CARBOHYDRATE PHOSPHATES

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In their classical studies of the effect of inorganic phosphate on cell-free sugar (glucose) fermentation, Harden and Young showed that carbohydrate phosphate is formed, in addition to alcohol and carbon dioxide, according to the equation:

$$2\ \text{Glucose} + 2\ \text{Phosphate} = 1\ \text{Hexose diphosphate} + 2\ \text{Alcohol} + 2\ \text{Carbon dioxide} \quad . \quad . \quad . \quad . \quad (1)$$

The phosphoric ester which accumulates is fructose 1 : 6-diphosphate, frequently termed the Harden–Young ester. These observations led to other important investigations, for example, the isolation of glucose monophosphates by Robison and Embden, studies of glycolysis in muscle extracts by Meyerhof and Lohmann, and Lundsgaard's observations on the chemical events which accompany the alactacid muscle contraction. This work clearly indicated that phosphoric esters play a central part in the biological world by linking processes of respiration and fermentation with other essential cellular reactions. Quite a large number of esters of this class are now known and the group is still growing as further compounds are isolated. Amongst these phosphoric esters, those of the carbohydrates form a principal class, and are known to function as intermediates in the network of enzymic reactions associated with the breakdown and interconversion of carbohydrates in plants and animals. Consequently it is not surprising that this class of compound has attracted widespread interest and there is a flood of papers annually on their biological function. Although strictly chemical studies are less numerous all facets of the subject could not be condensed adequately into a Review of the present type, so we propose to emphasise chemical synthesis, isolation, and reactions of sugar phosphates, and to mention only briefly their biological rôle. For more detailed accounts reference should be made to recent reviews.[1–3]

Detection and Estimation

Both in the intact cell and in the isolated enzyme systems in which biological reactions are studied, phosphoric esters usually occur as mixtures. Colour reactions are used to identify the sugar component of carbohydrate phosphates; *e.g.*, the reaction with resorcinol permits the estimation

[1] Leloir, *Fortschr. Chem. org. Naturstoffe*, 1951, **8**, 47.
[2] Foster, Overend, and Stacey, *Die Stärke*, 1953, **11**, 285.
[3] Benson, "Phosphorylated Sugars" in "Moderne Methoden der Pflanzenanalyse", Vol. II, Springer-Verlag, Berlin, 1955.

of ketose esters,[4] and pentose esters are detected with orcinol. The rate of colour development with the latter reagent serves to differentiate ribose 3- and 5-phosphate,[5] * and also compounds containing phosphoribose residues (and possibly related pentose phosphates). (The method is rapid and can be used on as little as 10 μg. of phosphate ester. It cannot be used precisely on crude plant and bacterial extracts containing polysaccharides since these alter the rate of colour development.)

As in other branches of carbohydrate chemistry, paper chromatography provides a valuable micromethod for identification. Removal of interfering ions by ion-exchange resins from hydrolysates of hexosephosphates improves the chromatograms.[6] Hanes and Isherwood [7] demonstrated that it is feasible to separate phosphoric esters, including compounds of very similar constitution, on a filter-paper chromatogram, and to detect them by spraying the papers with an acid molybdate solution and then heating them under conditions which hydrolyse the esters without unduly decomposing the paper. The orthophosphoric acid produced forms a phosphomolybdate complex and this is reduced to an intensely blue compound on exposure to hydrogen sulphide. Various solvent mixtures have been detailed for the chromatographic separation,[8] and lists of R_F values for sugar phosphates have been published. Addition of boric acid to the solvents helps to separate esters with *cis*-hydroxyl groups from esters in which this grouping is absent.[9]

The unidimensional chromatography described by Hanes and Isherwood does not always adequately resolve the complex mixtures obtained from some plant materials (cf. Mortimer [8]), and so two-dimensional chromatography, with successive development in an acid and in a basic solvent, has been worked out.[10] In addition, modifications of the original Hanes–Isherwood method have been described. It is claimed that by upward migration at 4° on acid-washed paper with appropriate solvents, it is possible to adopt shorter running times and achieve higher R_F values. This method gives more discrete spots than the two-dimensional procedure, and these spots are detected by dipping rather than spraying the papers.[11] Irradiation with ultraviolet light, resulting in colour differences,[10] has been used to differentiate between organic compounds containing bound phosphorus and those containing inorganic phosphate.

Two drawbacks to the widely used Hanes–Isherwood method are that

[4] Roe, *J. Biol. Chem.*, 1934, **107,** 15.

[5] Albaum and Umbreit, *ibid.*, 1947, **167,** 369.

[6] Dulberg, Roessler, Sanders, and Brewer, *ibid.*, 1952, **194,** 199.

[7] Hanes and Isherwood, *Nature*, 1949, **164,** 1107.

[8] Mortimer, *Canad. J. Chem.*, 1952, **30,** 653 ; see also Wright and Khorana, *J. Amer. Chem. Soc.*, 1956, **78,** 811, and Loring, Levy, and Moss, *Analyt. Chem.*, 1956, **28,** 539.

[9] Scott and Cohen, *J. Biol. Chem.*, 1951, **188,** 509 ; *Science*, 1950, **111,** 543.

[10] Bandurski and Axelrod, *J. Biol. Chem.*, 1951, **193,** 405.

[11] Burrows, Grylls, and Harrison, *Nature*, 1952, **170,** 800.

* In this Review, nomenclature of the type, ribose 5-phosphate, is used, as customary, when it is not desired to specify whether the compound is present as free acid R·O·PO_3H_2 or as salt. For particular derivatives the Anglo-American agreed nomenclature is used [see *J.*, 1952, 5111, rule 11(d)], *e.g.*, α-D-ribose 5-(barium phosphate).

the prolonged initial " digestion " necessary to break down the more resistant esters often leaves the paper in a fragile state, and that further analyses cannot be carried out on the spot after the detection treatment. To overcome the former difficulty Fletcher and Malpress [12] used an enzyme (alkaline phosphomonoesterase) to break down the esters resolved on the chromatogram. To counteract the latter a method has been used depending on fixation of ferric ions by the esters, and reaction of the free ferric ion with " salicylsulphonic acid ".[13] The phosphates appear as white spots on a pale mauve background, orthophosphoric acid having a band of deeper mauve surrounding it. Other spot indicators of ferric ions also have given good results but in some cases the colours fade in light.

In experiments with diphenylphosphoric esters, ultraviolet contact prints have been used to find the position of the spots on a chromatogram,[14] and radiograms of sugar phosphates labelled with ^{32}P have been examined by Calvin and his colleagues [15] during their work on photosynthesis.

Ion-exchange resin chromatography has been used to separate mixtures of sugar phosphates; *e.g.*, Horecker and Smyrniotis [16] used Dowex-1 formate for the separation of pentose phosphates formed from 6-phosphogluconate by yeast enzyme. Separations have also been achieved by ion-exchange with the aid of the borate complex,[17, 18] and by ionophoresis [14] in borate buffer at pH 10 and in acetate buffer at pH 5 at 800 v. Different phosphate esters have been separated by counter-current distribution: the solubility in the organic phase was increased by addition of long-chain amines.[19]

Procedures other than chromatography have also been used to estimate sugar phosphates. A method described by Slater [20] depends on enzymic conversion of these compounds into dihydroxyacetone phosphate which subsequently reacts with reduced diphosphopyridine nucleotide (DPN) in the presence of glycerol phosphate dehydrogenase. The amount of reduced nucleotide undergoing reaction is determined spectrophotometrically. The method is highly sensitive—0·05 millimole of phosphorylated sugar can be measured with an accuracy of a few per cent. Methods for the estimation of fructose diphosphate [21] and glucose 6-phosphate [22] have been outlined and very small amounts of glucose diphosphate can be estimated by taking advantage of its coenzyme activity for phosphoglucomutase.[23] A method

[12] Fletcher and Malpress, *Nature*, 1953, **171**, 838.
[13] Wade and Morgan, *ibid.*, p. 529.
[14] Matthews and Overend, unpublished results.
[15] Benson, Bassham, Calvin, Goodale, Haas, and Stepka, *J. Amer. Chem. Soc.*, 1950, **72**, 1710.
[16] Horecker and Smyrniotis, *Arch. Biochem. Biophys.*, 1950, **29**, 232.
[17] Khym and Cohn, *J. Amer. Chem. Soc.*, 1953, **75**, 1153.
[18] Khym, Doherty, Volkin, and Cohn, *ibid.*, p. 1262.
[19] Plaut, Kuby, and Lardy, *J. Biol. Chem.*, 1950, **184**, 243.
[20] Slater, *Biochem. J.*, 1953, **53**, 157.
[21] Meyerhof and Wilson, *Arch. Biochem. Biophys.*, 1948, **17**, 153.
[22] Haas, *J. Biol. Chem.*, 1944, **155**, 333.
[23] Cardini, Paladini, Caputto, Leloir, and Trucco, *Arch. Biochem. Biophys.*, 1949, **22**, 87.

proposed for the estimation of fructose diphosphate is based on the determination of the phosphate groups liberated during osazone formation.[24] Differences in the rate of hydrolysis of various sugar phosphates provide in some cases a method for their estimation in simple mixtures. Optical rotation has been used for distinguishing between ribose 3- and 5-phosphate and other pentose phosphates.

Isolation from Natural Sources and Preparation by Enzymic Methods

The pioneer investigations of Harden and his colleagues stimulated work on the isolation of sugar phosphates from natural sources. In addition to the changes formulated in equation (1) (the Harden–Young equation) it is possible under different conditions to obtain, by the use of dried yeast or yeast-juice fermentations, hexose monophosphate in amounts varying from 20 to 50% or more. The diphosphates can be separated from the monophosphates [25] and can be further differentiated by fractional crystallisation of their brucine salts.[26]

In 1937, Cori, Colowick, and Cori [27] showed that α-D-glucose 1-phosphate (Cori ester) is formed when a solution of glycogen, inorganic phosphate, and adenylic acid is incubated with a dialysed muscle extract. Phosphorylase is now known to be widespread in Nature. The reverse of this reaction, namely, the enzymic conversion of the Cori ester into 1 : 4-α-glucosans, is well known (cf. equation 2) and a recent review in this series by Barker and Bourne [28] on the enzymic synthesis of polysaccharides includes a full discussion of the formation of amylose and glycogen from glucose 1-phosphate.

$$\underset{\text{1 : 4-α-Glucosan}}{(C_6H_{10}O_5)_n} + n K_2HPO_4 \rightleftharpoons \underset{\text{K salt of Cori ester}}{n C_6H_{11}O_5 \cdot OPO_3K_2} \quad . \quad . \quad (2)$$

At equilibrium the ratio of total inorganic phosphate to total glucose 1-phosphate depends on the pH value of the system, but the ratio of the bivalent ions $[HPO_4]^{2-}/[C_6H_{11}O_5 \cdot O \cdot PO_3]^{2-}$ is independent of pH and is always constant [29, 30] at 2·2. Hence the conversion of an unbranched 1 : 4-α-glucosan into α-glucose 1-phosphate can be carried to virtual completion if the polysaccharide is treated with phosphorylase in the presence of a sufficiently large excess of inorganic phosphate to ensure that the equilibrium ratio of the bivalent ions is not attained before all the polysaccharide is degraded.[31–33] Since the enzymic degradation of amylose is so effective and easy to control, the preparation of α-glucose 1-phosphate by this method is popular. Glucose 1-phosphate can be rearranged by

[24] Deuticke and Hollman, *Z. physiol. Chem.*, 1939, **258**, 160.
[25] Robison and Morgan, *Biochem. J.*, 1930, **24**, 119.
[26] Robison and King, *ibid.*, 1931, **25**, 323.
[27] Cori, Colowick, and Cori, *J. Biol. Chem.*, 1938, **123**, 375, 381.
[28] Barker and Bourne, *Quart. Rev.*, 1953, **7**, 56.
[29] Hanes, *Nature*, 1940, **145**, 348; *Proc. Roy. Soc.*, 1940, *B*, **128**, 421; **129**, 174.
[30] Trevelyan, Mann, and Harrison, *Arch. Biochem. Biophys.*, 1952, **39**, 419, 440.
[31] Swanson, *J. Biol. Chem.*, 1948, **172**, 805, 825.
[32] Bourne, Sitch, and Peat, *J.*, 1949, 1448.
[33] Hestrin, *J. Biol. Chem.*, 1949, **179**, 943.

phosphoglucomutase to glucose 6-phosphate,[34] which can also be obtained in good yield directly from starch by using phosphorylase and phosphoglucomutase in conjunction.[35] In *Escherichia coli* a biosynthesis has been detected in which a transphosphorylation between two glucose 1-phosphate molecules gives glucose 1 : 6-diphosphate and free glucose.[36] Glucose 1 : 6-diphosphate has been isolated in small amount from crude fructose diphosphate preparations [37] obtained by fermentation procedures, the diphosphates being separated by destroying the fructose ester with alkali which leaves the glucose analogue unchanged. Hydrolysis of fructose 1 : 6-diphosphate with phosphatase splits off both phosphate residues at the same rate and thus half of the monophosphate formed is fructose 1-phosphate.[38] It is not advisable to use highly purified phosphatase since the crude enzyme also changes fructose 6-phosphate into glucose 6-phosphate. The glucose derivative can be oxidised to 6-phosphogluconic acid and separated as its insoluble barium salt. Consequently less fructose 6-phosphate remains to be separated from the l-isomer than is the case if purified enzyme is used. Fructose 1-phosphate was later obtained by an aldolase-induced condensation of phosphodihydroxyacetone and D-glyceraldehyde.[39] If DL-glyceraldehyde is used the products are fructose 1-phosphate and sorbose 1-phosphate.[39]

The action of kinases on the sugars is well established. To cite one example, fructose 6-phosphate is formed when fructose and adenosine triphosphate are incubated with yeast hexokinase.[40, 41] This ester is also produced by the action of a specific enzyme (phosphomannose isomerase) on mannose 6-phosphate, itself obtained by phosphorylation of mannose with hexokinase. There are many references to the enzymic preparation of other hexose phosphates but the products have not in all cases been fully purified or satisfactorily characterised.

Kalckar [42] observed that enzymic phosphorolysis of some ribonucleosides (inosine, guanosine) leads to the formation of a pentose phosphate, considered to be D-ribofuranose 1-phosphate. The yield is very low, possibly owing

$$\text{Inosine} + \text{Phosphate} \rightleftharpoons \text{Ribose 1-phosphate} + \text{Hypoxanthine} \quad . \quad (3)$$

to losses by acid hydrolysis, to specific and non-specific contaminant phosphatase action during the incubation with the enzyme, and to retention on the bulky barium phosphate precipitate during working-up. Moreover in reaction (3) the equilibrium favours formation of the nucleoside rather

[34] Colowick and Sutherland, *J. Biol. Chem.*, 1942, **144**, 423 ; Sutherland, Colowick, and Cori, *ibid.*, 1941, **140**, 309.

[35] Swanson, *ibid.*, 1950, **184**, 647.

[36] Leloir, Trucco, Cardini, Paladini, and Caputto, *Arch. Biochem. Biophys.*, 1949, **24**, 65.

[37] *Idem*, *ibid.*, 1948, **19**, 339 ; 1949, **22**, 87.

[38] MacLeod and Robison, *Biochem. J.*, 1933, **27**, 286.

[39] Meyerhof, Lohmann, and Schuster, *Biochem. Z.*, 1936, **286**, 301, 319.

[40] Kunitz and McDonald, *J. Gen. Physiol.*, 1946, **29**, 393.

[41] Berger, Slein, Colowick, and Cori, *ibid.*, p. 379.

[42] Kalckar, *J. Biol. Chem.*, 1945, **158**, 723 ; 1947, **167**, 477 ; *Fed. Proc.*, 1945, **4**, 248 ; *Symp. Soc. Expt. Biol.*, 1947, **1**, 38.

than of the pentose phosphate. Addition of xanthine-oxidase to the system leads to the removal of hypoxanthine by conversion into xanthine and uric acid: the pentose phosphate is then isolable as its barium salt. Naturally occurring nucleosides are derivatives of β-ribofuranose,[43] and apparently nucleoside phosphorylase produces inversion and this ribose 1-phosphate has been shown to have the α-configuration.[44a] Synthetic ribopyranose 1-phosphate [45] will not serve as substrate for the enzyme producing nucleosides, a result which suggests that the pentose phosphate produced according to equation (3) is of the furanose type. This has been confirmed by the chemical synthesis of α-D-ribofuranose 1-phosphate [44b] which was found to be identical with enzymically prepared samples, and to be fully active as a substrate for the fish-muscle purine-nucleoside phosphorylase.

Phosphorolysis of deoxyribonucleosides has also been achieved. Enzyme preparations from calf-thymus gland and rat liver act on hypoxanthine deoxyriboside to give an acid-stable phosphate ester; this is 2-deoxyribose 5-phosphate, and is formed from deoxyribose 1-phosphate by mutase action.[46] From the enzymic phosphorolysis product of guanine deoxy-D-riboside, Friedkin [47] isolated 2-deoxy-D-ribose 1-phosphate as the crystalline *cyclo*hexylamine salt. Recently a simplified procedure for the isolation of deoxyribose 1-phosphate has been developed: it involves phosphorolysis of thymidine in the presence of ammonium di*cyclo*hexyl hydrogen phosphate, followed by a fractionation with butan-1-ol-diethyl ether, which yields crystalline di*cyclo*hexylammonium deoxyribose 1-phosphate after a single filtration.[48] This ester is even more unstable than ribose 1-phosphate and is hydrolysed by the acid used in methods for phosphate estimation: it therefore appears in analyses as "inorganic phosphate".

By mutase action ribose 1-phosphate can be converted into ribose 5-phosphate. Klenow and Larsen [49] have shown that phosphoglucomutase acting with glucose 1 : 6-diphosphate (and probably ribose 1 : 5-diphosphate) as coenzyme will bring about this change. Preparations from liver also contain a mutase capable of transforming ribose 1-phosphate into the 5-isomer.[50]

Levene *et al.*[51] claimed to have prepared ribose 3-phosphate from nucleotides (xanthylic and yeast adenylic acid) but more recent work has shown that they were handling mixtures. In the light of present knowledge concerning the migrations of phosphate esters it is obvious that the experimental conditions employed by the Levene school could not have resulted in the retention of isomeric integrity in the compounds studied, but would lead

[43] Davoll, Lythgoe, and Todd, *J.*, 1946, 833.

[44] (*a*) Wright and Khorana, *J. Amer. Chem. Soc.*, 1956, **78**, 811; (*b*) Tener, Wright, and Khorana, *ibid.*, p. 506.

[45] Kalckar, *Biochim. Biophys. Acta*, 1950, **4**, 232.

[46] Manson and Lampen, *J. Biol. Chem.*, 1951, **191**, 95.

[47] Friedkin, *ibid.*, 1950, **184**, 449.

[48] Friedkin and Roberts, *ibid.*, 1954, **207**, 257.

[49] Klenow and Larsen, *Arch. Biochem. Biophys.*, 1952, **37**, 488.

[50] Wajzer and Baron, *Bull. Soc. Chim. biol.*, 1949, **31**, 750.

[51] Levene and Harris, *J. Biol. Chem.*, 1932, **95**, 755; **98**, 9; 1933, **101**, 419.

to mixtures. Ribose phosphates have been obtained in ingenious fashion by Khym *et al.*[18] Hydrolysis of the glycosylamine nitrogen–carbon linkage in adenylic acid " a " and " b " was achieved with the hydrogen form of a polystyrenesulphonic acid resin, at a rate comparable with the rate of isomerisation. The ribose phosphates were released from the resin at the moment of formation (in contrast to adenine and most of the adenylic acid) and little or no isomerisation takes place subsequent to their formation. In this way ribose 2-phosphate was obtained from adenylic acid " a " and ribose 3-phosphate from adenylic acid " b ". Subsequently the method was developed to obtain pure ribose 2- and 3-phosphate by hydrolysis of adenylic acids with a polystyrenesulphonic acid cation-exchange resin. The mixture of phosphate esters was separated by ion-exchange chromatography with borate complex-formation. Khym *et al.* also prepared ribose 5-phosphate by treating adenosine-5′ phosphate with resin [Dowex-50(H^+)] at 100° for 4 minutes. This ester had been obtained by Levene and Jacobs [52] by subjecting the barium salt of inosinic acid to acidic hydrolysis, thereby cleaving the sugar–base linkage. An improved method for the preparation from muscle of inosinic acid and then of ribose 5-phosphate has been described recently. Optimum conditions were determined for the hydrolysis.[53] This ester is also obtainable by acidic hydrolysis of cozymase [54] and it can be prepared in a high degree of purity from adenosine triphosphate by ion-exchange.[55] A fraction containing 70–80% of ribose 5-phosphate is afforded when xylose and adenosine triphosphate are incubated with a pentose phosphate isomerase from extracts of *Lactobacillus pentosus*.[56a] The enzymic conversion of 6-phosphogluconic acid into ribulose 5-phosphate and then ribose 5-phosphate is now well established.

In the past, to obtain ribose phosphates from ribonucleotides it has been necessary to work with purine nucleotides, but very recently Cohn and Doherty [56b] have developed a method for obtaining ribose from pyrimidine nucleosides, and ribose phosphates from pyrimidine nucleotides. The accessibility of sugars (and derivatives) of pyrimidine nucleosides and nucleotides is severely limited by the resistance of the glycosylamine linkage to acid hydrolysis. It has long been known that this stability is dependent on the ethylenic unsaturation between the adjacent carbon atoms in the ring and that reduction or bromination of the 4 : 5-double bond renders the glycosylamine linkage susceptible to acid hydrolysis. Cohn and Doherty completely hydrogenated pyrimidine ribonucleotides under mild conditions with a rhodium catalyst and cleaved the product by dilute alkali at room temperature to the phosphate of β-ribosylureidopropionic acid. Dilute acid at room temperature hydrolyses this substance to ribose phosphate and β-ureidopropionic acid without appreciable isomerisation of the

[52] Levene and Jacobs, *Ber.*, 1908, **41,** 2703 ; 1911, **44,** 746.
[53] Marmur, Schlenk, and Overland, *Arch. Biochem. Biophys.*, 1951, **34,** 209.
[54] Schlenk, *J. Biol. Chem.*, 1942, **146,** 619.
[55] Groth, Mueller, and LePage, *ibid.*, 1952, **199,** 389.
[56] (*a*) Lampen, *ibid.*, 1953, **203,** 999 ; (*b*) Cohn and Doherty, *J. Amer. Chem. Soc.*, 1956, **78,** 2863 ; (*c*) Bergmann and Burke, *Angew. Chem.*, 1955, **67,** 127.

phosphate group, thus making available the sugar phosphates of pyrimidine nucleotides. No previous isolation of a sugar phosphate from a pyrimidine nucleotide had been reported and even the reduction of such substances to achieve labilisation of the glycosylamine linkage has been achieved only rarely. The sodium–ethanol–liquid ammonia procedure, so effective with nucleosides, is seemingly ineffective with nucleotides.[56c] From uridylic acids " a " and " b " ribose 2- and 3-phosphate respectively were obtained, thus confirming the identity of the pyrimidine nucleotide isomers. The method is also applicable to deoxyribonucleotides and has been used with deoxycytidylic and thymidylic acid.

Evidence has been presented to show that xylose is phosphorylated at the expense of adenosine triphosphate by extracts of *Pseudomonas hydrophila*.[57]

Chemical Syntheses

Intrigued by the problems presented, and no doubt stimulated by the biological implications of sugar phosphates, organic chemists have developed chemical syntheses for many members of this class. In early experiments it was usual to phosphorylate unprotected sugars, and the products were probably mixtures. As far as we can trace, the first phosphorylation of a carbohydrate was carried out in 1858 by Berthelot,[58] who treated glucose with syrupy phosphoric acid at 140°. In the past, the most widely used reagent in synthesis of sugar phosphates was phosphoryl chloride. It was used by Neuberg and Pollak[59a, b] to prepare sucrose and dextrose phosphates, by Fischer[60] to obtain a phosphoric ester of methyl glucoside, and by Helferich *et al.*[61] to phosphorylate an unprotected disaccharide (trehalose). Neuberg and Pollak attempted to control the reaction by adding alkali to absorb the hydrogen chloride formed. Substances which have been added by others for the same reason include sodium hydroxide, magnesium oxide, anhydrous pyridine, and quinoline. Inconsistencies have been noted and it has been reported that phosphorylation of glucose was unsuccessful when barium or calcium hydroxide was replaced by calcium carbonate as the added base.[59b, 62] More examples need to be studied before all the inconsistencies can be satisfactorily explained.

Many phosphorylations have been carried out with suitably protected sugars. The following are a few representative examples : reaction between methyl 2 : 3-*O*-*iso*propylidene-D-ribofuranoside and phosphoryl chloride in pyridine at −40°, followed by hydrolysis of the *iso*propylidene and glycoside residues, yielded ribose 5-phosphate ;[63] arabinose 5-phosphate has also been prepared ;[64] phosphorylation of 1 : 2-5 : 6-di-*O*-*iso*propylideneglucose

[57] Hochster and Watson, *Nature*, 1952, **170**, 357.
[58] Berthelot, *Ann. Chim.* (*France*), 1858, **54**, 81.
[59] Neuberg and Pollak, (*a*) *Biochem. Z.*, 1910, **23**, 515 ; **26**, 514 ; (*b*) *Ber.*, 1910, **43**, 2060.
[60] Fischer, *Ber.*, 1914, **47**, 3193.
[61] Helferich, Löwa, Nippe, and Riedel, *Z. physiol. Chem.*, 1923, **128**, 141.
[62] Fawaz and Zeile, *ibid.*, 1940, **263**, 175.
[63] Levene and Stiller, *J. Biol. Chem.*, 1934, **104**, 299.
[64] Levene and Christman, *ibid.*, 1938, **123**, 607.

and 1 : 2 : 3 : 6-tetra-*O*-acetylglucose, and subsequent removal of the protecting groups, afforded glucose 3- [65, 66] and 4-phosphate [67] respectively.

Another reagent which has been used to some extent is phosphoric oxide. Di-*O*-*iso*propylidene-D-fructopyranose was phosphorylated with phosphoric oxide in ether, and the intermediate product, presumably a mixture of tri-, di-, and mono-*O*-*iso*propylidenefructose 1-phosphate, was subjected to hydrolysis : fructose 1-phosphate was isolated as the *cyclo*hexylammonium salt.[68]

Although it is usual, and frequently necessary, to use protected sugars in phosphorylations, selective reaction of free sugars can be achieved in some instances. After the conversion of glucose into glucose 6-phosphate [69] by metaphosphoric acid, Percival and Anderson [70a] directly phosphorylated glucosamine at position 6 with metaphosphoric acid in the presence of acetonitrile. Phosphate residues on other positions were removed by hydrolysis of the crude product with N-hydrochloric acid at 100°. (A purer product has since been prepared by these workers by an alternative route ; [70b] cf. Maley and Lardy.[70c]) Amino-sugar phosphate esters had previously only been obtained enzymically.

In addition to working with protected sugars, nowadays it is usual to use protected phosphorylating agents to eliminate undesirable side reactions. The value of such reagents was realised many years ago, since Langheld [71] in 1910 used ethyl metaphosphate in chloroform. To prevent the formation of di- and tri-esters it has become customary to use a disubstituted phosphoryl monochloride, usually in pyridine, as the phosphorylating agent. It is essential, of course, that the protecting groups should be removed easily under mild conditions. Compounds which have been suggested as useful include phosphorochloridic dianilide, the catechol ester of phosphorochloridic acid, and dibenzyl and diphenyl phosphorochloridate. The first-named compound was used for the phosphorylation of a series of compounds including a sugar ; [72] the aniline residues were removed as acetanilide by hydrolysis with acetic acid. It is claimed that catechol can be eliminated from the catechol ester of phosphorochloridic acid merely by treatment with water.[73] The most useful and widely used phosphorylating agents are dibenzyl and diphenyl phosphorochloridate. The benzyl or phenyl groups can be readily cleaved by hydrogenolysis. The diphenyl derivative, which is a stable liquid (for preparative details see Brigl and Müller [74]

[65] Nodzu, *J. Biochem.* (*Japan*), 1926, **6,** 31 ; *Chem. Abs.*, 1927, **21,** 924.

[66] Levene and Raymond, *J. Biol. Chem.*, (*a*) 1928, **79,** 621 ; (*b*) 1929, **83,** 619 ; (*c*) 1930, **89,** 479.

[67] Raymond, *ibid.*, 1936, **113,** 375.

[68] Pogell, *ibid.*, 1953, **201,** 645.

[69] Viscontini and Olivier, *Helv. Chim. Acta*, 1953, **36,** 466.

[70] (*a*) Percival and Anderson, *Chem. and Ind.*, 1954, 1018 ; (*b*) Anderson and Percival, *J.*, 1956, 814 ; (*c*) Maley and Lardy, *J. Amer. Chem. Soc.*, 1956, **78,** 1393.

[71] Langheld, *Ber.*, 1910, **43,** 1857.

[72] Zetzsche and Büttiker, *Ber.*, 1940, *B*, **73,** 47.

[73] Reich, *Nature*, 1946, **157,** 133.

[74] Brigl and Müller, *Ber.*, 1939, **73,** 2121.

or Baer [75]), has been used for the synthesis of monophosphates of aldo-,[76, 77] keto-,[66b, 74, 78] 2-deoxy-,[79] and 2-amino-2-deoxy-hexoses,[70b, c] and of pentoses [80, 81] and 2-deoxypentoses.[82] In addition, it has been used to prepare some *aldehydo*-sugar phosphates.[83] The initial reaction between the protected sugar and the phosphorylating agent proceeds in good yield and the products are frequently crystalline. The phenyl residues can be removed, not only by hydrogen and a catalyst, but also by dilute sodium hydroxide and in some cases by sodium in liquid ammonia. Illustrative of the use of this reagent are the following : phosphorylation of benzyl 3 : 4 : 6-tri-*O*-acetyl-β-D-glucoside yielded the 2-(diphenyl phosphate), which was treated with hydrogen over Adams catalyst to afford hexahydrobenzyl 3 : 4 : 6-tri-*O*-acetyl-β-D-glucoside 2-phosphate from which the free glycoside phosphate was obtained by deacetylation.[77] Similar phosphorylation of 1 : 3 : 4 : 6-tetra-*O*-acetyl-β-D-glucose followed by treatment of the product with potassium methoxide in methanol yielded glucose 2-(dipotassium phosphate). 1 : 2-*O*-*iso*Propylidene-D-xylose, when phosphorylated in anhydrous 2 : 6-lutidine at −20° with diphenyl phosphorochloridate, afforded pure crystalline 1 : 2-*O*-*iso*propylidene-D-xylofuranose 5-(diphenyl phosphate) ; hydrogenolysis in glacial acetic acid over Adams catalyst then quantitatively removed the phenyl groups ; mild hydrolysis in acetic acid cleaved the *iso*propylidene grouping, and D-xylofuranose 5-phosphate was obtained in 72% yield from D-xylose.[81] Phosphorylation of 2 : 3 : 4 : 5-tetra-*O*-acetyl-D-galactose diethyl mercaptal with diphenyl phosphorochloridate in pyridine proceeded readily at 0°, yielding crystalline 2 : 3 : 4 : 5-tetra-*O*-acetyl-D-galactose diethyl mercaptal 6-(diphenyl phosphate) which on scission of the ethylthio-residues afforded 2 : 3 : 4 : 5-tetra-*O*-acetyl-*aldehydo*-D-galactose 6-(diphenyl phosphate). A similar reaction sequence was successfully completed with the 2-deoxygalactose analogue.[83] 1 : 3 : 4-Tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine with the reagent yielded the 6-(diphenyl phosphate) which after hydrogenolysis and acidic hydrolysis of the acetyl groups afforded crystalline D-glucosamine 6-phosphate [70b] (cf. ref. 70*c*). Diphenyl phosphorochloridate was used in the nucleotide field by Bredereck and his collaborators.[84] Monophenyl [85] phosphorochloridate (and phosphorochloridic monoanilide [72]) has been used for the production of phosphate esters, but shows no advantage over the corresponding disubstituted derivative.

Although Zervas [86] mentioned the use of dibenzyl phosphorochloridate,

[75] Baer, " Biochemical Preparations ", Wiley and Sons, New York, 1949, Vol. I, p. 51.
[76] Reithel and Claycomb, *J. Amer. Chem. Soc.*, 1949, **71**, 3669.
[77] Farrar, *J.*, 1949, 3131.
[78] Mann and Lardy, *J. Biol. Chem.*, 1950, **187**, 339.
[79] Foster, Overend, and Stacey, *J.*, 1951, 980.
[80] Parker, Ph.D. Thesis, Birmingham, 1952.
[81] Barnwell, Saunders, and Watson, *Chem. and Ind.*, 1955, 173 ; *Canad. J. Chem.*, 1955, **33**, 711.
[82] Allerton, Overend, and Stacey, *Chem. and Ind.*, 1952, 952.
[83] Barclay, Foster, and Overend, *J.*, 1955, 2505.
[84] Bredereck, Berger, and Ehrenberg, *Ber.*, 1940, **73**, 269.
[85] Gulland and Hobday, *J.*, 1940, 746. [86] Zervas, *Naturwiss.*, 1939, **27**, 317.

he considered it too unstable to be of practical value. The reagent has been developed by Todd and his co-workers and is used extensively by them. If the sole purpose is the preparation of monoesters, then possibly the more stable diphenyl analogue is more convenient, but the use of dibenzyl phosphorochloridate is not limited to the preparation of simple phosphoric esters, and can be applied to the preparation of esters of pyrophosphoric acid and triphosphoric acid (see p. 76). A synthesis of ribose 5-phosphate provides an example of the use of this reagent. Methyl 2 : 3-*O*-*iso*propylidene-D-ribofuranoside with this phosphorylating agent in pyridine at low temperature affords methyl 2 : 3-*O*-*iso*propylidene-D-ribofuranoside 5-(dibenzyl phosphate), from which protecting groups were removed by the usual methods to give ribose 5-phosphate in high yield.[87] In the nucleotide field thymidine-3′ phosphate was synthesised by the phosphorylation of 5′-triphenylmethylthymidine with this reagent and subsequent elimination of the triphenylmethyl and benzyl residues.[88]

Some sugar phosphates have been prepared by phosphorylation at one site, the ester grouping being then caused to migrate to another. Levene and Raymond [89] tried to prepare xylose 3-phosphate by phosphorylation of 5-*O*-benzoyl-1 : 2-*O*-*iso*propylidenexylose but the product was xylose 5-phosphate. Likewise, phosphorylation of 5-*O*-benzyloxycarbonyl- or 5-*O*-acetyl-1 : 2-*O*-*iso*propylidenexylose also yielded xylose 5-phosphate after removal with mineral acid of the acyl and *iso*propylidene groups, and obviously a phosphate migration had occurred. Recently it was claimed by Watson and Barnwell [90a] that migration in the reverse direction (*i.e.*, from position 5 to position 3) afforded xylose 3-phosphate : xylose 5-phosphate was merely heated in water at pH 6·4 at 50° for 2 hours. This claim was soon shown to be incorrect by Moffatt and Khorana,[90b] who successfully prepared and fully characterised D-xylose 3-phosphate. Crystalline 1 : 2-*O*-*iso*propylidene-D-xylofuranose 5-(diphenyl phosphate) was converted by alkali into the 1 : 2-*O*-*iso*propylidenexylofuranose 3 : 5-(cyclic phosphate) which was hydrolysed quantitatively to a mixture of 1 : 2-*O*-*iso*propylidene xylose 3- and 5-phosphate from which the *iso*propylidene groups were readily cleaved by the aqueous acids at 100° for 10 minutes at their own pH. The xylose 3- and 5-phosphates were separated satisfactorily on a Dowex-2(formate) resin column, and the products differentiated structurally by standard carbohydrate reactions. The 3-isomer was obtained in 15% yield.

The nature of the reaction responsible for the change in the optical rotation of a solution of D-xylose 5-phosphate was re-investigated because the properties of the sample of D-xylose 3-phosphate, prepared as described above, were completely different from those of solutions of D-xylose 5-phosphate treated according to Watson and Barnwell's procedure.[90a] Moreover a migration under neutral conditions, as postulated by Watson and Barnwell,

[87] Michelson and Todd, *J.*, 1949, 2476. [88] *Idem*, *J.*, 1953, 951.

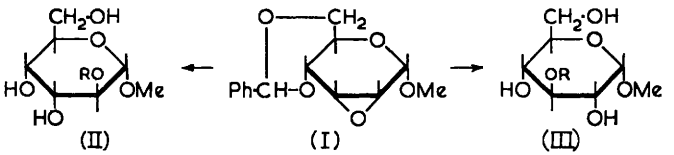
[89] Levene and Raymond, *J. Biol. Chem.*, 1934, **107**, 75 ; cf. *ibid.*, 1933, **102**, 317, 331, 347.

[90] (*a*) Watson and Barnwell, *Chem. and Ind.*, 1955, 1089 ; (*b*) Moffatt and Khorana, *J. Amer. Chem. Soc.*, 1956, **78**, 883 ; (*c*) Axelrod and Jang, *J. Biol. Chem.*, 1954, **209**, 847.

seemed highly improbable. The change was found to be really due to the formation of xylulose 5-phosphate from xylose 5-phosphate, a transformation analogous to that previously observed by Axelrod and Jang,[90c] who reported that ribose 5-(barium phosphate) can be partially converted at room temperature into a ribulose-containing compound.

When ribose 2- or 3-phosphate is heated for 2 hours with Dowex 50(H^+) resin or for 45 minutes with 0·1N-sulphuric acid it forms ribose 4-phosphate in low yield.

A method of preparing phosphoric esters which might be further exploited in carbohydrate chemistry is that employing ethylene oxide derivatives of sugars as initial materials. Lampson and Lardy [91] treated 5 : 6-anhydro-1 : 2-*O*-*iso*propylidene-D-glucofuranose in water with dipotassium or disodium hydrogen phosphate and cleaved the anhydro-ring. The phosphate residue was located at the terminal carbon atom of the sugar molecule, and by removal of the *iso*propylidene group, glucose 6-phosphate was obtained. Although the yield was lower than by other methods the authors recommend this procedure in the special case when it is desired to introduce labelled phosphate because it avoids the use of special phosphorylating agents. Todd and his co-workers [92] studied the action of dibenzyl hydrogen phosphate on methyl 2 : 3-anhydro-4 : 6-*O*-benzylidene-α-D-alloside (I). The product was a mixture of methyl benzylidenehexoside dibenzyl phosphates. After elimination of the benzyl and benzylidene residues this mixture was separated into methyl α-D-altropyranoside 2-phosphate (II),* which was the main product, and methyl α-D-glucopyranoside 3-phosphate (III).* The



general conclusion drawn by Todd and his colleagues was that the epoxide route is feasible for carbohydrate esters of phosphoric acid and compounds of the nucleotide type, but is limited in its application. The limitations were considered to be inaccessibility of appropriate anhydro-compounds and the tendency to formation of more than one product from other than 5 : 6-anhydro-sugar derivatives. The method probably warrants further study, however, especially in the light of the newer methods available for the separation of sugar phosphates.

Although the preparation of sugar phosphate esters with the substituent located at the glycosidic centre of the sugar is frequently achieved by enzymic methods, chemical syntheses have been developed. The reaction

[91] Lampson and Lardy, *J. Biol. Chem.*, 1949, **181**, 693.

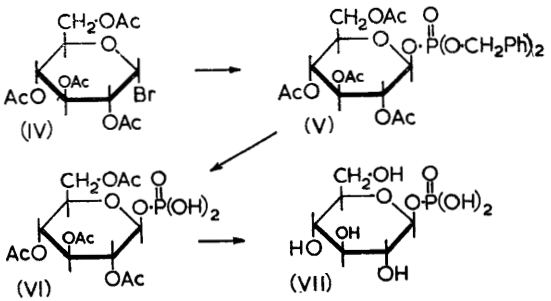
[92] Harvey, Michalksi, and Todd, *J.*, 1951, 2271.

* Depiction of this and other sugar phosphates as free acids does not imply that they were always isolated as such.

between the acetobromo-sugar and sodium or (more usually) silver phosphate, or silver diphenyl or dibenzyl phosphate is frequently employed. Depending on the experimental conditions and reagent either the α- or the β-derivative is formed. The α-form of glucose 1-phosphate was successfully obtained by treating trisilver phosphate with acetobromoglucose in benzene.[27, 93] The initial product, tris(tetra-*O*-acetylglucose-1) phosphate, was hydrolysed by acid in methanol until about 20% of the organic phosphate was liberated, and deacetylation was completed with alkali. The method has been described in detail by Krahl and Cori.[94] In like fashion, α-galactose 1-phosphate,[95] xylose 1-phosphate,[96] and maltose 1-phosphate [96] have been prepared. Usually α-acetobromoglucose reacts with inversion of configuration at the glycosidic centre, and in this respect the preparation of α-glucose 1-phosphate is anomalous.

Posternak [97] treated acetobromo-aldoses with silver diphenyl phosphate, and cleaved the phenyl groups from the product by hydrogenolysis. Treatment with alkali resulted in deacetylation and the glycoside phosphate was isolated. α-Glucose 1-phosphate and α-galactose 1-phosphate were synthesised in this way, and the yields are reported to be five times those obtained by the trisilver phosphate procedure. In similar fashion α-D-glucose 1 : 6-diphosphate was prepared from 2 : 3 : 4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucose 6-(diphenyl phosphate).[98, 99] Other compounds prepared by this route include α-D-mannose 1-phosphate and 1 : 6-diphosphate and α-lactose 1-phosphate.

If α-acetobromo-D-glucose (IV) is treated with silver dibenzyl phosphate reaction occurs with inversion of configuration and, after elimination of protecting groups, β-D-glucose 1-phosphate (VII) can be isolated.[86, 100] The compound is formed *via* the intermediates (V) and (VI).



[93] Cori, Colowick, and Cori, *J. Biol. Chem.*, 1937, **121**, 465.

[94] Krahl and Cori, "Biochemical Preparations", Wiley and Sons, New York, 1949, Vol. I, p. 33.

[95] Colowick, *J. Biol. Chem.*, 1938, **124**, 557.

[96] Meagher and Hassid, *J. Amer. Chem. Soc.*, 1946, **68**, 2135.

[97] Posternak, *ibid.*, 1950, **72**, 4824.

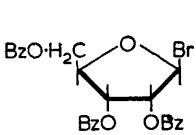
[98] Posternak, *J. Biol. Chem.*, 1949, **180**, 1269.

[99] See also Leloir, Repetto, Cardini, Paladini, and Caputto, *Anales Asoc. quim. argentina*, 1949, **37**, 187.

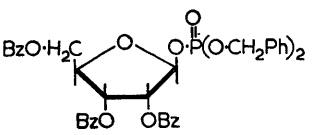
[100] Wolfrom, Smith, Pletcher, and Brown, *J. Amer. Chem. Soc.*, 1942, **64**, 23.

β-D-Galactose 1-phosphate can also be obtained by this procedure,[101] but α-D-mannose 1-phosphate is formed when acetochloromannose is treated with silver diphenyl phosphate or silver dibenzyl phosphate with subsequent removal of protecting groups.[102a] Likewise acetobromo-D-xylose affords finally α-D-xylose 1-phosphate when treated with either silver diphenyl or dibenzyl phosphate.[102b]

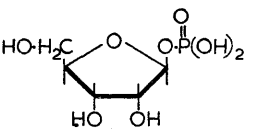
Khorana and his colleagues have successfully synthesised both α- and β-D-ribofuranose 1-phosphate.[103a, b] 2 : 3 : 5-Tri-*O*-benzoyl-β-D-ribose was converted into the corresponding ribofuranose 1-bromide (VIII) to which the β-configuration has been assigned. At low temperature this compound underwent some reaction with silver dibenzyl phosphate in a medium of chloroform and methylene dichloride, and chromatography of the product after hydrogenation showed the presence of a fast-moving labile phosphate, but much inorganic phosphate was also present. After debenzoylation only very small yields of ribofuranose 1-phosphate were obtained. To reduce losses a much shorter reaction period appeared advisable and, to achieve this, advantage was taken of the high solubility in benzene of triethylammonium dibenzyl phosphate. When a cooled benzene solution of this salt was added to a precooled solution of compound (VIII) a rapid reaction ensued. As expected, the product (IX) was extremely labile and direct hydrogenation appeared desirable in order to secure some stabilisation of the ester by the creation of phosphoryl dissociation. This viewpoint was borne out by experiment and after removal of the benzoyl groups a considerably improved yield of β-ribofuranose 1-phosphate (X) was obtained. (The β-configuration was based on enzymic studies and methods described



(VIII)



(IX)



(X)

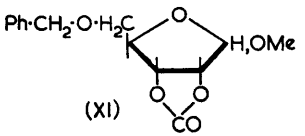
later.) In this case we have the formation of a β-glycose 1-phosphate from a β-glycose 1-halide on reaction with a salt of dibenzyl phosphoric acid. The importance of "neighbouring group" participation in the synthesis of purine nucleosides is well known and the configuration at $C_{(1)}$ appears to depend on the position of the 2-hydroxyl substituent in that, in all known cases, the base is on the opposite side of the ring from this 2-substituent regardless of the relative configuration at positions 1 and 2 in the original halogeno-sugar (see Baker *et al.*[103c] for a fuller discussion of this point).

[101] Reithel, *J. Amer. Chem Soc.*, 1945, **67**, 1056.

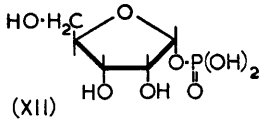
[102] (*a*) Posternak and Rosselet, *Helv. Chim. Acta*, 1953, **36**, 1614; (*b*) Antia and Watson, *Chem. and Ind.*, 1956, 1143.

[103] (*a*) Tener, Wright, and Khorana, *J. Amer. Chem. Soc.*, 1956, **78**, 506; (*b*) Wright and Khorana, *ibid.*, 1955, **77**, 3423; 1956, **78**, 811; (*c*) cf. Baker, Joseph, Schaub, and Williams, *J. Org. Chem.*, 1954, **19**, 1786; (*d*) Maley, Maley, and Lardy, *J. Amer. Chem. Soc.*, 1956. **78**, 5303.

An analogous explanation can be entertained for the nature of the products formed when salts of dibenzyl phosphoric acid are treated with acylglycose 1-halides. To prepare the other form of the anomeric pair of phosphates the blocking group at $C_{(2)}$ should not exercise the important neighbouring-group influence in the replacement reaction at position 1, and in addition should be readily removed at a later stage in the synthesis. α-D-Ribofuranose 1-phosphate was synthesised by taking account of these requirements. Methyl 5-*O*-benzyl-D-ribofuranoside 2 : 3-carbonate (XI) was converted by hydrogen bromide in acetic acid into an oily bromide which was directly treated in benzene with one equivalent of triethylammonium dibenzyl phosphate. Hydrogenation of the product followed by mild alkaline treatment afforded α-D-ribofuranose 1-phosphate (XII). The use of triethylammonium dibenzyl phosphate appears to be most promising for the synthesis of labile glycosidic phosphates. Lardy and his co-workers have recently prepared α-D-glucosamine 1-phosphate (and its *N*-acetyl derivative) by treating acetobromoglucosamine hydrobromide with the triethylamine salt of diphenyl phosphoric acid and subsequent removal of the protecting groups.[103d]



(XI)



(XII)

Reactions of acetohalogeno-sugars with monosilver phosphate (for preparation see Lipmann and Tuttle [104]) usually proceed with inversion and lead to the formation of β-glycosides. After removal of the protecting groups from the product of reaction of acetobromogalactose and monosilver phosphate, β-D-galactose 1-phosphate was obtained.[101] Methyl 2 : 3 : 4-tri-*O*-acetyl-1-bromoglucuronate with monosilver phosphate gave finally β-glucuronic acid 1-phosphate [105] (see also Pippen and McCready [106] for other attempts to prepare hexuronic acids with 1-phosphate substituents). A thorough study of the reactions for the preparation of aldose 1-phosphates would provide useful information. Although the nature of the products formed from acylglycosyl halides and salts of dibenzylphosphoric acid can be explained, the reactions with salts of diphenylphosphoric acid appear anomalous. Likewise, configurational assignments are demonstrated only for the aldose 1-phosphates finally isolated and not on the initial products of reaction of the acylglycosyl halides and phosphoric acid diester salts.*

[104] Lipmann and Tuttle, *J. Biol. Chem.*, 1944, **153**, 571.
[105] Touster and Reynolds, *ibid.*, 1952, **197**, 863.
[106] Pippen and McCready, *J. Org. Chem.*, 1951, **16**, 262.

* The reaction between the silver salt of these phosphoric acids and a halogeno-sugar in which the halogen grouping is located at positions other than 1 has apparently not been used to give simple sugar phosphates but has been used for nucleotides. Uridine-5′ phosphate was prepared by reaction of silver dibenzyl phosphate and 5′-deoxy-5′-iodo-2′ : 3′-*O*-*iso*propylideneuridine, with subsequent debenzylation. The method is not

In general, for the various syntheses described, the site of the phosphoryl residue in the sugar molecule has been confirmed by the classical methods of carbohydrate chemistry involving, *inter alia*, glycosidisation, methylation, periodate oxidation, optical rotation, and ion-exchange in the presence and absence of borate, and differences in the decomposition rates in alkali.

Work on the synthesis of esters of pyrophosphoric and triphosphoric acids has been limited to preparations of the nucleotides. Although in these compounds it is the sugar portion of the molecule which is esterified, this work will be described only briefly as it is more appropriately included in a review of nucleotides.

Methods have been developed which render it possible to eliminate selectively only one of the benzyl residues from the dibenzyl phosphate esters of sugars and nucleotides. If an alcohol of the general formula (XIII) is allowed to react with dibenzyl phosphorochloridate it affords the ester (XIV) which on hydrogenolysis yields a monoester (XV). Selective debenzylation of compounds of type (XIV) can be accomplished by " quaternisation "—a process depending on the transfer of a benzyl residue from oxygen to nitrogen with formation of a quaternary salt. A strong tertiary base such as 4-methylmorpholine [107] is satisfactory, but the method has been extended to include all classes of amines. Debenzylation can also be brought about by a base hydrochloride.[108] Lithium chloride in 2-ethoxyethanol proved most efficient and was recommended for the preparation of monobenzyl esters of the general formula (XVI). An equilibrium is set up between the triester and lithium chloride on the one hand, and the lithium salt of the diester and benzyl chloride on the other. Precipitation of this lithium salt from the solution leads to quantitative reaction. In both methods of debenzylation the monobenzyl ester is produced as an anion and therefore a second debenzylation, which would produce a doubly charged anion, is not favoured. Treatment of the silver salt of the monobenzyl ester (XVI) with dibenzyl phosphorochloridate gives the tribenzyl ester (XVII) and subsequently by hydrogenolysis the diphosphate (XVIII). Repetition of this sequence of reactions commencing with compound (XVII) yields the tetrabenzyl ester (XXI) and thence the triphosphate (XXII).

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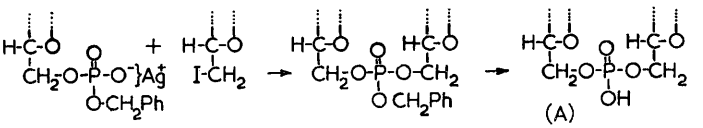
generally applicable because of the difficulties encountered in the preparation of halogeno-sugar moieties owing to formation of *cyclo*nucleoside salts. An unsymmetrical diester of phosphoric acid [a diribonucleoside phosphate (A)] has been synthesised by

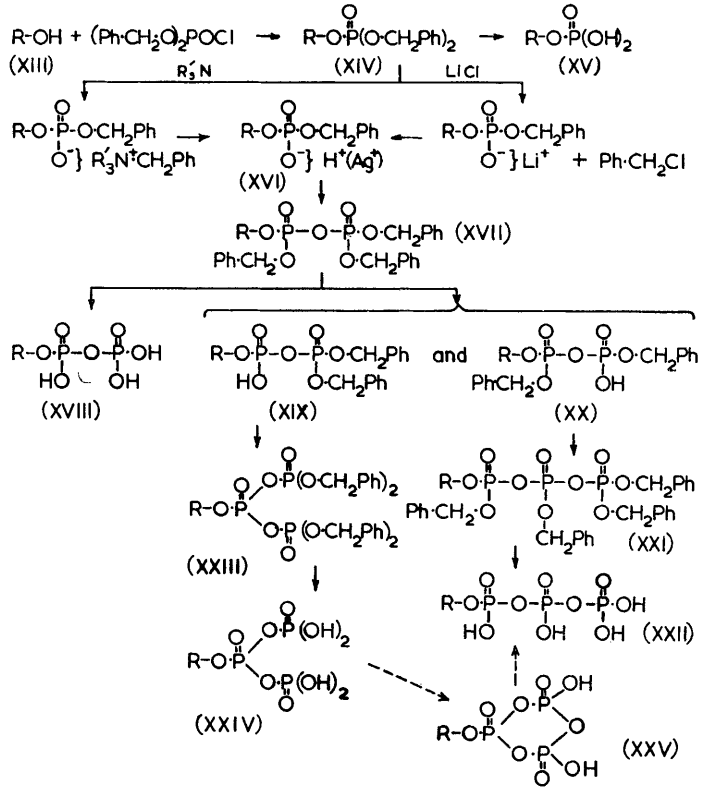


this reaction sequence. The silver salt of 2′ : 3′-*O*-*iso*propylideneadenosine-5′ benzyl phosphate was treated in boiling toluene with 5′-deoxy-5′-iodo-2′ : 3′-*O*-*iso*propylideneuridine to give, after removal of the protecting groups, adenosine-5′ uridine-5′ phosphate (Elmore and Todd, *J.*, 1952, 3681).

[107] Baddiley, Clark, Michalski, and Todd, *J.*, 1949, 815.

[108] Clark and Todd, *J.*, 1950, 2023, 2030.

By such methods Todd and his co-workers [109] synthesised adenosine diphosphate (ADP) and adenosine triphosphate (ATP). It might be expected that a mixture of isomers would be obtained from the monodebenzylation.



For example, the substance (XVII) could yield the triester (XIX) or (XX) Whereas further reaction of compound (XX) with dibenzyl phosphorochloridate would lead finally to the " unbranched " triphosphate (XXII), the isomer (XIX) would be expected to afford finally the " branched " substance (XXIV). If in compounds (XIX) and (XX), R were adenosine esterified at position 5′, then (XXII) would be natural ATP and (XXIV) an isomer of it. Practically, it has been shown [110] that the disilver salt of adenosine-5′ phosphate reacts with an excess of dibenzyl phosphorochloridate to give, after debenzylation, natural ATP in far better yield than is obtained by the original, alternative procedure.[109] Obviously a rearrangement is involved and probably compound (XXIV) is converted into ATP (XXII) *via* a cyclic intermediate (XXV).

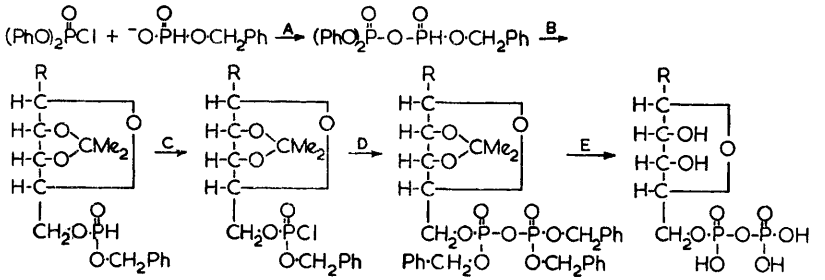
(At temperatures of 50° or above, benzyl pyrophosphates are rapidly debenzylated by phenol with the production of nuclear-benzylated phenols.[111]

[109] Todd and co-workers, *J.*, 1947, 648 ; 1949, 582.
[110] Michelson and Todd, *J.*, 1949, 2487.
[111] Quoted by Christie, Kenner, and Todd, *J.*, 1954, 46.

This is an acid-catalysed reaction, whereas anionic debenzylation occurs under neutral or alkaline conditions : it is an alternative to hydrogenolysis as a method of debenzylation.)

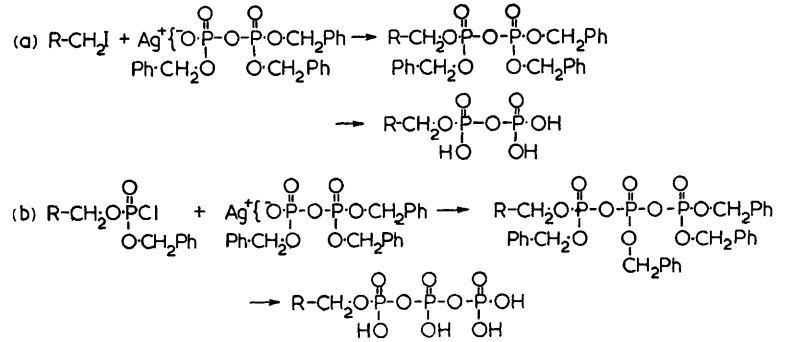
Other methods for the preparation of pyrophosphoric and triphosphoric esters have also been developed. Syntheses of ribonucleoside-5′ phosphites have been achieved.[112] Chlorination of phosphites can be effected with *N*-chlorosuccinimide, and the chloro-derivative is a valuable intermediate for further stages in the synthesis of ribonucleotides. *N* : 2 : 4-Trichloroacetanilide can also be used to chlorinate the phosphites and although it is less reactive it might be a useful reagent for the preparation of water-soluble phosphates and pyrophosphates from phosphites since both *N* : 2 : 4-trichloroacetanilide and 2 : 4-dichloroacetanilide produced from it are virtually insoluble in water and can be readily separated from the desired reaction products.

Uridine-5′ pyrophosphate has been synthesised in this way, as shown in Scheme I. R is uracil, and the reagents are (B) 2′ : 3′-*O*-*iso*propylideneuridine in acetonitrile containing 2 : 6-lutidine, (C) *N*-chlorosuccinimide, (D) triethylammonium dibenzyl phosphate, and (E) lithium chloride, hydrogenation, and hydrolysis.



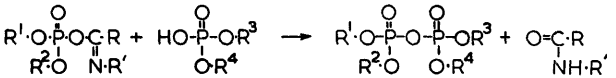
Scheme I

Esters of pyrophosphoric and triphosphoric acid can be synthesised by reactions of the following types :



Again the methods have been developed for use in nucleotide syntheses. In reactions of type (a), with a variety of carbohydrate derivatives in which the substituent to be replaced is on the terminal carbon atom, it was found that derivatives of open-chain *aldehydo*-sugars always react readily, whereas those containing a lactol ring (*e.g.*, methyl ribofuranoside derivatives) were so sluggish in reaction that they were of little preparative value.

Alternative methods of preparing these esters have also been developed. Adenosine monophosphate has been treated with phosphoric acid in the presence of di*cyclo*hexylcarbodi-imide to afford the di- and tri-phosphates:[113] the use of protected intermediates is avoided. Employment of carbodi-imides as reagents has proved remarkably effective for the synthesis of symmetrical pyrophosphates and, to a smaller degree, of unsymmetrical pyrophosphates, to which class most of the natural coenzymes belong. Although the method has been applied to the synthesis of, *inter alia*, uridine-diphosphate-glucose and the 5′-triphosphates of adenosine and uridine the unsymmetrical esters are always produced as components of complex mixtures with the corresponding symmetrical pyrophosphates. Recently attempts have been made to overcome this difficulty by the use of imidoyl phosphates,[114] which are analogous in structure to the hypothetical intermediates in the synthesis of pyrophosphates by use of carbodi-imides and consequently undergo phosphorolysis with the production of pyrophosphates, *e.g.*:



(R, R′, R^1, R^2, R^3, and R^4 are suitable protecting residues)

No doubt this method will be further exploited.

Exchange reactions with trifluoroacetic anhydride can be used for pyrophosphate syntheses, and fully esterified pyrophosphates can also be prepared by exchange reactions between diesters of phosphoric acid and a suitably reactive pyrophosphate. Exchange reactions with nucleosides were less successful than those with simple model compounds.

Although cyclic esters of phosphoric acid have been made from glycols, and their existence has been postulated as intermediates in various rearrangements, not much work has been done on the synthesis of such esters from simple sugars. Again, examples generally must be drawn from nucleotide chemistry. Sometimes direct phosphorylation of the sugar moiety leads to a cyclic ester. For example, treatment of riboflavin with phosphoryl chloride in pyridine containing a small amount of water yields a cyclic 4′ : 5′-phosphate.[115] An attempt [116] to synthesise a monobenzyl ester of flavin-adenine-dinucleotide consisted in bringing about an exchange

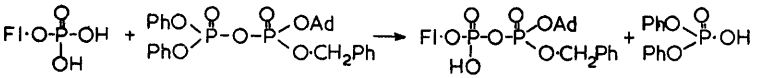
[113] Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3517.

[114] Atherton, Morrison, Cremlyn, Kenner, Todd, and Webb, *Chem. and Ind.*, 1955, 1183.

[115] Forest and Todd, *J.*, 1950, 3295.

[116] Forest, Mason, and Todd, *J.*, 1952, 2530

reaction between riboflavin-5′ phosphate and 2′ : 3′-*O*-*iso*propylidene adenosine-5′ (benzyl diphenyl pyrophosphate), *e.g.* :

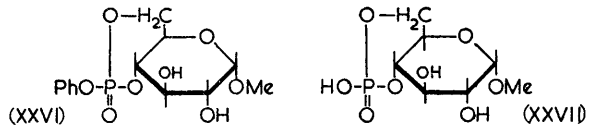


(Ad = adenosine residue used as 2′ : 3′-*O*-*iso*propylidene derivative ; Fl = riboflavin residue.)

From many reactions, in all cases the product was riboflavin-4′ : 5′ cyclic phosphate, a compound which could also be obtained by treating riboflavin-5′ phosphate with tetraphenyl or tetrabenzyl pyrophosphate in the presence of bases. It may be reasonably assumed that in these reactions the desired exchange did in fact occur and that the pyrophosphate of riboflavin initially produced then behaved in the presence of a base as a phosphorylating agent towards the adjacent hydroxyl group of the riboflavin residue. Riboflavin-5′ phosphate and trifluoroacetic anhydride afford 3 : 2′ : 3′-tristrifluoroacetylriboflavin-4′ : 5′ cyclic phosphate.[116]

Uridine-diphosphate-glucose, on treatment with alkali, yields glucose 1 : 2-(hydrogen phosphate) as a cleavage product.[117] The cyclic 2′ : 3′-phosphates derivable from the " a " and the " b " type of ribonucleotides have been well studied. The cyclic phosphates of this type were prepared by Brown *et al.*[118] from adenosine, cytidine, and uridine. The " a " and " b " nucleotides were treated with excess of trifluoroacetic anhydride, followed by ethanolic ammonia to remove the trifluoroacetyl residues. There is no doubt that intramolecular reaction occurs, as intermolecular reaction would have given diadenosine pyrophosphate. Reaction proceeds by the initial formation of a mixed anhydride of the phosphate with trifluoroacetic acid, and the mixed anhydride can react in intramolecular reaction as a phosphorylating agent towards the adjacent hydroxyl group. Adenylic acid " a " or " b " with di*cyclo*hexylcarbodi-imide yields the cyclic phosphate, although subsequent opening of the ring may occur in further reactions.[119]

Recently the synthesis was reported of six-membered cyclic phosphates derived from sugars.[120] Methyl α-D-glucoside and phenyl phosphorodichloridate afforded a crystalline neutral ester (XXVI) in 10—20% yield,



from which a phenyl group was removed by hydrogenolysis, thereby affording methyl α-D-glucoside 4 : 6-(hydrogen phosphate) (XXVII). From phenyl β-D-glucoside a better yield (40%) of phenyl β-D-glucoside 4 : 6-(phenyl

[117] Paladini and Leloir, *Biochem. J.*, 1952, **51**, 426.
[118] Brown, Magrath, and Todd, *J.*, 1952, 2708.
[119] Dekker and Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3522.
[120] Baddiley, Buchanan, and Szabó, *J.*, 1954, 3826.

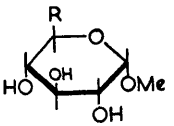
phosphate) was obtained which on hydrogenolysis afforded mainly glucose 4 : 6-(hydrogen phosphate).

Properties and Reactions

According to Leloir [1] sugar monophosphates are stronger acids than free phosphoric acid and both pK_1 and pK_2 have smaller values. (For a discussion of this point see Kumler and Eiler.[121])

Aldose 1-phosphates are very sensitive to acid and in this respect resemble the glycosides and glycosylamines. Further, like the glycosides, the β-anomers are usually more acid labile than the α-forms (*e.g.*, in comparative experiments hydrolysis constants for the α- and the β-form of glucose 1-phosphate are 5×10^{-3} and 15×10^{-3} respectively.[100]) Reasons for this difference are probably the same as those put forward for differences in hydrolysis rates of anomeric glycosides.[122] The rate of hydrolysis of the glycosidic phosphate residue in α-glucose 1-phosphate is greater than in α-glucose 1 : 6-diphosphate.[98] The phosphate substituent at $C_{(6)}$ reduces the rate of hydrolysis, again probably for the same reasons as in hydrolysis of methyl α-D-glucoside, methyl 6-deoxy-α-D-glucoside, and methyl α-D-xyloside (XXVIII ; R = CH_2·OH, Me, and H respectively) where the rate increases as the bulk of R diminishes.[122]

Ribose 1-phosphate is sufficiently acid-labile to undergo hydrolysis at the acidity employed in estimation of phosphate. The ester is somewhat more acid-labile than phosphocreatine but less so than acetyl phosphate. The pyranose form of ribose 1-phosphate is more stable towards acid than the furanose form.[103b] 2-Deoxy-D-ribose 1-phosphate is even more acid-labile than the ribose analogue.



(XXVIII)

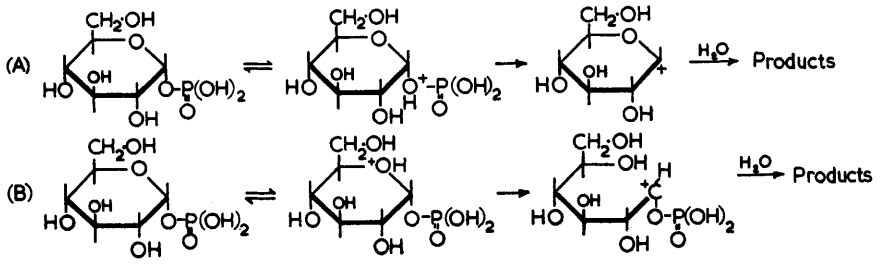
The mechanism of hydrolysis of aldose 1-phosphate has been studied by various workers. The curve of first-order rate coefficient against acidity for the hydrolysis of α-D-glucose 1-phosphate is quite different from that obtained for a simple phosphate such as methyl phosphate. At 72·9° and in the range pH 1—4, the logarithm of the rate coefficient is proportional to the pH of the medium. At higher acidities the rate increases more rapidly than the stoicheiometric acidity and at 25° in aqueous perchloric acid the logarithm of the rate coefficient is accurately proportional to Hammett's acidity function H_0. Isotope experiments at about pH 4 and in strong perchloric acid showed fission of the carbon–oxygen bond.[123] These results are consistent with a single unimolecular mechanism operative over the whole range of acidities studied. The first step must be a rapid and reversible proton-transfer to the α-D-glucose 1-phosphate, followed by a slow reaction not involving a water molecule. There are two possible formulations, one of which involves an opening of the hexose ring, *e.g.*, sequences (A) and (B). The two mechanisms possibly have different

[121] Kumler and Eiler, *J. Amer. Chem. Soc.*, 1943, **65**, 2355.

[122] Foster and Overend, *Chem. and Ind.*, 1955, 566.

[123] Barnard, Bunton, Llewellyn, Oldham, Silver, and Vernon, *ibid.*, 1955, 760 ; cf. Cohn, *J. Biol. Chem.*, 1949, **180**, 771.

stereochemical consequences: mechanism (B) necessarily involves the production, under kinetic control, of the equilibrium mixture of α- and β-glucose, but for mechanism (A) this is not necessarily so. Further information on this point would be desirable. Since under the experimental conditions the mutarotation of glucose to produce the equilibrium mixture is extremely rapid, this possible stereochemical distinction has no diagnostic



value. In methanol, however, where the methyl glucosides produced are stable under the experimental conditions, study of the steric course of the reaction may throw considerable light on the mechanism.

To achieve acidic hydrolysis of the phosphate ester resulting from the esterification of the primary hydroxyl group in a sugar, fairly drastic treatment is required which may lead to some decomposition of the sugar. Levene and Stiller [63] demonstrated that a pentose esterified at $C_{(3)}$ is hydrolysed more rapidly than the $C_{(5)}$-isomer. Thus, hydrolysis of 5-*O*-methyl- 1 : 2-*O*-*iso*propylidenexylose 3-phosphate is many times faster than that of xylose 5-phosphate. Ribose 3-phosphate is hydrolysed 5—9 times faster than the 5-phosphate, and 3-phosphoribonic acid is hydrolysed about twice as rapidly as 5-phosphoribonic acid.[51] This rate difference has been used to determine whether substances containing a ribose phosphate moiety have the phosphate residue at position 3 or 5. For the reasons given on p. 66 reservations must be made regarding the rates reported for the ribose phosphates obtained by Levene and his co-workers.

An examination [124] of the hydrolysis of fructose 6-phosphate revealed that the hydrolysis is markedly slower than that of the 1-phosphate, and indeed it is possible to obtain a good yield of fructose 6-phosphate by hydrolysis of the 1 : 6-diphosphate [125] with hydrochloric or hydrobromic acid at 35° under special conditions. Comparison of the rate constants for hydrolysis of fructose 6-phosphate, -pyrophosphate, and -triphosphate has shown that cleavage of the phosphate entity in the first substance is slower by a factor of 10^2—10^3 than is hydrolytic cleavage of a single phosphate group from either of the other two compounds. The hydrolysis of hexahydrobenzyl β-glucoside 2-phosphate by 0·1N-sulphuric acid at 100° was followed and it was found that k, calculated for a unimolecular reaction, increased from $2{\cdot}9 \times 10^{-5}$ after 30 minutes to $6{\cdot}1 \times 10^{-5}$ after 540 minutes. It might be inferred that the glycoside phosphate is more slowly hydrolysed

[124] Friess, *J. Amer. Chem. Soc.*, 1952, **74**, 5521.
[125] Neuberg, Lustig, and Rothenberg, *Arch. Biochem. Biophys.*, **1943**, **3**, 33.

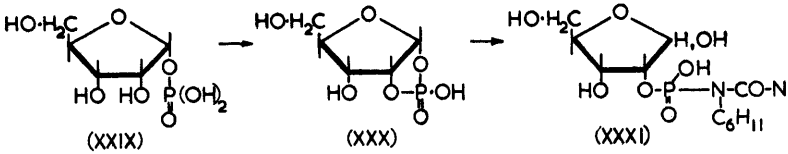
than glucose 2-phosphate ($k = 8{\cdot}4 \times 10^{-5}$) to which it will give rise on cleavage of the glycosidic substituent. Glucose 2-phosphate is far less acid-labile than methyl 3 : 5 : 6-tri-*O*-methylglucoside 2-phosphate, which is probably at least 80% hydrolysed by 0·1N-sulphuric acid after one hour at 100°. This difference is probably accounted for, at least partly, by the difference in lactol-ring forms in the two compounds, because generally sugar phosphates without a glycosidic substituent and with a 2-phosphate group are more readily hydrolysed. It has been suggested that this is possibly due to migration of the phosphate residue from position 2 to position 1, but proof has not been presented. Rates of hydrolysis of esters of 2-deoxygalactose have been compared with those for analogous derivatives of galactose.[126] Hydrolysis is faster in the 2-deoxy-series. The rates of hydrolysis (N-hydrochloric acid at 100°) of the phosphate groups in glucose 6-phosphate and glucosamine 6-phosphate were compared by Anderson and Percival.[70b] Whereas glucosamine 6-phosphate was only 50% hydrolysed during 73 hours, glucose 6-phosphate was hydrolysed to the same extent in only 23 hours. A list of acid-hydrolysis constants for some carbohydrate phosphates can be found in the review by Leloir.[1]

Sugar 1-phosphates are resistant to alkali. For example, D-ribofuranose 1-phosphate is completely stable to 0·5N-sodium hydroxide at 80° for one hour.[103] On the other hand, other sugar phosphates are rapidly changed: glucose 2-phosphate is 50% hydrolysed in 97 minutes by 0·1N-alkali at 100°, and glucose 6-phosphate is 60% hydrolysed by 0·2N-alkali at 100° in 3 minutes. In the ribose series the decreasing order of stability towards alkali (0·01N-sodium hydroxide at 22°) is ribose 2-phosphate (which is scarcely attacked), 3-phosphate, and 5-phosphate. It is reported [127] that phosphate residues are removed with greater difficulty than are sulphate residues in corresponding compounds.

Robinson [128] initially suggested that hydrolysis of phosphoric esters is accompanied by Walden inversion and that D-galactose and D-ribose might arise in hydrolysates from natural products by decomposition of glucose 4-phosphate and xylose 3-phosphate. The alkaline hydrolysis of methyl α-D-glucoside 6-(barium phosphate), methyl D-glucofuranoside 3-(barium phosphate), and *iso*propylidene-D-glucose 3- and 6-(barium phosphate) was studied by Percival and Percival [127] and in no case was any evidence found to support Walden inversion or anhydride formation. Levene *et al.*[129] however have claimed that treatment of fructose 3-phosphate with phenylhydrazine in acetic acid results in cleavage of the phosphate group with inversion, since the final product is 3 : 6-anhydroallosazone. Further, it is stated [66b] that glucose 3-phosphate, on treatment with phenylhydrazine, also gives this anhydro-compound. On the other hand hydrolysis of glucose 3-phosphate with phosphatase and subsequent osazone formation afforded glucosazone and not allosazone.

[126] Foster, Overend, and Stacey, *J.*, 1951, 987.
[127] Percival and Percival, *J.*, 1945, 874.
[128] Robinson, *Nature*, 1927, **120**, 44, 656.
[129] Levene, Raymond, and Walti, *J. Biol. Chem.*, 1929, **82**, 191.

Only very brief mention can be made of the effect of phosphatases on glycose phosphates. Using ^{18}O, Cohn [123] demonstrated that intestinal alkaline phosphatase ruptures the oxygen–phosphorus bond, a change apparently analogous to non-enzymic alkaline hydrolysis of sugar phosphates generally. Likewise, prostate acid phosphatase cleaves the same bond. Phosphorylases have been used in experiments designed to determine the anomeric configuration of glycosyl phosphates, but care must be exercised in interpreting the results. Changes in optical rotation have also been studied with this end in view (cf. Wolfrom *et al.*[100] and Wright and Khorana [103b]) : it appears that assignment of anomeric configuration can be based on Hudson's rules. A direct approach to this problem was suggested by the work of Dekker and Khorana [119] on the reactions of phosphate esters bearing an adjacent *cis*-hydroxyl function, *e.g.*, (XXIX) with di*cyclo*hexylcarbodi-imide. It was established that these esters give first the cyclic phosphates (XXX), which then form the phosphorylureas (XXXI). This reaction sequence may be followed readily by paper chromatography in suitable solvent systems, the mobilities of the reaction products following



(XXIX) (XXX) (XXXI)

the order (XXIX) > (XXX) > (XXXI) (examples drawn from the ribofuranose series). Owing to the more or less planar nature of the furanose ring, only the α-phosphate (XXIX) of the two anomeric ribofuranose 1-phosphates is able to form a 5-membered cyclic ester and subsequently give rise to (XXXI). The anomeric configurations of synthetic β- [103] and enzymically produced α-ribofuranose 1-phosphate can be assigned on the basis of these reactions. The phosphorylurea (XXXI) was much more stable than either of the samples of ribofuranose 1-phosphate. It is likely that the method developed by Smith and his colleagues [130] to determine the anomeric configuration of alkyl glycosides would be equally applicable for assignment of configuration in the glycose 1-phosphate series.

Properties of phosphate esters have been exploited in attempts to elucidate structural problems among natural products. To mention one example, Brown *et al.*,[131] in experiments directed towards the determination of nucleotide sequence in polyribonucleotides, made use of the fact that phosphates of β-aldehydo- and β-keto-alcohols undergo elimination reactions with alkali.

Reference has already been made to migration of phosphate groups and there are several observations in the literature concerning this. Tankó and Robison [132] suggested that this might explain certain changes in optical

[130] Abdel-Akher, Cadotte, Montgomery, Smith, Van Cleve, and Lewis, *Nature*, 1953, **171**, 474.

[131] Brown, Fried, and Todd, *Chem. and Ind.*, 1953, 352 ; *J.*, 1955, 2206.

[132] Tankó and Robison, *Biochem. J.*, 1935, **29**, 961.

rotation of samples of fructose 6-phosphate which had been subjected to various treatments. Indirect evidence was obtained of phosphoryl migration during mild acid hydrolysis of trehalose phosphate. In the migrations observed with glycerophosphates [133] and the " a " and " b " purine [134] and pyrimidine [135] nucleotides it is clear that the migration occurs *via* an intermediate cyclic ester. That interaction between neighbouring hydroxyl and phosphoryl groups takes place has been stressed by Kumler and Eiler,[121] who have shown that the polyol and sugar phosphates are abnormally strong acids in comparison with the monoalkyl phosphates. The difference in stability of ribo- and deoxyribo-nucleic acids towards alkali depends on the fact that only the former substance can form an internal cyclic triester.

Studies [90a] of the rates of oxidation of xylose 5- and 3-phosphate by periodic acid (and the reaction of these compounds with di*cyclo*hexylcarbodi-imide) have led to the conclusion that xylose 3-phosphate exists in solution in the pyranose form (CI conformation), a conclusion which necessitates a re-interpretation of some of Levene and Raymond's [89] results. Marked differences have been observed in the rates of periodate oxidation of some cyclic phosphates : methyl α-D-glucoside 4 : 6-(phenyl phosphate) is unaffected even by prolonged treatment with periodate, and methyl α-D-glucoside 4 : 6-(hydrogen phosphate) is oxidised rather slowly, but the rate of oxidation is greater for glucose 4 : 6-(hydrogen phosphate).[120] The periodate oxidation of sugar phosphates has been discussed recently by Loring *et al.*[135b]

A detailed description of the manifold enzymic reactions in which carbohydrate phosphates function as substrates is beyond the scope of this Review and only brief mention will be made of a few selected examples. Extensive investigations have established the importance of phosphate esters in carbohydrate metabolism, both at the pentose and hexose level and with the higher saccharides and polysaccharides. A recent development is the presentation of evidence that phosphoric esters of glucosamine and *N*-acetylglucosamine are concerned in the biosynthesis of mucopolysaccharides.[136] As well as this substrate function, some sugar phosphates have coenzyme activity, *e.g.*, glucose 1 : 6-diphosphate is a coenzyme for phosphoglucomutase and no interconversion of glucose 1- and 6-phosphate is effected by this enzyme if the diphosphate is absent from the reaction medium.

The rôle of phosphoglycosyl compounds in the biosynthesis of nucleosides and nucleotides has been reviewed by Kalckar.[137]

[133] Verkade, Stoppelenburg, and Cohen, *Rec. Trav. chim.*, 1940, **59**, 886.

[134] Brown and Todd, *J.*, 1952, 52.

[135] (*a*) Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 2811 ; (*b*) Loring, Levy, Moss, and Ploeser, *J. Amer. Chem. Soc.*, 1956, **78**, 3724.

[136] Glaser and Brown, *Proc. Nat. Acad. Sci., U.S.A.*, 1955, **41**, 253.

[137] Kalckar, *Biochim. Biophys. Acta*, 1953, **12**, 250.