A NEW METHOD OF
PHOSPHORYLATION AND THE SYNTHESIS
OF SOME PHOSPHATE ESTERS OF BIOLOGICAL INTEREST

by

JOHN GILBERT MOFFATT

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of

JOHN GILBERT MOFFATT
B.A. (U.B.C.), 1952
M.Sc. (U.B.C.), 1953

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General Introduction to Biologically Important Phosphate Esters.

During recent years biochemical investigations have clearly illustrated the extreme importance of phosphate, pyrophosphate, and polyphosphate esters in biological processes.

The general field of carbohydrate metabolism provides a particularly fruitful example of this importance and has been adequately reviewed on several occasions (75, 107). The glycolytic conversion of glucose to pyruvic acid via the well known Embden-Meyerhoff scheme is known to require hexokinase catalysed phosphorylation by adenosine triphosphate to give glucose-6-phosphate as its first step. Subsequent steps involve no less than seven different phosphorylated intermediates and are intimately associated with further reactions of adenosine di- and triphosphates as well as phosphate containing coenzymes. The more recent work of Horecker (75), Racker (155, 163), Gunsalus (75) and others has indicated the presence of alternate pathways which are equally rich in phosphate esters.

In the general field of nucleic acid and nucleotide chemistry, the monomeric nucleoside mono-, di- and triphosphates are of particular significance. For some time adenosine di- and triphosphates (I, n=0 and 1) have been known to function as a storehouse of "high energy phosphate" and to be intimately associated with enzymatic phosphorylations. The phosphagens such as N-phosphocreatine (II), serve a similar purpose. More recently the work of Potter (161), as well as of other workers (24, 126) has resulted in the isolation and characterization of other nucleoside di- and triphosphates possessing biological activity in phosphate transfer processes.
At the mononucleotide level many examples of nucleotide coenzymes are known. As typical of this fairly large class of compounds we might mention Coenzyme A$^8$ (III) active in fatty acid metabolism and synthesis, and uridine diphosphate glucose$^{11}$ (IV) an intermediate in the biosynthesis of sucrose$^{12}$.

Deoxyribonucleic acid (DNA) itself has recently been suggested as the controlling factor in hereditary transmission$^{167}$, while ribonucleic acid (RNA) appears definitely to be associated with protein biosynthesis$^{28}$. 
Finally we might mention the less clearly understood field of phospholipids\(^\text{(26)}\) as being yet another example of the many and diversified ways in which phosphate esters contribute to the functioning of biological systems.

In order that we may more fully understand the fundamental chemistry of such a biologically important class of compounds it is essential that general methods of phosphate ester synthesis be developed. With such methods at our disposal we may not only confirm the structures of certain naturally occurring substances but also produce them synthetically far more economically than they may be isolated from natural sources. Also non-naturally occurring model compounds may be prepared upon which to base the fundamental chemistry of more complex systems.

The present study may be logically divided into three main divisions. Firstly, the development of the use of tetra p-nitrophenyl pyrophosphate as a new and powerful phosphorylating agent, and a detailed study of the chemistry associated with its use; secondly, the specific use of this reagent in the first practical synthesis of guanosine-\(5'\)-phosphate; and thirdly, a study of the chemistry of certain xylose phosphates.
PART I.

A NEW METHOD OF PHOSPHORYLATION
AND THE SYNTHESIS OF GUANOSINE-5' PHOSPHATE.

A. Tetra-p-Nitrophenyl Pyrophosphate – a new Phosphorylating Agent.

1. Chemical Methods of Phosphorylation.

Since the discovery, during the latter part of the nineteenth century, of the biological importance of certain phosphate esters, a number of different laboratories have studied possible methods for the synthesis of such compounds. The phosphorylating agents which have been used may be classified into three main groups which are essentially analogous to the methods commonly used for the acylation of alcohols. These involve the use of free acids or salts, acid chlorides, and acid anhydrides.

(a) Free acids and salts.

The earliest phosphorylation technique involved merely warming an alcohol in 85% phosphoric acid. Due to the polyfunctional nature of phosphoric acid, however, mixtures of primary, secondary and tertiary esters inevitably resulted and the method had but little preparative significance. The use of mono- or disodium phosphate somewhat reduced the complexity of the reaction products and in many cases led to mixtures of only primary and secondary phosphates which could sometimes be converted to the pure primary esters by controlled hydrolysis. A modification of this technique involves the reaction of disodium phosphate with an epoxide to give a primary hydroxyalkyl phosphate. This approach has been further modified by Harvey et al. for the synthesis of certain sugar phosphates through the reaction of dibenzyl phosphate with protected sugar epoxides followed
by removal of the protecting groups.

The reaction of the silver salts of phosphoric acid with organic halides has been used with some success for the synthesis of phosphate esters, particularly in the case of the sugar-1-phosphates. By reacting acetobromo glucose (V) with trisilver phosphate in benzene, Cori et al.\(^{58}\) have isolated tri-(tetraacetyl glucose-1)-phosphate (VI) which after controlled acidic hydrolysis could be converted to glucose α-1-phosphate (VII). This method may be made much more specific by the use of silver dibenzyl phosphate\(^{158}\) or silver diphenyl phosphate\(^{154,23}\) followed by hydrogenolysis of the benzyl or phenyl groups. The use of benzene soluble triethylammonium dibenzyl phosphate for the synthesis of α- and α-D-ribofuranose-1-phosphates\(^{166,173}\) has been recently reported by Khorana and coworkers.

\[
\begin{align*}
\text{V} & \\
\text{VI} & \\
\text{VII}
\end{align*}
\]

Finally ethyl metaphosphate (VIII) has been used with limited success by Langheld\(^{106}\) and others for the preparation of ethyl alkyl phosphates according to the equation. This method, however, suffers from the necessity of selective hydrolytic removal of the ethyl group, and has received but limited use. It might also be better classified as an anhydride method since the metaphosphates are thought to exist in polymeric structures.

(b) Acid chlorides.

The earliest examples of carbohydrate phosphate synthesis were reported by Neuberg and Pollak\(^{146,147}\) who treated aqueous sugar solutions
with phosphorus oxychloride in the presence of calcium hydroxide or carbonate. This method was substantially improved by Fischer (64) who developed the use of phosphorus oxychloride in anhydrous pyridine at low temperatures. This technique was widely used by early workers, the classical efforts of Levene et al (109, 112, 113) being particularly noteworthy.

The use of phosphorus oxychloride, however, suffers from the serious drawback of the polyfunctional nature of the reagent. While conditions may be controlled so as to give mainly a specific primary ester, almost inevitably a mixture of primary and secondary phosphates results and leads to the necessity of separation of the products. This problem has been circumvented by the use of blocked phosphorochloridates in which only one reactive centre remains. Brigl and Müller (32) have developed the use of diphenyl phosphorochloridate (VII, \( R, R' = C_6H_5 \)) in pyridine and shown that phosphorylation of hydroxylic functions readily occurs, leading to the formation of neutral tertiary esters from which the protecting phenyl groups may be readily removed by catalytic hydrogenolysis over a platinum catalyst. This reagent has been used with some success by Zeile (177) for the synthesis of creatine phosphate, Bredereck (31) for adenosine-5'-phosphate, Baer (15) for phosphoryl choline, Fruton (63) for N-phospho amino acids, as well as by quite a number of more recent workers.

The use of dianilino phosphorochloridate (VIII, \( OR, OR' = NH_2C_6H_5 \)) in much the same manner has been described by Zetzsche (175), Malkin (25) and others, the advantage being that the protecting groups are acid labile. Also the less specific monophenyl phosphorodichloridate (32) (VIII, \( R = C_6H_5, OR' = Cl \)) and monoanilino phosphorodichloridate (175) (VIII, \( OR = NH_2C_6H_5, OR' = Cl \)) have been used in certain cases, especially where cyclic phosphate formation
is desired as in Baddiley's synthesis of glucose-\(\text{\textgreek{l}}\),\(\text{\textgreek{m}}\)-cyclic phosphate\(^{(10)}\) and in our synthesis of xylose-\(\text{\textgreek{b}},\text{\textgreek{c}}\)-cyclic phosphate\(^{(143)}\) (see Part II).

In order to obtain neutral phosphates from which the protecting groups may be removed by hydrogenolysis in the presence of other groups which would be reduced with platinum (e.g. the nucleotides), Todd and coworkers\(^{(4)}\) have developed the use of dibenzyl phosphoro dichloridate (VIII, \(R,R'\equiv \text{C}_6\text{H}_5\text{CH}_2\)) as a phosphorylating agent. The benzyl groups could be readily removed from compounds of the type IX (\(R,R'\equiv \text{C}_6\text{H}_5\text{CH}_2\)) by palladium catalysed hydrogenolysis which is inactive towards reduction of aromatic rings. Dibenzyl phosphoro dichloridate is unfortunately a very unstable  

\[
\begin{align*}
\text{RO} & \xrightarrow{0\text{P-Cl} + R'\equiv \text{CH}_2\text{OH}} \text{RO} & \xrightarrow{0\text{P-OCH}_2\text{R}} \xrightarrow{\text{H}_2 \text{catalyst}} \text{RCH}_2\text{O-}0\text{P-(OH)}_2 \\
\text{VIII} & \text{IX}
\end{align*}
\]

compound which must be generated immediately prior to use and cannot be conveniently isolated. It has, however, been used extensively by Todd's group for the synthesis of mononucleotides\(^{(13,134)}\) (see Section B) where it has proved to be of particular value since selective methods of monodebenzyla tion\(^{(11,50)}\) are available. This selective freeing of one phosphoryl dissociation has allowed the synthesis in moderate yield of adenosine di- and triphosphates (\(13,135\)). More recently Zervas\(^{(178)}\) and Miyano\(^{(139,140)}\) have reported the preparation of dibenzyl phosphoro dichloridates substituted in the para position with halide or nitro groups. These are superior to dibenzyl phosphoro dichloridate in that they are stable, crystalline compounds which may still be used as active phosphorylating agents\(^{(139,179)}\) leading to neutral esters which may be either mono- or di-debenzylated by conventional methods\(^{(139)}\).
The reagents listed above have completely eclipsed the use of compounds such as diisopropyl phosphorochloridate and diethyl phosphorochloridate (123, 170) which lead to products from which the selective cleavage of protecting groups is well nigh impossible.

(c) Acid anhydrides.

The use of pyrophosphates and other phosphate anhydrides as phosphorylating agents has received but little systematic study. Phosphorus pentoxide has been used for many years in the synthesis of phosphate esters, but almost inevitably leads to a mixture of mono- and diesters and thus is of limited use in most cases. Cherbuliez (148) has made perhaps the most comprehensive study of the use of this reagent. In certain cases the use of mixtures of phosphorus pentoxide and phosphoric acid (which may be considered as polyphosphoric acid) has proved singularly effective for the preparation of specific phosphates. In particular, Levene's preparation of amino acid phosphates (117), various studies on pyridoxal phosphates (12, 153), and Hall and Khorana's extremely efficient synthesis of uridine-5'-phosphate (76) may be mentioned. This method, however, requires the use of strongly acidic reaction conditions and thus is untenable for the phosphorylation of acid labile compounds such as purine nucleosides.

Few fully protected pyrophosphates (X) have been used as specific phosphorylating agents. During his studies on phosphorus containing enzyme inhibitors, Wagner-Jauregg (123, 170) has had occasion to phosphorylate amines and amino acid esters with tetraethyl pyrophosphate (X, R=C2H5). This reagent, however, suffers from the same defect as diethyl phosphorochloridate in that selective removal of the R blocking groups from the product XI cannot be accomplished. Atherton and Todd (5) have briefly mentioned the phosphorylative power of tetrabenzyl pyrophosphate (X, R=C6H5CH2) towards amines but have not
used this method preparatively other than as a test for pyrophosphate formation.

\[
\begin{align*}
\text{(RO)}_2P-O-P-(OR)_2 + R'NH_2 & \rightarrow (\text{RO})_2P-NHR' + (\text{RO})_2P-OH \\
\text{X} & \rightarrow \text{XI}
\end{align*}
\]

Mason and Todd have reported the use of tetraphenyl pyrophosphate (X, R=C\(_6\)H\(_5\)) for the phosphorylation of p-nitro-benzyl alcohol\(^{132}\), and have shown that after refluxing the reactants in chloroform for three hours a 23% yield of diphenyl p-nitrobenzyl phosphate could be isolated. This is none the less significant as it is the first example of the successful phosphorylation of an alcohol using one of the monofunctional or specific reagents (protected phosphorochloridates or pyrophosphates) without the necessity of base catalysis.

Finally, mention might be made of a reagent developed by Corby et al\(^{57}\) specifically for the synthesis of nucleotide esters and pyrophosphates. This is O-benzylphosphorous O,O-diphenylphosphoric anhydride (XII) prepared from monobenzyl phosphite and diphenyl phosphorochloridate in pyridine. The reaction of this reagent with an alcohol in the presence of a base leads to the formation of the secondary phosphite (XIII). (Typically cleavage of a mixed anhydride with an alcohol or amine leads to esterification of the weaker acid involved in the anhydride linkage.) Treatment of such a phosphite with N-chloro-succinimide provides a facile route to protected phosphorochloridates (XIV) which may be hydrolysed to the corresponding phosphates if desired. The particular value of this method, however, lies in the preparation of monobenzyl nucleoside phosphorochloridates (XIV, R=nucleoside) which are useful intermediates in the preparation of dinucleotides\(^{136}\), nucleotide coenzymes\(^{49}\), and nucleoside polyphosphates\(^{92}\).
This review of available methods of chemical phosphorylation is fairly complete with respect to relatively general techniques and ignores some methods which are of but limited use.

2. Development of the Present Method.

(a) Introduction.

As part of a general study of nucleoside polyphosphate synthesis which has been carried on in this laboratory, a synthetic source of guanosine-5'-phosphate was required. The problems involved, in this particular synthesis, will be discussed in detail in Section B of this thesis, and for the present it is sufficient to say that none of the standard methods of phosphorylation are applicable in this case. It was particularly felt that some of these difficulties might be overcome by the use of a phosphorylating agent which did not require base catalysis. It was, therefore, decided to investigate possible new methods of phosphorylation which might make the chemical synthesis of guanosine-5'-phosphate possible.

A useful phosphorylating agent should possess the following criteria:

(1) Relative ease of formation of the reagent itself.

(2) Monofunctional nature so as to lead to a single phosphorylated product.

(3) Ready reaction with alcoholic functions under mild conditions.

(4) Ease of subsequent removal of blocking groups.

(5) If possible, no necessity for base catalysis.
Since in the absence of base catalysis the phosphorylation of an alcohol involves nucleophilic attack of the alcoholic oxygen upon a relatively positive phosphorus atom, the qualities of a useful phosphorylating agent might be met by the pyrophosphate of a particularly strongly acidic aromatic diester of phosphoric acid, i.e. one in which the esterifying groups are strongly electron withdrawing. In order that the protecting groups might be readily removed by either hydrolytic means or by hydrogenolysis, it is desirable that they be aromatic in nature. These criteria suggest that the pyrophosphate formed from di-p-nitrophenyl phosphate (XV), i.e. tetra-p-nitrophenyl pyrophosphate (XVI), would be a suitable compound to study.

Corby, Kenner and Todd have attempted to prepare tetra-p-nitrophenyl pyrophosphate by the "exchange reaction" technique. Very briefly the principle of this technique is that treatment of an anhydride with the anionic form of a second weaker acid leads to an exchange giving as the final product a less reactive anhydride. Thus treatment of tetra-phenyl pyrophosphate (XVII) with two moles of triethylammonium dibenzyl phosphate (diphenyl phosphate is a stronger acid than dibenzyl phosphate) in an inert solvent, leads, in two stages, through the less reactive diphenyl dibenzyl pyrophosphate (XVIII) to the still less reactive tetra-benzyl pyrophosphate (XIX) which may be isolated in high yield.
This type of reaction may be further facilitated by initial formation of a mixed anhydride between a diester of phosphoric acid and another, stronger acid. Thus reaction of a metal salt of dibenzyl phosphate with p-nitrobenzenesulfonyl chloride, or with trifluoroacetic anhydride, results in the formation of a mixed anhydride (e.g. XX from trifluoroacetic anhydride). Addition of a second mole of dibenzyl phosphate leads to the formation of the less reactive tetrabenzyl pyrophosphate as above.

\[
\text{On treatment of di-p-nitrophenyl phosphate salts with trifluoroacetic anhydride, however, Corby failed to obtain any evidence for the formation of tetra-p-nitrophenyl pyrophosphate}^{(56)} \text{. On the other hand, use of p-nitrobenzenesulfonyl chloride did give evidence of pyrophosphate formation since treatment of the reaction mixture with cyclohexylamine resulted in isolation of both cyclohexylammonium di-p-nitrophenyl phosphate and di-p-nitrophenyl N-cyclohexylphosphoramidate. The pyrophosphate, however, was never isolated.}
\]

Khorana's observation that the reaction of carbodiimides with mono- and diesters of phosphoric acid leads to the formation of essentially quantitative yields of the corresponding pyrophosphates\(^{(100)}\) has vastly facilitated the synthesis of such compounds. The carbodiimides constitute one of the classes of organic compounds containing "twinned" double bonds such as are also found in the allenes, ketenes, isocyanates and ketenimines. The chemistry of carbodiimides has recently been comprehensively reviewed by
Khorana (96) and need not be mentioned in any detail here.

Khorana and Todd (100) have succeeded in synthesising tetra-p-nitrophenyl pyrophosphate (XVI) in crystalline form by the reaction of two moles of di-p-nitrophenyl phosphate with one of di-p-tolyl carbodiimide (XXI) in dioxane. This reaction is envisioned as proceeding through initial; addition of one mole of the acid across one of the N=C bonds to give an intermediate isoureia phosphate (XXII) which accepts a second proton and then undergoes anionic attack by a second phosphate ion to liberate the pyrophosphate (XVI) and the highly insoluble di-p-tolyl urea (XXIII).

\[
\begin{align*}
(A) \quad & \text{CH}_3\text{C}_6\text{H}_4\text{N}=\text{C}=\text{N}-\text{C}_6\text{H}_4\text{CH}_3 + (\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{POH} \rightarrow \text{CH}_3\text{C}_6\text{H}_4\text{NH}-\text{C} \equiv \text{N}-\text{C}_6\text{H}_4\text{CH}_3 \\
& \quad \text{O} \equiv \text{P}-(\text{OC}_6\text{H}_4\text{NO}_2)_2 \\
(B) \quad & \text{CH}_3\text{C}_6\text{H}_4\text{NH}-\text{C} \equiv \text{N}-\text{C}_6\text{H}_4\text{CH}_3 \\
& \quad \text{O} \equiv \text{P}-(\text{OC}_6\text{H}_4\text{NO}_2)_2 \\
\text{XXI} & \quad \rightarrow \text{CH}_3\text{C}_6\text{H}_4\text{NH}-\text{C} \equiv \text{N}-\text{C}_6\text{H}_4\text{CH}_3 \\
& \quad \text{O} \equiv \text{P}-(\text{OC}_6\text{H}_4\text{NO}_2)_2 \\
\text{XXII} & \quad \rightarrow \text{CH}_3\text{C}_6\text{H}_4\text{NH}-\text{C} \equiv \text{N}-\text{C}_6\text{H}_4\text{CH}_3 \\
& \quad \text{O} \equiv \text{P}-(\text{OC}_6\text{H}_4\text{NO}_2)_2 \\
\text{XVI} & \quad (\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{PO} - \text{O} \equiv \text{P}-(\text{OC}_6\text{H}_4\text{NO}_2)_2
\end{align*}
\]

For this particular synthesis the use of di-p-tolyl carbodiimide (97) rather than the more common dicyclohexyl carbodiimide is dictated by the greater insolubility of the di-p-tolyl urea in dioxane which thus permits ready separation of the urea and the tetra-p-nitrophenyl pyrophosphate. Khorana did not, however, study the phosphorylative power of this compound.

(b) Preparation of the Reagent.

In order that the chemistry of tetra-p-nitrophenyl pyrophosphate might be studied, an efficient synthesis of di-p-nitrophenyl phosphate was necessary and this has led to some interesting observations.
Some six preparations of di-p-nitrophenyl phosphate were carried out according to the procedure reported by Corby et al.\(^{[56]}\), namely the reaction of two moles of p-nitrophenol with one mole of phosphorus oxychloride in benzene-acetonitrile containing two moles of pyridine. This reaction led in each case to the isolation of 30-35% yields of the desired crystalline product melting at 173-5\(^{0}\). All subsequent preparations, however, gave none of this product but only a roughly similar amount of a low melting compound (m.p. 90-91\(^{0}\)) of decidedly different crystal form. This compound was indistinguishable from authentic di-p-nitrophenyl phosphate by paper chromatography in six solvent systems and formed a crystalline cyclohexylammonium salt which was identical to that from di-p-nitrophenyl phosphate in melting point, mixed melting point, and infra red spectrum. The authentic free acid could be recovered from this salt by removal of the cyclohexylamine with a cation exchange resin and evaporation of the acidic solution to dryness.

The low melting compound, however, was markedly different from authentic di-p-nitrophenyl phosphate in its infra red spectrum, the most striking difference being the complete absence of a strong peak at 10.3 microns. The ultra violet spectra (in 0.1N HCl) were similar in form but that of the low melting compound exhibited a slightly increased extinction in the range 230-270 m\(\mu\). (U.V. 1 and 2). Titration to the phenolphthalein end point showed the low melting compound to have a neutralization equivalent of 389 while di-p-nitrophenyl phosphate gave the theoretical value of 340.

It was also observed that treatment of tri-p-nitrophenyl phosphate with hot 50% aqueous pyridine followed by ether extraction at pH5 of the p-nitrophenol produced, led to the isolation in high yield of a crystalline compound identical in every way with the previously described 90-91\(^{0}\) melting material.
This suggested that in some way pyridine was present in a loosely bound form, and in an effort to confirm this the following experiment was devised. Roughly equal weights of the low melting compound and di-p-nitrophenyl phosphate were separately dissolved in water, the free acids absorbed on Dowex 2 formate form anion exchange resin, and the resin carefully washed with water. The ultra violet spectrum of the solution resulting from the low melting compound was determined using the solution from authentic di-p-nitrophenyl phosphate as a blank. The resulting spectrum (U.V.3) was superimposable upon that of authentic pyridine, and by comparison with an experimentally determined extinction coefficient the percentage of pyridine in the unknown compound could be determined with remarkable reproducability. A combination of elemental analysis, pyridine content, and neutralization equivalent served to characterize the low melting material as a complex between two molecules of di-p-nitrophenyl phosphate and one molecule of pyridine, apparently occurring as a stable monohydrate. No more exact picture of the actual nature of this compound has yet been obtained.

In an effort to eliminate pyridine from the preparation of di-p-nitrophenyl phosphate the reaction of two moles of sodium p-nitrophenolate with one mole of phosphorus oxychloride was investigated. This led, however, to a mixture of mono-, di- and tri-p-nitrophenyl phosphates which required rather tedious separation and hence the method was considered undesirable.

The preferred route to di-p-nitrophenyl phosphate appears to lie in the selective hydrolysis of one p-nitrophenyl group from tri-p-nitrophenyl phosphate (XXIV). Yoshida(174) has introduced the use of di-p-nitrophenyl phosphate as a substrate for quantitative studies on phosphomesterase activity and has synthesized the pure compound as its calcium salt by hydrolysis.
of tri-p-nitrophenyl phosphate with boiling alcoholic sodium hydroxide. Ketelaar's study of the alkaline hydrolysis of p-nitrophenyl phosphates, as well as the observations reported in this thesis, indicate that the hydrolysis of the first grouping from tri-p-nitrophenyl phosphate is so rapid at room temperature that there is no need to use as vigorous conditions as those reported by Yoshida. The use of an alcoholic solvent is also questionable since during the present work paper chromatographic evidence suggested a transesterification reaction during alkaline hydrolysis of di-p-nitrophenyl methyl phosphate in ethanol leading to a certain amount of methyl ethyl phosphate.

The preparation of tri-p-nitrophenyl phosphate has been recorded several times in the early literature, but never by a particularly efficient method. Rapp originally prepared this compound by addition of phosphorus oxychloride to solid anhydrous potassium p-nitrophenolate without any diluant. The desired product was indeed obtained but in poor yield. Some time later Hoeflake studied the nitration of di- and tri-phenyl phosphates and obtained modest yields of di- and tri-p-nitrophenyl phosphates. We have found that tri-p-nitrophenyl phosphate may be obtained in essentially quantitative yield and in a high state of purity by the gradual addition of an excess of scrupulously dry sodium p-nitrophenolate to a dilute solution of phosphorus oxychloride in dry ether. As the reaction proceeds the orange-red phenolate disappears and is replaced by a white crystalline mixture of the desired tertiary ester and sodium chloride. By merely filtering the solids and repeatedly washing with water to dissolve salts and excess phenolate, the white, water insoluble, neutral ester is left and is quite suitable for subsequent reactions without further purification.
The alkaline hydrolysis itself is best conducted using three equivalents of lithium hydroxide in aqueous dioxane at room temperature and is complete in a matter of minutes. At room temperature the hydrolysis leads exclusively to di-p-nitrophenyl phosphate, no sign of mono-p-nitrophenyl phosphate being formed even after several days. The p-nitrophenol released is extracted with ether at pH5, and the free acid precipitated by addition of concentrated hydrochloric acid. One recrystallization from water gives pure di-p-nitrophenyl phosphate (XV) in 90% yield.

\[
\text{(NO}_2\text{C}_6\text{H}_4\text{O})_3\text{P} = \text{OH} \xrightarrow{\text{dioxan}} (\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{P} \xrightarrow{\text{OH}^-} \text{NO}_2\text{C}_6\text{H}_4\text{OH}
\]

With a satisfactory method for the synthesis of di-p-nitrophenyl phosphate at our disposal, the conversion of this compound to its pyrophosphate, and the phosphorylating power of the latter, was investigated. The crystalline pyrophosphate was prepared according to Khorana in 70% yield, but due to its sensitivity towards water, especially while in solution, it was necessary to use a dry box and scrupulously dry equipment throughout the entire preparation.

(c) The Phosphorylation Technique.

Crystalline tetra-p-nitrophenyl pyrophosphate was readily shown to be an active phosphorylating agent towards simple alcohols but in view of the precautions necessary during its preparation and isolation it was considered preferable to generate the reagent directly for use without isolation. Accordingly the pyrophosphate was generated by the reaction of two moles of di-p-nitrophenyl phosphate and one of di-p-tolyl carbodi-imide in anhydrous dioxan, and the compound to be phosphorylated was
directly added to the crude mixture of pyrophosphate and urea. After storage overnight under anhydrous conditions the highly insoluble di-p-tolyl urea was removed by filtration, and the mole of di-p-nitrophenyl phosphate released upon cleavage of the pyrophosphate extracted with water from a chloroform solution. Evaporation of the chloroform left an almost quantitative yield of the highly water insoluble neutral ester (XXV) which in most cases could be readily crystallized from aqueous alcohol.

\[
\begin{align*}
\text{H}_2\text{N}-\text{C}-\text{N-C}_6\text{H}_4\text{CH}_3 & \quad \text{CH}_3\text{C}_6\text{H}_4\text{N}-\text{C}=\text{N-C}_6\text{H}_4\text{CH}_3 \\
(\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{P}-\text{O} & \quad (\text{OC}_6\text{H}_4\text{NO}_2)_2\text{P}-\text{O} \quad \text{CH}_3\text{C}_6\text{H}_4\text{N}-\text{C}=\text{N-C}_6\text{H}_4\text{CH}_3
\end{align*}
\]

XXV

The phosphorylation of methyl alcohol under these standard conditions resulted in the isolation of a 90% yield of crystalline di-p-nitrophenyl methyl phosphate (XXVI), (XXV, R=CH₃). This compound was identical in every way with a sample of the compound prepared according to Ketelaar and Gersmann⁹ by addition of two moles of sodium p-nitrophenolate and one of sodium methoxide to an ethereal solution of phosphorus oxychloride. Di-p-nitrophenyl methyl phosphate was used as a model for the study of methods for the removal of the p-nitrophenyl protecting groups, and of other fundamental chemical behaviour of this type of compound.

(d) Removal of the Protecting Groups.

By comparison with the methods which have been used for the removal of protecting phenyl groups after use of diphenyl phosphorochloridate, it might be expected that fairly vigorous alkaline hydrolysis⁹, it might be expected that fairly vigorous alkaline hydrolysis,
platinum catalysed hydrogenolysis \(^{(32)}\), or reductive cleavage by sodium in liquid ammonia \(^{(93)}\) would remove the protecting groups from di-p-nitrophenyl alkyl phosphates.

Treatment of di-p-nitrophenyl methyl phosphate in aqueous dioxane made 0.1-1.0 M in lithium hydroxide resulted in the instantaneous appearance of the characteristic yellow colour of the p-nitrophenolate ion. Paper chromatographic examination of the reaction mixture after five minutes showed the complete disappearance of the fast moving starting material and the formation of a single slower moving spot identified as mono-p-nitrophenyl methyl phosphate (XXVII). Continued treatment at room temperature for up to a week resulted in no further hydrolysis to primary phosphates. This selective removal of one p-nitrophenyl group was routinely conducted for thirty minutes, the liberated p-nitrophenol extracted with ether at pH5, and the free secondary phosphate isolated by evaporation of the solvent after removal of cations with a cation exchange resin in the hydrogen form. Most of the mono-p-nitrophenyl alkyl phosphates obtained by this method tended to crystallize on prolonged evacuation, but recrystallization was frequently tedious and wasteful and it was generally more convenient to isolate these compounds as their readily crystallizable cyclohexylammonium salts (Table II).

If, however, mono-p-nitrophenyl methyl phosphate was treated with 1.0 N lithium hydroxide at 100°, a further hydrolysis occurred and could be followed quantitatively by spectrophotometric determination of the p-nitrophenol released (Figure I). This release reached a constant and essentially theoretical value in roughly two and one-half hours after which paper chromatographic examination revealed mono methyl phosphate (XXVIII) and p-nitrophenol as the sole products. On a preparative scale it is impossible to follow the hydrolysis by p-nitrophenol release since in strongly alkaline solution the latter compound is insoluble as its alkali salts. Use
has been made of this property for the convenient preparation of fairly large amounts of sodium p-nitrophenolate (see experimental). In preparative hot alkaline hydrolyses the completeness of reaction is best judged by paper chromatographic examination of the hydrolysis mixture after two hours reaction. On Whatman #4 paper using BuOH:HOAc:H2O (4:1:5) system (151), a half hour development is sufficient to qualitatively demonstrate the presence or absence of unreacted mono-p-nitrophenyl methyl phosphate. If after two hours only a very weak ultra violet absorbing spot (Rf 0.55) remains then it is almost certain that the hydrolysis is complete by the time the chromatogram is studied.

For complete hydrolysis of both p-nitrophenyl groups from a di-p-nitrophenyl alkyl phosphate, the extreme insolubility of the neutral esters in aqueous solutions makes direct alkaline treatment without an organic solvent extremely slow. For this case it has been found convenient to first remove one protecting group with three equivalents of lithium hydroxide in dioxane as previously described, evaporate the dioxane to dryness, and take up the water soluble residue in 1.0N lithium hydroxide for the hot hydrolysis. On a 1.0 gm. scale using this approach, di-p-nitrophenyl methyl phosphate was converted in 90% yield to the crystalline bis-(cyclohexylammonium) salt of mono methyl phosphate (XXVIII).

Hydrogenolysis of di-p-nitrophenyl methyl phosphate in ethanol using Adams platinum catalyst, resulted in complete hydrogen uptake in four hours. On exposure to air, however, the reaction mixture tended to become brown quite rapidly. After removal of the released cyclohexylamine with a cation exchange resin and evaporation of the solvent, a brown powder was obtained which after decolourization with charcoal could be crystallized as beautiful white needles. This compound was weakly ultra violet absorbing
and did not behave chromatographically like methyl phosphate. Potentiometric titration showed primary phosphate buffering at low pH's and a second dissociation at roughly pH 5. The ultra violet spectrum in 0.01N HCl showed strong end adsorption at 200 mÅ (U.V. 6). The infra red spectrum contained a characteristic N-H stretching band at 2.98 microns. Elemental analysis identified this compound as p-aminophenyl methyl phosphate (XXIX). This same compound was readily obtained by rapid reduction of the nitro group in mono-p-nitrophenyl methyl phosphate using Adams catalyst in neutral ethanol.

The addition of a small amount of concentrated hydrochloric acid to the ethanol during the platinum catalysed hydrogenolysis of di-p-nitrophenyl methyl phosphate resulted in the complete removal of both p-nitrophenyl groups and led to the isolation of a good yield of methyl phosphate (XXVIII) as its crystalline bis-(cyclohexylammonium) salt. The catalytic effect of added mineral acid during the catalytic reduction of aromatic rings, particularly aromatic amines, in the presence of platinum, is well established, and since the hydrogenolysis of phenyl esters has been postulated by Kenner as proceeding through an initial partial reduction of the ring to give a readily cleaved allyl ester, the addition of acid is justified. During the hydrogenolysis of simple diphenyl alkyl phosphates, the cleavage of the first group leaves a free phosphoryl dissociation which may then autocatalyse the cleavage of the second group. In the case of the p-nitrophenyl analogs, however, the initial reaction is the reduction of the nitro groups to amines. The phosphoryl dissociation liberated upon cleavage of one group is then neutralized and the normal autocatalytic effect is lost, resulting in the isolation of mono-p-aminophenyl methyl phosphate if no mineral acid is added.
For the preparation of certain phosphates which would be sensitive to both platinum catalysed hydrogenolysis and hot alkaline hydrolysis (as guanosine-5'-phosphate was originally thought to be — see section B) it was considered that the p-nitrophenyl protecting groups might be conveniently removed by exchanging them for benzyl groups. These could then be readily removed by palladium catalysed hydrogenolysis under conditions where aromatic groups are stable. Since the p-nitrophenyl group is stabilised in the anionic form such an exchange should be readily accomplished through a transesterification reaction. Indeed, treatment of a benzyl alcohol solution of di-p-nitrophenyl methyl phosphate with three equivalents of sodium benzoate resulted in the immediate appearance of the characteristic yellow colour of the p-nitrophenolate ion. After a short time neutralization of the benzoate with acetic acid and evaporation of the benzyl alcohol left a residue from which a quantitative amount of p-nitrophenol could be extracted with ether. The resulting oil contained considerable dibenzyl ether which boils at roughly the same point as dibenzyl methyl phosphate (XXXIII) and, therefore, prevented purification by distillation. Rather than removing both benzyl groups from the product by the well established palladium catalysed hydrogenolysis, it was decided to monodebenzylate it according to the conditions of Brown et al[35]. Accordingly the oil was dissolved in aqueous acetic acid and heated at 100° for one hour. Following this treatment the crystalline cyclohexylammonium salt of monobenzyl methyl phosphate (XXXIV) was isolated in good yield. This method is, therefore, practical, if somewhat tedious, and might prove to be of use in certain cases. (See its application to the guanosine series.)
Kenner(93) has utilized the reductive cleavage of aromatic phosphate esters with sodium and liquid ammonia as a method of reducing phenols to the corresponding aromatic hydrocarbons. On applying this technique to p-nitrophenyl methyl phosphate (or to nitrobenzene itself), however, a dark reddish brown colour was formed which tended to make the reaction very dirty. After destruction of excess sodium and sodamide with ammonium chloride, evaporation of the ammonia, and removal of as much of the colour as possible by extraction and charcoal treatment, the residue was examined chromatographically. It proved to contain a certain amount of mono methyl phosphate as well as p-aminophenyl methyl phosphate and a third unidentified product. Because of the time consuming nature of the reaction, the rather trying workup, and the formation of byproducts, this approach was considered unsuitable for preparative use.

In general, if the mono phosphate which is ultimately desired is stable to hydrogenation in the presence of a platinum catalyst (i.e. if it contains no reducible functional groups or aromatic systems), acid catalysed hydrogenolysis is considered to be the most convenient method for the removal of p-nitrophenyl protecting groups. For the preparation of nucleoside-5'-phosphate (see Section B), and benzyl phosphate, both of which contain reducible rings, and of compounds such as p-nitrophenyl-N-cyclohexyl phosphoramidic acid which are extremely acid labile, hot alkaline hydrolysis is the preferred route.

(e) The Scope of the Phosphorylation Technique.

The general phosphorylation procedure using tetra-p-nitrophenyl pyrophosphate has been found to give excellent yields of the tertiary esters derived from a variety of simple alcohols, amines, and to a lesser degree
mercaptans. The various products obtained are described in Table I. Its application to the phosphorylation of nucleosides and other suitably protected carbohydrate derivatives will be discussed in Sections B and C of this thesis.

The phosphorylation of ethyl mercaptan to give di-p-nitrophenyl-S-ethyl thiophosphate (XXV, $\Theta_R=SC_2H_5$) resulted in only a 35% yield of the crystalline product. It is uncertain whether this low yield is due solely to the lower nucleophilic power of the mercaptan in comparison with an alcohol, or to traces of moisture in the mercaptan.

The various tertiary esters referred to in Table I were converted to their mono-p-nitrophenyl derivatives by mild alkaline hydrolysis and isolated as the crystalline free acids or cyclohexylammonium salts (Table II). Most of the tertiary esters were converted to the corresponding monoalkyl phosphates (Table III) by hydrogenolysis, benzyl phosphate being obtained by hot alkaline hydrolysis.

Of the compounds listed in Tables I, II and III, all the mono-p-nitrophenyl alkyl phosphates and di-p-nitrophenyl alkyl phosphates with the exception of di-p-nitrophenyl methyl phosphate and di-p-nitrophenyl-N-cyclohexylphosphoramidate (56, 97) are previously unreported. Most of the monoalkyl phosphates in Table III have been described in the early literature as their barium salts. These were usually obtained in very low yield and questionably purity by phosphorylation with phosphorus pentoxide and separation of the products by fractional precipitation. In this way methyl phosphate has been prepared by Cavalier (143), Harlay (79), Bailly (18), and others; n-hexyl phosphate by Ohmoi (148), cyclohexyl phosphate by King (103) and Komatsu (104), and benzyl phosphate by Zetzsche (180) and Langheld (106).

While the use of tetra-p-nitrophenyl pyrophosphate provides infinitely superior results to those obtained by these earlier workers,
it is to be pointed out that other reagents such as diphenyl phosphorochloridate could equally well be used for preparing simple phosphates. Tetra-p-nitrophenyl pyrophosphate may be considered as a "specialty reagent" to be used primarily when other methods are unsuitable, as in the synthesis of guanosine-5'-phosphate. The numerous simple compounds which have been phosphorylated served mainly as a test of the generality of the reaction and as a means of becoming thoroughly familiar with its use.

(f) Miscellaneous Reactions Related to the Phosphorylation Technique.

If a solution of di-p-nitrophenyl methyl phosphate in anhydrous pyridine was allowed to stand at room temperature for several hours, a beautifully crystalline compound was observed to separate from solution. On paper chromatographic examination this compound was observed to separate into two ultra violet absorbing spots. One of these was identical in Rf to di-p-nitrophenyl phosphate in several solvent systems. The second, slower moving spot did not contain phosphorus as shown by a negative test with the Hanes and Isherwood perchloric acid - molybdate spray. Examination by paper electrophoresis using an apparatus similar to that recently described by Paigen except that the buffer impregnated paper was immersed in a bath of carbon tetrachloride to prevent local heating, showed the non-phosphorus containing spot to be a cation, migrating towards the cathode. The ultra violet spectrum of this spot after elution with 0.1N hydrochloric acid was identical with that of the N-methyl pyridinium ion obtained by paper chromatography of N-methylpyridinium methyl sulfate prepared from dimethyl sulfate and pyridine in ether (U.V.7). The paper chromatographic mobilities of these ions were also identical in several solvents. Elemental analysis confirmed the identification of the crystalline compound as N-methylpyridinium di-p-nitrophenyl phosphate (XXX).
This reaction is entirely analogous to the tertiary base promoted monodebenzylation of benzyl tertiary phosphates described by Todd and co-workers (11, 50). The mechanism described by these workers involves a nucleophilic attack of the tertiary nitrogen atom upon the inductively positive charged carbon atom of the benzyl phosphate followed by a redistribution of charge to give an N-benzylpyridinium secondary phosphate ester. These workers have attributed the location of the nucleophilic attack on the carbon rather than on the more positive phosphorus atom to a steric effect.

The same mechanism would apply to the present reaction and may be described by:

\[(\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{P} - \text{OCH}_3 + \text{XXX} \rightarrow (\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{P} - \text{OCH}_3 + \text{XXX} \]

While in benzyl phosphates the carbon atom is particularly predisposed towards nucleophilic attack because of resonance with the aromatic ring, in the case of di-p-nitrophenyl methyl phosphate the methyl group is the centre of attack because of the induced slight positive charge due to the extremely positive phosphorus atom in this compound. Once again we must explain the lack of attack on the phosphorus atom itself by steric repulsion by the p-nitrophenyl groups.

If di-p-nitrophenyl methyl phosphate was dissolved in dry diethyl amine rather than in pyridine, a crystalline compound once again separated. By analogy with the pyridine reaction, this product might be expected to be diethyl methyl ammonium di-p-nitrophenyl phosphate (XXXI), but elemental analysis and comparison with an authentic sample indicated it to be diethyl ammonium di-p-nitrophenyl phosphate (XXXII). This may be explained by
either a proton transfer from the diethyl methyl ammonium ion of XXXI to
the more strongly basic diethyl amine solvent, or by the characteristically
lower solubility of secondary amine salts in comparison with those of
tertiary amines. A simple mass effect due to the large excess of diethyl
amine could also be responsible in part.

\[
(\text{NO}_2 \text{C}_6 \text{H}_4 \text{O})_2 \text{P} - \text{OCH}_3 + (\text{C}_2 \text{H}_5)_2 \text{NH} \rightarrow \left[ (\text{NO}_2 \text{C}_6 \text{H}_4 \text{O})_2 \text{P} - \text{OCH}_3 - \text{N}^+ (\text{C}_2 \text{H}_5)_2 \right]
\]

\[
(\text{NO}_2 \text{C}_6 \text{H}_4 \text{O})_2 \text{P} - \text{O} - \text{CH}_3 + (\text{C}_2 \text{H}_5)_2 \text{NH} - (\text{C}_2 \text{H}_5)_2
\]

XXXI

excess (\text{C}_2 \text{H}_5)_2 \text{NH}

(\text{NO}_2 \text{C}_6 \text{H}_4 \text{O})_2 \text{P} - \text{O} - \text{NH}_2 (\text{C}_2 \text{H}_5)_2

XXXII

While alkaline hydrolysis of di-p-nitrophosphoryl methyl phosphate
was rapid and led invariably to removal of only p-nitrophosphoryl groups,
acidic hydrolysis by 50% acetic acid at 100° was somewhat slow and led to
a mixture of di-p-nitrophosphoryl phosphate and mono-p-nitrophosphoryl methyl
phosphate. The course of this reaction could be readily followed by paper
chromatography in the BuOH:HOAc:H₂O (4:1:5) system which cleanly separates
the starting material and p-nitrophosphoryl (coincident) from di-p-nitrophosphoryl
phosphate and mono-p-nitrophosphoryl methyl phosphate (Rf's 0.92, 0.73, 0.49
respectively). By elution of the ultra violet absorbing spots with .01N
hydrochloric acid and comparison of the optical densities at 285 m\u00b5 with
standard absorption spectra of these compounds, the examination may be put
on a readily reproducible semi quantitative basis. The formation of both
products reached a maximum in three hours (Figure II) after which both fell
off slightly due to further hydrolysis to give mono-p-nitrophosphoryl phosphate
FIGURE I
1.0 N LITHIUM HYDROXIDE HYDROLYSIS OF
MONO p-NITROPHENYL METHYL PHOSPHATE @ 100°.

FIGURE II
HYDROLYSIS OF DI-p-NITROPHENYL METHYL
PHOSPHATE BY 50% ACETIC ACID AT 100°.
and mono methyl phosphate (Rf's 0.36 and 0.11). The relative proportions of the two main products was remarkably constant throughout the course of the reaction, the average values being 77.6% di-p-nitrophenyl phosphate and 22.4% mono-p-nitrophenyl phosphate. After three hours hydrolysis the starting material was completely absent since at this point the ultra violet absorption spectrum of the fast moving (Rf 0.92) material was almost exactly that of pure p-nitrophenol (U.V.8) and was seemingly uncontaminated with di-p-nitrophenyl methyl phosphate (U.V.4). Also at this point the p-nitrophenol release had essentially reached a maximum (except for slight further hydrolysis of the initial products) as demonstrated by its determination at 340 m/μ where the other products do not absorb. The same general distribution of products appeared to result from hydrolysis by aqueous dioxane made 1.0N with hydrochloric acid, but quantitative data was made difficult by the presence of chloride ions which move very close to mono-p-nitrophenyl methyl phosphate in the desired solvent systems, and due to some type of reaction with the paper tend to fluoresce, thus partially obscuring absorption readings.

The possible extension of this general method of phosphorylation to the preparation of specific diesters of phosphoric acid has also been considered. Since it has been shown that mild alkaline hydrolysis of di-p-nitrophenyl alkyl phosphates allows the isolation of excellent yields of the corresponding mono P-nitrophenyl alkyl phosphates, the possibility exists that these compounds could be converted to the corresponding \( P^1P^2\)-di-p-nitrophenyl-\( P^1P^2\)-dialkyl pyrophosphates by reaction with di-p-tolyl carbodiimide. This pyrophosphate might well be active as a phosphorylating agent itself, and by reaction with a second alcohol would lead to a p-nitrophenyl dialkyl phosphate from which the protecting group could be removed by
FLOW SHEET I

ESSENTIAL CHEMICAL REACTIONS OF DI-p-NITROPHENYL METHYL PHOSPHATE

\[
\begin{align*}
\text{NH}_2\text{C}_6\text{H}_4\text{O}^+\text{P-OCH}_3 & \quad \xleftarrow{\text{H}_2, \text{Pt}} \quad \xrightarrow{\text{EtOH}} \quad \text{NO}_2\text{C}_6\text{H}_4\text{O}^-\text{P-OCH}_3 \\
\text{XXXIX} & \quad \text{XXVII} \\
\xleftarrow{\text{H}_2, \text{Pt}} \quad \xrightarrow{\text{EtOH}} \quad \xrightarrow{\text{Aqueous LiOH, Dioxane, 200°}} \quad \text{1N LiOH, 100°} \\
\text{(NO}_2\text{C}_6\text{H}_4\text{O})_2\text{P-OCH}_3 & \quad \xrightarrow{\text{1N LiOH, 100°}} \quad \text{CH}_3\text{O}^-\text{P-(OH)}_2 \\
\text{XXVI} & \quad \text{XXVIII} \\
\xleftarrow{\text{Dry Pyridine, 200°}} \quad \xrightarrow{\text{2 C}_6\text{H}_5\text{CHOH}} \quad \xrightarrow{\text{50% HOAc, 100°}} \\
\text{-OP-(C}_6\text{H}_5\text{NO}_2)_2 & \quad \text{(C}_6\text{H}_5\text{CH}_2\text{O})_2\text{P-OCH}_3 \\
\text{XXX} & \quad \text{XXXIII} \\
\xrightarrow{\text{Ag, HOAc, 100°}} \\
\text{(NO}_2\text{C}_6\text{H}_4\text{O})_2\text{P-OH} & \quad 77.4\% \\
\text{NO}_2\text{C}_6\text{H}_4\text{O}^-\text{P-OCH}_3 & \quad 22.6\% \\
\text{XXXIV}
\end{align*}
\]
mild alkaline hydrolysis. Such a method might prove useful for the synthesis of certain unsymmetrical diesters of phosphoric acid.

Such a technique would, of course, suffer from the disadvantage that one-half of the mono-p-nitrophenyl alkyl phosphate obtained in the first stage would be recovered unchanged after reaction of the pyrophosphate with the second alcohol.

As a check on the feasibility of such a scheme, crystalline mono-p-nitrophenyl methyl phosphate (XXVII) was treated in a dry box with di-p-tolyl carbodiimide in anhydrous acetonitrile. Di-p-tolyl urea rapidly separated and was removed by filtration. Evaporation of the filtrate left a syrup which was readily crystallized from acetonitrile-ether to give a good yield of \( P^1P^2-di-p\)-nitrophenyl-\( P^1P^2\)-dimethyl pyrophosphate (XXXV). This compound was water insoluble but on addition of acetonitrile slowly became acidic in nature indicating the relatively stable nature of this pyrophosphate.

Unlike tetra-p-nitrophenyl pyrophosphate this compound does not appear to act as a phosphorylating agent in the absence of a tertiary base. Overnight treatment of a dioxane solution of the pyrophosphate with benzyl alcohol followed by aqueous extraction of any acidic components, and mild alkaline hydrolysis of the expected mono-p-nitrophenyl methyl benzyl phosphate (XXXVI) led to the recovery of a nearly quantitative yield of the starting material, mono-p-nitrophenyl methyl phosphate. In the presence of pyridine, however, phosphorylation did apparently take place since a similar work up of the reaction mixture gave methyl benzyl phosphate (XXXVII) as the main product. It was contaminated, however, by a moderate amount of an ultra violet absorbing material identified as mono-p-nitrophenyl benzyl phosphate. This byproduct presumably arose through a partial quatern-
ization reaction between the intermediate p-nitrophenyl methyl benzyl phosphate and pyridine similar to that previously described between di-p-nitrophenyl methyl phosphate and pyridine. Sterically hindered pyridine derivatives such as 2,6-lutidine and 2,4,6-collidine have been shown to be inactive in monodebenzylation reactions\(^{50}\) and hence their use as the basic catalyst during the phosphorylation reaction would be expected to lead to only the ultimately desired product.

\[
2 \text{NO}_2\text{C}_6\text{H}_4\text{O-P-OCH}_3 + \text{CH}_3\text{C}_6\text{H}_4\text{N-CN-C}_6\text{H}_4\text{CH}_3 \rightarrow \text{NO}_2\text{C}_6\text{H}_4\text{O-P-P-OOC}_6\text{H}_4\text{NO}_2\text{OCH}_3\text{OCH}_3
\]

\[
\text{C}_6\text{H}_5\text{CH}_2\text{OH} \rightarrow \text{NO}_2\text{C}_6\text{H}_4\text{O-P-OCH}_2\text{C}_6\text{H}_5 + \text{NO}_2\text{C}_6\text{H}_4\text{O-P-OCH}_3
\]

As previously mentioned, for the synthesis and isolation of tetra-p-nitrophenyl pyrophosphate, di-p-tolyl carbodiimide rather than the more common dicyclohexyl carbodiimide was the preferred reagent because of the greater insolubility of the di-p-tolyl urea. This same argument makes di-p-tolyl carbodiimide the preferred reagent during the in situ preparation of the pyrophosphate for use as a phosphorylating agent. This is so since the ease of isolation of the di-p-nitrophenyl alkyl phosphates in a pure state is inversely related to the amount of urea which failed to separate from solution, large amounts of urea being difficult to eliminate by
crystallization. Another observation has led to the same choice of reagent. During an early attempted phosphorylation of methanol using dicyclohexyl carbodiimide rather than the usual di-p-tolyl compound, a highly crystalline material separated from solution admixed with finely divided dicyclohexyl urea. After ready hand separation of these large crystals they were purified by crystallization and chromatographically shown to still contain di-p-nitrophenyl phosphate as well as indications of a fast moving component in the solvent systems used. Elemental analysis showed this compound to agree in formula with O-methyl-N,N'-dicyclohexyl isouuronium di-p-nitrophenyl phosphate (XXXVIII). This compound could also be obtained in high yield by the reaction of equimolar amounts of di-p-nitrophenyl phosphate and dicyclohexyl carbodiimide in anhydrous methanol. Virtually no dicyclohexyl urea was concomitantly formed during this reaction. This acid catalysed addition of an alcohol to a carbodiimide is to be compared with the base catalysed addition previously described by Khorana\(^{(97)}\). No analogous addition has as yet been observed during phosphorylations using the less strongly basic di-p-tolyl carbodiimide.

By treatment of the isourea salt with an anion exchange resin in the free base form the di-p-nitrophenyl phosphate was readily removed, and by high vacuum distillation O-methyl-N,N'-dicyclohexyl isourea (XXXIX) was isolated in high yield as the free base.

\[
\begin{align*}
\text{XXXVIII} \\
\text{XXXIX}
\end{align*}
\]
3. **Summary of the Experimental Work.**

Tetra-p-nitrophenyl pyrophosphate has been prepared by the reaction of di-p-nitrophenyl phosphate and di-p-tolyl carbodiimide in dioxane. This reagent has been found to be extremely effective for the phosphorylation of hydroxyl functions and has also been applied to some cases of amines and mercaptans.

The p-nitrophenyl protecting groups may be removed by hydrogenolysis in the presence of platinum and a trace of mineral acid or by hot alkaline hydrolysis. The removal of protecting groups has also been studied by means of transesterification with sodium benzoate and by reductive cleavage with sodium in liquid ammonia. One p-nitrophenyl group may be selectively removed by mild alkaline hydrolysis. Acidic hydrolysis of di-p-nitrophenyl methyl phosphate leads to a mixture of di-p-nitrophenyl phosphate (77%) and mono-p-nitrophenyl methyl phosphate (23%) while treatment with anhydrous pyridine results in a quaternization reaction to form N-methyl pyridinium di-p-nitrophenyl phosphate. The acid catalysed addition of methanol to dicyclohexyl carbodiimide has been found to lead to O-methyl N,N'-dicyclohexyl isocuronium di-p-nitrophenyl phosphate.
B. The Synthesis of Guanosine-5'-Phosphate.


Since Miescher's original isolation of deoxyribonucleic acid from the nuclei of pus cells, the study of the chemistry and biochemistry of the nucleic acids has attracted many workers. Two main types of nucleic acids, Deoxyribonucleic acid (DNA), and Ribonucleic acid (RNA), are now recognised and their general structures have been elucidated. Our fundamental knowledge of this field has recently been comprehensively reviewed in two excellent volumes entitled "The Nucleic Acids" edited by Chargaff and Davidson. For the present work it is sufficient to define some of the terms encountered in this field and to present the structures of the fundamental units.

(a) Nucleosides.

The nucleosides have been shown to be β-N-glycosides of various purines and pyrimidines. The carbohydrate moiety of the ribonucleosides (derived from RNA) has been shown to be D-ribose, while that of the deoxyribonucleosides (derived from DNA) is D-2-deoxyribose. For convenience during further discussion the structures of the common ribonucleosides are given (XL and XLI) without any attempt to indicate preferred tautomeric forms.

\[
\text{XL} \quad \text{XLI}
\]

Uridine, \(X = \text{OH}\)  
Cytidine, \(X = \text{NH}_2\)  
Adenosine, \(X = \text{NH}_2, Y = \text{H}\)  
Guanosine, \(X = \text{OH}, Y = \text{NH}_2\)

(b) Nucleotides.

Nucleotides are defined as phosphate esters of the carbohydrate moiety
of nucleosides. From a ribonucleoside one may clearly obtain three possible isomeric monophosphates with the phosphate group occupying the 2', 3' or 5' positions.

In the polymeric RNA and DNA the monomeric unit is known to consist of the various nucleotides, the linkage being from the 3' position of one unit to the 5' position of the next through a phosphodiester bond as shown in the structures below where R represents the various purine and pyrimidine bases.

The following discussion will be limited to the components of RNA.

![RNA structure](image1)

![DNA structure](image2)

Alkaline hydrolysis of ribonucleic acid leads to an essentially complete conversion to a mixture of mononucleotides\(^{(47)}\) which may be separated by ion exchange chromatography to give the 2' and 3' phosphates of each of the common ribonucleosides\(^{(53,54)}\). Enzymatic hydrolysis of
ribonucleic acid with purified snake venom diesterase, however, leads essentially to ribonucleoside-5'-phosphates as well as to lesser amounts of pyrimidine nucleoside 2',5'-and 3',5'-diphosphates (55).

The profound biological activity of ribonucleotides is almost always associated with derivatives of ribonucleoside-5'-phosphates, and very seldom with the 2' or 3'-isomers. Free ribonucleoside-5'-phosphates seldom possess biological activity, such activity being largely associated with enzymatic conversion to a higher degree of phosphorylation as in the nucleoside-5'-di- and triphosphates. Until recently the only nucleoside polyphosphates known were the classically important adenosine di- and triphosphates originally isolated by Lohmann (127) from muscle. The function of these compounds as the carriers of high energy bonds in biological systems is so well known as to require no further discussion. More recently, however, Potter (161) has isolated the 5'-di- and triphosphates of uridine, cytidine, and guanosine from the acid soluble extracts of rat tissue, and specific biological activities are becoming apparent. Uridine-5'-triphosphate has, for example, been shown to be active as the phosphate donor during the enzymatic conversion of fructose-6-phosphate to fructose-1,6-diphosphate (124). The four ribonucleoside-5'-diphosphates have been shown by Ochoa and coworkers (69A) to be the direct biosynthetic precursors of ribonucleic acid.

The rapidly growing general class of nucleotide coenzymes provides another example of the great importance of 5'-nucleotide derivatives. These compounds may be represented by the general formula (XLII)
where $R$ is a purine or pyrimidine base and $R'$ a variety of structures. The nucleotide coenzymes may be thought of as arising through the enzymatically controlled reaction of a compound $R'OPO_{3}H_{2}$ with a nucleoside-5'-triphosphate, resulting in the cleavage of pyrophosphoric acid and the formation of an unsymmetrical pyrophosphate. As representative of this fairly extensive class of compound we might mention the reaction of glucose-α-1-phosphate and uridine-5'-triphosphate to give the previously mentioned uridine diphosphate glucose (IV). In an analogous manner the adenosine nucleotide coenzymes, and others such as cytidine diphosphate choline and guanosine diphosphate mannose are produced.

For the particular case of the guanosine polyphosphates several biological functions have recently been described. Thus guanosine-5'-triphosphate has been shown to be active in the interconversion of inosinic and adenylic acids, in phosphopyruvate synthesis and in the incorporation of amino acids into proteins. Guanosine-5'-diphosphate has been shown to be a cofactor in the phosphorylation of adenosine diphosphate to adenosine triphosphate coupled with the breakdown of succinyl coenzyme A.

2. Nucleotide Syntheses.

Syntheses of nucleotides of known structure have been frequently attempted during the last twenty years and have met with mixed success. The methods which have been used will now be briefly reviewed, the particular case of the guanosine phosphates being considered separately at the end.

(a) Phosphorylation of free Nucleosides.

The direct phosphorylation of unprotected nucleosides is, of course, an unspecific method since there are three hydroxyl groups available for esterification. By using phosphorus oxychloride and pyridine, however,
very poor yields of adenosine-5'-phosphate and uridine-5'-phosphate have been obtained by Jachimowicz\(^{(89)}\) and Gulland\(^{(71)}\) respectively. If, however, barium hydroxide was used as the basic catalyst during the phosphorylation of uridine, the product contained a considerable proportion of uridine-3'-phosphate.

(b) Nucleoside-2'- and -3'-Phosphates.

Specific syntheses of nucleoside-2'- and -3'-phosphates have, in general, met with little success. Bredereck has reported the isolation of very low yields of the 3'-phosphates of uridine\(^{(30)}\) and cytidine\(^{(31)}\) by treatment of the 5'-trityl nucleosides with diphenyl phosphorochloridate followed by hydrolysis of the protecting groups. The location of the phosphate groups was, of course, ambiguous and Brown and Todd\(^{(36)}\) have shown that in the case of 5'-trityl adenosine, phosphorylation with dibenzyl phosphorochloridate leads to a mixture of the 2'- and 3'-phosphates.

Only one example of the synthesis of a nucleoside-2'- or -3'-phosphate of predetermined structure has been reported, namely, that of adenosine-2'-phosphate. For this case Brown et al\(^{(34)}\) have isolated 3',5'-diacetyl adenosine by counter current distribution and proved its structure by classical means. Phosphorylation of this compound with O-benzyl phosphorus O,O-diphenyl phosphoric anhydride\(^{(61)}\) followed by conversion of the resulting phosphite (XLI111) to the phosphate and removal of the protecting groups gave a crystalline product identical with naturally occurring adenylic acid "a", thus proving its identity as adenosine-2'-phosphate.
Both Gulland\(^{(72,73)}\) and Todd\(^{(134)}\) have reported specific syntheses of the various nucleoside-2'- and -3'-phosphates. All of these, however, were based upon starting materials designated as the 3,5'-benzylidene nucleosides. We now know such compounds to be stereochemically impossible and to be in fact the 2',3'-benzylidene nucleosides\(^{(35)}\) analogous to the 2',3'-isopropylidene compounds to be described in the next section. The compounds believed to be the nucleoside-2'-phosphates were, therefore, in fact the 5'-phosphates, and those designated as 3'-phosphates actually a mixture of the 2'- and 3'-isomers.
(c) Nucleoside-5'-Phosphates.

Much of the early structural and synthetic work on nucleoside-5'-phosphates was the result of the remarkable efforts of Levene and his collaborators. In order to obtain specific syntheses of nucleoside-5'-phosphates, Levene suggested the use of the isopropylidene group as a means of blocking the 2'- and 3'-hydroxyl groups. This expedient has been used in the majority of subsequent successful nucleoside-5'-phosphate syntheses.

The general procedure used by Levene was to prepare the 2',3'-O-isopropylidene nucleoside by condensation of the nucleoside with acetone in the presence of an acidic catalyst, followed by phosphorylation of the free 5'-hydroxyl group with phosphorous oxychloride and pyridine at -20°. Following acidic hydrolysis of the isopropylidene group a mixture of products was inevitably obtained from which the desired nucleoside-5'-phosphate could be obtained only be a tedious and wasteful isolation. Levene did, however, succeed in preparing low yields of the 5'-phosphates of uridine, adenosine, and inosine all of which were identical with naturally occurring samples. The method is illustrated by the preparation of adenosine-5'-phosphate (XLI).

\[
\begin{align*}
\text{HOC}_2\text{H}_2\text{O} & \quad \text{Acetone} \quad \text{H}^+ \\
\text{NH}_2 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{HOCH}_2 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{OH} & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{HOCH}_2 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{OH} & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{HOCH}_2 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{OH} & \\
\text{HOCH}_2 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{OH} & \\
\text{HOCH}_2 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{OH} & \\
\text{HOCH}_2 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{CH}_3 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\text{CH}_3 & \quad \text{HOC}_2\text{H}_2\text{O} \\
\end{align*}
\]

(1) \(\text{POCl}_3\) \text{PYRIDINE} \\
(2) \(\text{H}_2\text{O}\)
Other workers have on occasion used the less directly obtained 2',3'-diacetyl nucleosides rather than the isopropylidene derivatives and have in general obtained only very small yields of the ultimate nucleotides (31,121).

The use of a monofunctional reagent such as diphenyl phosphoro-chloridate rather than phosphorus oxychloride was developed by Bredereck but suffered from the difficulty of removal of the protecting phenyl groups. Platinum catalysed hydrogenolysis was unsuitable due to simultaneous reduction of the purine ring. By subsequent alkaline and acidic hydrolysis Bredereck (31) obtained only a 0.5% yield of adenosine-5'-phosphate from 2',3'-diacetyl adenosine.

The development of the use of dibenzyl phosphorochloridate by Atherton et al (14) was particularly suited to nucleotide synthesis and in the hands of the Cambridge group permitted the synthesis of the 5'-phosphates of adenosine (13), uridine (134) and cytidine (134) in yields of roughly 40-45% from the isopropylidene derivative. The benzyl protecting groups were removed by palladium catalysed hydrogenolysis, conditions under which the heterocyclic bases were stable.
One further method might be mentioned for the synthesis of pyrimidine nucleoside-5'-phosphates. This is Hall and Khorana's(76) elegant synthesis of uridine-5'-phosphate in 65% yield by treatment of 2',3'-O-isopropylidene uridine with a mixture of phosphorus pentoxide and ortho phosphoric acid at 60°. This method is, however, untenable for the preparation of the more acid labile purine nucleotides.

(d) Guanosine Phosphates.

From the above discussion it can be seen that fairly efficient syntheses are available for the 5'-phosphates of uridine, adenosine and cytidine. The application of these techniques to the preparation of guanosine derivatives has, however, been most unrewarding. Bredereck(30) has attempted to phosphorylate compounds designated as 5'-trityl-2'-acetyl guanosine with diphenyl phosphorochloridate but was unable to isolate any product. These starting materials were, however, prepared from what Bredereck referred to as 3',5'-benzylidene guanosine and were in fact probably N2-trityl guanosine and N2-trityl-5'-acetyl guanosine. Gulland(71) has attempted the direct phosphorylation of guanosine in pyridine with both phosphorus oxychloride and phenyl phosphorodichloridate and with both reagents obtained less than 1% yields of a compound tentatively identified as guanosine-5'-phosphate. Using barium hydroxide in place of pyridine, a trace of a compound reported to be guanosine-3'-phosphate was obtained.

Michelson and Todd(134) have attempted to phosphorylate 2',3'-O-isopropylidene guanosine with both diphenyl phosphorochloridate and dibenzyl phosphorochloridate. Unlike the corresponding reactions with the other 2',3'-O-isopropylidene nucleosides, however, no product could be isolated. Some reaction had apparently occurred but both before and after removal of the protecting groups the products were extremely heterogeneous and could
not be purified. The reaction with dibenzyl phosphorochloridate has been repeated in this laboratory and the extreme complexity of the reaction mixture confirmed. Michelson (134) then turned to the use of phosphorus oxychloride and pyridine in dimethyl formamide for the phosphorylation of 2',3'-O-isopropylidene guanosine. After quenching the reaction mixture with aqueous pyridine and acidic hydrolysis of the isopropylidene group, guanosine-5'-phosphate (XLV) was obtained in a reported yield of 20%.

Dr. R. W. Chambers of this laboratory has made an exhaustive study of the synthesis described above using ion exchange chromatography as an accurate, analytical means of determining the nature and distribution of the reaction products (45). Using the conditions described by Michelson and Todd and many variations of this procedure, it was shown that inevitably some 50% of the 2',3'-O-isopropylidene guanosine was recovered unchanged. As well as guanosine-5'-phosphate, the reaction products included two other guanosine phosphates which were isolated by ion exchange chromatography and shown to be guanosine-5'-di- and triphosphates. The maximum isolatable yield of guanosine-5'-phosphate was only 10% and the isolation extremely
tedious. Until the present work this has been the only synthetic route to guanosine-5'-phosphate.

Our interest in an improved synthetic route to guanosine-5'-'phosphate was based largely upon the need for substantial amounts of this compound in order to study the preparation of guanosine-5'-di- and tri-phosphates. Khorana has recently shown that the reaction of a 5'-nucleotide with an excess of phosphoric acid and dicyclohexyl carbodiimide in aqueous pyridine provides a facile route to the otherwise difficult to prepare nucleoside-5'-di- tri- and polyphosphates which may be readily separated by ion exchange chromatography. This technique has been highly successful for the synthesis of the biologically important uridine(77) and adenosine(98) di- and triphosphates.

Before proceeding to a discussion of our new synthesis of guanosine-5'-phosphate, it is interesting to speculate upon the reason for the chemical intertness of the 5'-hydroxyl group in guanosine in comparison with the other nucleosides. No explanation of this phenomenon has yet been proposed and no concrete experiments have been devised to provide definite information. The only suggestion which appears to be reasonable is that some type of interaction (probably strong hydrogen bonding) exists between the 5'-hydroxyl group and the amino group at carbon 2 of the guanine ring. The stereochemistry of the guanosine molecule certainly tends to throw these groups fairly close to one another and since guanosine is the only nucleoside bearing an amino group on the 2 position, its anomalous behaviour might be explained.

A certain amount of indirect support for this type of argument is provided by a reinterpretation of some experiments performed by Bredereck(30). This worker prepared what he referred to as 3',5'-benzylidene guanosine by acid catalysed condensation of guanosine with benzaldehyde. The location of
the benzylidine group was thought to be confirmed by the fact that a trityl
derivative could not be prepared thus indicating occupation of the 5'-hydroxyl
group: After acetylation of the benzylidine compound and hydrolysis of the
benzylidine group, however, a trityl derivative could be obtained, giving
what Bredereck referred to as 2'-acetyl,5'-trityl guanosine (XLVI). With
the realization that benzylidine guanosine is actually the 2',3' derivative
then acetylation followed by removal of the benzylidine group would lead to
5'-acetyl guanosine, a compound which was then readily tritylated to give
presumably 5'-acetyl-N2-trityl guanosine (XLVII). This ability to form a
trityl derivative only when the 5'-hydroxyl group is blocked is indicative
of a fairly strong interaction between the two groups which may be broken
by acetylation under unspecified conditions. The fact that 2',3'-0-isopropylidine
guanosine may be tritylated, supposedly on the 5' position (134), is
difficult to reconcile with this theory and cannot at the moment be justified.

If such hydrogen bonding does exist, then one might predict that
if the phosphorylation of 2,3-0-isopropylidine guanosine were attempted
using a reagent which could be used in acidic solution, such that the amino
group (pK2.4) were charged, the hydrogen bonding might be materially
weakened and the 5'-hydroxyl would then be available for reaction.
3. **Discussion of the Experimental Method.**

It was felt that the powerful phosphorylating agent tetra-p-nitrophenyl pyrophosphate described in Section A of this work might prove successful for the phosphorylation of guanosine derivatives where other reagents requiring base catalysis have failed. This has indeed proved to be the case, and our successful synthesis of guanosine-5'-phosphate has been reported.

(a) **The Phosphorylation Technique.**

2',3'-O-Isopropylidene guanosine (XLVIII) was prepared in 78% yield by a modification of the method of Michelson and Todd, zinc chloride being conveniently removed by use of a cation exchange resin. This product was readily phosphorylated by addition to a dioxane solution of tetra-p-nitrophenyl phosphate generated *in situ* as described in Section A. An extra mole of di-p-nitrophenyl phosphate was routinely used in order to keep the guanine amino group charged. Later experiments in which the excess di-p-nitrophenyl phosphate was omitted appear to indicate that this precaution is unnecessary in order to obtain successful phosphorylation. After standing overnight in a sealed flask, the di-p-tolyl urea was removed by filtration and excess di-p-nitrophenyl phosphate removed by extraction with water leaving a nearly quantitative yield of 2',3'-O-isopropylidene guanosine-5'-di-p-nitrophenyl phosphate (XLIX) which could be obtained in a crystalline state if desired.

(b) **Removal of Protecting Groups.**

As in the case of the di-p-nitrophenyl alkyl phosphates described in Section A, mild alkaline hydrolysis at room temperature removed only one p-nitrophenyl group from this neutral ester as shown by paper chromatography and p-nitrophenol release. When using lithium hydroxide or sodium hydroxide
for this hydrolysis, however, isolation of the resulting 2',3'-O-isopropylidene
guanosine-5'-mono-p-nitrophenyl phosphate (L) free from inorganic salts proved
extremely difficult since in order to extract the p-nitrophenol released the
initial alkaline solution must be first neutralized to roughly pH5, resulting
in considerable amounts of sodium chloride for example. Cations could not
be removed by use of a cation exchange resin since guanosine derivatives are
also held by the resin at acid pH's. The salt removal problem could be met
with some success by conducting the hydrolysis with lithium hydroxide and
neutralizing with hydrochloric acid, thereby obtaining lithium chloride as
the contaminating salt. Lithium chloride has a marked solubility in methanol
and by repeated extraction of the dry reaction mixture with this solvent, some
measure of purification could be achieved. The preferred method, however, was
to conduct the hydrolysis with barium hydroxide, followed by extraction of the
p-nitrophenol and quantitative precipitation of the barium ions by addition of
a stoichiometric amount of sulfuric acid. 2',3'-O-Isopropylidene guanosine-5'
mono-p-nitrophenyl phosphate (L) could then be isolated by lyophilization of
the resulting solution. The most serious difficulty in this approach is to
obtain hydrolysis conditions such that both the highly water insoluble neutral
ester, and organic solvent insoluble barium hydroxide, are both in solution.
This problem was best met by use of aqueous acetone as described in the
experimental section. Even then, however, some of the tertiary ester was
inevitably thrown out of solution and had to be retreated.

If, after barium hydroxide hydrolysis, the reaction mixture was
adjusted to pH2.7 and heated at 100° for ninety minutes followed by removal
of barium ions and lyophilization, an excellent yield of guanosine-5'-mono-
p-nitrophenyl phosphate (LI) was obtained. Both these mono-p-nitrophenyl
derivatives were shown to be homogeneous by paper chromatography in several solvent systems (Table V) but only phosphorus analyses could be obtained since, as is sometimes found in nucleotide derivatives, complete combustion of the samples required temperatures above the normal working range.

The removal of the second p-nitrophenyl group in order to obtain the unprotected guanosine-5'-phosphate (LII) initially appeared to be a serious problem. Guanosine-5'-phosphate has been considered as a particularly labile nucleotide and it was considered that hot alkaline hydrolysis such as was used in the case of the di-p-nitrophenyl alkyl phosphates was untenable, especially since deamination of cytidylic acid to uridylic acid has been
reported in hot alkaline solution\((33)\).

No other immediately obvious chemical method appeared available and recourse was taken to an enzymatic method. Purified rattlesnake venom phosphodiesterase (\textit{Crotalus adamanteus}) is generally considered to be specific for the cleavage of nucleotide esters to free nucleotides and hence might be expected to remove the p-nitrophenyl group from guanosine-5'-mono-p-nitrophenyl phosphate. The commercial diesterase, however, always contains a 5'-nucleotidase which would at the same time decompose guanosine-5'-phosphate to guanosine. The purification of the crude diesterase so as to eliminate the 5'-nucleotidase activity is a tedious and wasteful process which would be desirable to avoid. Some evidence does exist in the literature, however, that 5'-nucleotidase activity requires free hydroxyl groups on the ribose moiety, presumably to enable attachment to the enzyme surface. Thus, while the 5'-phosphates of adenosine, thymidine and deoxycytidine are readily dephosphorylated by bull semen 5'-nucleotidase, adenosine-2',5'-diphosphate\((105)\), thymidine-3',5'-diphosphate\((61)\), and deoxycytidine-3',5'-diphosphate\((61)\) are unaffected. It was, therefore, considered possible that incubation of 2',3'-O-isopropylidine guanosine-5'-mono-p-nitrophenyl phosphate, which contains no free ribose hydroxyl, with crude venom diesterase, would result in cleavage of the p-nitrophenyl group with complete inhibition of the 5'-nucleotidase activity.

Accordingly 2',3'-O-isopropylidine guanosine-5'-di-p-nitrophenyl phosphate (XLIX) was initially hydrolysed with dilute lithium hydroxide at room temperature to give 2',3'-O-isopropylidine guanosine-5'-mono-p-nitrophenyl phosphate (L). Without isolation this compound was incubated at 37\(^{\circ}\) and pH8.8.
with crude snake venom diesterase in the presence of magnesium ions. The cleavage of the second p-nitrophenyl group occurred readily without the formation of any 2',3'-0-isopropylidene guanosine. Spectrophotometric determination of the p-nitrophenol release showed the reaction to be complete within four hours leading to 2',3'-0-isopropylidene guanosine-5'-phosphate (LI) and p-nitrophenol as the sole products as shown by paper chromatography. Removal of magnesium ions followed by extraction of p-nitrophenol and acidic hydrolysis of the isopropylidene group led to the isolation of a 64% yield of pure guanosine-5'-phosphate (LII) as its amorphous barium salt. The homogeneity of this product was shown by analytical ion exchange chromatography, paper chromatography in several solvent systems, and paper electrophoresis. The product directly obtained by precipitation of the barium salt followed by vacuum drying at room temperature was shown to consistently contain eight moles of water which could be removed by overnight vacuum drying at 105°C. This treatment, however, led to slight colouration of the product and some release of inorganic phosphate and was hence avoided.

The synthetic guanosine-5'-phosphate as the free acid (prepared from the barium salt by treatment with Dowex-50 resin and lyophilization)
was identical in ultra violet and infra red spectra with another synthetic sample prepared in low yield by phosphorylation of 2',3'-0-isopropylidene guanosine with phosphorus oxychloride\(^{45}\) according to Michelson and Todd\(^{134}\). This latter sample was shown to be enzymatically fully active in the nucleoside monophosphate kinase system by Dr. D. R. Sanadi\(^{7}\) of the University of California.

After this successful synthesis had been completed and briefly reported\(^{44}\) it was observed that guanosine-5'-phosphate was completely stable for several hours in 1N lithium hydroxide at 100\(^\circ\). This made the possible removal of the second p-nitrophenyl group from 2',3'-0-isopropylidene guanosine-5'-di-p-nitrophenyl phosphate by hot alkaline hydrolysis of interest. Accordingly one p-nitrophenyl group was hydrolysed from the neutral ester (XLIX) as before and the resulting mono-p-nitrophenyl compound (L) was heated at 100\(^\circ\) in 1N lithium hydroxide for two and one-half hours. The pH was then adjusted to 2.7 with hydrochloric acid and the isopropylidene group hydrolysed by heating at 100\(^\circ\) for seventy-five minutes. After extraction of the p-nitrophenol with ether a 59% yield of guanosine-5'-phosphate was obtained as its barium salt identical with that from the enzymatic hydrolysis. While very simple in principle the alkaline hydrolysis method is slightly complicated by the release of quite a large amount of inorganic phosphate which coprecipitates a lesser amount of guanosine-5'-phosphate on addition of barium ions. The recovery of this coprecipitated product is somewhat tedious since once precipitated barium guanosine-5'-phosphate is difficult to redissolve. By repeatedly redissolving the mixed barium salts in cold acid followed by adjustment of the pH to 8.5 and removal of barium phosphate, the desired product may be largely recovered. This difficulty is largely significant only if one is concerned with obtaining the highest
possible yield of guanosine-5'-phosphate. A 51% yield of the pure product may be obtained directly without coprecipitation, the extraction process only serving to raise this yield to 59%.

The reason for this cleavage of inorganic phosphate on alkaline hydrolysis of 2',3'-0-isopropylidene guanosine-5'-mono-p-nitrophenyl phosphate is not clear in view of the stability of guanosine-5'-phosphate under the same conditions. One must, therefore, postulate a certain amount of cleavage of the nucleoside-phosphate bond as well as of the expected p-nitrophenyl-phosphate bond. The formation of a cyclonucleoside salt, which would facilitate this cleavage, (see later), does not appear to occur once a free phosphoryl dissociation is present.

The guanosine-5'-phosphate obtained by hot alkaline hydrolysis was carefully checked as to whether any deamination had occurred during the treatment. Deamination apparently did not occur since no ammonia could be detected during the hydrolysis and since the ultra violet spectra of both the guanosine-5'-phosphate (U.V.13), and of the guanine (U.V.16) obtained from it by acid hydrolysis, were superimposable upon authentic spectra and distinctly different from those of xanthosine and xanthine respectively.

(c) Miscellaneous Reactions of 2',3'-0-Isopropylidene Guanosine-5'-di-p-Nitrophenyl Phosphate.

During crystallization of 2',3'-0-isopropylidene guanosine-5'-di-p-nitrophenyl phosphate (XLIX), which may best be done from acetonitrile, care must be taken to avoid excessive heating of the solution in order to prevent the separation of a very fluffy, highly insoluble compound. This compound was isolated in excellent yield by refluxing an acetonitrile solution of the neutral ester (XLIX) for several hours. Elemental analysis showed it to
be isomeric with the neutral ester but paper chromatographic examination showed it to separate into two ultra violet absorbing spots, one of which was identical in Rf and ultra violet spectrum with di-p-nitrophenyl phosphate. The second spot was shown by paper electrophoresis to be a cation, the ultra violet spectrum (U.V.15) of which showed a single broad maxima at 260 m\(\mu\). By analogy with the studies of Clark et al on the formation of cyclonucleoside salts\(^1\) upon heating 2',3'-O-isopropylidene-5'-tosyl adenosine and cytidine, this compound has been designated according to Clark and Todd's nomenclature as 2',3'-O-isopropylidene-3,5'-cycloguanosine di-p-nitrophenyl phosphate (LTV).

\[ \text{LTV} \]

In view of the previously suggested interaction between the hydroxyl group on Carbon 5 of the ribose unit and the amino group on C2 the possibility of the cycloguanosine salt having the \(N_2-5'\) structure somewhat similar to the \(O_2-5'\) cyclocytidine reported by Clark et al. cannot be excluded but is probably less likely on steric grounds.

Before either the enzymatic or alkaline hydrolysis methods were conceived it was considered possible that the p-nitrophenyl groups could be exchanged for benzyl groups by transesterification as described in Section A.
These could then be hydrogenolysed in the presence of a palladium catalyst, the guanine ring remaining unchanged. Accordingly the neutral ester (XLIX) was dissolved in dry benzyl alcohol and treated with an excess of sodium benzoxide. A yellow colour immediately appeared but after removal of benzyl alcohol the residue was spectrophotometrically shown to contain only 6% of the theoretical free p-nitrophenol. Paper chromatographic examination of the product showed it to be identical with the cycloguanosine salt obtained above by heating the neutral ester, but the presence of a considerable amount of sodium acetate prevented further purification. The base catalysed formation of the cyclic salt might be explained by the formation of the sodium derivative of the guanine phenolic group (pK9.4) which by resonance would impart a greater than normal negative charge upon the N3 position. This nitrogen could then attack the relatively positive carbon atom at the 5' position and eliminate the di-p-nitrophenyl phosphate anion giving the cyclonucleoside salt.

One further reaction of 2',3'-O-isopropylidene guanosine-5'-di-p-nitrophenyl phosphate which markedly differs from that of di-p-nitrophenyl methyl phosphate is found in the reaction with anhydrous pyridine. In Section A it was shown that several hours reaction of di-p-nitrophenyl methyl phosphate in pyridine at room temperature resulted in loss of the alkyl group and formation of N-methyl pyridinium di-p-nitrophenyl phosphate (XXX). One might expect that similar treatment of the corresponding isopropylidene guanosine neutral ester would lead to cleavage of the di-p-nitrophenyl phosphate group. In practice, however, the reaction was somewhat slow and led instead to cleavage of one p-nitro-phenyl group and the formation of 2',3'-O-isopropylidene guanosine-5'-mono-p-nitrophenyl phosphate (I) as
shown by paper chromatography and ultra violet spectrum. Some three days were required for complete reaction as determined by elution of standard chromatographic spots and determination of optical density. The second component of the reaction, presumably the N-(p-nitrophenyl)pyridinium ion appeared as a fluorescent front during paper chromatography and was not further investigated.

(d) The Preparation of Mono-p-Nitrophenyl-Uridine-5'-Phosphate.

At this point workers in several laboratories in the United States expressed interest in obtaining samples of mono-p-nitrophenyl nucleotides for use as model substrates in the study of various enzymes. These compounds would be particularly convenient examples of nucleoside esters since p-nitrophenol release may be so readily followed spectrophotometrically. Accordingly it was decided to synthesize uridine-5'-mono-p-nitrophenyl phosphate (LVII) as a model pyrimidine nucleotide ester. 2',3'-O-Isopropylidine uridine (LV) was readily prepared, essentially by the method of Levene(119), and phosphorylated in the usual manner to give an excellent yield of highly crystalline 2',3'-O-isopropylidine uridine-5'-di-p-nitrophenyl phosphate (LVI). This compound was hydrolysed by cold, dilute lithium hydroxide and the p-nitrophenol extracted with ether. Acid hydrolysis at pH2.7 and 100° for ninety minutes resulted in the complete removal of the isopropylidine group after which analytically pure uridine-5'-mono-p-nitrophenyl phosphate (LVII) was isolated as its barium salt.

The results of enzymatic tests on this compound will be reported shortly by Dr. C.A. Dekker of the University of California and Dr. L.A. Heppel of the National Institutes of Health. Dr. Dekker has recently informed us that uridine-5'-mono-p-nitrophenyl phosphate is attacked by
snake venom diesterase some eighty times faster than is di-p-nitrophenyl phosphate which is the usual standard substrate for this enzyme.

\[
\begin{align*}
\text{LV} & \quad \xrightarrow{(\text{N}_{2}\text{C}_{6}\text{H}_{4}\text{O})_2\text{H}^+} \quad \text{LVI} \\
\text{LVII} & \quad \xrightarrow{\text{H}^+} \quad \text{LVII}
\end{align*}
\]

Hot alkaline hydrolysis of \(2',3'-\text{O-}	ext{isopropylidine uridine-5'}\)-di-p-nitrophenyl phosphate also appears to lead to excellent yields of uridine-5'-phosphate but this reaction has not been fully studied.

4. **Summary of Experimental Results.**

Phosphorylation of \(2',3'-\text{O-}	ext{isopropylidine guanosine with tetra-p-nitrophenyl pyrophosphate has led to the isolation of an almost quantitative yield of readily crystallizable \(2',3'-\text{O-}	ext{isopropylidine guanosine-5'}\)-di-p-nitrophenyl phosphate. The p-nitrophenyl groups have been successfully removed both enzymatically using commercial snake venom diesterase and
chemically by hot alkaline hydrolysis. Acidic hydrolysis of the resulting 2',3'-Cvisopropylidine guanosine-5'-phosphate has resulted in the isolation of 60-65% yields of guanosine-5'-phosphate. This constitutes the first synthesis of this compound in practical yield. Mild alkaline hydrolysis of the tertiary ester followed by acidic treatment gave excellent yields of guanosine-5'-mono-p-nitrophenyl phosphate. Quaternization of the tertiary ester to give 2',3'-0-isopropylidene-cycloguanosine di-p-nitrophenyl phosphate has been achieved by both mild heating and reaction with sodium benzoate. The action of anhydrous pyridine results in slow cleavage of one p-nitrophenyl group to give 2',3'-0-isopropylidene guanosine-5'-mono-p-nitrophenyl phosphate.

Phosphorylation of 2',3'-0-isopropylidene uridine gave an excellent yield of crystalline 2',3'-0-isopropylidene uridine-5'-di-p-nitrophenyl phosphate which was converted by mild alkaline hydrolysis followed by acidic treatment to uridine-5'-mono-p-nitrophenyl phosphate.
C. Experimental.

1. Methods.

Throughout this work paper chromatography was conducted on 10 cm. wide strips of untreated Whatman No. 1 paper using the descending technique with all systems other than D.

In Part I the following solvent systems were employed:

Solvent A. n-Butyl alcohol : acetic acid : water (4:1:5)(151)

Solvent B. i-Propyl alcohol : 28% ammonium hydroxide : water (7:1:2)(38)

Solvent C. i-Propyl alcohol : 5% ammonium sulfate (2:1)(2)

Solvent D. Pyridine : i-amyl alcohol : water (7:7:6)(63)

Phosphorus-containing spots were visualized by the use of the perchloric acid - molybdate spray of Hanes and Isherwood(78), the blue colour being developed by ultra violet irradiation(20). Ultra violet absorbing materials were detected by means of a Gates Raymaster lamp fitted with a cobalt filter.

For paper electrophoresis an apparatus similar to that described by Paigen(150) was employed except that the starch troughs were replaced by a shallow dish of carbon tetrachloride in which the buffer sprayed paper strips (Whatman No. 3 Mm. previously washed with 2M formic acid, then with water, and air dried) were suspended.

Total phosphorus determinations were done by the method of King(102), and inorganic phosphate according to Lowry and Lopez(128). Carbon, hydrogen, and nitrogen analyses were done by W. Manser, Zurich, Switzerland.
2. Tetra-p-Nitrophenyl Pyrophosphate - A New Phosphorylating Agent.

1. Sodium p-Nitrophenolate.

p-Nitrophenol (150 g.) was suspended in boiling water (700 mls.) and taken into solution by the addition of 10 N sodium hydroxide (110 mls., 1 equiv.). Gradual addition of further 10N sodium hydroxide (100 mls.) to the hot solution followed by rapid cooling with constant stirring resulted in the crystallization of the yellow sodium salt which was removed by filtration and quickly washed three times with 50 ml. portions of ice water. After drying for three days at 110° with intermediate pulverization, sodium p-nitrophenolate (165 g., 95%) was obtained as a red, anhydrous salt which rapidly became hydrated and assumed a yellow colour.

2. Tri-p-Nitrophenyl Phosphate (XXIV).

Freshly dried, finely powdered sodium p-nitrophenolate (112 g.) was slowly added to a well stirred solution of freshly distilled phosphorus oxychloride (16.0 mls., 26.8 g.) in anhydrous ether (400 mls.). The mixture was stirred at room temperature for thirty minutes and refluxed for two hours. The mixed orange and white solids were removed by filtration and washed repeatedly with cold water (1500 mls. total) until the washings were colourless. The white crystalline residue was dried in vacuo over phosphorus pentoxide to constant weight giving 76.6 g. (96%) of tri-p-nitrophenyl phosphate which melted at 153-5°. The melting point could be raised to 155-156° by one recrystallization from ethyl acetate or acetone. (Reported m.p. 155° (85,156).) The crude material was entirely suitable for the next step.

3. Di-p-Nitrophenyl Phosphate (XV).

Tri-p-nitrophenyl phosphate (80 g.) was dissolved in warm dioxane (800 mls.) and quickly cooled to room temperature. Lithium hydroxide (125 mls.
of 4.0 N) was added plus sufficient water to provide a homogeneous solution which was mechanically shaken for thirty minutes. The yellow solution was roughly neutralized by the portionwise addition of Amberlite (1R-120 (H⁺)) ion exchange resin, the resin removed by filtration and thoroughly washed with water. The combined filtrate and washings were evaporated to dryness under reduced pressure. The resulting gum was dissolved in water (400 mls.) and adjusted to pH4 by the addition of 1R-120 (H⁺) resin. The resin was removed by filtration, washed with water, and the combined filtrates extracted repeatedly with ether until the extracts gave no yellow colour on shaking with aqueous alkali. The aqueous solution was then heated to 80° and concentrated hydrochloric acid slowly added with constant stirring until the separation of a heavy oil was complete. On cooling and scratching the oil crystallized, and, after filtration, was recrystallized from the minimum volume of boiling water giving di-p-nitrophenyl phosphate (51 g.) as white needles. The addition of concentrated hydrochloric acid (10 mls.) to the mother liquors resulted in the crystallization of a further 2.0 g. of product. Total yield 53 g. (90%) which after drying at 100° under vacuum melted at 175.0-175.5°. Corby⁵⁶ has reported m.p. 176-8°, Hoeflake⁵⁵, 175°. The highly crystalline cyclohexylammonium salt of di-p-nitrophenyl phosphate was obtained as long needles (from ethanol) of m.p. 188.2-188.6°. (Corby⁵⁶ reports 188-190°.)

4. Di-p-Nitrophenyl Phosphate by the Method of Corby et al.⁶⁵.

p-Nitrophenol (38.7 g.) and freshly distilled phosphorus oxychloride (12.6 mls.) were dissolved in a mixture of acetonitrile (45 mls.) and benzene (360 mls.). The solution was cooled in an ice bath and pyridine (34.5 mls.) added dropwise with constant stirring. The mixture, which contained a heavy deposit of pyridine hydrochloride, was stirred at room
temperature for five hours. The pyridine hydrochloride was removed by filtration and the solvent removed by evaporation under reduced pressure. The resulting oil was dissolved in chloroform (25 mls.) and shaken for several hours with 5% aqueous sodium hydroxide (120 mls.). A yellow crystalline deposit was removed by filtration, air dried, and dissolved in boiling water (300 mls.). Tri-p-nitrophenyl phosphate (6.7 g.) was removed by filtration, and the filtrate strongly acidified with concentrated hydrochloric acid. A white crystalline material was thrown out of solution, collected, and recrystallized from ethyl acetate, giving di-p-nitrophenyl phosphate (15 gms., 32%) as white needles of m.p. 174-5°.

After some six successful preparations by this method, the only product which could be isolated in subsequent runs was a chunky crystalline material of m.p. 90-91°. This compound was acidic but quite different from di-p-nitrophenyl phosphate in its solubility characteristics and crystal form. Its properties and nature will be considered under the next two headings.

5. Pyridine Hydrolysis of Tri-p-Nitrophenyl Phosphate.

Tri-p-nitrophenyl phosphate (7.0 g.) was dissolved in dry, refluxing pyridine (20 mls.) giving a colourless solution. Water (20 mls.) was added dropwise over ten minutes giving a clear, yellow solution which was refluxed for a further thirty minutes. The solvent was evaporated under reduced pressure, the oily residue taken up in water (100 mls.) and adjusted to pH5. The resulting suspension was extracted with ether until the extracts gave no colour with alkali. The clear aqueous solution was concentrated to a volume of 25 mls. and strongly acidified with hydrochloric acid. A light coloured oil separated and crystallized on scratching. It was removed by filtration and recrystallized from ethyl acetate giving 5.1 g.
of a very chunky crystalline compound of m.p. 90-91°, unchanged by repeated recrystallization from a number of solvents.

This compound was identical in melting point, mixed melting point, ultra violet (U.V.2) and infra red spectra, with the low m.p. compound obtained during the direct phosphorylation of p-nitrophenol with phosphorus oxychloride according to Corby(56) et al. The addition of excess cyclohexylamine to an acetonitrile solution of this compound resulted in the separation of an insoluble salt which was washed well with ether and recrystallized from ethanol giving white needles of m.p. 188.2-188.6°, undepressed on admixture with authentic cyclohexylammonium di-p-nitrophenyl phosphate. The infra red spectra of the cyclohexyl-ammonium salts were also identical.

Treatment of a hot aqueous suspension of the cyclohexylammonium salt obtained from 40 gms. of the low melting material with an excess of 1R-120 (H+) resin, followed by filtration, evaporation, and crystallization of the solid residue, gave authentic di-p-nitrophenyl phosphate (35 g.) of M.P. 173-5°.

6. Identification of Pyridine in the Low Melting Compound.

The 91° m.p. compound (5.81 mgs.) and authentic di-p-nitrophenyl phosphate (5.71 mgs.) were separately dissolved in 5 ml. portions of water and stirred for five minutes with 1 ml. portions of freshly washed Dowex 2 (formate) resin. The resin was filtered from each and washed with water until the optical densities of the effluents at 260 m\(\mu\) were zero. Each combined filtrate and washings was diluted to 100 mls. and the ultra violet spectrum of the solution originating with the low melting compound determined using that from authentic di-p-nitrophenyl phosphate as a blank. The resulting spectrum (U.V.3) was superimposable upon that of
pyridine in 0.0001 M formic acid.

Using an experimentally obtained $E_{\text{max}}$ of 5.400 for pyridine, the compound was shown to contain 10.07% pyridine which would correspond to the monohydrate of a molecular compound consisting of two molecules of di-p-nitrophenyl phosphate and one molecule of pyridine.

Calculated for $C_{29}H_{23}N_5P_2O_{16} \cdot H_2O$: C, 44.90; H, 3.24; N, 9.02;
pyridine, 10.16; N.E. 388.

Found: C, 45.17; H, 3.27; N, 9.24; pyridine, 10.07; N.E. 389.


Di-p-tolyl thiourea (60 g.) was dissolved in carbon disulfide (500 mls.) and yellow mercuric oxide (100 g.) added. The mixture was gently heated and shaken for thirty minutes. Anhydrous calcium chloride (25 g.) was added and the mixture shaken for a further thirty minutes. The calcium chloride and mixed mercury salts were filtered and washed thoroughly with ether. After removal of the solvent and evacuation on an oil pump the residue crystallized and was dissolved in petroleum ether (30-60°, 300 mls.). A small insoluble residue was removed by filtration. On storage at -15°, di-p-tolyl carbodiimide crystallized as large white crystals of m.p. 52-56°. The yield was 0.45 g. (84%). One further crystallization gave a recovery of 0.41.3 g. and a melting point of 53-56°. The product should be stored in a refrigerator as it very slowly polymerizes at room temperature to a very insoluble compound.

8. Tetra-p-Nitrophenyl Pyrophosphate (XVI)(100).

Thoroughly dry di-p-nitrophenyl phosphate (2.33 g.) was dissolved in anhydrous dioxane (10 mls.) and di-p-tolyl carbodiimide (800 mgs.) added. The stoppered flask was stored for one hour and filtered in a dry box. The insoluble di-p-tolyl urea (800 mgs., 92%)
was washed with dioxane and the combined filtrates evaporated to dryness with exclusion of moisture. The oily residue was dissolved in anhydrous benzene (5 mls.) and after a few minutes a white crystalline compound separated. This was filtered in the dry box and recrystallized from a mixture of benzene and dioxane to give tetra-p-nitrophenyl pyrophosphate (1.60 g., 70%) as clusters of fine needles.

Calculated for \( \text{C}_{24}\text{H}_{16}\text{N}_{4}\text{P}_{2} \text{O}_{15} \): C, 34.52; H, 2.44; N, 8.46.

Found: C, 34.25; H, 2.64; N, 8.29.


Di-p-nitrophenyl phosphate (1.0 g., 3.0 m. moles) was dissolved in warm, anhydrous dioxane (8 mls.) and quickly cooled to room temperature. Di-p-tolyl carbodiimide (330 mgs., 1.5 m. moles) was added and the stoppered flask shaken for ten minutes during which time a heavy deposit of di-p-tolyl urea separated from solution. Dry methanol (50 mgs., 1.5 m. moles) was added and the sealed flask stored overnight in a desiccator.

Di-p-tolyl urea (335 mgs.) was removed by filtration and washed three times with small portions of dioxane. The combined filtrates were evaporated to a gum under reduced pressure, dissolved in chloroform (10 mls.), and extracted with water until the washings were neutral. Addition of concentrated hydrochloric acid (5 mls.) to the aqueous extracts resulted in the crystallization of regenerated di-p-nitrophenyl phosphate (475 mgs.). Evaporation of the chloroform solution to dryness left a dry white solid which was crystallized from aqueous ethanol to give di-p-nitrophenyl methyl phosphate (490 mgs., 90%) as white needles of m.p. 142.0-143.0°.

Calculated for \( \text{C}_{13}\text{H}_{11}\text{N}_{2}\text{PO}_{8} \): C, 44.08; H, 3.13; N, 7.90.

Found: C, 44.25; H, 3.07; N, 7.98.
By completely analogous procedures several other alcohols, mercaptans and amines were phosphorylated to give the corresponding di-p-nitrophenyl phosphoryl derivatives listed in Table I.

10. Mono-p-Nitrophenyl Methyl Phosphate (XXVII).

Di-p-nitrophenyl methyl phosphate (4.5 g.) was dissolved in acetonitrile (50 mls.) and aqueous lithium hydroxide (38 mls. of 1N) added. The resulting yellow solution was shaken mechanically for thirty minutes, roughly neutralized with acetic acid and the acetonitrile removed by evaporation. The aqueous solution was adjusted to pH5 with acetic acid and repeatedly extracted with ether until the extracts gave no colour on shaking with aqueous alkali. The aqueous solution was passed through a column (2.5 x 15 cm.) of 1R-120 (H+ ) resin. The column was washed with water until the effluent was neutral, and the total effluent evaporated to dryness under reduced pressure, leaving a colourless syrup which crystallized on prolonged evacuation. This material was recrystallized from benzene - light petroleum ether to give mono-p-nitrophenyl methyl phosphate (2.77 g., 94%) as stout, white needles of m.p. 121-3°. One further recrystallization raised the m.p. to 123.5-123.5° with excellent recovery.

Calculated for C9H18NPO5 : C, 36.09; H, 3.46; N, 6.01.

Found: C, 36.27; H, 3.46; N, 5.91.

The crystalline cyclohexylammonium salt prepared by addition of excess cyclohexylamine to an ether-acetonitrile solution of the free acid, melted at 150.5-151.0° after one recrystallization from benzene.

Calculated for C13H21N2PO5 : C, 47.00; H, 6.37; N, 8.44.

Found: C, 47.18; H, 6.23; N, 8.44.

By similar procedures most of the tertiary esters listed in Table I were hydrolysed to the corresponding mono-p-nitrophenyl alkyl
phosphates and analogs listed in Table II. These compounds were isolated as either crystalline free acids or cyclohexylammonium salts.

Only p-nitrophenyl N-cyclohexyl phosphoramidic acid required any special precautions because of its extreme acid lability. In this case, following mild alkaline hydrolysis of di-p-nitrophenyl N-cyclohexyl phosphoramidate, the pH was adjusted to 5 with 1R-120 (H⁺), p-nitrophenol extracted with ether and the aqueous solution passed rapidly through an ice cold column of 1R-120 (H⁺) resin into an excess of cyclohexylamine. Evaporation of the basic solution and crystallization of the residue afforded cyclohexylammonium p-nitrophenyl N-cyclohexyl phosphoramidic acid in good yield.

11. Mono Methyl Phosphate (XXVIII).

(a) By Alkaline Hydrolysis.

Di-p-nitrophenyl methyl phosphate (1.0 g.) was dissolved in dioxane (10 mls.), and one p-nitrophenyl group hydrolysed by the addition of aqueous lithium hydroxide (9.0 mls. of 1N). After shaking for thirty minutes the solution was evaporated to dryness and the residue transferred to a polyethylene tube with aqueous lithium hydroxide (15 mls. of 1N). The alkaline solution was heated at 100⁰ for three hours after which time paper chromatography in Solvent A showed the absence of mono-p-nitrophenyl methyl phosphate (Rf 0.55), and the presence of only mono methyl phosphate (Rf 0.11) and p-nitrophenol (Rf 0.93).

The pH was then adjusted to 5 with acetic acid, and the solution extracted with ether until the extracts gave no colour with alkali. The colourless, aqueous solution was passed through a column (1.5 x 15 cm.) of 1R-120 (H⁺) resin and the column washed with water until the effluent was neutral. The total effluent was taken to dryness and last traces of acetic
acid removed by evaporation with dioxane. The oily residue was taken up in water (5 mls.), brought to pH 8.5 with cyclohexylamine, and evaporated to dryness leaving a white solid which was crystallized from alcohol-ether. Bis-(cyclohexylammonium) mono methyl phosphate (790 mgs., 90%) was obtained as white needles which decomposed at 195-198°.

Calculated for C_{13}H_{31}N_{2}PO_{4}·H_{2}O: C, 47.54; H, 10.13; N, 8.53.
Found: C, 47.53; H, 10.13; N, 8.64.

Cyclohexylammonium salts of this type were found to lose cyclohexylamine slowly on vacuum drying at 90° and have, therefore, been routinely vacuum dried at room temperature.

(b) By Catalytic Hydrogenolysis.

Di-p-nitrophenyl methyl phosphate (500 mgs.) was dissolved in ethanol (200 mls.) and shaken under a slight pressure of hydrogen in the presence of Adam's platinum catalyst (100 mgs.) and concentrated hydrochloric acid (0.5 ml.). Hydrogen uptake (530 mls.) ceased after roughly four hours and the catalyst was removed by filtration. The colourless alcoholic solution was evaporated to dryness leaving a white solid which was dissolved in water (5 mls.) and passed through a column (1 x 10 cm.) of IRC-120 (H+) resin. The column was washed with water until neutral, the total effluent evaporated to a small volume, and converted to the bis-(cyclohexylammonium) salt of mono methyl phosphate (345 mgs., 78%) identical to the product obtained by alkaline hydrolysis.

Hydrogenolysis in acidic solution was also used to prepare n-hexyl and cyclohexyl phosphates (Table III), but hot alkaline hydrolysis was necessary for the readily hydrogenated benzyl phosphate.


Di-p-nitrophenyl methyl phosphate (1.0 g.) was dissolved in ethanol (200 mls.) and shaken at room temperature under a slight pressure of
hydrogen in the presence of Adams platinum catalyst (200 mgs.). Hydrogen uptake (624 mls.) was complete in four hours, after which the catalyst was removed by filtration and washed with ethanol. The combined filtrate and washings were quickly evaporated to a colourless oil which was taken up in water (10 mls.) and passed through a column (1 x 10 cm.) of 1R-120 ($H^+$) resin. The effluent gradually turned brown on exposure to air. The column was washed with water until the effluent was neutral, and the total aqueous solution evaporated to dryness under reduced pressure. The resulting brown powder (442 mgs.) was washed three times with small volumes of ether and the ether extracts treated with cyclohexylamine to give bis-(cyclohexylammonium) mono methyl phosphate (27 mgs.). The ether insoluble residue (1430 mgs.) was decolourized with charcoal and crystallized from aqueous ethanol, giving mono-p-aminophenyl methyl phosphate (300 mgs., 53%) as white needles of m.p. 212.5-212.8°.

Calculated for $C_{7}H_{10}NO_{4}$: C, 41.62; H, 5.57; N, 6.90.

Found: C, 41.62; H, 5.57; N, 6.93.

Hydrogenolysis of mono-p-nitrophenyl methyl phosphate (500 mgs.) in 50% aqueous ethanol with Adams platinum catalyst (150 mgs.) resulted in complete hydrogen uptake (142 mls., 3 moles) in thirty minutes. After working the reaction up as above, mono-p-aminophenyl methyl phosphate (340 mgs., 78%) was obtained in an identical form to that previously described. It moved as a single spot in several solvent systems (Table IV) and on potentiometric titration exhibited dissociations at roughly pH 1 and at pH 4.9.


Di-p-nitrophenyl methyl phosphate (1.0 g.) was dissolved in anhydrous pyridine (5 mls.) and allowed to stand overnight at room
temperature. During this time, N-methyl pyridinium di-p-nitrophenyl phosphate (1060 mgs.) separated as beautiful white crystals. Evaporation of the mother liquors gave a further 100 mgs. of the same product, m.p. 134-5°, unchanged on recrystallization from ethyl acetate-ethanol. Total yield, 1160 mgs., 95%.  

Calculated for C$_{18}$H$_{16}$N$_3$P$_8$: C, 49.89; H, 3.72; N, 9.70.  
Found: C, 49.87; H, 3.74; N, 9.72.  

Paper chromatography and paper electrophoresis (pH4) resulted in a separation of the product into its ions, one identical in mobility and spectrum to di-p-nitrophenyl phosphate, and the other to the N-methyl pyridinium ion from the reaction of dimethyl sulfate and pyridine (U.V.7).  

14. **Diethylammonium di-p-Nitrophenyl Phosphate.**  

Di-p-nitrophenyl methyl phosphate (1.0 g.) was dissolved in dry diethylamine (20 mls.) with warming. A crystalline material immediately settled out and was removed by filtration, giving diethylammonium di-p-nitrophenyl phosphate (1.10 g., 91%) as white needles of m.p. 158-158.5°, unchanged on recrystallization from ethanol and identical with an authentic sample prepared from di-p-nitrophenyl phosphate and diethylamine in acetonitrile.  

Calculated for C$_{16}$H$_{20}$N$_3$P$_8$: C, 46.49; H, 4.88; N, 10.16.  
Found: C, 46.29; H, 5.03; N, 10.28.  

15. **Acidic Hydrolysis of di-p-Nitrophenyl Phosphate.**  

Di-p-nitrophenyl methyl phosphate (40 mgs.) was dissolved in glacial acetic acid (2.0 mls.) and suspended in a boiling water bath. Water (2.0 mls.) was added portionwise at such a rate as to maintain a homogeneous solution. The final mixture was held at 100° under a small condenser. At intervals 5 α aliquots were spotted on sheets of Whatman No. 1 filter paper and developed with Solvent A. The ultra violet absorbing spots were cut out
and eluted for one hour with 3.0 mls. of 0.01N HCl. The relative molar proportions of the products (di-p-nitrophenyl phosphate and mono-p-nitrophenyl methyl phosphate) were obtained by determination of the optical densities at 285 m$\mu$ and comparison with standard absorption spectra (U.V.1 and 5). The results of this experiment are shown in Figure 2.

16. Methyl Benzyl Phosphate (XXXIV).

Di-p-nitrophenyl methyl phosphate (1.0 g.) was dissolved in dry benzyl alcohol (5 mls.) and sodium benzoide (from 200 mgs. of sodium in 10 mls. of benzyl alcohol) was added. An immediate yellow colour appeared and the solution was stored overnight. Some sodium p-nitrophenolate which crystallized was removed by filtration and washed with a little benzyl alcohol. Anhydrous acetic acid was added to the combined filtrates until they became colourless. The benzyl alcohol was evaporated to dryness under high vacuum, the oily residue taken up in ether and extracted with very dilute alkali (pH 8.0) until the extracts were colourless. Evaporation of the ether left a considerable residue, much of which appears to be di-benzyl ether. The oil was taken up in 35% acetic acid (30 mls.), held at 100° for one hour, and evaporated to dryness. The residue was equilibrated with very dilute alkali (20 mls.) and benzyl alcohol polymers extracted three times with ether. The aqueous solution was passed through a column (1 x 10 cm.) of 1R-120 (H$^+$) resin which was then washed with water until neutral. Removal of the water left a colourless oil (288 mgs.) which was dissolved in ether (5 mls.) and treated with an excess of cyclohexylamine. Addition of light petroleum ether and chilling resulted in the crystallization of the cyclohexylammonium salt of methyl benzyl phosphate (400 mgs., 50%) which melted at 99-101° and was raised to 100-101.5° by one recrystallization from ether.

Di-p-nitrophenyl methyl phosphate (500 mgs.) was dissolved in liquid ammonia (50 mls.) giving a clear, yellow solution. Sodium (465 mgs.) was added in small pieces over one hour giving an orange-brown solution which was allowed to stand for four hours. Ammonium chloride (1.2 g.) was slowly added and the ammonia evaporated. The brown residue was dissolved in water (25 mls.) and extracted with ether six times from acidic solution and six times from alkaline solution. The aqueous solution, which was still orange, was treated with charcoal and passed through a column of LR-120 (H⁺) which was then washed with water until neutral. The effluent was concentrated to a small volume and examined paper chromatographically. This indicated the presence of some mono methyl phosphate, p-aminophenyl methyl phosphate, and a third unidentified compound. The yield appeared to be very poor and the method seems to be of little preparative value.


Di-p-nitrophenyl phosphate (500 mgs.) was dissolved in anhydrous methanol (5 mls.) and dicyclohexyl carbodiimide (310 mgs.) was added. After four hours the solvent was evaporated to dryness leaving a dry, white solid which was crystallized from dioxane to give O-methyl N,N'-dicyclohexyl isouronium di-p-nitrophenyl phosphate (710 mgs., 84%) as white needles of m.p. 152.8-153.5°.

Calculated for C₂₅H₃₅N₄PO₉: C, 54.00; H, 6.10; N, 9.70.

Found: C, 54.20; H, 6.30; N, 9.91.

O-Methyl-N,N'-dicyclohexyl-isouronium di-p-nitrophenyl phosphate (500 mgs.) was dissolved in warm, 50% ethanol (20 mls.) and well stirred with freshly washed IRA-400 (OH⁻) resin. The resin was removed by filtration, washed twice with alcohol, and the combined filtrate and washings evaporated to dryness leaving a colourless oil. This oil was distilled under high vacuum giving O-methyl-N,N'-dicyclohexyl isourea (185 mgs., 90%) as a colourless, mobile liquid boiling at a bath temperature of 90° under 0.1 mm. pressure.

Calculated for C₁₄H₂₆N₂O : C, 70.45; H, 11.00.

Found: C, 70.10; H, 11.08.

On exposure to air the isourea rapidly absorbed carbon dioxide to give a crystalline carbonate.

20. P₁,P₂-di-p-Nitrophenyl-P₁,P₂-dimethyl Pyrophosphate (XXXV).

Mono-p-nitrophenyl methyl phosphate (466 mgs.) was dissolved in dry acetonitrile (5 mls.), and di-p-tolyl carbodiimide (240 mgs.) was added. Di-p-tolyl urea (223 mgs., 86%) was immediately precipitated and after standing for one hour was filtered in a dry box. The filtrate was evaporated to dryness giving an oil which was suspended in ether (5 mls.) and taken into solution with acetonitrile. On chilling P₁,P₂-di-p-nitrophenyl-P₁,P₂-dimethyl pyrophosphate (350 mgs., 78%) was obtained as colourless, chunky crystals of m.p. 81-83°, unchanged after recrystallization from the same solvents.

Calculated for C₁₄H₁₄N₂P₂O₁₁ : C, 37.50; H, 3.15; N, 6.25.

Found: C, 37.31; H, 3.25; N, 6.35.
### TABLE I
DI-β-NITROPHENYL ALKYL PHOSPHATES \((\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{PO}_\text{OR})\)

<table>
<thead>
<tr>
<th>-OR</th>
<th>Yield</th>
<th>M.P.</th>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>90</td>
<td>142-3°C</td>
<td>44.25</td>
<td>3.07</td>
</tr>
<tr>
<td>O-Benzyl</td>
<td>96</td>
<td>101.5-2.5°C</td>
<td>53.17</td>
<td>3.51</td>
</tr>
<tr>
<td>O-Cyclohexyl</td>
<td>85</td>
<td>81.5-2.5°C</td>
<td>51.17</td>
<td>4.70</td>
</tr>
<tr>
<td>O-n-Hexyl</td>
<td>95</td>
<td>Non Distillable Oil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Ethyl</td>
<td>35</td>
<td>131-3°C</td>
<td>44.06</td>
<td>3.59</td>
</tr>
<tr>
<td>NH-Cyclohexyl</td>
<td>91</td>
<td>175.5-6.5°C</td>
<td>51.49</td>
<td>4.86</td>
</tr>
</tbody>
</table>

### TABLE II
CYCLOHEXYLAMMONIUM SALTS OF MONO-P-NITROPHENYL ALKYL PHOSPHATES \((\text{NO}_2\text{C}_6\text{H}_4\text{O})_2\text{PO}_\text{OR})\)

<table>
<thead>
<tr>
<th>-OR</th>
<th>Yield</th>
<th>M.P.</th>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>94</td>
<td>150.5-1.5°C</td>
<td>47.18</td>
<td>6.23</td>
</tr>
<tr>
<td>(Free Acid)</td>
<td></td>
<td>121-3°C</td>
<td>36.27</td>
<td>3.46</td>
</tr>
<tr>
<td>O-Benzyl</td>
<td>90</td>
<td>168.9°C</td>
<td>55.64</td>
<td>6.17</td>
</tr>
<tr>
<td>O-Cyclohexyl</td>
<td>85</td>
<td>202-3°C</td>
<td>54.03</td>
<td>7.50</td>
</tr>
<tr>
<td>O-n-Hexyl</td>
<td>85</td>
<td>109-10°C</td>
<td>54.08</td>
<td>7.37</td>
</tr>
<tr>
<td>NH-Cyclohexyl</td>
<td>74</td>
<td>196.5-7.0°C</td>
<td>53.94</td>
<td>8.00</td>
</tr>
</tbody>
</table>
TABLE III
BIS-(CYCLOHEXYLAMMONIUM) SALTS OF MONOALKYL PHOSPHATES

<table>
<thead>
<tr>
<th>R</th>
<th>% Yield</th>
<th>M.P.</th>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>Methyl •H₂O</td>
<td>90</td>
<td>195-8°</td>
<td>47.53</td>
<td>10.13</td>
</tr>
<tr>
<td>Benzyl</td>
<td>94</td>
<td>232-4°</td>
<td>58.91</td>
<td>8.91</td>
</tr>
<tr>
<td>(Free Acid)</td>
<td>94-6°</td>
<td></td>
<td>44.84</td>
<td>4.87</td>
</tr>
<tr>
<td>Cyclohexyl</td>
<td>85</td>
<td>218-9°</td>
<td>57.31</td>
<td>10.38</td>
</tr>
<tr>
<td>(Free Acid)*</td>
<td>~73°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Butyl</td>
<td>91</td>
<td>195-6°</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* This free acid was difficult to crystallize from solvents and was not obtained analytically pure.
### TABLE IV

**PAPER CHROMATOGRAPHIC BEHAVIOUR OF ALKYL PHOSPHATES AND THEIR p-NITROPHENYL DERIVATIVES**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf's in Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td><strong>(1) Tertiary Esters</strong></td>
<td></td>
</tr>
<tr>
<td>di-p-nitrophenyl methyl phosphate</td>
<td>0.92</td>
</tr>
<tr>
<td>di-p-nitrophenyl-n-hexyl phosphate</td>
<td>0.96</td>
</tr>
<tr>
<td>di-p-nitrophenyl cyclohexyl phosphate</td>
<td>0.80</td>
</tr>
<tr>
<td>di-p-nitrophenyl benzyl phosphate</td>
<td>0.90</td>
</tr>
<tr>
<td>di-p-nitrophenyl S-ethyl thiophosphate</td>
<td>0.95</td>
</tr>
<tr>
<td>di-p-nitrophenyl N-cyclohexyl phosphoramidate</td>
<td>0.91</td>
</tr>
<tr>
<td><strong>(2) Secondary Esters</strong></td>
<td></td>
</tr>
<tr>
<td>di-p-nitrophenyl phosphate</td>
<td>0.74</td>
</tr>
<tr>
<td>mono-p-nitrophenyl methyl phosphate</td>
<td>0.55</td>
</tr>
<tr>
<td>mono-p-nitrophenyl n-hexyl phosphate</td>
<td>0.78</td>
</tr>
<tr>
<td>mono-p-nitrophenyl cyclohexyl phosphate</td>
<td>0.80</td>
</tr>
<tr>
<td>mono-p-nitrophenyl benzyl phosphate</td>
<td>0.78</td>
</tr>
<tr>
<td>mono-p-nitrophenyl N-cyclohexyl phosphoramidic acid</td>
<td>0.77</td>
</tr>
<tr>
<td>mono-p-nitrophenyl-S-ethyl thiophosphate</td>
<td>0.64</td>
</tr>
<tr>
<td>mono-p-aminophenyl methyl phosphate</td>
<td>0.14</td>
</tr>
<tr>
<td>benzyl methyl phosphate</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>(3) Primary Phosphates</strong></td>
<td></td>
</tr>
<tr>
<td>mono-p-nitrophenyl phosphate</td>
<td>0.36</td>
</tr>
<tr>
<td>mono methyl phosphate</td>
<td>0.11</td>
</tr>
<tr>
<td>mono n-hexyl phosphate</td>
<td>0.76</td>
</tr>
<tr>
<td>mono cyclohexyl phosphate</td>
<td>0.69</td>
</tr>
<tr>
<td>mono benzyl phosphate</td>
<td>0.26</td>
</tr>
</tbody>
</table>
3. The Synthesis of Guanosine-5'-Phosphate.

1. 2',3'-O-Isopropylidene Guanosine (XLVIII).

Freshly fused zinc chloride (56 g.) was dissolved in anhydrous acetone (350 mls.), and dried guanosine (22 g.) was added. The mixture was then heated under reflux overnight with exclusion of moisture. The clear solution was evaporated to a thick syrup and 8N ammonium hydroxide added until the initially formed precipitate redissolved. Amberlite 1R-120 (NH₄⁺) resin (100 mls.) was added and the precipitate which sometimes formed dissolved by the addition of concentrated ammonia. The mixture was stirred for fifteen minutes, the resin removed by filtration, and washed twice with 50 ml. portions of very dilute ammonia. This whole process was repeated four times using 100 ml. portions of the wet resin each time. The total volume of the final filtrate was roughly 1 liter. Ammonia was removed by heating the solution on a water bath, and on allowing it to cool 2',3'-O-isopropylidene guanosine crystallized and was washed with water. The yield was 15.2 g.. The mother liquors were concentrated, treated twice more with 100 ml. portions of 1R-120 (NH₄⁺) resin and freed of ammonia as above to give a further 4.2 g. of pure product. Total yield 19.4 g. (78%). This material could be recrystallized from water with negligible loss to give 2',3'-O-isopropylidene guanosine which melted and resolidified at 296° (Corby (56) reports 299°). Paper chromatography (Table V) showed the presence of a single component, free from guanosine.

2. 2',3'-O-Isopropylidene Guanosine-5' -di-p-nitrophenyl Phosphate (XLIX).

Carefully dried di-p-nitrophenyl phosphate (4.2 g.) was dissolved in warm, anhydrous dioxane (20 mls.) and the solution quickly cooled to room temperature with exclusion of moisture. Di-p-tolyl carbodiimide (1.10 g.) was added and the stoppered solution stored for ten minutes.
Dry 2',3'-O-isopropylidene guanosine (1.0 g.) was quickly added and the stoppered mixture stored in a desiccator overnight. The mixture was then filtered and the white solid washed three times with 3 ml. portions of dioxane leaving di-p-tolyl urea (1.060 g.), theory 1.190 g.) after drying under vacuum. The filtrate was evaporated under reduced pressure leaving a viscous gum which was dissolved by twice equilibrating it between chloroform (30 mls.) and pH6.5, 1M lithium acetate buffer (20 mls.). The combined chloroform layers were extracted three further times with pH6.5 buffer. During the third extraction a white solid settled from solution. This was removed by filtration, washed well with water and dried to a constant weight (1.106 g.) under vacuum. The chloroform mother liquors were washed three times with water and evaporated to dryness leaving a pale yellow resin which was also dried to constant weight (0.982 g.). Assuming the combined products to still contain 110 mgs. of di-p-tolyl urea, the yield of 2',3'-O-isopropylidene guanosine-5'-di-p-nitrophenyl phosphate was 1.898 g. (95%).

Paper chromatographic examination of this product showed it to contain a single very fast moving ultra violet absorbing and phosphorus-containing component, and to be completely free of 2',3'-O-isopropylidene guanosine and di-p-nitrophenyl phosphate. (See Table V for Rf's.)

Small portions of this compound could be readily crystallized with excellent recovery from acetonitrile as very pale yellow needles which underwent a sharp transition at 161-3° to an amorphous solid which decomposed at 263-4°. Prolonged heating must be avoided during the crystallization. (See 6 in this section.)

Calculated for C_{25}H_{24}N_{7}P_{12}O_{12}·H_{2}O: C, 45.28; H, 3.95; N, 14.80; P, 4.68.
3. 2',3'-O-Isopropylidene Guanosine-5'-mono-p-nitrophenyl Phosphate (L).

2',3'-O-Isopropylidene guanosine-5'-di-p-nitrophenyl phosphate (180 mgs.) was dissolved in acetone (6 mls.) and aqueous barium hydroxide (16 mls. of 0.12 N) was slowly added with constant shaking. The total mixture was shaken for thirty minutes, after which a small amount of unreacted neutral ester was removed by filtration and retreated with acetone and barium hydroxide as above. The combined yellow solutions were taken to dryness, the residue dissolved in water (5 mls.) and adjusted to pH5 with acetic acid. p-Nitrophenol was extracted with ether until an ether extract gave no yellow colour on shaking with aqueous alkali. A stoichiometric amount of 1N sulfuric acid (based on the total barium hydroxide used) was added to precipitate the barium ions. The completeness of precipitation was insured by alternate, dropwise addition of 0.01N sulfuric acid and barium hydroxide until no further precipitation occurred. The barium sulfate was removed by centrifugation and washed with water. The combined supernatants were lyophilized to give 2',3'-O-isopropylidene guanosine-5'-mono-p-nitrophenyl phosphate (hexahydrate) (155 mgs., 88\%) as a fluffy, white solid which could not be crystallized either as the free acid or as several of its salts.

Calculated for C_{19}H_{21}N_{6}PO_{10}·6H_{2}O : P, 4.89\%.

Found: P, 4.88\%. This compound could not be completely combusted for other elemental analyses, but behaved chromatographically as a single component. (See Table V) The ultra violet spectrum is shown in U.V.12.

* Typical of purine nucleotides, this compound was exceedingly difficult to combust and required a greater than normal furnace temperature. The mono-p-nitrophenyl derivatives L and LI could not be completely combusted even with increased temperatures.
4. Guanosine-5'-mono-p-nitrophenyl Phosphate (LI).

2',3'-O-Isopropylidene guanosine-5'-di-p-nitrophenyl phosphate (180 mgs.) was hydrolysed with aqueous barium hydroxide in acetone as above. After extraction of the p-nitrophenyl with ether, the aqueous solution was adjusted to pH 2.7 with acetic acid and held in a boiling water bath for ninety minutes. Barium ions were removed as barium sulfate as above and the aqueous solution lyophilized to give guanosine-5'-mono-p-nitrophenyl phosphate (pentahydrate) (145 mgs., 90%) as a noncrystallizable, fluffy white solid.

Calculated for C_{16}H_{17}N_{6}P_{10}·5H_{2}O : P, 5.38%.

Found: P, 5.34%.

This compound was chromatographically homogeneous and had an ultra violet spectrum (U.V.II) identical with that of (L).

5. Guanosine-5'-Phosphate (LII).

2',3'-O-isopropylidene guanosine (1.0 g.) was phosphorylated as described in 2 to give 2',3'-O-isopropylidene guanosine-5'-di-p-nitrophenyl phosphate (1.915 g., 96%). This product was suspended in dioxane (25 mls.) and aqueous lithium hydroxide (12 mls. of 1N) was added followed by water (12 mls.) to obtain a homogeneous solution. The yellow solution was shaken for thirty minutes, roughly neutralized with hydrochloric acid, and a small amount of residual di-p-tolyl urea removed by filtration. The clear solution was then treated in either of the two following ways to remove the second p-nitrophenyl group.

A. Enzymatically.

The solution was evaporated to a viscous gum which was dissolved in 0.1M, pH 8.8 tris-(hydroxymethyl)-aminomethane buffer (25 mls.). Magnesium acetate (25 mls. of 0.3M) was added and the total volume brought
to 150 mls. with more buffer. Crude rattlesnake venom* (62 mgs.) was added and the solution incubated at 37° for four hours at which time the p-nitrophenol liberation was complete as measured spectrophotometrically at 440 m/.

The solution was cooled to room temperature, adjusted to pH5 with glacial acetic acid, and stirred for twenty minutes with IR-120 (NH4+) resin (25 mls.) to remove magnesium ions. The resin was removed by filtration and washed thoroughly with water. Glacial acetic acid (10% of the total volume) was added, and the solution adjusted to pH2.7 by the dropwise addition of concentrated hydrochloric acid. The solution was heated in a boiling water bath for one hour, cooled to room temperature, and repeatedly extracted with ether until the extracts gave no colour on shaking with aqueous alkali. The aqueous solution was evaporated to dryness under reduced pressure, the residue taken up in water and adjusted to pH6.5 with lM lithium hydroxide (Total volume about 40 mls.). Aqueous barium acetate (4.0 mls. of 2M) was added and after one hour a small precipitate of barium phosphate was removed by centrifugation and washed with water. Two volumes of 95%ethyl alcohol were added to the combined supernatants and the precipitation of the product completed by storage at -10° for one hour. The precipitate was collected by centrifugation, washed twice with 70% ethyl alcohol, and once with 95% alcohol, acetone, and ether. After drying at room temperature in vacuo over phosphorus pentoxide for three hours barium guanosine-5'-phosphate (1.263 g.; 64%) was obtained as its octahydrate. A sample (117.48 mgs.) of this material was dried overnight in vacuo at 105° over phosphorus pentoxide. The weight loss was 25.79 mgs., corresponding to 7.8 moles of water. Both before and after drying the E_{max} at pH2 was 12.3 x 10^3 based on the octahydrate and anhydrous sample respectively. Inorganic phosphate contamination was negligible and the product homogeneous by both

* Crotalus Adamanteus - obtained from Ross Allen's Reptile Farm, Silver Springs, Florida.
paper chromatography (Table V)* and paper electrophoresis. An analytical sample was obtained by one further precipitation.

Calculated for $C_{10}H_{12}N_5PO_8Ba·8H_2O$: C, 18.69; H, 4.39; P, 4.82.

Found: C, 18.59; H, 3.55; P, 4.67.

B. By Alkaline Hydrolysis.

The aqueous dioxane solution was evaporated to dryness leaving a gummy residue which was transferred in a total of 20 mls. of 1N lithium hydroxide into a polyethylene test tube. This alkaline solution was suspended under a reflux condenser in a boiling water bath for 2.5 hours, then cooled, adjusted to pH 2.7 with concentrated hydrochloric acid, and held in a boiling water bath for a further 75 minutes. After cooling, the acidic solution was extracted repeatedly with ether until the extracts gave no colour with alkali. The pH was adjusted to 8.5 with 1N lithium hydroxide and the volume was reduced to 25 mls. The addition of 2.0 M barium acetate (4.0 mls.) gave a fairly heavy precipitate which was spun down and washed with water. Two volumes of ethyl alcohol were added to the combined supernatant and washings, and the precipitation completed by storage at $-10^\circ$ for one hour. The white precipitate was washed twice with 70% alcohol, once with 95% alcohol, acetone, and ether, and dried in vacuo.

* With the exception of C the solvents listed in Table V are unsatisfactory for examination of guanosine-5'-phosphate.

The homogeneity of our synthetic sample was better indicated by the use of the following systems:

1. 5% aqueous potassium dihydrogen phosphate : i-amyl alcohol. 
   $R_f = 0.63$.

2. i-Propyl alcohol : water : trichloroacetic acid (15:5:1).
   $R_f = 0.35$.

3. i-Butyric acid : 1N ammonium hydroxide : 0.1 M disodium ethylene-
   diamine tetraacetic acid (10:6:0.16). $R_f = 0.28$. 
at room temperature to give essentially pure barium guanosine-5' -phosphate (1.00 g.) as a white, amorphous solid.

While tedious, the recovery of some further product from the initial precipitate of barium phosphate containing coprecipitated barium guanosine-5' -phosphate could be effected in the following way. The precipitate was taken into solution by suspending it in water (15 mls.) cooled in an ice bath, and adding concentrated hydrochloric acid dropwise until a clear solution resulted. The pH was then adjusted to 8.5 with 4 M lithium hydroxide, and the resulting precipitate collected and retreated two further times in the same manner. Two volumes of alcohol were added to the combined filtrates and the precipitated barium guanosine-5' -phosphate (163 mgs.) collected, washed, and dried as before. The total yield was 1.163 g. (59%). This product was pure except for the presence of 1.7% barium phosphate as determined by the Lowry-Lopez method. One further reprecipitation gave pure, inorganic-free barium guanosine-5' -phosphate, identical in every way with the product obtained by enzymatic hydrolysis.

\[ E_{\text{max}} = 12.4 \times 10^3 \text{ at } pH_2, \quad \text{and } = 12.1 \times 10^3 \text{ at } pH_{11}. \]

The accepted \( E_{\text{max}} \) at \( pH_2 = 12.3 \times 10^3 \).

6. \( 2',3'-O\)-Isopropylidine-3,5'-cycloguanosine di-p-nitrophenyl Phosphate (LIV).

   A. By heating \( 2',3'-O\)-isopropylidine guanosine-5' -di-p-nitrophenyl phosphate.

\( 2',3'-O\)-Isopropylidine guanosine-5' -di-p-nitrophenyl phosphate (45 mgs.) was dissolved in acetonitrile (10 mls.) and heated under reflux for three hours. On cooling, very fluffy, fine, white needles (30 mgs.) settled out, and by evaporation of the solvent a further 10 mgs. was obtained. The total yield of the cyclonucleoside quaternary salt which decomposed at 261-3\(^o\) was 40 mgs. (90%).
Calculated for $\text{C}_{25}\text{H}_{24}\text{N}_7\text{PO}_{12} : \text{C}, 46.51; \text{H}, 3.75; \text{N}, 15.20.$

Found: C, 46.13; H, 4.10; N, 14.99.

Paper chromatography in several solvents, and paper electrophoresis at pH 3.4 (800 V, 3.5 m. amps.) resulted in the separation of this compound into two ions, one identical in migration to di-p-nitrophenyl phosphate and the other to a mononized cation. See spectra U.V. I and U.V. II.

B. By Attempted Base Catalysed Transesterification.

2',3'-0-Isopropylidine guanosine-5'-di-p-nitrophenyl phosphate (300 mgs.) was dissolved in dry benzyl alcohol (2 mls.). Sodium benzoate (from 40 mgs. of sodium in 3 mls. of benzyl alcohol) was added, and the orange solution set aside for thirty minutes. Glacial acetic acid was added to neutralize the solution, and the benzyl alcohol was evaporated under high vacuum. The oily residue was taken up in 5% acetic acid, a trace of insoluble material removed by filtration, and the acidic solution extracted with ether until the extracts gave no colour with alkali. The ether extracts were reextracted with 0.01 N sodium hydroxide and shown to contain only 8.0 mgs. of p-nitrophenol by spectrophotometric analysis. The aqueous solution was evaporated to dryness leaving 360 mgs. of a dry, white powder after washing with ether. Chromatographically this material was shown to be identical with the cyclonucleoside salt obtained by heating the neutral ester. It was, however, contaminated with sodium acetate and was not further purified.

7. The Action of Anhydrous Pyridine on 2',3'-0-Isopropylidine Guanosine-5'-di-p-nitrophenyl Phosphate.

Crystalline 2',3'-0-isopropylidine guanosine-5'-di-p-nitrophenyl phosphate (10 mgs.) was dissolved in anhydrous pyridine (1.0 mls.) and stored at room temperature with exclusion of moisture. At intervals 0.005 ml. aliquots were removed, spotted on Whatman No. 1 paper, and developed
in Solvent A. Within one hour a spot corresponding to 2',3'-O-isopropylidene guanosine-5'-mono-p-nitrophenyl phosphate in mobility and ultraviolet spectrum (U.V.10) appeared, and reach a maximum intensity after three days as estimated by elution of the spots with .01N hydrochloric acid and determination of the optical density at 260 μ. At this point the starting material had completely disappeared and was replaced by a fast moving fluorescent zone. Trace amounts of di-p-nitrophenyl phosphate and the 2',3'-O-isopropylidene cyclo-guanosine cation were also observed.

8. 2',3'-O-Isopropylidene Uridine(119) (LV).
Finely powdered, freshly dried uridine (10 g.) was suspended in anhydrous acetone (250 mls.) and concentrated sulfuric acid (0.25 mls.) and anhydrous copper sulfate (20 g.) were added. The suspension was stirred at 37° for forty-eight hours with exclusion of moisture. The copper sulfate was removed by filtration, thoroughly washed with acetone, and the combined filtrates neutralized by shaking with calcium hydroxide (10 g.) for one hour. The calcium salts were removed by filtration, well washed with acetone and the filtrates evaporated to dryness under reduced pressure leaving colourless, crystalline 2',3'-O-isopropylidene uridine (10.8 g., 93%) which was shown by paper chromatography to contain a trace of uridine. One recrystallization from benzene containing a little acetone gave the pure compound melting at 159-60° with excellent recovery. Levene(110) reports m.p. 159-60°.

9. 2',3'-O-Isopropylidene Uridine-5'-di-p-nitrophenyl Phosphate (LVI).
Dry di-p-nitrophenyl phosphate (2.85 g.) was dissolved in anhydrous dioxane (15 mls.) by warming and then rapidly cooled to room temperature.
Di-p-tolyl carbodiimide (900 mgs.) was added and the mixture stored for ten minutes. Dried 2',3'-O-isopropylidine uridine (1.0 g.) was added and the sealed mixture stored overnight. The di-p-tolyl urea (880 mgs.) was removed by filtration and washed three times with small portions of dioxane. The combined filtrates were evaporated to dryness, taken up in chloroform (25 mls.) and extracted with water until the extracts were neutral. The chloroform was then evaporated under reduced pressure leaving chromatographically pure 2',3'-O-isopropylidine uridine-5'-di-p-nitrophenyl phosphate (1.916 g., 90%) after subtraction of 96 mgs. of residual di-p-tolyl urea. This compound was entirely satisfactory for subsequent steps. A sample was crystallized from methanol as beautiful white needles melting at 118.5-120.5°.

Calculated for C_{24}H_{23}N_{4}PO_{13} : C, 47.53; H, 3.82; N, 9.2%. Found: C, 47.3%; H, 3.8%; N, 9.03.

10. Uridine-5'-mono-p-nitrophenyl Phosphate (LVII).

2',3'-O-Isopropylidine uridine-5'-di-p-nitrophenyl phosphate (1.22 g.) was suspended in acetonitrile (30 mls.) and shaken with 1N lithium hydroxide (6.5 mls.) followed by water (15 mls.) for thirty minutes. The acetonitrile was removed by evaporation under reduced pressure and the residual aqueous solution brought to pH 5 with acetic acid. A little di-p-tolyl urea was removed by filtration and the aqueous solution was extracted with ether until the extracts gave no yellow colour with alkali. The pH was then adjusted to 2.7 with acetic acid and the acidic solution was heated in a boiling water bath for ninety minutes. The solution was cooled and evaporated to dryness, acetic acid being completely removed by twice evaporating a solution of the residue in dioxane. The oily residue was dissolved in water (10 mls.)
and slowly passed through a column (2 x 10 cm.) of Dowex 50 (H⁺) resin, the column being then thoroughly washed with water. The total effluent was concentrated to a volume of roughly 5 mls. and brought to pH 4.0 with saturated aqueous barium hydroxide. A trace of insoluble material was removed by centrifugation and the supernatant concentrated to an oil which solidified on trituration with ethyl alcohol. This solid was washed with acetone and ether and dried under vacuum at room temperature to give the barium salt of uridine-5'-mono-p-nitrophenyl phosphate (746 mgs., 67%) as the dihydrate.

Calculated for C₁₅H₁₆N₃P₀₁₁Ba₁/₂·₂H₂O: C, 32.75; H, 3.66; N, 7.64.  
Found: C, 33.34; H, 3.75; N, 7.19.

Paper chromatographically the compound behaved as a single component (Table V). The ultra violet spectrum is shown as U.V. 17.
TABLE V.

Paper Chromatographic Behaviour of Compounds Involved in the Syntheses of Guanosine and Uridine Nucleotides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf's in Solvent Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.00</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.18</td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene guanosine</td>
<td>0.63</td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene guanosine-5'-di-p-nitrophenyl phosphate</td>
<td>0.95</td>
</tr>
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</tr>
<tr>
<td>Guanosine-5'-mono-p-nitrophenyl phosphate</td>
<td>0.14</td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene guanosine-5'-phosphate</td>
<td>0.19</td>
</tr>
<tr>
<td>Guanosine-5'-phosphate</td>
<td>0.0</td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene cycloguanosine cation</td>
<td>0.47</td>
</tr>
<tr>
<td>di-p-nitrophenyl phosphate</td>
<td>0.73</td>
</tr>
<tr>
<td>p-nitrophenol</td>
<td>0.90</td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene uridine-5'-di-p-nitrophenyl phosphate</td>
<td>0.88</td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene uridine-5'-mono-p-nitrophenyl phosphate</td>
<td>0.54</td>
</tr>
<tr>
<td>Uridine-5'-mono-p-nitrophenyl phosphate</td>
<td>0.25</td>
</tr>
</tbody>
</table>
PART II.
THE CHEMISTRY OF SOME XYLOSE PHOSPHATES.

A. Discussion.

1. Introduction to Xylose Phosphates.

Chemical interest in xylose-3-phosphate originated with Robinson's suggestion\(^{(159)}\) in 1927 that the fundamental carbohydrate unit of pentose nucleic acid was xylose rather than ribose. Robinson postulated that during alkaline hydrolysis of the pentose phosphate isolated by acid hydrolysis of nucleotides, an inversion of the esterified hydroxyl group took place similar to that observed during tosyl group hydrolysis. Since the ultimate products of the pentose phosphate hydrolysis were D-ribose and phosphoric acid\(^{(111)}\), the original compound could have been D-xylose-3-phosphate, D-arabinose-2-phosphate or L-xylose-4-phosphate. The latter two were considered unlikely since the parent sugars were found in only small amounts in nature.

In an attempt to cast light upon this problem, Levene and Raymond devoted considerable effort to the development of an unambiguous synthesis of xylose-3-phosphate\(^{(113,114,115,116)}\). Their approach involved phosphorylation, with phosphorus oxychloride and pyridine, of 5-acetyl, 5-benzoyl, and 5-carbo benzoxyl 1,2-O-isopropylidene-D-xylofuranose (I, \(R=\text{CH}_3\text{CO, C}_6\text{H}_5\text{CO}\) and \(\text{C}_6\text{H}_5\text{CH}_2\text{O-CO}\) respectively). In each case a compound was isolated which gave a correct analysis for the expected fully protected xylose-3-phosphate (II). After acid hydrolysis (2N sulfuric acid at 80° for two hours) of the isopropylidene and acyl groups, however, the only product which could be isolated and identified was xylose-5-phosphate (III, identical with the compound obtained by direct phosphorylation of 1,2-O-isopropylidene-D-xylofuranose
(I, R=H) with one mole of phosphorus oxychloride.

The starting materials were rigorously shown to have the assigned structures and hence the isolation of xylose-5-phosphate must have been the result of either acyl migration during phosphorylation or phosphoryl migration during removal of protecting groups. It now appears fairly obvious that the latter is the correct explanation, the acidic conditions used by Levene for removal of the isopropylidene group being vastly more severe than necessary.

With these failures, no subsequent reference to xylose-3-phosphate has occurred in the literature until 1955 with the exception of a mention of its chromatographic behaviour by S.S. Cohen\(^{(52)}\). Dr. Cohen has, however, informed us that this information was erroneous, being based upon a sample from the Levene collection which was actually xylose-5-phosphate.

Watson and Barnwell\(^{(171)}\) have recently reported that the optical rotation of an aqueous solution of disodium salt of D-xylofuranose-5-phosphate at pH 6.5 slowly decreases over the course of several days from +10° to -1°. The free acid did not undergo this phenomenon. The process could be greatly accelerated by holding pH 6.2 phthalate buffer solutions of the sodium salt at 50°. This change in rotation was accompanied by an increase in formaldehyde release on periodate oxidation and has been inter-
preted by Watson in terms of a partial phosphoryl group migration from the 5 to the 3-position. By ion exchange chromatography on a Dowex-1 (sulfate) column, the heated product could be separated into two fractions, the first (43%) being xylose-5-phosphate, and the second (57%) releasing 47% of the formaldehyde expected to result from periodate oxidation of xylose-3-phosphate.

While phosphoryl migrations accompanied by ester cleavage are well known during either acidic or alkaline treatment of phosphate diesters bearing suitably placed hydroxyl functions (14, 19, 37), migrations of primary phosphates are normally restricted to strongly acidic solution. Thus the suggested migration of a primary phosphate from the 5 to the 3 position of xylose under essentially neutral conditions is in direct contrast to our previous knowledge of the subject. This is especially so since Watson has reported that the supposed migration occurred less readily under more acidic conditions.

Our interest in xylose phosphates (14, 15) originated with a general programme of study in this laboratory of the chemistry of cyclic phosphates (60, 165) and in particular of the stereochemical requirements for cyclic phosphate formation (99). Studies of cyclic phosphate formation and phosphoryl migration are to a degree complimentary in that if cyclic phosphate formation is possible, migration is also stereochemically possible, since the accepted mechanism of phosphate migration involves an intermediate cyclic structure (125). Levene's early observations on migration from the 3 to 5 positions of xylose suggest that xylose-3,5-cyclic phosphate would be an interesting compound to study.

The present work has resulted in the synthesis of D-xylofuranose-3,5-cyclic phosphate and various derivatives of this compound by several routes, and have further led to the first synthesis of authentic D-xylose-
2. The Stereochemical Requirements of Cyclic Phosphate Formation.

Before proceeding to a discussion of this work it might be well to summarize the stereochemical requirements which we have established to be necessary for the formation of cyclic phosphates in simple carbohydrates.\(^{(99)}\)

Dekker and Khorana\(^{(60)}\) have shown that the reaction of a phosphate ester bearing a true cis adjacent hydroxyl group (IV) with dicyclohexyl carbodiimide in 80% pyridine, results in the rapid formation of a cyclic phosphate (VI). This cyclization is envisioned as proceeding by way of a highly reactive dicyclohexyl isourea phosphate (V). For the case of five membered cyclic phosphate formation a further reaction with dicyclohexyl carbodiimide occurs leading to the formation of N-phosphoryl ureas (VII).

\[
\begin{align*}
\text{IV} & \quad \text{COH}_{11}N=\text{C}=\text{NCOH}_{11} \\
\text{V} & \quad \text{COH}_{11}N=\text{C}=\text{NCOH}_{11} \\
\text{VI} & \quad \text{COH}_{11}N=\text{C}=\text{NCOH}_{11}
\end{align*}
\]
We have now shown that six membered cyclic phosphate formation may also be accomplished by reaction with dicyclohexyl carbodiimide under the same conditions. The six membered cyclic phosphates may be readily distinguished from the five membered analogues since they do not react further to give N-phosphoryl ureas. The simple phosphates, cyclic phosphates and N-phosphoryl ureas may be readily differentiated by their paper chromatographic behaviour in the iPrOH:NH₄OH:H₂O (7:1:2) system, the Rf's invariably following the order VII > VI > IV and being highly characteristic. This technique has been routinely used to determine whether cyclic phosphate formation may occur, and if so whether the product is five or six membered.

For the ease of phosphate esters of sugars occurring in the planar furanose form, five membered cyclic phosphate formation requires that the adjacent hydroxyl group be on the same side of the lactol ring as the phosphate. Thus the anomeronic D-ribofuranose-1-phosphates may be rapidly differentiated by the fact that on reaction with dicyclohexyl carbodiimide the α-anomer (VIII) forms D-ribofuranose-1,2-cyclic phosphate (IX) and then a phosphoryl urea, whereas the β-anomer (X) remains unchanged.

![Diagrams](VIII, IX, X)

Six membered cyclic phosphates in furanose sugars appear to occur only when a carbon atom not involved in the lactol ring (and, therefore, allowed much greater freedom of position) is present in the cyclic phosphate ring. This is illustrated by the ready cyclization of D-xylofuranose-5-phosphate (XI)
to give D-xylofuranose-3,5-cyclic phosphate (XII) (see later) while D-arabino-

furanose-α-1-phosphate (XIII) gives no cyclic phosphate. D-Ribofuranose-5-

phosphate (XIV) in which the groups at carbons 3 and 5 are trans also fails
to cyclize.

\[ \text{XI} \]

\[ \text{XII} \]

\[ \text{XIII} \]

\[ \text{XIV} \]

For the case of sugar phosphates possessing the pyranose ring, the

conformational structure must be considered. Five membered cyclic phosphates

may be formed when the phosphate and adjacent hydroxyl groups are in the
equatorial-equatorial or in the equatorial-axial arrangements. Thus both

D-glucopyranose-β-1-phosphate (XV) and its α-anomer (XVI) (both of which

almost certainly exist in the stable Cl conformation\(^{(157)}\)) readily form

D-glucopyranose-1,2-cyclic phosphate.
The formation of an equatorial-equatorial five membered cyclic phosphate is interesting in view of the fact that it is fairly well established that neither n-acyl migration (which presumably involves an intermediate five membered ortho ester) nor isopropylidene formation (once again involving a five membered ring) normally occur between adjacent equatorial-equatorial positions. This has been explained in terms of the considerable strain which must be imposed upon the pyranose ring in order to bring the adjacent equatorial hydroxyl groups into a coplanar arrangement for five membered ring formation. This strain may be apparently reduced by the incorporation of a nitrogen atom into the five membered ring since acetyl migration has been reported from O₃ to N₂ in certain glucosamine derivatives, and nitrate migration from O₃ to O₂ in 3-nitro-glucopyranose derivatives.

The large size of the phosphorus atom compared to that of carbon allows bridging of the equatorial-equatorial gap without the introduction of serious strain.

Cyclization is not possible when the phosphate and adjacent hydroxyl groups are both axial. Thus D-mannopyranose-α-l-phosphate (XVII) gives no cyclic phosphate on reaction with dicyclohexyl carbodiimide.

Six membered cyclic phosphate formation in pyranose sugars has not as yet been exhaustively studied due to the difficulty of obtaining suitable compounds for study. D-glucopyranose-6-phosphate (XVIII), however, is readily cyclized to D-glucopyranose-1,6-cyclic phosphate (XIX). It might
also be predicted that a compound such as the hypothetical \(\text{D-altropyranose-\(\alpha\)-l-phosphate (XX), which in the Cl conformation would possess an axial hydroxyl}^{3}\) to an axial phosphate, might form a six membered cyclic phosphate as would certain sugar phosphates in a boat conformation.

While exceedingly stable, seven membered cyclic phosphates may be prepared from aliphatic \(\gamma\)-hydroxy phosphates such as \(4\)-hydroxybutyl phosphate, they have not as yet been observed in carbohydrate derivatives.


In order to confirm the predicted, cyclization of D-xylofuranose-5-phosphate (XI) it was decided to prepare this compound by a published method \((21,69)\). \(1,2;3,5\)-Di-O-isopropylidine-D-xylofuranose (XXI) was readily prepared by condensation of D-xylose with acetone in the presence of zinc chloride and polyphosphoric acid according to Freudenberg \((70)\). Mild acidic hydrolysis of this compound selectively removed one isopropylidine group \((145)\) to give an excellent yield of \(1,2\)-O-isopropylidine-D-xylofuranose (XXII)
Phosphorylation of this compound with slightly more than one equivalent of diphenyl phosphorochloridate\(^{132}\) gave crystalline \(1,2-0\)-isopropylidene-\(D\)-xylofuranose-5-diphenyl phosphate (XXIII) in excellent yield.

Hydrogenolysis of this compound in the presence of a platinum catalyst resulted in the ready cleavage of both phenyl groups to give \(1,2-0\)-isopropylidene-\(D\)-xylofuranose-5-phosphate (XXIV) which could be isolated as its barium salt. The isopropylidene group could be readily removed by heating an aqueous solution of the free acid directly obtained by hydrogenolysis (pH 1.5) at 100° for ten minutes. This is of interest since Jones\(^{69}\) has emphasized that removal of this group requires conditions similar to those reported by Levene (2N sulfuric acid at 100° for three hours). We have repeated this experiment under the conditions described by Jones and shown by paper chromatography that within five minutes the isopropylidene group was completely removed. Prolonged treatment resulted only in marked phosphoryl migration and the formation of a mixture of xylose phosphates—(See later.).

The \(D\)-xylofuranose-5-phosphate (XI) obtained by the reaction sequence above was isolated in excellent yield as its amorphous barium
FLOW SHEET II

SOME REACTIONS OF XYLOSE PHOSPHATES

XXII

CH\sub{2}OH

XXV

Aqueous Pyridine

pH 1.5
100°, 10 min.

C\sub{6}H\textsubscript{5}O\textsubscript{2}C\textsubscript{2}H\textsubscript{2}

XXVI

OH\textsuperscript{−}
or

H\textsubscript{2}/Pt

C\sub{6}H\textsubscript{11}N=CN=C\sub{6}H\textsubscript{11}

Aqueous Pyridine

XXIV

pH 1.5
100°, 10 min.

C\sub{6}H\textsubscript{11}N=CN=C\sub{6}H\textsubscript{11}

Aqueous Pyridine

XXIII

(\text{HO})\textsubscript{2}PO\textsubscript{2}CH\textsubscript{2}

XI

H\textsubscript{2}OH
salt. A portion of this salt was converted to the pyridine salt and treated with dicyclohexyl carbodiimide in 80% pyridine. Examination of the reaction mixture by paper chromatography in the usual way showed that within six hours the slow moving D-xylofuranose-5-phosphate had been completely replaced by a stable faster moving component identified as D-xylofuranose-3,5-cyclic phosphate (XII).

With the realization that 3,5-cyclic phosphates of xylose may indeed be prepared, the possibility arose that hydrolysis of a suitably blocked derivative of this compound might be expected to result in opening of the phosphate ring in either of two directions so as to give a mixture of derivatives of xylose-3- and -5-phosphates. Since primary phosphate esters are more stable in alkaline solution than in acid, alkaline hydrolysis would be the preferred route and 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate (XXVI) might prove to be a suitable starting material. The formation of this compound could be readily demonstrated by the reaction of 1,2-O-isopropylidene-D-xylofuranose-5-phosphate (see above) with dicyclohexyl carbodiimide in the usual way, but a more direct synthesis of this compound was desirable. Several such routes appeared feasible and will be separately discussed below.

Treatment of 1,2-O-isopropylidene-D-xylofuranose (XXII) with monophenyl phosphorodichloridate in anhydrous pyridine resulted in the almost quantitative isolation of 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic monophenyl phosphate (XXV) as an extremely viscous, distillable syrup. There was no indication of the formation of any bis-(1,2-O-isopropylidene-D-xylofuranose) monophenyl phosphate. Mild alkaline hydrolysis or catalytic hydrogenolysis of the neutral ester (XXV) resulted in cleavage of the phenyl group and the formation of 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate (XXVI).
which was isolated in good yield as its crystalline cyclohexylammonium salt and could be converted to the crystalline free acid by removal of barium with a cation exchange resin followed by lyophilization. The catalytic method is extremely rapid and clean and is the preferred route if the cyclic phosphate is to be isolated directly.

The alkaline hydrolysis method did not result in direct exclusive cleavage of the phenyl group. Examination of the reaction by paper chromatography showed that the starting material was rapidly hydrolysed to give two distinct phosphorus containing products (Rf's in the i-propyl alcohol : ammonium hydroxide : water (7:1:2) system 0.72 and 0.65). The slower moving of these was identical in migration to 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate (XXVI) obtained by hydrogenolysis, while the faster compound was presumably 1,2-O-isopropylidene-D-xylofuranose-3 (or 5) monophenyl phosphate arising through ring cleavage rather than phenyl group hydrolysis. Typical of phosphate diesters, or triesters, containing a stereochemically suitably placed hydroxyl function, in alkaline solution this compound underwent transesterification to form the cyclic phosphate (XXVI). This is completely analogous to the formation of ribonucleoside-2',3'-cyclic phosphates upon alkaline hydrolysis of ribonucleic acid(131). Paper chromatography indicated that the complete conversion of the faster moving compound to the cyclic phosphate required seventy-two hours at room temperature. Treatment of the cyclic phosphate as the free acid under its own pH(1.5) at 100° for ten minutes resulted in quantitative removal of the isopropylidene group and led to the isolation of analytically pure D-xylofuranose-3,5-cyclic phosphate (XII) as its amorphous barium salt. This compound was chromatographically identical with the product from the reaction of D-xylofuranose-5-phosphate with dicyclohexyl carbodiimide as
The phosphorylation of 1,2-O-isopropylidene-D-xylofuranose was also attempted using tetra-p-nitrophenyl pyrophosphate as the phosphorylating agent in the usual manner. On extraction of the free di-p-nitrophenyl phosphate with buffer and water, however, the characteristic yellow colour of the p-nitrophenolate ion was observed. The extractions were continued with \(10^{-1}\) M sodium hydroxide (conditions under which di-p-nitrophenyl alkyl phosphates are stable) until the yellow colour no longer appeared. Evaporation of the solvent and crystallization of the resulting oil gave 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic mono-p-nitrophenyl phosphate (XXVIII) as a highly crystalline material which could be rapidly hydrolysed with 0.5 M lithium hydroxide at room temperature to 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate. In this case no intermediate ring opening occurred as in the case of the phenyl analog. The formation of the cyclic p-nitrophenyl phosphate may be once again explained by a transesterification of the highly reactive initial reaction product 1,2-O-isopropylidene-D-xylofuranose-5-di-p-nitrophenyl phosphate (XXVII).

The spontaneous transesterification of the di-p-nitrophenyl compound compared with the ready isolation of its diphenyl analog (XXIII) provides a striking example of the activating influence of the p-nitrophenyl group.
4. The Ring Opening of 1,2-O-Isopropylidene-D-xylofuranose-3,5-Cyclic Phosphate.

The ring opening of 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate was studied using 1N sodium hydroxide at 100°C. Paper chromatography showed that after twenty hours under these conditions the starting material had completely disappeared and been replaced by a single phosphorus containing, non-reducing compound with an Rf identical to that of 1,2-O-isopropylidene-D-xylofuranose-5-phosphate (XXIV). The alkaline ring opening was studied quantitatively by the potentiometric determination of the release of secondary phosphoryl dissociations as described in the Experimental section. The results are shown in Figure III. After removal of sodium ions with Dowex-50 (H⁺) resin the product was isolated as the amorphous barium salt. After conversion to the pyridine salt, treatment with dicyclohexyl carbodiimide in aqueous pyridine resulted in complete reconversion of this product to 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate.

The barium salt obtained after hot alkaline hydrolysis was converted to the free acid by use of Dowex-50 (H⁺) resin and heated under its own pH (1.5) at 100°C for ten minutes. The product of this reaction was isolated in almost quantitative yield as the barium salt and shown by paper chromatography in the i-propanol : ammonia : water (7:1:2) system to contain two distinctly separated components. The slower moving (Rf 0.04) major product was identical in migration to D-xylofuranose-5-phosphate and gave a brown colour with the aniline hydrogen phthalate spray of Partridge (152) modified by the addition of 0.5% concentrated hydrochloric acid. The faster moving component (Rf 0.09) gave a pink colour with the aniline hydrogen phthalate spray, and, as will be shown later, was identified as D-xylopyranose-3-phosphate (XXIX).
FIGURE III
RING OPENING OF 1,2-ISOPROPYLDINE-D-XYLOSE-
3,5-CYCLIC PHOSPHATE BY 1.0 N N\textsubscript{a}OH @ 100°.
5. The Isolation and Proof of Structure of D-xylofuranose-3,5-cyclic Phosphate.

The most convenient route to this mixture of phosphates was found to involve a two stage transesterification and hydrolysis of 1,2-O-isopropylidene-D-xylofuranose-5-diphenyl phosphate (XXIII) which may be obtained in quantity in crystalline form from the reaction of 1,2-O-isopropylidene-D-xylofuranose and diphenyl phosphorochloridate as previously described. Treatment of this neutral ester with 1N aqueous sodium hydroxide in dioxane resulted in an initial transesterification to form 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic monophenyl phosphate (XXV) which was then further hydrolysed during seventy-two hours at room temperature to give 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate (XXVI), partially by way of the open chain -3 (or 5)-monophenyl esters as previously described. When paper chromatography showed this reaction to be complete, the alkaline solution was heated in a boiling water bath for twenty hours as described above in order to open the cyclic phosphate ring.
Ten minutes hydrolysis at pH 1.5 and 100° after ether extraction of the phenol released led to the isolation of an essentially quantitative yield of the mixed barium salts of D-xylose-3- and -5-phosphates. Before proceeding to a discussion of the separation of the xylose-3- and -5-phosphates, it may be mentioned that the acidic ring opening of D-xylofuranose-3,5-cyclic phosphate was also qualitatively examined by paper chromatography. Treatment of the cyclic compound in 1N hydrochloric acid at 100° for two and one-quarter hours, resulted in the complete disappearance of the starting material. In addition to moderate amounts of xylose and inorganic phosphate, the reaction products contained a mixture of xylose phosphates which appeared similar in distribution to that obtained by alkaline hydrolysis. In view of the extensive phosphoryl migration which we have shown to occur under these acidic conditions (see later), this mixture, however, almost certainly contained other xylose phosphates and the method is not of preparative value.

The separation of the mixture of D-xylose-3- and -5-phosphates arising from alkaline ring opening of 1,2-O-isopropylidine-D-xylofuranose-3,5-cyclic phosphate followed by mild acidic treatment was effected by ion exchange chromatography. The separation was initially attempted using a Dowex-1 (sulfate) column and elution with 0.001 M ammonium sulfate as suggested by Watson (17) but gave a poor separation. The two products were cleanly separated, however, by use of a Dowex-2-(formate) column, eluting with 0.1 N formic acid adjusted to pH 3.0 with sodium hydroxide. The peaks were conveniently located by spotting each fifth tube on long strips of filter paper and spraying with the aniline hydrogen phthalate spray. The pooled fractions were concentrated to a small volume, deionized by passage through a cold IR-120 (H+) column, and the effluent and washings lyophilized. The resulting gums were taken up in water, neutralized with barium hydroxide, and the barium salts precipitated with alcohol after removal
of inorganic phosphate. Each fraction was purified by reprecipitation and shown to be chromatographically homogeneous. D-xylose-3-phosphate once again gave a pink colour with the aniline hydrogen phthalate spray, and D-xylose-5-phosphate a brown colour. Pure xylose-3-phosphate was obtained in this way in 18% yield and the 5-phosphate in 45% yield.

Since the mixture obtained by Watson(171) on heating D-xylose-5-phosphate had a small negative rotation, whereas both the D-xylose-3- and -5-phosphates obtained in the present study were dextrorotatory two problems remained, namely proof of structure of our synthetic D-xylose-3-phosphate, and of the compound reported by Watson as being D-xylose-3-phosphate.

The synthetic D-xylose-3-phosphate was analytically pure as based upon total phosphorus(182) and xylose determinations on a freshly dried, anhydrous sample and upon carbon and hydrogen analysis on an air equilibrated sample. The xylose determination was essentially according to Mejbaum(133) and Potter(88) except that both the sample and standards were incubated with Wheat Germ Acid Phosphatase prior to development of the orcinol-ferric chloride colour. The sample contained xylose as the sole reducing sugar as demonstrated by enzymatic dephosphorylation and paper chromatography in several solvents, known to separate the pentoses.

Degradations designed to confirm the position of the phosphate group were considered to be simplified by an initial reduction of the xylose-3-phosphate to xylitol-3-phosphate (XXX). This reduction was readily carried out using either sodium borohydride or catalytic hydrogenation in the presence of platinum. The former method is extremely rapid but requires the fairly tedious removal of boric acid as methyl borate(181) in order to obtain a pure product.

* Worthington Biochemical Corporation, Freehold, N.J.
The catalytic method is, therefore, to be preferred because of its simplicity and the purity of the products. Both xylose-3- and -5-phosphates were reduced to the corresponding xylitol phosphates by this method. While xylitol-5-phosphate (XXXI) gave a small, but consistent, optical rotation of -1.2°, xylitol-3-phosphate, as expected from its symmetry, was completely optically inactive. Similar evidence has been used previously during the proof of structure of ribose-3-phosphate\(^1\)\(^{110,101}\).

The periodate oxidation of the various xylose and xylitol phosphates was of interest. The technique used was essentially that of Percival et al\(^{(62)}\), and was entirely satisfactory for obtaining complete oxidation curves on as little as 5 mgs. of compound. Due to the insolubility of barium periodate it was necessary to deionize barium salts prior to oxidation. The results of the various oxidations (pH 4.5, 20°) are shown in Figure IV. The oxidations of xylose-5-phosphate and xylose-3,5 cyclic phosphate agree closely with the expected results, total uptakes of 2.8 and 0.9 moles of periodate being achieved in five and one hours respectively. The uptake of 2.8 moles by xylose-5-phosphate is somewhat greater than the value of 2.5 reported by Rouser\(^{(130)}\) and indicates a quite marked lability of the intermediate 2-formyl-3-phosphoglyceraldehyde (XXXII) under the
conditions used. As might be expected, the uptake of the third mole of periodate is somewhat slower than that of the first two, being dependant upon initial hydrolysis of the formyl ester\(^{(114)}\).

The oxidation of xylitol-3-phosphate is interesting in that it resulted in the fairly rapid uptake of 4.8 moles of periodate. This may be explained by overoxidation of the intermediate 2-hydroxy malondialdehyde phosphate (XXXIII), a phenomena that is fairly well established whenever a C-H linkage is activated by two adjacent carbonyl groups\(^{(62)}\). This labile intermediate was originally postulated by Courtois and Ramet\(^{(59)}\) during periodate oxidation of a phosphorylated glucose obtained from sucrose phosphate and has recently been mentioned by Cohn\(^{(101)}\) as an intermediate in the periodate oxidation of ribose-3-phosphate and ribitol-3-phosphate. Cohn has reported the uptake of six moles of oxidant by the latter compounds whereas we have observed only five moles from the seemingly analogous case of xylose-3-phosphate and xylitol-3-phosphate.
FIGURE IV
PERIODATE OXIDATION OF XYLOSE PHOSPHATES.

XYLITOL-3-PHOSPHATE

XYLOSE-3-PHOSPHATE

XYLOSE-5-PHOSPHATE

XYLOSE-3,5-CYCLIC PHOSPHATE

TIME (Hours)

PERIODATE UPTAKE (MOLES)
The oxidation of xylose-3-phosphate once again approaches the uptake of five moles of periodate but is decidedly slow after the consumption of one mole of oxidant. This may be explained by the greater stability of the formyl ester (XXXIV) resulting from the initial oxidation compared with that (XXXII) from xylose-5-phosphate which is labilized by the presence of an adjacent carbonyl group.

![Chemical diagram of reactions involving periodate oxidation of xylose-3-phosphate](image)

XXXIV

Conclusive evidence that our synthetic xylose-3-phosphate did indeed bear the phosphate group on the 3 position was obtained by a degradative technique. D-Xylose-3-phosphate was catalytically reduced to xylitol-3-phosphate and treated with just over two equivalents of sodium periodate at pH7.0 in order to just reach the 2-hydroxymalondialdehyde phosphate (XXXIII) stage. This extremely labile compound was stabilised by immediate reduction with sodium borohydride to the stable glycerol-2-phosphate (XXXV). The latter compound was freed from inorganic ions by removal of borate as methyl borate and paper chromatography as a long streak developed in the i-propanol : ammonia : water (7:1:2) system. The stable phosphate ester was located by examination of a marker strip and eluted with water. The eluted material was dephosphorylated with wheat germ acid phosphatase and examined by paper chromatography in the pyridine : ethyl acetate : water (2:7:1) system. Vicinal hydroxyl containing compounds were visualized
by the use of the periodate-benzidine spray of Viscontini\(^{(169)}\) et al. In this way it was shown that the sole observable product was glycerol which could only have originated from a xylose-3-phosphate. In an exactly analogous way xylitol-5-phosphate was treated with three equivalents of periodate to give glycolaldehyde-2-phosphate (XXXVI) which was reduced, purified and dephosphorylated. In this case the sole product was, as expected, ethylene glycol.

![Chemical structures of glycerol, xylitol, and ethylene glycol]

The dephosphorylation step was necessary since no chromatographic system could be readily found which would separate glycerol-2-phosphate and ethylene glycol monophosphate. The parent alcohols, however, were readily separated in the system described.

The assignment of the pyranose ring structure to our synthetic D-xylose-3-phosphate was made on the basis of its reaction with dicyclohexyl carbodiimide in 80% pyridine. Within two hours the slow moving starting
material had completely disappeared and been replaced by a spot typical of a cyclic phosphate. Its Rf \((0.40)\), however, was consistently lower than that of D-xylose-3,5-cyclic phosphate \((0.47)\), both on Whatman No. 1 paper. This cyclic phosphate fairly rapidly disappeared and was replaced by a fast moving component \((Rf 0.88)\) typical of an N-phosphoryl urea. This behaviour is typical of 5 membered cyclic phosphate formation and is incompatible with the D-xylofuranose-3-phosphate (XXXVII) structure in which the hydroxyl group adjacent to the phosphate is on the opposite side of the planar ring. It is, however, readily explained by assuming the structure to be D-xylopyranose-3-phosphate which would exist in the stable C1 conformation\(^{157} \). In this case five membered cyclic phosphate formation would readily occur to give a mixture of D-xylopyranose-3,4- and 2-3- cyclic phosphates.

\[
XXXVII
\]

One might also predict the pyranose ring form for xylose-3-phosphate in view of the considerable steric strain which might be expected between the bulky phosphate group on carbon 3 and the C5 hydroxymethyl group in the furanose form. In the pyranose form, however, all functional groups are thrown into the energetically favoured equatorial position and
little steric strain would be expected. Pitzer strain between the phosphate group and the hydroxyl group at Carbon 2 would also be reduced by assuming the pyranose ring.

Further confirmation of this structure has been provided by Dr. A.S. Perlin of the National Research Council, Prairie Regional Laboratory who has kindly studied the lead tetraacetate oxidation of our synthetic D-xylopyranose-3-phosphate. He has reported that it rapidly consumes slightly less than one equivalent of the oxidant with no release of formic acid. The product of this reaction was the formate ester of a tetrose phosphate (XL) which, after acidic hydrolysis and enzymatic dephosphorylation, gave threose (XLI) as the sole product. Dr. Perlin has interpreted these results as verifying the assigned structure. His results will be published shortly in greater detail.

6. The Acid Hydrolysis of Xylose and Ribose Phosphates.

The rates of acidic hydrolysis of xylose-3- and -5-phosphates as compared with those of ribose 3(2) phosphates leads to some interesting observations. The ribose-3(2) and 5-phosphates were prepared from adenosine-3' and 5' phosphates by hydrolysis with IR-120 (H⁺) resin at 100° as described by Cohn(101) and isolated as their barium salts. Hydrolysis curves for
ribose-3- and -5-phosphates have previously been reported by several workers\(^1\,113\) but were repeated in order to use the same conditions as were employed for the xylose phosphates. Ribose-5-phosphate and xylose-5-phosphate were found to have very similar rates of hydrolysis in 1N hydrochloric acid at 100°, but the 3-phosphates were distinctly different. Ribose-3(2) phosphate was very rapidly hydrolysed, inorganic phosphate release being 97% complete in one hour. Xylose-3-phosphate, however, was hydrolysed at almost the same rate as xylose-5-phosphate, requiring nine to ten hours to reach completion. The results of these studies are shown in Figure V and VI.

A tentative explanation of this difference in hydrolytic behaviour may be advanced if we assume that under the strongly acidic conditions of the hydrolysis, the ribose and xylose-3-phosphates occur in the normal furanose form. In this form, ribose-3-phosphate may be hydrolysed through either simple cleavage of the phosphate group or acid catalysed phosphate migration towards the extremely acid labile 1-position. Xylofuranose-3-phosphate on the other hand is stereochemically capable of migration only to the generally more stable 5-position. This argument would indeed predict stabilization of xylose-3-phosphate relative to ribose-3-phosphate.

Experimental support for this hypothesis has been obtained by chromatographic examination of the xylose phosphate hydrolysis mixtures after roughly 50% hydrolysis had occurred (one and one-half hours). At this time the unhydrolysed sugar phosphate in the xylose-5-phosphate experiment behaved largely like authentic xylose-5-phosphate but did contain a small amount of a compound which exhibited the same Rf and pink colour reaction as xylose-3-phosphate. The unhydrolysed material from the xylose-3-phosphate
FIGURE V
HYDROLYSIS OF XYLOSE-3- and -5-PHOSPHATES BY 1.0 N HCl @ 100°.

FIGURE VI
HYDROLYSIS OF RIBOSE-3(2)- AND -5-PHOSPHATES BY 1.0 N HCl @ 100°.
hydrolysis, however, was very largely converted to a slower moving spot exhibiting the same Rf and brown colour reaction as xylose-5-phosphate. The lack of authentic samples of xylopyranose-2- and -4-phosphates prevents the positive identification of this migration product, but this experiment is entirely analogous to Levene's observed migrations to the 5-position during attempted preparations of D-xylofuranose-3-phosphate(115,116). A more extensive ion exchange chromatographic examination of the intermediate products of sugar phosphate hydrolysis is planned for a later date.

7. The Partial Conversion of D-Xylose-5-Phosphate to D-Xylulose-5-Phosphate.

The only outstanding problem in connection with the present work is the characterization of the compound obtained by Watson on heating xylose-5-phosphate and identified by him as xylose-3-phosphate. The experiment described by Watson has been repeated, the optical rotation changes verified, and the mixed product isolated as the amorphous barium salt. Paper chromatography in the system known to separate xylose-3- and -5-phosphates showed only a single spot identical in Rf with the 5-phosphate. Enzymatic dephosphorylation of the mixture followed by paper chromatography in several solvent systems (see experimental) showed the presence of two components. The major spot was identified as xylose while the minor component gave colour reactions characteristic of a ketose with the orcinol-hydrochloric acid(27) and cysteine-carbazole sprays(162). It was identical in Rf to xylulose in all solvents studied. Treatment of a small portion of the dephosphorylated mixture with bromine water(160) resulted in the disappearance of the xylose spot but, as expected, no apparent loss of xylulose.

The two dephosphorylated products were further identified as xylose and xylulose by separation and purification by paper chromatography and determination of the characteristic orcinol-ferric chloride spectra (8h,138,164) in the range 440-760 m/. The spectra were superimposable
upon those of authentic xylose and xylulose (Figures VII and VIII) obtained under the same conditions.

Qualitative periodate oxidation of the mixed phosphates showed that oxidation was complete within one hour, thus indicating the absence of xylose-3-phosphate. By use of the degredative technique described earlier ethylene glycol was the sole ultimate product indicating that both components bore the phosphate group on the 5-position. Treatment of the sodium salt of xylose-5-phosphate at pH 6.2 for either a week at room temperature or at 50° for two hours, was thus shown to result in a partial isomerization to xylulose-5-phosphate (XLII) rather than in phosphoryl group migration. Prolonged heating or increased temperature does not appear to increase the degree of isomerization. It is interesting to note that the dry barium salt of xylose-5-phosphate on storage at room temperature for several months spontaneously isomerizes to give roughly the same proportion (11-12%) of xylulose-5-phosphate as is obtained in aqueous solution of the sodium salt. The analogous partial conversion of dry barium ribose-5-phosphate to ribulose-5-phosphate has been reported by Axelrod and Jang(6).
FIGURE VII
ORCINOL-FeCl₃ SPECTRA

--- XYLOSE FROM WATSON MIXTURE

----- AUTHENTIC XYLOSE

FIGURE VIII
ORCINOL-FeCl₃ SPECTRA

--- XYLULOSE FROM WATSON MIXTURE

----- AUTHENTIC XYLULOSE
Drs. B.L. Horecker and G. Ashwell of the National Institutes of Health, Bethesda, Maryland, have been kind enough to assay the mixture of barium salts obtained by treatment of xylose-5-phosphate according to Watson's conditions. This was done both by a quantitative cysteine-carbazole method used in their laboratory, and enzymatically, using a recently isolated liver transketolase which specifically requires xylulose-5-phosphate as substrate. Both these methods indicated the presence of 13-14% xylulose-5-phosphate in the mixture obtained under Watson's conditions. This figure is somewhat lower than that suggested by Watson's formaldehyde liberation data but appears in our hands to be representative.

In view of the recently discovered biological importance of xylulose-5-phosphate as an intermediate in the pentose cycle metabolism of carbohydrates, several attempts were made to isolate this compound by ion exchange chromatography after partial isomerization of xylose-5-phosphate. A clean separation could not be achieved, however, and this simple approach appears to be unsuitable.


1,2-O-Isopropylidene-D-xylofuranose-3,5-cyclic phosphate has been prepared by several methods as follows:

1. The reaction of 1,2-O-isopropylidene-D-xylose-5-phosphate with dicyclohexyl carbodiimide in 80% pyridine.

2. Phosphorylation of 1,2-O-isopropylidene-D-xylofuranose with monophenyl phosphorodichloridate followed by catalytic hydrogenolysis of the resulting tertiary ester.

3. Phosphorylation of 1,2-O-isopropylidene-D-xylofuranose with tetra-p-nitrophenyl pyrophosphate or with diphenyl phosphorodichloridate followed by base catalysed cyclization and mild alkaline hydrolysis.
Hot alkaline hydrolysis of this compound gave a mixture of 1,2-O-isopropylidene-D-xylofuranose-3- and 2,5-phosphates which was converted to a mixture of D-xylose-3- and -5- phosphates by mild acidic treatment. These compounds were separated by ion exchange chromatography and the 3-phosphate fully characterized as D-xylopyranose-3-phosphate. A compound described previously in the literature as D-xylose-3-phosphate was shown to be D-xylulose-5-phosphate.

The experimentally determined stereochemical requirements of cyclic phosphate formation are discussed.
B. **Experimental.**

The chromatographic and analytical methods described in Part I were employed, reducing sugars being detected by means of the aniline hydrogen phthalate spray of Partridge\(^{(152)}\), modified by the addition of 0.5% concentrated hydrochloric acid.

The following solvent systems were used, all by the descending technique.

Solvent A: n-Butyl alcohol : acetic acid : water \((4:1:5)\)\(^{(151)}\).

Solvent B: i-Propyl alcohol : 28% ammonium hydroxide : water \((7:1:2)\)\(^{(38)}\).

Solvent E: Pyridine : ethyl acetate : water \((2:7:1)\)\(^{(65)}\).

Solvent F: n-Butyl alcohol : ethyl alcohol : water \((5:1:4)\)\(^{(29)}\).

Solvent G: Methyl ethyl ketone plus 1% concentrated ammonium hydroxide\(^{(151)}\).

1. **1,2,3,5-tetra-O-Isopropylidene-D-xylofuranose (XXI).**

D-xylose (25 g.) was suspended in anhydrous acetone (500 ml.) and freshly fused zinc chloride (30 g.) was added followed by a mixture of phosphorus pentoxide (5 g.) dissolved in 85% phosphoric acid (10 g.). The mixture was shaken mechanically for three hours and the homogeneous solution allowed to stand overnight. Sufficient saturated aqueous sodium carbonate was added to make the solution distinctly basic and after ten minutes the heavy precipitate of mixed inorganic salts was removed by filtration and washed well with acetone. The combined filtrates were evaporated under reduced pressure until the acetone was removed leaving water and an insoluble oil. This mixture was extracted three times with 50 ml. portions of ether and the ether extracts dried over magnesium sulfate. The dried ether solution was evaporated leaving a viscous oil
which was distilled through a short column under high vacuum. The distillate (b.p. 84-86°/0.025 mm.) rapidly crystallized to give 1,2;3,5-di-O-isopropylidene-D-xylofuranose (29.5 g., 78%) as a white crystalline mass melting at 43.0-44.5°. (Reported[113] m.p. 44-45°.

2. 1,2-O-Isopropylidene-D-xylofuranose (XXII).

1,2;3,5-di-O-Isopropylidene-D-xylofuranose (15 g.) was suspended in 0.16% (w/w) sulfuric acid (500 mls.) and shaken at room temperature for three hours. The homogeneous solution was then made slightly basic with aqueous sodium carbonate and taken to dryness. The resulting oily solid was extracted three times with acetone, the acetone evaporated under reduced pressure and the residue distilled through a short column to give a very small amount of unreacted starting material b.p. 90°/0.05 mm.) and 9.7 g. (80%) of 1,2-O-isopropylidene-D-xylofuranose which crystallized on standing but, as reported[145], could not be recrystallized from solvents. M.p. 67.-70°, \([\alpha]_D^{22} = -19.6° (c = 5.5 in water). Watson reports \([\alpha]_D^{26} = -19.3°[21].

3. 1,2-O-Isopropylidene-D-xylofuranose-5-diphenyl Phosphate (XXIII).

1,2-O-Isopropylidene-D-xylofuranose (3.5 g.) was dissolved in anhydrous pyridine (25 mls.) and cooled in an ice bath. Diphenyl phosphor-chloridate (5.0 g., 3.9 mls.) was added dropwise with constant agitation and exclusion of moisture. The mixture was stored overnight at 5° and then evaporated to dryness. The residue was dissolved in chloroform (40 mls.) and extracted six times with water. The chloroform was evaporated to dryness leaving a dry white solid which was readily crystallized from carbon tetrachloride to give 1,2-O-isopropylidene-D-xylofuranose-5-diphenyl phosphate (6.6 g., 88%) as white needles melting at 102-3°. Watson[21] reports m.p. 102.2-102.4°, and Jones[69], 99-100°.
4. **D-Xylofuranose-5-Phosphate (XI).**

1,2-0-Isopropylidene-D-xylofuranose-5-diphenyl phosphate (2.0 g.) was dissolved in methanol (20 mls.) and shaken at room temperature under a slight pressure of hydrogen in the presence of Adams platinum catalyst (200 mgs.). The hydrogen uptake reached a constant, theoretical value of 930 mls. after two hours, after which the catalyst was removed by filtration and the solvent evaporated under reduced pressure. The residue was taken up in water (5 mls.) giving a solution of pH 1.4 which was heated in a boiling water bath for ten minutes to remove the isopropylidine group. The solution was cooled to room temperature and brought to pH 7.5 with saturated aqueous barium hydroxide. A trace of barium phosphate was removed by centrifugation and barium D-xylofuranose-5-phosphate precipitated by the addition of three volumes of ethyl alcohol. The white precipitate was washed twice with 70% ethyl alcohol, and once with 95% ethyl alcohol, acetone and ether prior to drying under high vacuum at room temperature for two hours. The yield of the amorphous hydrated barium salt was 1.50 g. (85%).

Calculated for C_{12}H_{20}O_{7}Ba·2H_{2}O: P, 7.82%.

Found: P, 7.86%.

The dry barium salt must be stored at -10° to prevent partial rearrangement to D-xylulose-5-phosphate. Conversion to the half barium salt by portion-wise addition of 1R-120(H^+) resin to an aqueous solution of the mono barium salt until the pH was 3.5, followed by evaporation of the solvent and trituration of the resulting gum with acetone also gave a stable product.

5. **1,2-0-Isopropylidene-D-xylofuranose-5-Phosphate (XXIV).**

1,2-0-Isopropylidene-D-xylofuranose-5-diphenyl phosphate was hydrogenated with 50 mgs. of Adams platinum catalyst as described above. After removal of the catalyst and evaporation of the solvent, the oily
residue was taken up in 2.0 mls. of water and brought to pH 7.0 with saturated barium hydroxide. Addition of two volumes of alcohol or acetone failed to precipitate the barium salt and so the aqueous solution was evaporated to dryness and triturated with 95% ethyl alcohol giving barium 1,2-0-isopropylidene-D-xylofuranose-5-phosphate as a dry, white powder which behaved chromatographically as a single component. Rf's in Solvent A (0.15), B (0.32).

6. 1,2-0-Isopropylidene-D-xylofuranose-3,5-cyclic monophenyl Phosphate (XXV).

Monophenyl phosphorodichloridate (2.3 g., 11 m.moles) was added dropwise at 0° to an agitated solution of 1,2-0-isopropylidene-D-xylofuranose (1.75 g., 9.2 m. moles) in anhydrous pyridine (10 mls.). The mixture was stored overnight at 5°, evaporated to dryness, and taken up in chloroform (25 mls.). The chloroform solution was extracted eight times with water and the solvent evaporated under reduced pressure. After evacuation under high vacuum an extremely viscous syrup (2.86 g., 95%) was left which was distilled through a short path apparatus virtually without loss to give the neutral cyclic ester 1,2-0-isopropylidene-D-xylofuranose-3,5-cyclic monophenyl phosphate which boiled at 170°/0.01 mm. and on cooling set to a completely imobile glass.

Calculated for C11H17PO7: C, 51.20; H, 5.22.

Found: C, 50.55; H, 5.49.

7. 1,2-0-Isopropylidene-D-xylofuranose-3,5-cyclic mono-p-nitrophenyl Phosphate (XXVIII).

Carefully dried di-p-nitrophenyl phosphate (3.95 g.) was dissolved in anhydrous dioxane (20 mls.) by warming. The clear solution was quickly cooled to room temperature and di-p-tolyl carbodiimide (1.31 g.) was added. Di-p-tolyl urea immediately precipitated and the stoppered mixture was stored for ten minutes. 1,2-0-Isopropylidene-D-xylofuranose (1.0 g.) was
then added and the sealed mixture stored overnight. Di-p-tolyl urea (1.24 g., 88%) was removed by filtration, washed with a little dioxane, and the combined filtrates evaporated to dryness. The resulting syrup was taken up in chloroform (25 mls.) and extracted three times with pH 6.5 acetate buffer (1M) to remove di-p-nitrophenyl phosphate. During these extractions the typical yellow colour of p-nitrophenol was observed. The chloroform solution was then repeatedly extracted with 10⁻⁴ molar sodium hydroxide until no further yellow colour was produced. The chloroform was then evaporated under reduced pressure leaving an oil which was directly crystallized from acetone-petroleum ether (65-110°) to give 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic mono-p-nitrophenyl phosphate (1.30 g., 67%) as stout, colourless needles which melted sharply at 148°.

Calculated for C₁₄H₁₆NPO₅ : C, 45.10; H, 4.40; N, 3.71.

Found: C, 45.05; H, 4.32; N, 3.76.

8. 1,2-O-Isopropylidene-D-xylofuranose-3,5-cyclic Phosphate (XXVI).

A. By hydrogenolysis of 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic monophenyl phosphate.  

1,2-O-Isopropylidene-D-xylofuranose-3,5-cyclic monophenyl phosphate (940 mgs.) was dissolved in methanol (30 mls.) and hydrogenated as before in the presence of Adams platinum catalyst (200 mgs.). Hydrogen uptake (288 mls., theoretical) was complete in twenty-five minutes after which time the catalyst was removed by filtration and the solvent evaporated. The resulting gum was dissolved in water (5 mls.), brought to pH 8.5 with cyclohexylamine and evaporated to dryness, last traces of moisture being removed by evaporation with absolute alcohol. The solid residue was dissolved in a little ethyl alcohol and diluted with four volumes of ether. The white crystalline cyclohexylammonium 1,2-O-isopropylidene-D-xylofuranose-
3,5-cyclic phosphate (850 mgs., 85%) was collected and washed with ether. It decomposed at 217-9°C and had \[ \alpha_D^{21} = +5.5 \ (0, 1.90, H_2O). \]

Calculated for C_{14}H_{26}NPO_7: C, 47.84; H, 7.66; N, 3.99.

Found: C, 47.79; H, 7.50; N, 3.61.

Paper chromatography indicated the presence of a single component. Rf's in Solvent B, 0.63; E, 0.37; F, 0.67.

B. By alkaline hydrolysis of 1,2-0-isopropylidine-D-xylofuranose-3,5-cyclic monophenyl phosphate.

1,2-0-Isopropylidine-D-xylofuranose-3,5 cyclic monophenyl phosphate (2.85 g.) was dissolved in dioxane (8 mls.) in a polyethylene tube and sodium hydroxide (24.5 mls. of 1N) was added. An oil separated and redissolved within fifteen minutes at which time more sodium hydroxide (2.0 mls. of 8N) was added and the stoppered tube kept at room temperature for seventy-two hours, the progress of the reaction being followed chromatographically in Solvent B. The fast moving starting material was rapidly replaced by two spots of Rf 0.72 and 0.65 (1,2-0-isopropylidine-D-xylofuranose-3(5)-monophenyl phosphate and 1,2-0-isopropylidine-D-xylofuranose-3,5-cyclic phosphate respectively). On prolonged storage the faster of these spots gave way in favour of the slower, the conversion being complete in seventy-two hours. The solution was adjusted to pH 5 by portionwise addition of Dowex 50 (H⁺) resin, the resin removed by filtration and thoroughly washed with water. The combined filtrates were extracted five times with ether and the aqueous solution passed slowly through a Dowex 50 (cyclohexylammonium form) column (1 x 10 cm.). The column was well washed with water and the total effluent evaporated to dryness leaving a residue which crystallized after thorough drying. It was crystallized from ethyl alcohol ether (1:4) giving cyclohexylammonium 1,2-0-isopropylidine-D-xylofuranose-3,5-cyclic phosphate.
(2.08 g., 68%) identical in melting point and chromatographic behaviour to that obtained by hydrolgenolysis.

The crystalline cyclohexylammonium salt (200 mgs.) was dissolved in water and passed slowly through a Dowex 50 (H\(^+\)) column (1 x 3 cm.) at 5°. The column was washed with water until the effluent was neutral, and the total effluent was lyophilized leaving a partially crystalline residue. This was crystallized from a mixture of ethyl alcohol and ether giving 1,2-O-isopropylidine-D-xylofuranose-3,5-cyclic phosphate (100 mgs., 70%) as the free acid which turned brown at 161° and decomposed at 163°.

Calculated for C\(_9\)H\(_{13}\)PO\(_7\): C, 38.11; H, 5.20.

Found: C, 37.81; H, 5.11.

Potentiometric titration showed the absence of any secondary phosphoryl dissociation in the range pH3-8.


A. By Acidic Hydrolysis of 1,2-O-Isopropylidine-D-xylofuranose-3,5-cyclic Phosphate.

The crystalline cyclohexylammonium salt of 1,2-O-isopropylidine-D-xylofuranose-3,5-cyclic phosphate (250 mgs.) was dissolved in water (2 mls.) and passed through a Dowex 50 (H\(^+\)) column (1 x 3 cm.) the column being washed with water until the effluent was neutral. The total effluent (c.a. 15 mls., pH 1.4) was heated in a boiling water bath for 10-15 minutes, rapidly cooled, and adjusted to pH 4.0 with barium hydroxide. The solution was concentrated to a small volume and four volumes of acetone were added. A gum initially separated but on trituration solidified to a dry white solid which was washed with ether and dried under vacuum at room temperature giving the barium salt of D-xylofuranose-3,5-cyclic phosphate (162 mgs., 86%). \[\alpha_D^{18} = -11.16°\] (C = 2.16, H\(_2\)O)
Calculated for \( \text{C}_{12}\text{H}_{15}\text{PO}_{7}\text{Ba}_2 \): C, 21.47; H, 2.88.


This product consumed one mole of periodate, (Figure IV) showed no secondary phosphoryl dissociation after deionization with LR-120 (H\(^+\)) resin, and behaved chromatographically as a single component. Rf's in Solvent B, 0.33; E, 0.00; F, 0.14.

B. By the reaction of D-xylofuranose-5-phosphate with dicyclohexyl carbodiimide.

Barium D-xylofuranose-5-phosphate (5 mgs.) was well stirred with Dowex 50 (pyridinium form) resin (0.2 mls.) and a little water. The clear supernatant solution was evaporated to dryness and taken up in pyridine containing 20% water (1 ml.). Dicyclohexyl carbodiimide (50 mgs.) was added and the initially clear solution set aside at room temperature. At intervals chromatographic samples were removed and developed with Solvent B after initially washing the spotted papers with petroleum ether (30-60\(^\circ\)) to remove carbodiimide. Within six hours the spot corresponding to D-xylofuranose-5-phosphate (Rf 0.05) had disappeared and been replaced by one of Rf identical to that of D-xylofuranose-3,5-cyclic phosphate.

If samples of barium D-xylofuranose-5-phosphate which had been stored at room temperature for some time were tested as above, a second weak spot (Rf 0.88) appeared corresponding to the phosphoryl urea derived from traces of D-xylulose-5-phosphate.

10. **Mixed 1,2-0-Isopropylidine-D-xylose-3- and -5-phosphates.**

Crystalline cyclohexylammonium 1,2-0-isopropylidine-D-xylofuranose-3,5-cyclic phosphate (1.0 g.) was dissolved in 1N sodium hydroxide (10 mls.) and heated in a polyethylene tube at 100\(^\circ\) for twenty hours. Paper chromatography in Solvent B then showed the complete conversion of the starting material (Rf 0.67) to a mixture of 1,2-0-isopropylidine-D-xylose-3- and -5-phosphates which moved as a single spot of Rf 0.40. The alkaline
solution was then cooled, brought to pH 5 with Dowex 50 (H⁺) resin, extracted four times with ether, and passed through a cold column (2 x 10 cm.) of Dowex 50 (H⁺) resin, washing well with water. The acidic effluent was neutralized to pH 7.5 with barium hydroxide and the amorphous mixed barium salts of 1,2-O-isopropylidine-D-xylose-3- and -5-phosphates (1.04 g., quantitative) isolated by evaporation of the neutralized solution and trituration of the residue with acetone.

A small sample of the mixed barium salts was converted to the pyridinium salts and treated with dicyclohexyl carbodiimide as previously described. Paper chromatography in Solvent B showed that within two hours the mixed starting products (Rf 0.1+0) were completely reconverted to a single spot of 1,2-O-isopropylidine-D-xylofuranose-3,5-cyclic phosphate (Rf 0.67).

   A. By alkaline hydrolysis of 1,2-O-isopropylidine-D-xylofuranose-3,5-cyclic phosphate.

   The crystalline cyclohexylammonium salt of 1,2-O-isopropylidine-D-xylofuranose-3,5-cyclic phosphate (1.95 g.) was hydrolysed exactly as above in 1N sodium hydroxide (20 mls.) at 100° for twenty hours. The solution was then cooled, adjusted to pH 5 with Dowex 50 (H⁺) resin, extracted with ether and passed through a column of Dowex 50 resin. The acidic effluent (pH 1.5) was heated in a boiling water bath for ten minutes, cooled and brought to pH 8 with barium hydroxide. After removal of a trace of barium phosphate two volumes of ethyl alcohol were added to precipitate the barium salts of D-xylose-3- and -5-phosphates (2.19 g., 96%) which were washed in the usual manner. At this stage the two components could be cleanly separated in Solvent B using an overnight run on Whatman No. 4 paper. D-Xylose-5-phosphate was the major component (Rf 0.64) and gave
a brown colour with the aniline hydrogen phthalate spray, while D-xylose-3-phosphate (Rf 0.09) gave a pink colour.

B. By direct alkaline hydrolysis of 1,2-O-isopropylidene-D-xylofuranose-5-diphenyl phosphate.

1,2-O-Isopropylidene-D-xylofuranose-5-diphenyl phosphate (3.0 g.) was dissolved in dioxane (10 mls.) and shaken for a few minutes in a polyethylene bottle with 1N sodium hydroxide (29 mls.). An oil initially separated and then dissolved. The overall alkali concentration was adjusted to 1N by the addition of 7N sodium hydroxide (4.0 mls.) and the solution was stored at room temperature for seventy-two hours as previously described. The solution was then heated in a boiling water bath for twenty hours, cooled, ether extracted at pH5, deionized, and heated at its own pH for ten minutes as above. After neutralization to pH 8.0 with barium hydroxide and addition of two volumes of alcohol the mixed barium salts of D-xylose-3- and -5-phosphates (2.14 g., 93%) were isolated in the usual manner.


The mixed barium salts of D-xylose-3- and -5-phosphates (1.5 g.) were dissolved in water (5 mls.) and applied to the top of a column (4 x 11 cm.) of Dowex 2 (formate) resin. The column was washed with water (1 li.) and the products eluted with 0.1N formic acid adjusted to pH 3.0 with sodium hydroxide, 20 ml. fractions being collected by means of an automatic fraction collector. The appearance of reducing sugars was followed by multiple spotting of each fifth tube on long strips of filter paper and spraying with the aniline hydrogen phthalate spray. No reducing materials appeared during the first five liters of effluent. The next 2350 mls. contained D-xylose-5-phosphate (brown colour with spray) followed by a liter of non-reducing effluent. The next 2500 mls. contained xylose-3-phosphate (pink colour with spray).
The two pooled reducing sugar fractions were separately concentrated below 20° to 25 mls. and passed through columns (1.5 x 10 cm.) of Dowex 50 (H⁺) resin. The columns were washed with water until the effluent was neutral, and the total effluents lyophilized giving viscous gums which were dissolved in the minimum volume of water and adjusted to pH 7.5 with barium hydroxide. Barium phosphate (29 mgs. and 140 mgs. from D-xylose-3- and -5-phosphates respectively) was removed by centrifugation, and the barium salts of the sugar phosphates precipitated by the addition of two volumes of cold ethyl alcohol. The white precipitates were washed and dried at room temperature in the usual manner to give:

1. Barium-D-xylofuranose-5-phosphate (680 mgs., 45.4%) identical to that previously described.
   Calculated for C₅H₁₀P₂O₅Ba.2H₂O: P, 7.82.
   Found: P, 7.86.

2. Barium-D-xylose-3-phosphate (230 mgs., 18%) as a white amorphous solid.
   Calculated for C₅H₁₀P₂O₅Ba: P, 8.47; xylose, 11.0.
   Found (freshly dried sample): P, 8.40; xylose, 11.5.
   \[ \int\alpha \int_D^{22} = \times 1.27^0 \] (C=5.13, H₂O).
   Calculated for C₅H₁₀P₂O₅Ba.3H₂O: C, 14.33; H, 3.60.
   Found (air equilibrated sample): C, 14.70; H, 3.63.
   (The xylose determination was done generally according to Potter except that the samples (H₂O) and standard xylose samples were dephosphorylated by incubation at 37° and pH5 for twenty-four hours with 20 ° of wheat germ acid phosphatase and a trace of magnesium acetate prior to development of the orcinol-ferric chloride colour at 100° for fifteen minutes.)

The D-xylose-3- and -5-phosphates were readily separated in Solvent B (Rf's 0.09 and 0.04) and each was shown to be free from
contamination by the other.

13. Xylitol-3- and -5-Phosphates (XXX and XXXI).

Barium-D-xylose-3-phosphate (30 mgs.) was dissolved in water (2 mls.) and shaken under a slight pressure of hydrogen in the presence of Adams platinum catalyst (20 mgs.). Hydrogen uptake was largely complete in two hours after which the solution was concentrated to about 0.5 mls. and a trace of insoluble material removed by centrifugation. Addition of 1.5 volumes of ethyl alcohol threw out barium xylitol-3-phosphate as a white solid which was dried and reprecipitated once giving the pure product (25 mgs., 83%).

\[ [\alpha]_D^{21} = 0.00^\circ \] \( (c = 4.8, H_2O) \)

Calculated for \( C_5H_9PO_4Ba \) : P, 8.42%.

Found: P, 8.44%.

In an exactly similar fashion the barium salt of xylitol-5-phosphate was obtained in 91% yield. \[ [\alpha]_D^{21} = +1.21^\circ \] \( (c=1.24, H_2O) \).

Calculated for \( C_5H_{11}PO_4Ba \) : P, 8.42%.

Found: P, 8.38%.

14. Periodate Oxidations of the Xylose and Xylitol Phosphates.

The barium salt of the phosphate ester (10-15 mgs.) was dissolved in the minimum volume of water and thoroughly stirred with freshly washed 1R-120 (\( H^+ \)) resin (0.2 mls.). The supernatant solution was withdrawn into a 25 ml. volumetric flask and the resin repeatedly washed with water (10 x 0.5 mls.). pH4.5 Acetate buffer (0.1M) was added to the combined aqueous solution to a volume of 20 mls. and 0.10 M periodic acid in pH4.5 acetate buffer (2.0 mls.) was added. The solution was diluted to 25 mls. with water. A blank was prepared in exactly the same way only omitting the phosphate sample. The solutions were stored at room temperature in the dark. At intervals 2.0 mls. aliquots were removed from both sample and blank, and sodium
bicarbonate (0.5 g.), 0.1N sodium arsenite (2.0 mls.), and 20% aqueous potassium iodide (1.0 ml.) were added. The resulting solutions were stoppered and stored for fifteen minutes and then titrated to the starch end point with standardized 0.0995 N iodine in aqueous potassium iodide. Aliquots were removed and examined in this way until the periodate consumption had reached a constant value. The results are shown in Figure IV.

15. Degradation of D-Xylitol-3- and -5-Phosphates to Glycerol and Ethylene Glycol.

Barium-D-xylitol-3-phosphate (10 mgs.) was converted to the sodium salt by stirring with an aqueous suspension of 1R-120(Na) resin. The clear supernatant was concentrated to 0.5 mls. and 0.10 M sodium periodate (0.60 mls., pH7.0) was added. After thirty minutes solid sodium borohydride (20 mgs.) was added and after five hours the pH was adjusted to 8.0 with sodium hydroxide. The neutral solution was stored overnight. Paper chromatography indicated the complete conversion of the extremely labile 2-hydroxy-malondialdehyde phosphate to glycerol-2-phosphate during this time. The solution was made acidic with acetic acid and evaporated to dryness. Boric acid was removed as methyl borate by repeated evaporation of methyl alcohol and the resulting white powder was purified by chromatography as a long streak developed in Solvent B. The zone corresponding to a stable phosphate ester was eluted with water and evaporated to dryness. The residue was dissolved in pH4.5 acetate buffer (0.2 mls.) and .2M magnesium acetate (0.1 mls.) and wheat germ acid phosphatase (2 mgs.) were added. The resulting solution was incubated at 37° for two hours and examined by paper chromatography in Solvent D. The only spot visualized with the periodate-benzidine spray (169) corresponded in Rf to authentic glycerol.

By exactly the same technique barium D-xylitol-5-phosphate (10 mgs.) was degraded with sodium periodate (0.75 mls. of 0.10N), and after
reduction and dephosphorylation was shown to give rise to only ethylene glycol which was cleanly separated from glycerol in Solvent D.


These were prepared according to Cohn(101) by treatment of aqueous adenosine-3'- and -5'- phosphates (500 mgs.) with 1R-120 (H⁺) resin at 100° for four minutes. The sugar phosphates were precipitated as the amorphous barium salts and shown spectrophotometrically to contain less than 0.5% unhydrolysed adenylic acid. Similar to the xylose phosphates these were readily separated in Solvent B, D-ribose-5-phosphate having Rf 0.05 and giving a brown colour with the aniline hydrogen phthalate spray while D-ribose-3(2)-phosphate had Rf 0.15 and gave a pink colour on spraying.

17. Acidic Hydrolysis of the Xylose and Ribose Phosphates.

Samples of the barium salts of D-xylose-3- and -5-phosphates and D-ribose-3(2)- and -5-phosphates(101) (5 mgs.) were dissolved in the minimum volume of water and stirred with 1R-120 (H⁺) resin (0.1 ml.). The supernatants were transferred into 5 ml. volumetric flasks and the resin thoroughly washed with small volumes of water. The volumes were brought to about 4 mls. and concentrated hydrochloric acid (0.43 mls.) was added. The solutions were quickly diluted to 5.0 mls. with water (overall acid concentration 1N) and transferred to hydrolysis flasks consisting of small flasks (bulb capacity about 6 mls.) with long necks (1 x 25 cm.) fitted with an efficient condenser. These flasks were then placed in a boiling water bath and after equilibration fitted with a stopper. At intervals the flasks were inverted to wash down the walls, and 0.20 ml. aliquots were removed. A predetermined amount of 2.0 N sodium hydroxide was added sufficient to neutralize the acid and the inorganic phosphate release was measured by the method of Lowry and Lopez(128). The hydrolysis curves are shown in Figures V and VI.
18. Quantitative Alkaline Ring Opening of 1,2-O-Isopropylidene-D-xylofuranose-3,5-cyclic Phosphate.

Crystalline cyclohexylammonium 1,2-O-isopropylidene-D-xylofuranose-3,5-cyclic phosphate (137 mgs.) was dissolved in 1N sodium hydroxide (4 mls.) contained in a polyethylene tube. The solution was heated in a boiling water bath under a reflux condenser fitted with a soda lime tube. At intervals one ml. aliquots were removed (the volume need not be accurate) and passed slowly through a small column (1 x 0.5 cm.) of Dowex 50 (H⁺) resin, washing with water. The acidic effluent was titrated potentiometrically to pH 8.0 with 0.100 N sodium hydroxide. The results are shown in Table VI below. Column A shows the volume of alkali (mls.) necessary to neutralize the acidic solution to pH 4.0 (primary phosphoryl dissociations) while column B shows the volume of alkali required to neutralize the solution from pH 4.0 to 8.0 (secondary phosphoryl dissociations released). The ratio of B to A will, therefore, directly indicate the percentage of ring opening since at 100% B/A. The hydrolysis curve is shown in Figure III.

**TABLE VI**

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>A</th>
<th>B</th>
<th>% Ring opening (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.95</td>
<td>0.18</td>
<td>19.0</td>
</tr>
<tr>
<td>5</td>
<td>0.94</td>
<td>0.59</td>
<td>62.8</td>
</tr>
<tr>
<td>11.5</td>
<td>1.02</td>
<td>0.91</td>
<td>89.2</td>
</tr>
<tr>
<td>23</td>
<td>0.76</td>
<td>0.77</td>
<td>100</td>
</tr>
</tbody>
</table>

Barium D-xylose-5-phosphate (250 mgs.) was converted to the sodium salt by passing it through a column (1 x 3 cm.) of 1R-120 (Na⁺) resin. The effluent (20 mls.) had a pH of 8.5 and was adjusted to pH 6.2 with dilute acetic acid. The resulting solution was held in a water bath at 50° for 2.5 hours giving a very pale yellow solution which was passed through a column (1 x 5 cm.) of 1R-120 (H⁺) resin and washed well with water. The effluent was concentrated to dryness, taken up in water (3 mls.) and adjusted to pH 8.0 with barium hydroxide. The addition of two volumes of ethyl alcohol caused the precipitation of the white, amorphous mixed barium salts of D-xylose-5-phosphate and D-xylulose-5-phosphate (230 mgs., 92% recovery).

By enzymatic dephosphorylation (see below) this sample was shown to be very similar to that prepared by use of a pH 6.2 phthalate buffer as described by Watson (171). In the latter case it was necessary to extract the phthalic acid with ether from the deionized solution prior to precipitation of the barium salts in order to prevent contamination with barium phthalate.

20. Enzymatic Dephosphorylation of the Xylose and Xylulose Phosphates.

The barium salts of D-xylose-3- and -5-phosphates (2-3 mgs.) and of the mixed xylose and xylulose-5-phosphates (5 mgs.) were dissolved in pH 4.5 acetate buffer (0.5 mls.) and magnesium acetate (.02 mls. of .1M) and wheat germ acid phosphatase (1 mg.) were added. The solutions were incubated for two hours at 37° and examined by paper chromatography in solvent systems E, F and G.

In solvent E, the sole product from dephosphorylation of D-xylose-3-phosphate and D-xylose-5-phosphate was a spot of Rf 0.26 identical in mobility to authentic xylose. The mixture obtained by heating xylose-5-phosphate at 50°
however, led to two spots of Rf 0.26 and 0.42 identical to xylose and xylulose respectively.

In solvent F, dephosphorylation of xylose-3- and -5-phosphates gave only xylose, Rf 0.26, while the Watson treated mixture gave two spots of Rf 0.26 and 0.33 once again identical to xylose and xylulose.

In solvent G using a long run, the spots identified as xylose were shown to indeed be xylose and not ribose or lyxose, the three being cleanly separated.

The spots identified as xylulose gave characteristic colour tests for ketoses with the orcinol-hydrochloric acid (27) and cysteine-carbazole (162) sprays, and were stable to bromine water oxidation while xylose was destroyed.


The mixed barium salts (5 mgs.) obtained by heating D-xylofuranose-5-phosphate at 50° were enzymatically dephosphorylated as above and applied as a long streak to a strip of Whatman No. 4 paper. The chromatogram was developed overnight by the descending technique in Solvent E. By spraying marker strips out from the edges of the paper with the aniline hydrogen phthalate spray two distinct zones were located and eluted from the main paper with water. Each eluted material was concentrated and rechromatographed as above. The entire faster moving material and one-quarter of the slower compound were concentrated to about 1.0 ml. and added to 4.0 mls. of a solution of freshly crystallized orcinol (85 mgs.) and ferric chloride (8.5 mgs.) in concentrated hydrochloric acid (25 mls.). The yellow solutions were held in a boiling water bath for ten minutes, rapidly cooled, and their spectra (λmax 760 mμ) determined using the eluate from a strip of Whatman No. 4 paper treated in exactly the same way as a blank. The resulting spectra (Figures VII and VIII) were identical with those of authentic xylose
and xylulose, the characteristic ratios at 540/670 nm being 0.23 and 0.39 for the treated material and 0.22 and 0.39 for authentic xylose and xylulose.
ULTRA VIOLET ABSORPTION SPECTRA

U.V.1
Di-p-NITROPHENYL PHOSPHATE
pH2
$E_{287} = 17600$

U.V.2
91° M.P. COMPOUND FROM PREPARATION OF
Di-p-NITROPHENYL PHOSPHATE
pH2
$E_{287} = 18450$
U.V. 3
PYRIDINE
0.0001 M FORMIC ACID
$E_{256} = 5400$

Authentic

--- From 91° M.P. Compound

U.V. 4
Di-p-NITROPHENYL METHYL PHOSPHATE
pH 2
$E_{271} = 17390$
U.V. 5
MONO-p-NITROPHENYL METHYL PHOSPHATE
pH 2
$E_{288} = 9300$

U.V. 6
p-AMINOPHENYL METHYL PHOSPHATE
pH 2
$E_{263} = 480$
U.V. 7
N-METHYL PYRIDINIUM ION
pH 7

U.V. 8
N-METHYL PYRIDINIUM-di-p-NITROPHENYL PHOSPHATE
pH 7
$E_{287} = 17950$
U.V. 9
p-NITROPHENOL
pH 2
$E_{317} = 9800$

U.V. 10
2',3'-ISOPROPYLIDINE GUANOSINE-
5'-MONO-p-NITROPHENYL PHOSPHATE
pH 2
$E_{258} = 15100$
GUANOSINE-5'-MONO-
p-NITROPHENYL PHOSPHATE

pH 2

$\varepsilon_{258} = 15700$

U.V.11

GUANOSINE-5'-PHOSPHATE

pH 2

$\varepsilon_{258} = 15700$

U.V.12

2', 3' -ISOPROPYLIDINE
GUANOSINE 5'-PHOSPHATE

pH 2

U.V.12
GUANOSINE-5'-PHOSPHATE

\[ \varepsilon_{250} (\text{pH} 2) = 12400 \]
\[ \varepsilon_{260} (\text{pH} 12) = 12100 \]

Wave Length (m\(\mu\))

U. V. 14

2', 3'-ISOPROPYLIDINE-CYCLO-
GUANOSINE DI-p-NITROPHENYL PHOSPHATE

\[ \varepsilon_{258} = 20000 \]
U.V. 17
URIDINE-5'-MONO-p-NITROPHENYL PHOSPHATE
pH 2
$E_{266} = 13900$

OPTICAL DENSITY

WAVE LENGTH (m$\mu$)
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