatography		
			Deoxyadenosine-3'-phosphoric acid counts sec^{-1}	Thymidine-3'-phosphoric acid counts sec^{-1}	Ratio $\frac{dAp}{Tp}$
Thymidine-5'-phosphoric acid	80280	3576	395	2648	1 : 6.703
Deoxyadenosine-5'-phosphoric acid	15893	1427	147	968	1 : 6.585

* With 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate.

RESULTS OF NEAREST NEIGHBOUR SEQUENCE ANALYSIS

*Polymerisation of thymidine-5'-phosphoric acid, and deoxyadenosine-5'-phosphoric acid, in the absence of primer

Labelled Nucleotide	Initial Activity counts sec^{-1}	Activity after Dialysis counts sec^{-1}	Separation of Mononucleotides by paper chromatography		
			Deoxyadenosine-3'-phosphoric acid counts sec^{-1}	Thymidine-3'-phosphoric acid counts sec^{-1}	Ratio $\frac{dAp}{Tp}$
Thymidine-5'-phosphoric acid	78206	5143	572	3446	1 : 6.024
Deoxyadenosine-5'-phosphoric acid	18347	2829	259	1691	1 : 6.528

* With Ethoxyacetylene.

RESULTS OF NEAREST NEIGHBOUR BASE SEQUENCE ANALYSES

*Polymerisation of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid in the presence of polyuridylic acid

Initial Activity counts sec^{-1}	Activity after Dialysis counts sec^{-1}	Separation of Mononucleotides by paper chromatography		
		Deoxyadenosine-3'-phosphoric acid counts sec^{-1}	Thymidine-5'-phosphoric acid counts sec^{-1}	Ratio $\frac{dAp}{Tp}$
63750	2131	491	1099	1 : 2.238
12045	1395	291	592	1 : 2.034

* With 1-Cyclohexyl-3-(2-morpholinoethyl) Carbodiimide metho-p-toluenesulphonate.

RESULTS OF NEAREST NEIGHBOUR BASE SEQUENCE ANALYSES

*Polymerisation of deoxyadenosine-5'-phosphoric acid-P-32
and thymidine-5'-phosphoric acid
in the presence of polyuridylic acid

Initial Activity counts sec^{-1}	Activity after Dialysis counts sec^{-1}	Separation of Mononucleotides by paper chromatography		
		Deoxyadenosine-3'-phosphoric acid counts sec^{-1}	Thymidine-3'-phosphoric acid counts sec^{-1}	Ratio $\frac{dAp}{Tp}$
17772	7025	2336	4554	1 : 1.95
27040	4717	598	1689	1 : 2.824

* With 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate.

RESULTS OF NEAREST NEIGHBOUR BASE SEQUENCE ANALYSES

*Polymerisation of thymidine-5'-phosphoric acid-P-32 and deoxyadenosine-5'-phosphoric acid in the presence of polyuridylic acid

Initial Activity counts sec^{-1}	Activity after Dialysis counts sec^{-1}	Separation of Mononucleotides by paper chromatography		
		Deoxyadenosine-3'-phosphoric acid counts sec^{-1}	Thymidine-5'-phosphoric acid counts sec^{-1}	Ratio $\frac{dAp}{Tp}$
12044	2415	608	1323	1 : 2.175
9401	506	147	270	1 : 1.836

* With Ethoxyacetylene

RESULTS OF NEAREST NEIGHBOUR BASE SEQUENCE ANALYSES

*Polymerisation of deoxyadenosine-5'-phosphoric acid-P-32
and thymidine-5'-phosphoric acid
in the presence of primer

Initial Activity counts sec^{-1}	Activity after Dialysis counts sec^{-1}	Separation of Mononucleotides by paper chromatography		
		Deoxyadenosine-3'-phosphoric acid counts sec^{-1}	Thymidine-3'-phosphoric acid counts sec^{-1}	Ratio $\frac{dAp}{Tp}$
19387	2142	628	1245	1 : 1.982
28961	1330	309	742	1 : 2.401

* With Ethoxyacetylene

(β) α-Ketotriesters of Phosphoric Acid

Diazomethane[291] prepared from N-nitrosomethyl urea[292] was used for the preparation of diazoketones (ω-diazoacetophenone,[293,294] diazoacetone,[294,295] and 1-diazo-3,3-dimethylbutanone.[296]

Di-p-nitrophenyl,[258] diphenyl,[250,251] and dibenzyl[134] phosphoric acids were either prepared by standard methods or obtained from R.N. Emanuel or Sigma Chemicals U.S.A.

The α-ketotriesters of phosphoric acid were prepared by the reaction of an α-diazoketone with an appropriate diester of phosphoric acid as described:[284]

(a) Preparation of α-ketotriesters of phosphoric acid

(i) Phenacyl di-p-nitrophenyl phosphate

Di-p-nitrophenyl phosphoric acid (3 g; 0.08 M) was slowly added to a solution of ω-diazoacetophenone (1.4 g; 0.01 M) in a benzene-chloroform (2:1, 15 ml.) mixture at reflux temperature. After a further hour at this temperature, the solution was cooled, washed rapidly with ice-cold sodium bicarbonate solution and dried ($MgSO_4$). The solution was evaporated to dryness and the product recrystallised from benzene/light petroleum (b.p. 60-80°) to yield pure phenacyl di-p-nitrophenyl phosphate, (2.86 g; 70•74%) m.p. 118-118.5° (lit.[284] m.p. 121°).

mass spectrum : m/e 275 (M-93) (base peak), 196, 119, 105

Found : C 65.6; H = 4.47%

Calculated for

$C_{20}H_{17}PO_5$: C = 65.1; H = 4.61%

(iii) Phenacyl dibenzyl phosphate

Dibenzyl phosphoric acid (2.5 g; 0.009 M) was allowed to react with ω-diazoacetophenone (1.47 g; 0.01 M) in dry dioxan (40 ml.) at 100° for 8 hrs. The solution was evaporated to dryness (reduced pressure) and the product was dissolved in chloroform, washed with ice-cold sodium bicarbonate solution, dried ($MgSO_4$) and the triester was obtained as a pure liquid (2.78 g; 78.3%), b.p. 182-184°/ 0.15 m.m. (previously described as light yellow oil[284]).

λ_{max} (95% EtOH) : 248.5, 280 nm.

ν_{max} (film) : 1715 (C=O), 1460 (CH_2 vibration), 1295-1280 cm^{-1} (P=O)

n.m.r. ($CDCl_3$) : 2.18-2.85 (m, 15 H), 4.85τ (2d, superimposed, J = 9 Hz; 12 Hz; 6 H)

mass spectrum : m/e 289 (M-107), 196, 119, 105, 91 (base peak)

Found : C = 67.9; H = 5.6%

Calculated for

$C_{22}H_{21}PO_5$: C = 66.7; H = 5.3%

(iv) Pivaloyl di-p-nitrophenyl phosphate

This was prepared by a method similar to that used for phenacyl di-p-nitrophenyl phosphate using di-p-nitrophenyl phosphoric acid (3.0 g; 0.009 M) and 1-diazo-3,3-dimethylbutanone (1.56 g; 0.0124 M). The recrystallisation from benzene/light petroleum (b.p. 60-80°) yielded pure pivaloyl di-p-nitrophenyl phosphate (2.95 g; 76%), m.p. 104° (lit.[284] m.p. 103-4°).

λ_{max} (95% EtOH) : 268, 231 nm.

ν_{max} (KBr) : 1730 (C=O), 1435 (CH_2 vibration), 1310 cm^{-1} (P=O)

n.m.r. ($CDCl_3$) : 1.65-2.65 (m, Cs, 8 H), 4.9τ (d, J = 12 Hz; 2 H), 8.8τ (s, 9H)

mass spectrum: : m/e 300 (M-138), 123, 255, 176, 57 (base peak)

Found : C = 49.6; H = 4.63, N = 6.4%

Calculated for

$C_{18}H_{19}N_2PO_9$: C = 49.32; H = 4.34; N = 6.39%

(v) Pivaloyl diphenyl phosphate

Diphenyl phosphoric acid (1.22 g; 0.005 M) was added to a solution of 1-diazo-3,3-dimethylbutanone (0.65 g; 0.005 M) in dry benzene (20 ml.) at room temperature. After 30 min., the solution was heated under reflux for 3 hrs. and the product was worked up as above to yield the triester (1.2 g; 71%), b.p. 178-180°/0.02 mm.

ν_{max} (film) : 1730 (C=O), 1495 (CH_2 vibration). 1300 cm^{-1} (P=O)

n.m.r. ($CDCl_3$) : 2.7-3.3 (m, 10 H), 5.0τ (d, J = 11 Hz; 2 H), 8.9τ (s, 9 H)

mass spectrum : m/e 348 (M^+), 255 (M-93), 175, 94, 91, 85, 57 (base peak)

Found : C = 62.4; H = 6.29%

Calculated for

$C_{18}H_{21}PO_5$: C = 62.1; H = 6.03%

(vi) Acetonyl dibenzyl phosphate

Dibenzyl phosphoric acid (2.78 g; 0.01 M) and diazoacetone (0.96 g; 0.011 M) were heated in dry dioxan at 75° for 3 hrs. after which the solvent was removed under reduced pressure. Working up as above yielded triester as yellow liquid (0.78 g; 23%), b.p. 168°/0.02 mm. with decomp. (previously described as pale yellow liquid[284]).

λ_{max} (95% EtOH)	:	263 nm.
ν_{max} (film)	:	1750 (C=O), 1450 (CH_2 vibration), 1300 cm^{-1} (P=O)
n.m.r. ($CDCl_3$)	:	2.7 (m, 10 H), 4.9τ (2d, superimposed, J = 10, 9 Hz; 4 H), 5.6τ (d, J = 11 Hz; 1 H), 8.0τ (s, 3 H)
mass spectrum	:	m/e 227 (M-107), 205, 198, 148, 107, 91, 18 (base peak)
Found	:	C = 61.2; H = 5.7%
Calculated for $C_{17}H_{19}PO_5$	:	C = 61.1; H = 5.68%

(vii) Acetonyl di-p-nitrophenyl phosphate

Di-p-nitrophenyl phosphoric acid (1.5 g; 0.0045 M) was added portionwise to a solution of diazoacetone (1.68 g; 0.002 M) in benzene (20 ml.) and chloroform (10 ml.). The solution was refluxed for a further three hours, cooled and washed with sodium bicarbonate solution. The organic layer was dried ($MgSO_4$) and worked up as above. Recrystallisation from benzene/light petroleum (b.p. 60-80°) yielded the impure triester (0.714 g; 40.8%), m.p. 164-165°.

Found : C = 44.7; H = 3.71; N = 6.1%

Calculated for

$C_{15}H_{13}N_2PO_9$: C = 45.45; H = 3.3; N = 7.1%

(b) Alkaline Hydrolysis of phenacyl di-p-nitrophenyl phosphate

All titrations, including the blank experiments, were performed under an atmosphere of CO_2-free nitrogen. The titrations were carried out at pH 9 and pH 11.

(i) At pH 9

Phenacyl di-p-nitrophenyl phosphate (22.9 mg.) was dissolved in aqueous tetrahydrofuran (1:1 v/v; 20 ml.) and titrated against N/5.191 sodium hydroxide using the automatic titrator set at pH 9. At pH 6 the solution became yellow and the uptake of alkali ceased after 264 seconds. The volume of alkali (0.273 ml.; 1 mole = 0.259 ml.) used at this pH remained constant for 10 minutes; $t_{\frac{1}{2}}$ = 33 sec.

(ii) At pH 11

The procedure was the same as above except that the titrations were carried out at pH 11. The volume of alkali used at this pH was 0.52 ml. (2 mole = 0.519 ml.); $t_{\frac{1}{2}}$ = 18 seconds.

(C) Attempted cyclisation reactions of α-ketotriesters of phosphoric acid

(i) 2,4-Dinitrophenylhydrazine (0.19 g; 1 mM) was dissolved in boiling ethanol (10 ml.) and the solution was allowed to cool after the addition of a few drops of conc. HCl. Phenacyl di-p--nitrophenyl phosphate (0.459 g; 1 mM) was then added, the mixture warmed and allowed to stand at room temperature to yield the 2,4--dinitrophenylhydrazine derivative of phenacyl di-p-nitrophenyl phosphate (0.34 g; 52.3%), m.p. 202-4°.

Found: C = 47.8; H = 2.93; N = 13.4%

Calculated for

$C_{26}H_{19}N_6PO_{12}$: C = 48.9; H = 2.97; N = 13.16%

(ii) 2,4-Dinitrophenylhydrazine reagent prepared as above was reacted with phenacyl diphenyl phosphate (0.378 g; 1 mM) and the hydrazone was collected after filtration, washing with ethanol and drying under vacuum. Yellow solid (0.313 g), m.p. 178-80°.

Found : C = 53.0; H = 3.63; N = 15.6%

Calculated for

$C_{26}H_{21}N_4PO_8 \cdot C_6H_3(NO_2)_2NHNH_2$: C = 51.47; H = 3.61; N = 15.01%

(iii) Phenacyl di-p-nitrophenyl phosphate (0.23 g; 0.0005 M) dissolved in deuterochloroform (2 ml.) was shaken with sodium hydroxide (2 ml., 20 mg., 0.0005 M) at room temperature for 15 min. The $CDCl_3$

layer, after drying ($MgSO_4$), was used to obtain a n.m.r. spectrum. The same pattern as that of the starting material was observed except for (in $CDCl_3/D_2O$) the appearance of a new singlet at 5.18τ (1 H).

(iv) Using an equimolar mixture of the reactants, the above reaction was repeated (reflux temp. for 10 min.) to obtain a yellow solid, m.p. 114-16°. N.m.r. and i.r. spectra showed the solid to be the starting material.

(v) Phenacyl diphenyl phosphate (0.368 g; 1 mM) was allowed to react with sodium methoxide (0.054 g; 1 mM) in methanol (5 ml.) for half an hour at room temperature. The solution was evaporated (reduced press.), the residue dissolved in $CDCl_3$ and filtered. The filtrate was used for n.m.r., i.r. and T.L.C. (thin layer chromatography). No cyclic phosphate could be identified.

(vi) Phenacyl di-p-nitrophenyl phosphate was allowed to react with Grignard reagents (C_2H_5MgBr; C_6H_5MgCl; or t-BuMgCl; prepared by the conventional methods) for 12-16 hrs. under anhydrous conditions at room temperature. The products were hydrolysed with cracked ice to precipitate magnesium hydroxide which was again dissolved by addition of a few drops of conc. HCl. Extraction with diethyl ether always gave complex mixtures containing 4-6 fractions (T.L.C. or column chromatography).

(vii) Phenacyl di-p-nitrophenyl phosphate was allowed to react with the sodio derivative of dimethyl malonate (prepared from dimethyl malonate and sodium methoxide in methanol). The product was a mixture of 6 fractions but no cyclic phosphate.

(viii) Phenacyl di-p-nitrophenyl phosphate was reacted with phenyl lithium under anhydrous conditions and 7 fractions were obtained from the reaction product. No evidence of cyclic phosphate.

(ix) Phenacyl di-p-nitrophenyl phosphate was reacted with lutidine at reflux temperature (benzene solvent) for 3 hours. The solution (changed from yellow to brown colour) was cooled and evaporated under reduced pressure to yield a brown solid (m.p. 114-119°). Recrystallisation from methanol and then benzene/petroleum (b.p. 60-80°) yielded the starting material, phenacyl di-p-nitrophenyl phosphate. The mother liquor was found to be a mixture of 5 components but no desired product.

(x) Phenacyl di-p-nitrophenyl phosphate was also reacted with dimethylamine; p-nitrophenol and a complex mixture of products was obtained but no cyclic compound could be identified.

(xi) Phenacyl di-p-nitrophenyl phosphate dissolved in tetrahydrofuran was stirred (5 hrs.) with aqueous potassium hydroxide under N_2 atmosphere. The resulting solution was evaporated under reduced pressure and at room temperature. The solid was dissolved in water

and the solution shaken with chloroform. The water layer was acidified with conc. HCl and p-nitrophenol (m.p. 110-113°) was extracted with diethyl ether.

The chloroform layer was used for n.m.r. and i.r. spectra but there was no evidence of cyclic phosphate.

(xii) An equimolar mixture of phenacyl di-p-nitrophenyl phosphate and lithium hydroxide (as the hydrate) in $CDCl_3$ was shaken for 36 hours at room temperature. The $CDCl_3$ solution was dried ($MgSO_4$) and used for T.L.C. and a n.m.r. spectrum which showed the presence of a mixture. A new doublet at 3.3τ (J = 13 Hz) could have been due to the cyclic intermediate. The integration of the protons in the n.m.r. spectrum indicated a mixture of phenacyl di-p-nitrophenyl phosphate and the postulated cyclic compound (75:25). No pure cyclic compound was isolated.

PART IV

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