STUDIES ON DEOXY SUGAR PHOSPHATES

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ABSTRACT

Synthesis of the only unknown deoxy fructose, 4-deoxy-D-threohexulose (4-deoxy-D-fructose), is reported. Its preparation involved reductive lithium aluminum hydride ring opening of the epoxide, 3,4-anhydro-1,2;5,6-di-O-isopropylidene-D-talitol prepared from D-mannitol. A mixture of epimeric deoxy diisopropylidene hexitols, 4-deoxy-1,2;5,6-di-O-isopropylidene-D-lyxohexitol (-D-mannitol) and 4-deoxy-1,2;5,6-di-O-isopropylidene-D-arabinohexitol (-D-altritol) resulted. Hydrolysis followed by selective bacterial oxidation of free 4-deoxy-D-mannitol by Acetobacter suboxydans produced the desired compound. Phosphorylation of 4-deoxy-D-fructose in the presence of ATP was shown to be catalyzed by yeast hexokinase and the product 4-deoxy-D-threohexulose 6-phosphate (4-deoxy-D-fructose 6-phosphate) was isolated. The relative influence of the four hydroxyl group on formation of an enzyme-substrate complex was assessed by kinetic studies. Subsequent phosphorylation of the monophosphate ester by rabbit muscle phosphofructokinase ensued, and isolation of 4-deoxy-D-threohexulose 1,6-diphosphate (4-deoxy-D-fructose 1,6-diphosphate) was carried out. Neither of these phosphate esters have been previously reported.

A new improved method of synthesis of 3-deoxy-D-erythropentose 5-phosphate (3-deoxy-D-ribose 5-phosphate) is described. It entails treatment of the fully protected 1,2;5,6-di-O-isopropylidene-α-D-allose with triphenyl-

phosphine in carbon tetrachloride. The 3-chloro-3-deoxy-1,2;5,6-di-O-isopropylidene-α-D-glucose produced in high yield can be converted into the deoxy analogue by treatment with lithium aluminum hydride. The key intermediate, 3-deoxy-1,2;5,6-di-O-isopropylidene-α-D-glucose, then readily yields 3-deoxy-D-*erythro*pentose 5-phosphate. The attempted preparation of 3-deoxy-D-*glycero*pentulose 1,5-diphosphate (3-deoxy-D-ribulose 1,5-diphosphate) from the above compound is also outlined.

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CONTENTS

Introduction — Page

Purpose — 1

Approach — 5

Discussion

Synthesis of 4-Deoxy-D-fructose — 9

Yeast Hexokinase Action on 4-Deoxy-D-fructose — 19

Sugar Phosphate Synthesis — 26

Preparation of 4-Deoxy-D-fructose 6-phosphate — 28

Preparation of 4-Deoxy-D-fructose 1,6-diphosphate — 32

Synthesis of 3-Deoxy-D-ribose 5-phosphate — 35

Attempted Synthesis of 3-Deoxy-D-ribulose 5-phosphate — 39

Conclusions — 44

Experimental

General Methods — 45

1,2;5,6-Di-O-isopropylidene-D-mannitol — 48

1,2;5,6-Di-O-isopropylidene-3-O-p-toluenesulfonyl-D-mannitol — 48

3,4-Anhydro-1,2;5,6-di-O-isopropylidene-D-talitol — 48

4-Deoxy-D-threohexulose — 48

Assay System for Yeast Hexokinase — 51

4-Deoxy-D-threohexulose 6-phosphate — 51

Page

4-Deoxy-D-threohexulose 1,6-diphosphate 53

1,2;5,6-Di-O-isopropylidene-α-D-glucofuranose 54

1,2;5,6-Di-O-isopropylidene-α-D-ribohexofuranos-3-ulose 54

1,2;5,6-Di-O-isopropylidene-α-D-allofuranose 54

3-Chloro-3-deoxy-1,2;5,6-di-O-isopropylidene-α-D-glucose 55

3-Deoxy-1,2;5,6-di-O-isopropylidene-α-D-ribohexose 55

3-Deoxy-1,2-O-isopropylidene-D-ribohexofuranose 56

3-Deoxy-1,2-O-isopropylidene-D-erythropentose 56

3-Deoxy-D-erythropentose 5-phosphate 56

Attempted Preparation of 3-Deoxy-D-glycero-pentulose 5-phosphate 57

Attempted Preparation of 3-Deoxy-D-glycero-pentulose 1,5-diphosphate 59

References 61

Page

TABLES

Table 1. Michaelis constants, relative affinities and relative rates of hexose phosphorylation by yeast hexokinase 23

FIGURES

Fig. 1. Substrates of yeast hexokinase 2

Fig. 2. Groups in D-mannitol and 4-deoxy-D-fructose involved in the interconversions 5

Fig. 3. Groups in D-glucose and 3-deoxy-D-ribose 5-phosphate involved in the interconversions 7

Fig. 4. Stereochemistry of reductive opening of the epoxide, 3,4-anhydro-1,2;5,6-di-O-isopropylidene-D-talitol 16

Fig. 5. Double reciprocal Lineweaver-Burk plot for yeast hexokinase 24

Fig. 6. Graph of base uptake and time taken for phosphofructokinase reaction 33

Fig. 7. Action of ribosephosphate isomerase on ribose 5-phosphate 39

Fig. 8. Ion exchange chromatography of the isomerase product 41

Fig. 9. Ion exchange chromatography of the base treated product 43

INTRODUCTION

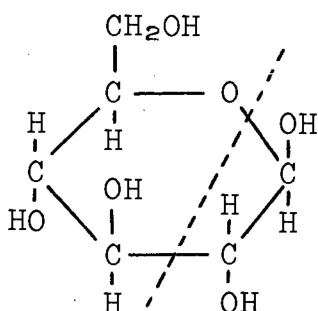
Purpose

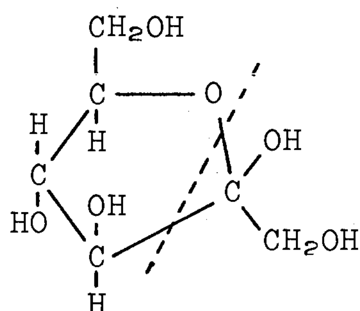
One of the most outstanding characteristics of enzymatic reactions is the high degree of specificity exhibited towards one substrate or structurally related substrates. Biologically, the importance of enzyme specificity cannot be overemphasized as it allows metabolic processes to proceed in an orderly fashion.

Elucidation of the minimal substrate structure necessary for enzyme catalysis to occur constitutes the prime reason for studying enzyme specificity. Hopefully from such studies active site conformation can be visualized, and with polyhydroxy compounds, such as carbohydrates, intermolecular contacts between the enzyme and substrate in the enzyme-substrate complex become evident at various points. In several instances, for example yeast hexokinase, considerable progress has been made and a common chemical structure can be recognized on which the enzyme acts[1,2]. In many cases the enzyme is extremely specific and only acts on one substrate or a pair of substrates. However to persue a thorough investigation of spatial requirements necessary for substrate binding, slight substrate changes must be tolerated by the enzyme.

As mentioned previously, crystalline yeast hexokinase, an enzyme responsible for the initial phosphorylation of sugars has been investigated in some detail[1,2]. Studies

have shown that the enzyme is able to phosphorylate a number of different sugars and is specific for the 3,4,5,6,-region of the carbohydrate molecule, but not the 1,2-section, provided that larger groups are not attached[1]. β-D-Fructofuranose, the α and β forms of D-glucose and D-mannose serve as the main substrates, Figure 1.





β-D-glucopyranose β-D-fructofuranose

Figure 1. Substrates of yeast hexokinase[1].

However in the 3,4,5,6-region 3-deoxy-D-glucose and 3-deoxy-D-mannose were the only compounds tested[2] in which selected hydroxyl groups had been replaced by hydrogen. In the four position, only the structural analogues D-galactose, the four epimer of D-glucose and a 1,4-anhydrohexitol, 1,4-sorbitan have been tested as substrates. A structural analogue containing a deoxy function at the four position could supply further valuable information concerning the specificity of yeast hexokinase. This research consists of preparing the deoxy fructose analogue, 4-deoxy-D-*threo*-

hexulose (4-deoxy-D-fructose), the only unknown deoxy fructose as yet not synthesized and testing it as a substrate for yeast hexokinase.

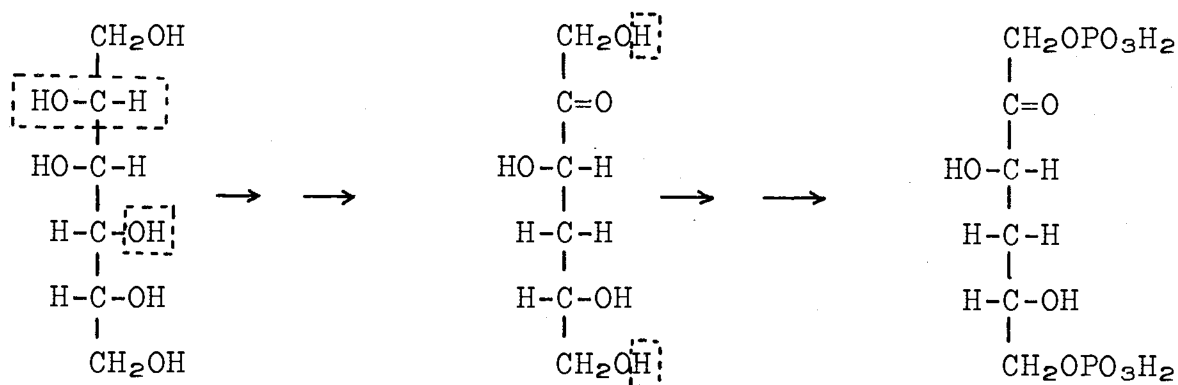
Few reports exist on the enzymatic preparation of deoxy sugar mono- and diphosphates[3,4,5]. Only readily accessible deoxy sugars and deoxy sugar phosphates have been used in these conversions. However phosphorylation of 4-deoxy-D-fructose by yeast hexokinase provided 4-deoxy-D-*threo*hexulose 6-phosphate (4-deoxy-D-fructose 6-phosphate). This substrate analogue of D-fructose 6-phosphate was tested with the highly specific rabbit muscle phosphofructokinase which phosphorylates only D-fructose 6-phosphate and D-sedoheptulose 7-phosphate in the presence of ATP[6]. Successful synthesis and isolation of the endproduct of these studies provided a deoxy phosphorylated sugar, 4-deoxy-D-*threo*-hexulose 1,6-diphosphate (4-deoxy-D-fructose 1,6-diphosphate) which has not itself been found in nature. It should be capable of blocking carbohydrate metabolism specifically since aldolase cleavage cannot occur.

In an analogous fashion preparation of a deoxy sugar phosphate analogue of D-ribulose 1,5-diphosphate, the primary acceptor of carbon dioxide in photosynthesis was attempted. Currently no overall synthesis of 3-deoxy-D-*glycero*pentulose 1,5-diphosphate (3-deoxy-D-ribulose 1,5-diphosphate) in which the three hydroxyl group is replaced by hydrogen has appeared in the literature, however, a

possible precursor 3-deoxy-D-*erythro*pentose 5-phosphate (3-deoxy-D-ribose 5-phosphate) has been synthesized[7]. 3-Deoxy-D-ribose 5-phosphate was prepared by a new improved method of synthesis which has not been extensively applied to carbohydrates and involves the use of triphenylphosphine-carbon tetrachloride[8]. Then enzymatic or chemical isomerization of 3-deoxy-D-ribose 5-phosphate was attempted followed by phosphorylation with phosphoribulokinase. The product should competitively inhibit D-ribulose 1,5-diphosphate carboxylase, specifically blocking the first step of photosynthesis.

Approach

To explore the effect of selectively replacing hydrogen for the C-4 hydroxyl group of fructose on yeast hexokinase-substrate affinity, synthesis of 4-deoxy-D-fructose must first be accomplished. Deoxy sugar synthesis has recently been reviewed by Hanessian[9] and Overend[10]. In most cases, non-terminal deoxy sugar preparation is possible only by the introduction of appropriate protecting groups, since most sugars are polyhydroxy compounds with more than one free hydroxyl group. Readily available D-mannitol (Figure 2) whose vicinal cis hydroxyl groups



D-mannitol 4-deoxy-D-fructose 4-deoxy-D-fructose 1,6-diphosphate

Figure 2. Groups in D-mannitol and 4-deoxy-D-fructose involved in the interconversions.

react with acetone provides predominantly 1,2;5,6-di-O-isopropylidene-D-mannitol[11] leaving the three and four hydroxyl groups unprotected. The most widely used method for preparation of non-terminal deoxy sugars appears to be reductive ring opening of epoxides. Monotosylation of the partially substituted D-mannitol provides an intermediate, 1,2;5,6-di-O-isopropylidene-3-*O*-*p*-toluenesulphonyl-D-mannitol[12], necessary for the formation of the 3,4-epoxide. Subsequently the tosyl derivative was converted to the 3,4-epoxide, 3,4-anhydro-1,2;5,6-di-O-isopropylidene-D-talitol[12]. The expected products after reductive scission of the epoxide with lithium aluminum hydride were 4-deoxy-1,2;5,6-di-O-isopropylidene-D-*lyxo*-hexitol (-D-mannitol) and 4-deoxy-1,2;5,6-di-O-isopropylidene-D-*arabino*hexitol (-D-altritol). Separation was not necessary, since following hydrolysis, selective bacterial oxidation of free 4-deoxy-D-mannitol by *Acetobacter suboxydans* provided 4-deoxy-D-fructose. The resulting mixture after oxidation was then separated on bisulphite ion exchange resin. The pure 4-deoxy-D-fructose produced was treated with yeast hexokinase and the reaction kinetics followed by observing acid liberation. From these studies the relative influence of the C-4 hydroxyl group on the formation of an enzyme-substrate complex and hence the binding requirements became evident. For isolation of the product, 4-deoxy-D-fructose 6-phos-

phate, relatively mild conditions were used so that phosphate migration does not occur. In an analogous manner 4-deoxy-D-fructose 1,6-diphosphate was isolated after treatment of 4-deoxy-D-fructose 6-phosphate with rabbit muscle phosphofructokinase.

As in the previous case, the synthesis of 3-deoxy-D-*erythro*pentose 5-phosphate first involved preparation of the deoxy sugar (Figure 3). The key intermediate 3-deoxy-

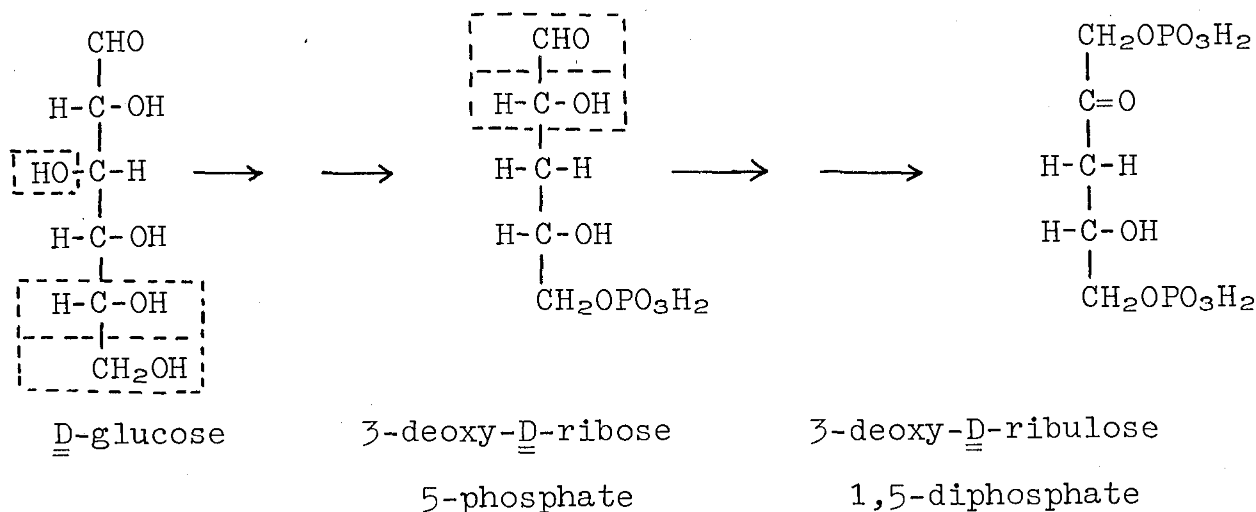


Figure 3. Groups in D-glucose and 3-deoxy-D-ribose 5-phosphate involved in the interconversions.

1,2;5,6-di-O-isopropylidene-α-D-glucofuranose, has already been prepared through a xanthate rearrangement followed by desulphurization; however the yield is low[13,14]. The utility of halodeoxy sugars in providing attractive synthetic precursors for the preparation of deoxy sugars has been reviewed recently by Hanessian[15]. Methods for

introducing halo substituents into carbohydrates are varied. Lee and Nolan[8] demonstrated that an isolated primary hydroxyl group reacted readily with triphenylphosphine-carbon tetrachloride. Isolated secondary hydroxyl groups have not shown the same reactivity. Therefore it was of interest to apply the triphenylphosphine-carbon tetrachloride reaction to a secondary center and the product, the 3 chloro derivative, was then reduced to the 3 deoxy intermediate. Thence by the method of the Szabós[7], 3-deoxy-1,2;5,6-di-O-isopropylidene-α-D-glucofuranose was converted to 3-deoxy-D-*erythro*-pentose 5-phosphate. Isomerization of 3-deoxy-D-*erythro*-pentose 5-phosphate mediated by the enzyme phosphoribo-isomerase was expected to produce 3-deoxy-D-*glycero*-pentulose 5-phosphate. Alternatively, base catalyzed isomerization was also a possibility. However in either of these conversions the product could be separated by borate anion-exchange chromatography. Finally phosphorylation at the one position might be carried out with phosphoribulokinase.

DISCUSSION

Synthesis of 4-Deoxy-D-threohexulose (4-Deoxy-D-fructose) (8)

Generally, reductive ring opening of epoxides appears to be the method of choice for 4-deoxyhexose preparation[9]. Two of the 4-deoxyaldohexoses, 4-deoxy-D-arabinohexose (4-deoxy-D-altrose)[16] and 4-deoxy-D-xylohexose (4-deoxy-D-glucose)[13], and the only available 4-deoxyhexulose, 4-deoxy-D-erythrohexulose (4-deoxy-D-allulose)[17] have been prepared in this manner. Preparation of the 4-deoxyhexulose involved lithium aluminum hydride reduction of 3,4-anhydro-1,2-O-isopropylidene-α-D-ribohexulose resulting in 4-deoxy-1,2-O-isopropylidene-α-D-erythrohexulose. This was readily hydrolyzed to free 4-deoxy-D-erythrohexulose. However, as yet an analogous synthesis of 4-deoxy-D-fructose can not occur since a suitable hexulose epoxide is not available. Instead an alternative pathway, through an intermediate hexitol epoxide, is possible.

Epoxide formation usually involves the readily available sulponate esters and entails base treatment of a compound containing a trans hydroxyl group to the vicinal departing sulphonate group. C-O bond scission results with elimination of the sulphonyloxy group, and Walden inversion occurs at the carbon atom bearing the sulphonate group as the epoxide forms.

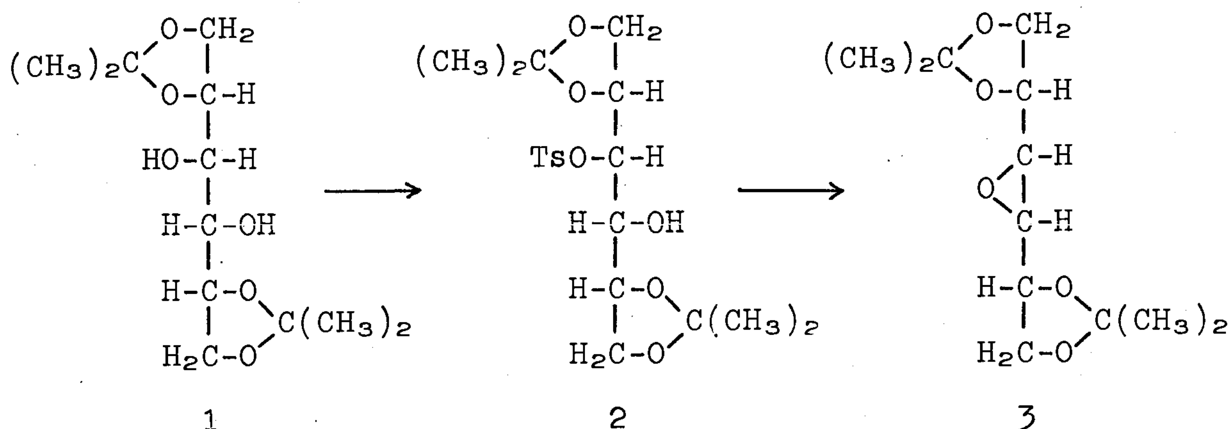
D-Mannitol constitutes a suitable hexitol with trans 3,4-hydroxyl groups to use as starting material. However,

tosylation is known to occur preferentially at primary rather than secondary hydroxyl groups[18] and the polyhydroxy nature of this compound adds further complications. Therefore synthesis of the specifically substituted carbohydrate, 4-deoxy-D-fructose, from D-mannitol requires the introduction of appropriate blocking groups. Cyclic acetals formed from aldehydes and ketones appear to be the most widely used. They are easily prepared, stable to a wide variety of reagents and their removal is effected under mildly acidic conditions[13]. In the presence of condensing agents the vicinal cis hydroxyl groups of D-mannitol react with acetone in such a manner to form 1,2;5,6-di-O-isopropylidene-D-mannitol (1). In the present work the method of Tipson[11] was used to prepare this compound (1).

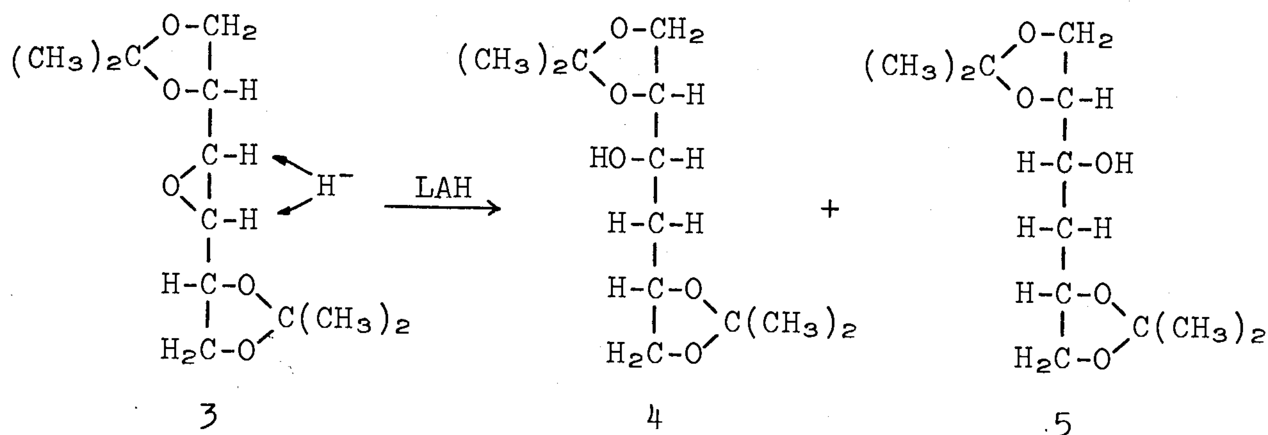
Monotosylation of protected D-mannitol (1) with one mole of toluene-p-sulphonyl chloride in pyridine resulted in the formation of crystalline 1,2;5,6-di-O-isopropylidene-3-O-p-toluenesulphonyl-D-mannitol (2) by the method of Bladen and Owen[12]. However when the reaction was followed on thin-layer chromatography a discrepancy became apparent. A two day reaction period was necessary for maximum yield of the 3 tosyl derivative (2) instead of the recommended 20 hours, which gave roughly a 60 per cent conversion.

3,4-Anhydro-1,2;5,6-di-O-isopropylidene-D-talitol (-D-altritol) (3) was prepared by the method of Bladen and Owen[12], in which the formation of the 3,4-epoxide is brought

about by base treatment of the suitably protected 3 tosyl compound (2). The product is the three epimer of D-mannitol, D-talitol (D-altritol).



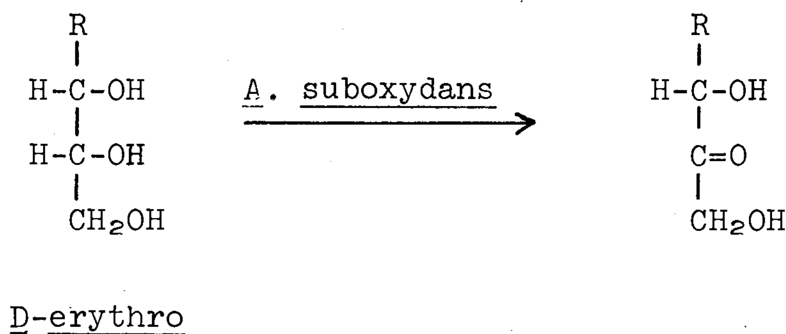
Formation of the deoxy compounds can now be brought about by reductive ring opening of the epoxide 3,4-anhydro-1,2;5,6-di-O-isopropylidene-D-talitol (3) with lithium aluminum hydride in ethereal solution. A chromatographically pure syrup in approximately quantitative yield resulted. This syrup consists of a mixture of the epimeric deoxy diisopropylidene hexitols, 4-deoxy-1,2;5;6-di-O-isopropylidene-D-lyxohexitol (-D-mannitol) (4) and 4-deoxy-1,2;5,6-di-O-isopropylidene-D-arabinohexitol (-D-altritol) (5). Hydride attack, as shown below, can occur at either carbon atoms three or four yielding a mixture of deoxy diisopropylidene hexitols (4,5). The ketal ring systems are stable to reducing agents and alkaline and neutral pH so cleavage does not occur. Hydrolysis of the mixture of epi-



meric diisopropylidene deoxy hexitols (4,5) was carried out in mildly acidic conditions. The products 4-deoxy-D-mannitol (6) and 4-deoxy-D-altritol (7) were not separated by paper chromatography and ketal cleavage occurred in almost quantitative yield. In general deoxyhexitols with the deoxy function at a secondary carbon atom are prepared by reduction of the corresponding deoxyaldose[19], however in this case the deoxyaldose is not available.

At present, very few cases are known of successful selective oxidation of a secondary hydroxyl group of monosaccharides by chemical means and the yields are extremely poor. Such a reaction requires appropriately blocked derivatives and a suitable sterical arrangement of the free hydroxyl group. On the other hand, microbial oxidation of hexitols is a much more specific method of ketose preparation. Organisms such as Acetobacter suboxydans are well

known for their capacity to dehydrogenate hexitols and accumulate the oxidation products in the culture medium. This enzymic oxidation proceeds according to Bertrand-Hudson's rule[20,21] in the pH range 5-6.5. It states that the favourable configuration for oxidation is a cis-arrangement of two secondary hydroxyl groups in the D-configuration adjacent to a terminal primary alcohol grouping. Furthermore the rule is applicable to terminal deoxy hexitols if the CH_3CHOH group is considered as the primary alcohol group[22].

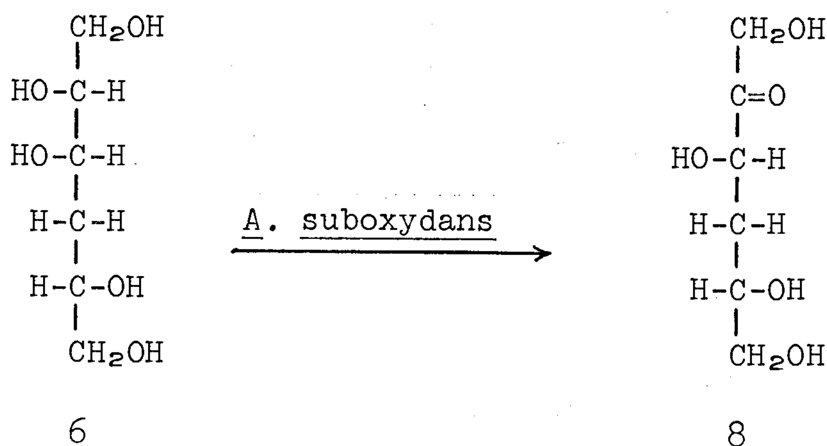


D-erythro

Preparations of non-terminal deoxyhexuloses has essentially not been attempted using Acetobacter suboxydans. However in the commercial oxidation of D-glucitol to L-sorbose by Acetobacter suboxydans a side product 5-deoxy-D-threohexulose has resulted[23]. 2-Deoxy-D-glucitol, an impurity in D-glucitol is thought to be the substrate acted on by the bacteria. Because of their much greater ease of preparation, a great deal more work has been carried

out on Acetobacter suboxydans oxidation of terminal deoxy hexitols[23,24,25]. Hexitol oxidation by Acetobacter suboxydans has provided a method to prepare ketoses whose synthesis is otherwise difficult.

Separation of the mixture of 4-deoxy-D-mannitol (3-deoxy-D-mannitol) (6) and 4-deoxy-D-altritol (3-deoxy-D-talitol) (7) is not necessary as Acetobacter suboxydans selectively oxidizes 4-deoxy-D-mannitol (6). The product is a mixture of 4-deoxy-D-threohexulose (8) and 4-deoxy-D-



altritol (7). The reaction was conveniently followed on thin-layer chromatography and care has to be taken when withdrawing samples from the incubation mixture to maintain asceptic conditions. Also, as pointed out by Lockwood[26] the bacterial pellicle must not be allowed to sink, as the reaction ceases. A maximum yield was obtained after a seven day incubation period.

Preparative separation of ketoses from aldoses by the use of a strongly basic, anion-exchange resin in the bisulphite form has been successfully demonstrated by Lindberg and Slessor[27]. The basis of the procedure is that aldehyde bisulphite complexes are more stable than ketone complexes, which allows the ketone to be removed from the bisulphite column more readily than the aldehyde. Similarly hexuloses and hence deoxyhexuloses should form a bisulphite complex whereas hexitols and hence deoxyhexitols should not form a bisulphite complex. Therefore deoxyhexitols should be more readily removed from the bisulphite column.

Separation of 4-deoxy-D-fructose (8) and 4-deoxy-D-altritol (7) by the above method was successful and both sugars were obtained chromatographically pure. 4-Deoxy-D-altritol (7) was eluted from the column in Fraction 20-27 and 4-deoxy-D-fructose (8) in Fraction 29-42. The by-product 4-deoxy-D-altritol (3-deoxy-D-*lyxo*hexitol) (7) has as yet not been reported in the literature. This synthesis constitutes a reasonable means of obtaining it.

Approximately three times as much 4-deoxy-D-fructose (8) was isolated from the bisulphite column as the deoxyhexitol (7). Assuming that all of the 4-deoxy-D-mannitol (6) in the deoxyhexitol mixture was converted to 4-deoxy-D-fructose (8) by *Acetobacter suboxydans*, then lithium aluminum hydride reduction of the 3,4-epoxide (3) must have taken place more readily at carbon atom four. Furthermore three times the

amount of 4-deoxy-1,2;5,6-di-O-isopropylidene-D-mannitol (4) as 4-deoxy-1,2;5,6-di-O-isopropylidene-D-altritol (5) must have been produced. This agrees with the stereochemistry of the reaction (Figure 4). A likely conformation in which

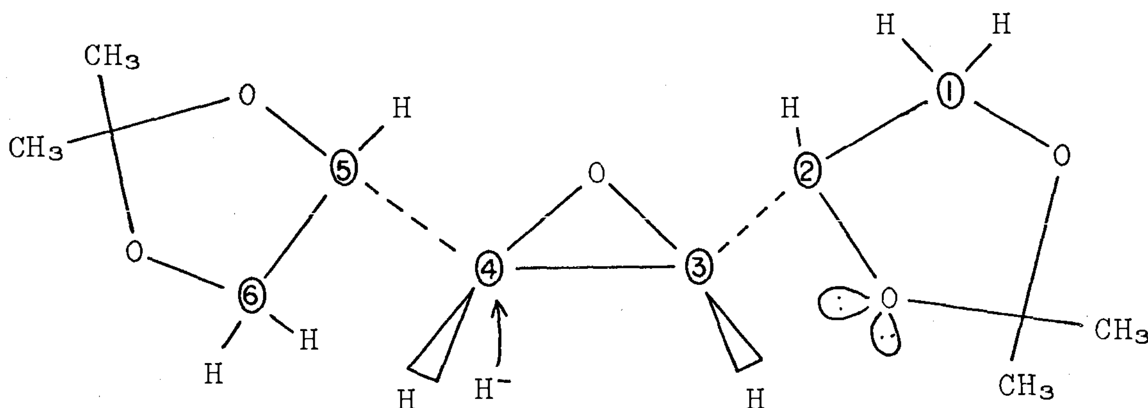


Figure 4. Stereochemistry of reductive opening of the epoxide, 3,4-anhydro-1,2;5,6-di-O-isopropylidene-D-talitol (3).

there is the least interaction between the dioxalane ring systems causes the lone pairs of electrons on the C-2 oxygen atom to interfere with C-3 hydride attack. Therefore attack occurs primarily at C-4 giving mainly the mannitol derivative (4).

The structure of 4-deoxy-D-fructose (8) was confirmed by several methods, including mass spectroscopy, N.m.r. and a chemical method. Curtius et al.[28] have separated pertrimethylsilyl (TMS) derivatives of fructose into five components by gas chromatography which were then subjected to mass spectroscopy. The molecular ion at M^+=540 was not

observable. The highest mass number was at m/e 437 which could be attributed to the loss of a $-CH_2OSi(CH_3)_3$ group and amounted to at least 10% of the base peak. The mass spectra of the trimethylsilyl ether of α-D-glucose had a visible molecular ion at mass 540[29]. A peak of low intensity (<1%) was present at m/e 432. Pertrimethylsilyl-4-deoxy-D-fructose had a clearly visible molecular ion $M^+=452$. Furthermore a peak of high intensity arising from the splitting of a $-CH_2OSi(CH_3)_3$ fragment analogous to TMS-fructose was present at m/e 349. This amounted to 25% of the base peak at m/e 73. The high intensity peak at m/e 349 in TMS-4-deoxy-D-fructose analogous to the m/e 437 peak in TMS-fructose confirms the ketose structure.

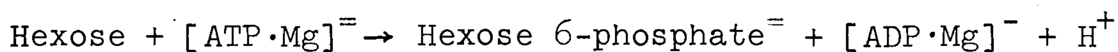
Further evidence was provided by N.m.r. spectroscopy. 4-Deoxy-D-fructose (8) in deuterium oxide showed no low field anomeric proton characteristic of aldoses. Condensation of 4-deoxy-D-fructose (8) with acetone lends further support. 4-Deoxy-D-fructose (8) can form a maximum of six substituted isopropylidene compounds. However 4-deoxy-D-glucose can form only one substituted isopropylidene derivative and 4-deoxy-D-mannose two. After isolation of the product from acetone condensation three distinct isopropylidene derivatives of 4-deoxy-D-fructose (8) were found on thin layer chromatography. This data, along with the specificity of action of *Acetobacter suboxydans* confirms the structure of 4-deoxy-D-fructose (8).

Now that 4-deoxy-D-fructose (8) has been obtained in pure form it can be used as a possible substrate for yeast hexokinase.

Yeast Hexokinase Action on 4-Deoxy-D-fructose (8)

It has previously been established that yeast hexokinase, the first hexokinase identified, is a multisubstrate enzyme and can phosphorylate many different structurally related sugars. These include glucose, mannose, fructose, glucosamine, 2-deoxyglucose, glucosone[30], 2,5-anhydro-D-mannitol[31], 1-deoxy-D-fructose[31] and 5-keto-D-fructose [5]. Phosphorylation of these compounds, and the low Km values involved, furnishes substantial evidence that the structural makeup about C-1 and C-2 does not play a significant role in enzyme-substrate complex formation, providing excessively large groups are not attached[30]. The 3,4,5,6-region of the substrate glucose has also been extensively studied, mainly by Sols et al.[30], by testing glucose epimers and some of their respective deoxy derivatives as substrates. Comparison of Km values of glucose and its structural derivatives illustrates the influence of the hydroxyl groups on enzyme-substrate binding. These, along with studies by other workers[2,31] led to the conclusion that the enzyme is specific for the 3,4,5,6-region of the molecule (Figure 1). So far, galactose, the four epimer of glucose is the only substrate modified specifically at C-4 that has been tested as a substrate for yeast hexokinase. Fructose analogues have recieved even less attention and therefore 4-deoxy-D-fructose (8) would be a reasonable compound to try.

Since product recovery, after completion of the reaction, is anticipated, the assay system should perform two basic functions, a) allow the kinetics to be followed relatively easily and b) allow easy isolation of the product. The assay system most extensively used is that of Darrow and Colowick[32], in which the phosphoryl group transferred is determined as its acid equivalent. However,



$$\text{Hexose} + [\text{ATP}\cdot\text{Mg}]^{=} \rightarrow \text{Hexose 6-phosphate}^{=} + [\text{ADP}\cdot\text{Mg}]^{-} + \text{H}^{+}$$

an acid-base indicator (cresol red) and a buffer (glycyl-glycine) are necessary to determine the amount of acid produced and may interfere in the subsequent isolation. Less frequently applied methods include sugar disappearance, non-nucleotide organic phosphate formation and enzymic determination of hexose 6-phosphate formed[30]. A more suitable and easier method, from the standpoint of product isolation, simply involves titration of the acid liberated in an unbuffered medium. A more sophisticated modification of this method, to obtain maximum sensitivity, involves using a titrimetric pH-stat to maintain constant pH. ATP-creatine transphosphorylase[33], phosphofructokinase[34] and hexokinase[35] assays have made use of this procedure.

This method allows the reaction rate to be determined by measurement of the rate of base uptake necessary

to maintain constant pH. At pH 8.5 and above Alberty, Smith and Bock[36] have shown that the reaction $ATP^{4-} \longrightarrow ADP^{3-} + Pi^{2-}$ produces one equivalent of H^+ and this relationship has been shown to exist at pH 8.0[35]. Yeast hexokinase exhibits an optimal activity between pH 8 and 9. Therefore, the hexokinase assay was run at pH 8.5 so that pH would not extend beyond the region of optimal activity and linearity of acid production upon base addition. The conversion of known amounts of fructose to fructose 6-phosphate was tested for stoichiometry under the experimental conditions by following sodium hydroxide uptake. The values obtained approximate one (0.96) at pH 8.5 after a correction factor has been applied. The correction factor is necessary since there is a small but significant spontaneous nonenzymic ATP hydrolysis and also leakage of KCl from the electrode[34]. Base uptake by the blank was relatively constant at 4.4×10^{-2} μequiv./min. for 6.0 ml. of reaction mixture. Most studies[33,35] using the pH stat method found that an initial deviation of the curve is due mostly to instrumental recovery from pH disturbances in the reaction mixture. This is brought about by addition of the enzyme aliquot, whose size was kept to a minimum, and is unrelated to the reaction velocity. In the present work, this was overcome by previous adjustment of the enzyme solution pH to that of the reaction mixture prior to reaction. Furthermore, use of relatively dilute

sodium hydroxide solution provided a high degree of sensitivity in the initial portion of the enzyme reaction, as changes from dilution are relatively small. This allows initial velocities needed for kinetic studies to be determined relatively accurately.

Table 1 shows the values of the Michaelis-Menton constants, the affinities (relative to that for D-glucose) and the maximum velocities (relative to D-glucose) obtained by the pH-stat method and Figure 5 the Lineweaver-Burk plot. The relative affinities are based on the assumption that the reciprocal of Km is a measure of enzyme-substrate affinity[1]. The Michaelis constants for glucose and fructose agree quite favourably with those obtained by Sols et al.[30] at pH 7.4 and Zewe et al.[37] at pH 7.6, which have been arrived at by other assay procedures. The relative maximal rate value obtained for fructose also agrees with that of Sols et al.[30].

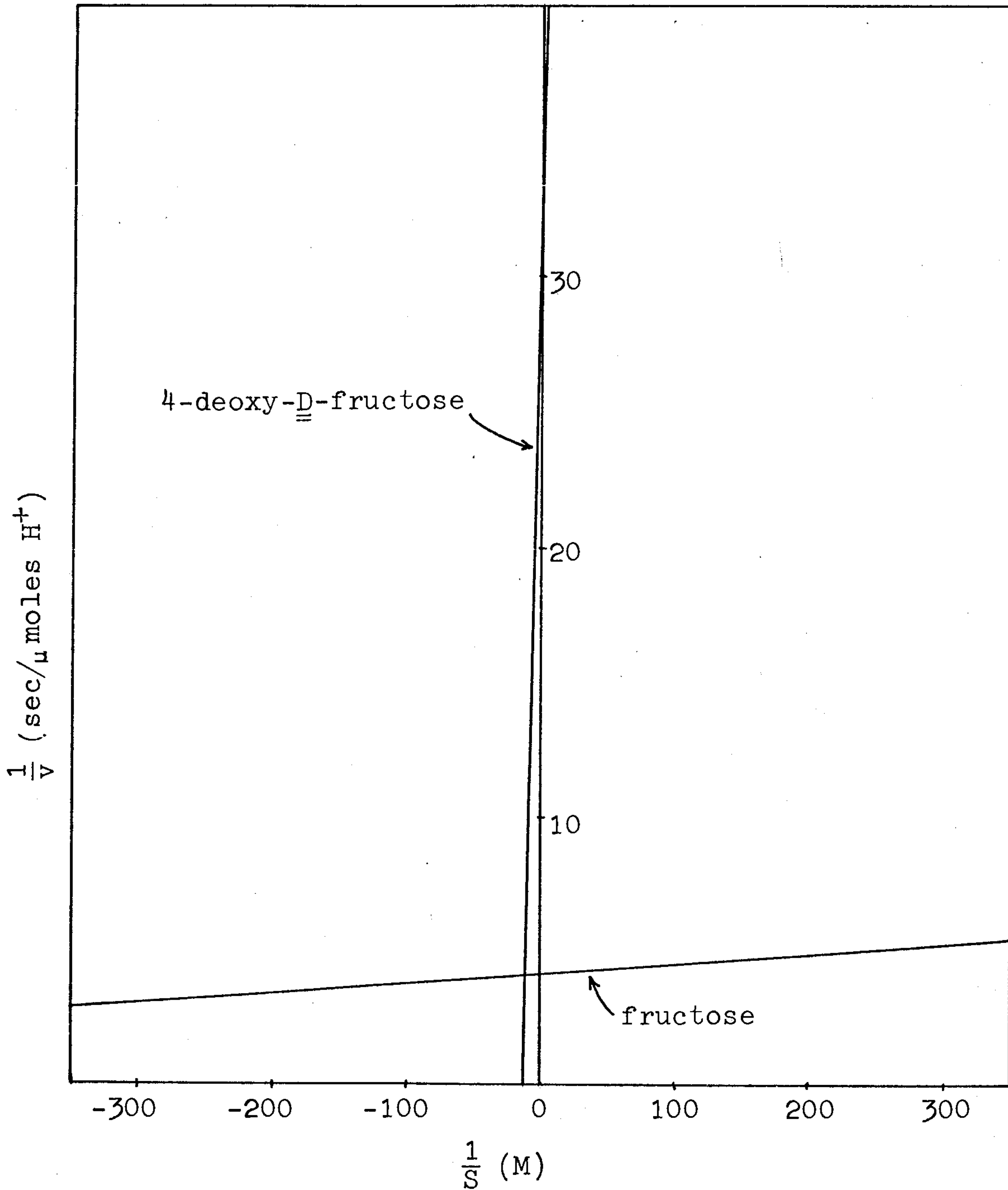
From the affinity data, galactose, structurally the four epimer of glucose, had a relative affinity compared to glucose of approximately 0.002. This suggests that the C-4 hydroxyl group of glucose is a binding group in the enzyme-substrate complex as already has been postulated[1]. Further comparison of the data from Table 1 shows that 4-deoxy-D-fructose (8) has a significantly higher Km for yeast hexokinase than glucose and fructose, although the relative Vmax was 0.1 of that achieved by

Table 1

Michaelis constants, relative affinities and relative rates of hexose phosphorylation by yeast hexokinase

Substrate	Km (M)	Relative Affinity	Relative Vmax
Glucose	8×10^{-5}	1.0	1.0
Fructose	9×10^{-4}	0.14	2.0
4-Deoxy-D-fructose	8×10^{-2}	0.001	0.2
Galactose[30]	$>5\times10^{-2}$	<0.002	<0.002

Figure 5. Double reciprocal Lineweaver-Burk plot of initial reaction velocity and substrate concentration for yeast hexokinase.



fructose. The relative affinity of 4-deoxy-D-fructose (8) compared to that of fructose was 0.007. The results from the studies with 4-deoxy-D-fructose (8) in which the possible binding group at carbon four has been removed lend further support to the contention that the C-4 hydroxyl is a binding group. This stems from the fact that the relative affinity of 4-deoxy-D-fructose (8) compared to fructose is in the same order of magnitude as galactose compared to glucose.

Now that many substrate epimers and their respective deoxy derivatives have been tested as substrates for yeast hexokinase, along with a few analogues bearing larger groups it would be useful to evaluate steric requirements of the active site more fully. If the hydroxyl group is inactive in binding then steric requirements at that site can be investigated by increasing the size of substituents, such as in the series F, Cl, Br, I. In recent years considerable interest in monohalogenated derivatives has led to the synthesis of many halogenated carbohydrates and fruitful studies such as those used successfully in the carrier-mediated transport of sugars across the intestinal membrane[38] may emerge.

Sugar Phosphate Synthesis

In the preparation of carbohydrate phosphate esters it is imperative that the possibility of phosphate group migration be considered. In cases where reaction conditions may allow migration to occur a careful check should be maintained on sample purity. Migration of phosphomonoesters generally occurs in acid conditions[39,40] as base catalyzed migration usually involves only phosphodiesters. Under appropriate conditions the proximity of a neighbouring hydroxyl group to a phosphate group allows migration to proceed through the intramolecular formation of an intermediate cyclic phosphate. This intermediate can then undergo ring opening to the migrated ester.

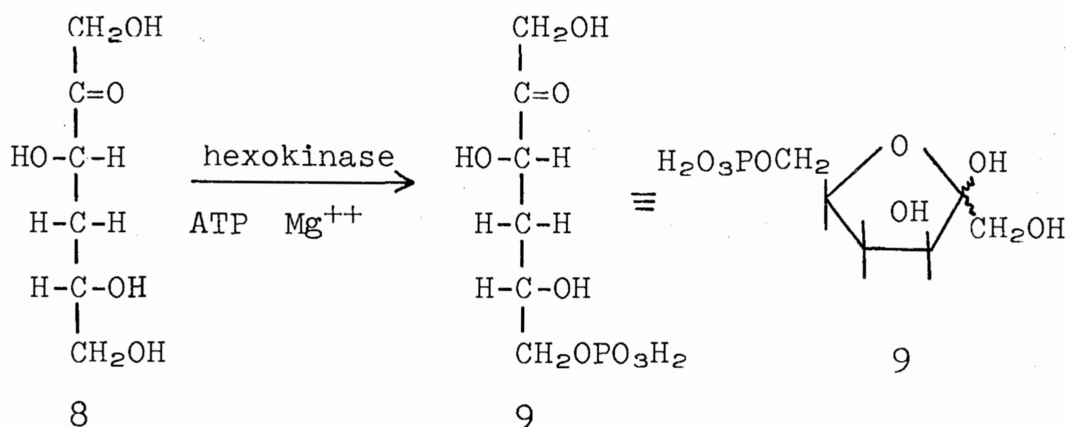
Synthesis of deoxy sugar phosphates usually involves all the numerous general chemical methods used for the synthesis of sugar phosphates[40]. The basic difficulty lies in preparing the appropriately blocked sugar and further making sure that no migration occurs. Introduction of phosphate groups to unprotected sugars by kinase enzymes and isolation of products constitutes another method used in sugar phosphate preparation. The only deoxy sugar phosphates isolated after the action of kinase enzymes on the deoxy derivatives were 2-deoxy-D-ribose 5-phosphate from 2-deoxy-D-ribose[3], L-fucose 1-phosphate from L-fucose[4], L-fuculose 1-phosphate from

L-fuculose[5] and L-rhamnulose 1-phosphate from L-rhamnulose[41]. However all these compounds occur naturally and their phosphorylation is mediated by the appropriate deoxy sugar kinases. Deoxy sugars, which do not serve as normal substrates to the kinase enzymes, have not as yet been isolated from reactions as the corresponding deoxy sugar phosphates.

Isolation of the sugar phosphate products after kinase action usually consists of three basic steps, a) removal of the enzyme, b) removal of the nucleotide coenzyme and c) precipitation of the phosphate from the reaction mixture. The first step can be accomplished by heating or adding acid (trichloroacetic or perchloric). Step two consists of nucleotide adsorption onto activated charcoal or ion-exchange separation. The last step can be carried out by precipitation of the barium salt of the alcohol insoluble phosphomonoester from the reaction mixture.

Preparation of 4-Deoxy-D-fructose 6-phosphate (9)

Yeast hexokinase action on hexoses lends itself well to product isolation because the equilibrium position of the reaction lies so far in the direction of product formation as to render the reaction virtually irreversible.



As previously pointed out phosphate migration may occur. However in the furanoid ring there is no free hydroxyl group present in a sterically favourable orientation as long as the phosphate group is attached to the terminal primary alcohol. Fructose 6-phosphate has recently been shown by Swenson[42] to contain 5 per cent of the keto form in solution. If a similar situation exists in 4-deoxy-D-fructose 6-phosphate (9) then this free keto form could undergo migration since the C-5 hydroxyl is in the correct orientation to form a cyclic intermediate. After cleavage, the phosphate group would reside on the

C-5 hydroxyl leaving the primary hydroxyl free to form a pyranoid ring. From the above discussion two possibilities become evident, either the reaction and isolation conditions can be carefully controlled so ester migration is not possible or the product can be rigorously checked afterwards to ensure migration has not occurred. In some cases the exact position of the phosphate ester on the carbohydrate molecule still is unclear[40], so the first possibility is preferred.

In the synthesis of 4-deoxy-D-fructose 6-phosphate (9) from 4-deoxy-D-fructose (8) basically the same conditions were maintained as in the assay, only on a larger scale. The reaction was carried out at pH 8.0, the lower end of the pH optimum because of the presence of reducing sugar. Since relatively long reaction times are involved and dilution of the enzyme occurs as the reaction proceeds, an inert protein, bovine serum albumin was used to stabilize the enzyme. The reaction was stopped when rate of base consumption equalled the blank titration value, which usually requires about twelve hours. Product isolation was carried out at near neutrality to avoid acid conditions favoured by phosphate group migration. Deproteinization was not attempted by the usual methods previously described because they are too vigorous and could induce phosphate migration. Instead dialysis tubing was used to contain the reaction mixture, which retains the protein

and allows passage of the sugar phosphate across the membrane, as a continuous flow of water passes through a column containing the tubing. The water surrounding the tubing and reaction mixture was held to a minimum, so large volumes of eluant were not produced as the sugar phosphate is eluted.

4-Deoxy-D-fructose 6-phosphate (9) was isolated from the reaction mixture, after dialysis and removal of nucleotides, as its barium salt by precipitation with five volumes of ethanol. The overall yield was in the order of 10 per cent. The barium salt of the ketohexose phosphate was very water soluble and for its successful precipitation the solution containing it should be reasonably concentrated. Decomposition was observed unless the barium salt was stored at pH 7.0 in a frozen state. This has also been observed for phosphorylated sugars in the form of their sodium salts[43]. The sodium salt of 4-deoxy-D-fructose 6-phosphate (9) necessary for paper chromatography and the reaction catalyzed by phosphofructokinase was prepared with ion-exchange resin in the sodium form so the solution does not become acidic.

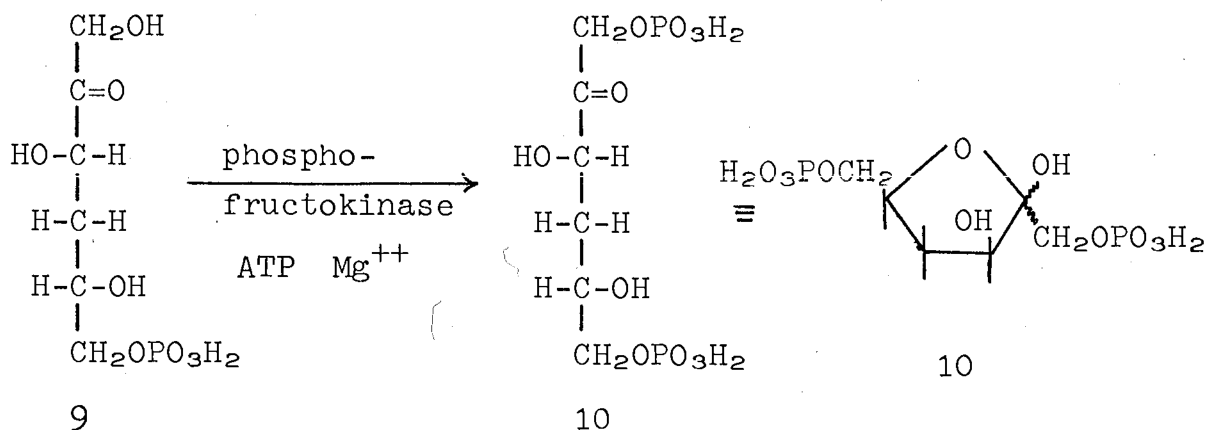
Paper chromatography of the sodium salt of the product (9) in two solvent systems, followed by treatment with ammonium molybdate specific for phosphorylated sugars, showed mainly one phosphorylated compound. In solvent system A the major component had R_{PO_4} 0.56 and the minor

0.42. Wood[44] has shown that glucose 6-phosphate R_{PO_4} 0.41 runs slower than fructose 6-phosphate R_{PO_4} 0.50 in this solvent system. Therefore in view of the slightly basic conditions of the reaction mixture this minor component may be 4-deoxy-D-glucose 6-phosphate. N.m.r. studies on the sodium salt in deuterium oxide showed this to be the case as the low field proton characteristic of an anomeric C-1 hydrogen was found to be present in small amounts. If pure 4-deoxy-D-fructose 6-phosphate (9) or 4-deoxy-D-glucose 6-phosphate are required, they can probably be separated by anion-exchange chromatography with triethylammonium borate[45] since separation by paper chromatography is relatively easy. However, in this case anion-exchange chromatography is unnecessary unless 4-deoxy-D-glucose 6-phosphate inhibits phosphofructokinase.

Identification of sugar phosphate can be confirmed by phosphatase hydrolysis followed by paper chromatography and production of characteristic color reactions of the free sugars[43]. However, color reactions of deoxyketoses have not been studied very extensively and as the parent sugar had not previously been prepared, this method is unreliable. Instead, after phosphatase treatment and deionization of the resulting mixture, the mobility of the free sugar and the parent deoxy sugar, 4-deoxy-D-fructose (8), can be compared on paper chromatography. The residue liberated by phosphomonoesterase was identical to 4-deoxy-D-fructose (8).

Preparation of 4-Deoxy-D-fructose 1,6-diphosphate (10)

Completion (Figure 6) and workup of the phosphofructokinase reaction was carried out in a similar manner as for yeast hexokinase. This alleviates the possibility of

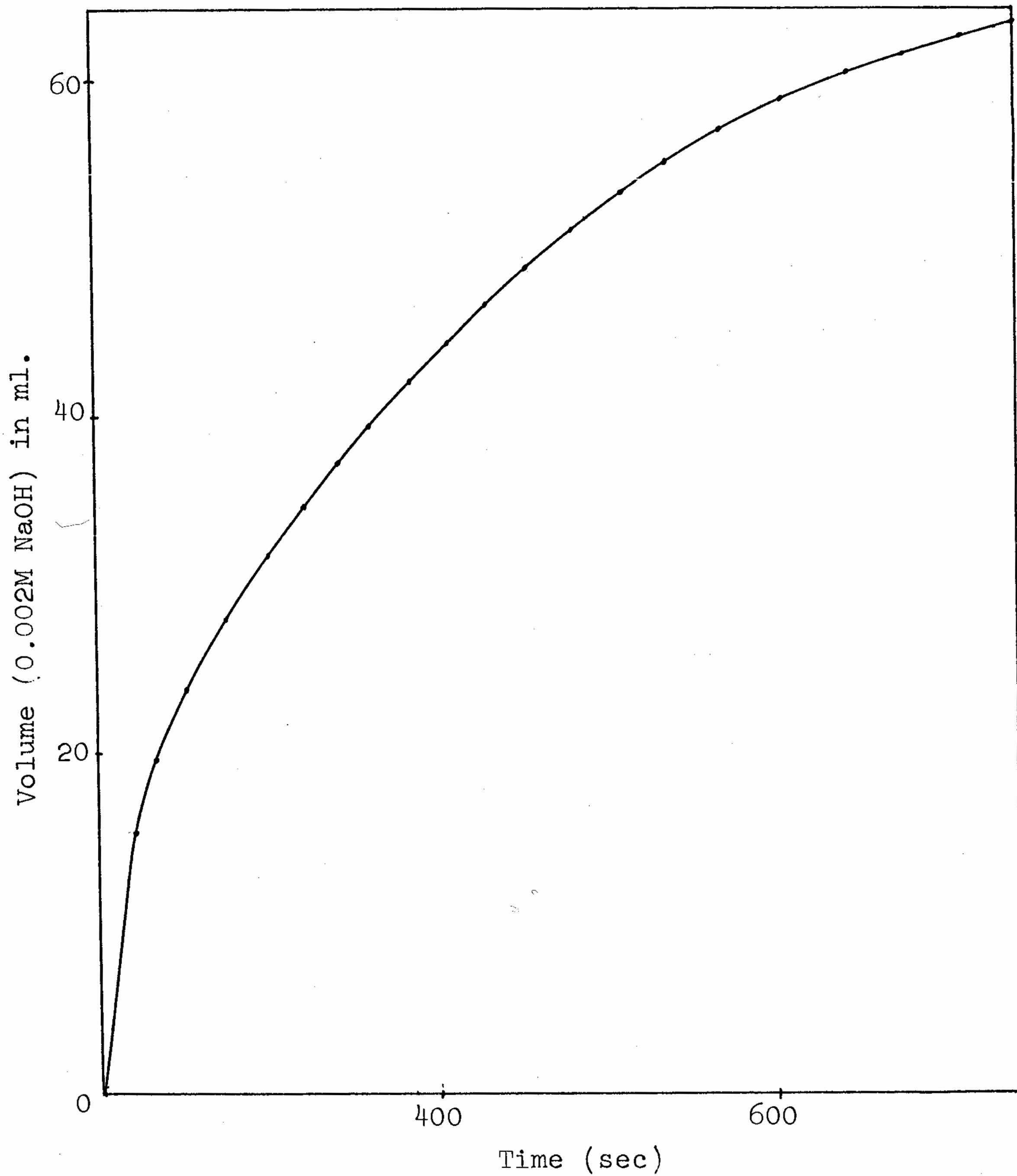


phosphate migration and also the probability of cleavage of the alkali labile 1-phosphate group.

Unlike sugar phosphomonoesters, the barium salts of sugar phosphates bearing more than one phosphate group are water insoluble. The dibarium salt of 4-deoxy-D-fructose 1,6-diphosphate (10) was precipitated out of the reaction mixture simply by cooling, as the deoxy sugar and deoxy sugar monophosphate remain in solution. The product (10) was obtained chromatographically pure in two solvent systems and the overall yield was 20 per cent.

Phosphomonoesterase treatment of fructose 1,6-diphosphate has been used in its analysis[46]. Similarly treatment of 4-deoxy-D-fructose 1,6-diphosphate (10) with

Figure 6. Graph of base uptake and time taken for phosphofructokinase reaction.



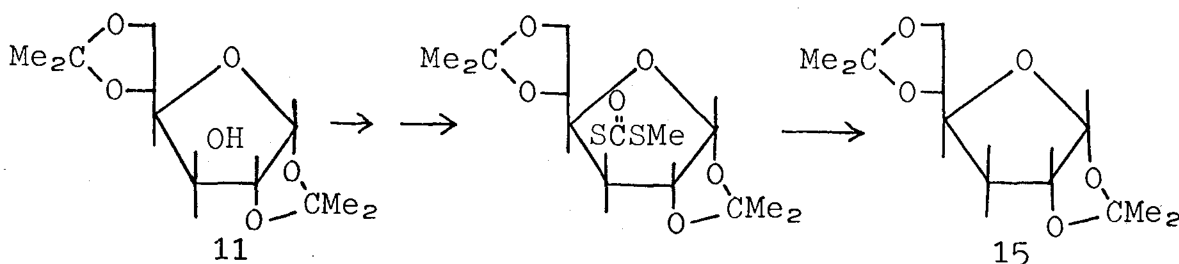
phosphatase followed by isolation and chromatography showed the free sugar had the same mobility as the parent sugar, 4-deoxy-D-fructose (8). As the sugar diphosphate was not exposed to acid conditions the assignment of the phosphate groups to the terminal hydroxyl functions is unambiguous.

Although 4-deoxy-D-fructose 1,6-diphosphate (10) is not known to occur naturally, it is of potential interest because of the general importance of ketose diphosphates in biological systems. Its deoxy function should enable it to act as a competitive inhibitor in the aldolase catalyzed reaction and thereby be a possible specific block of carbohydrate metabolism.

Synthesis of 3-Deoxy-D-ribose 5-phosphate (21)

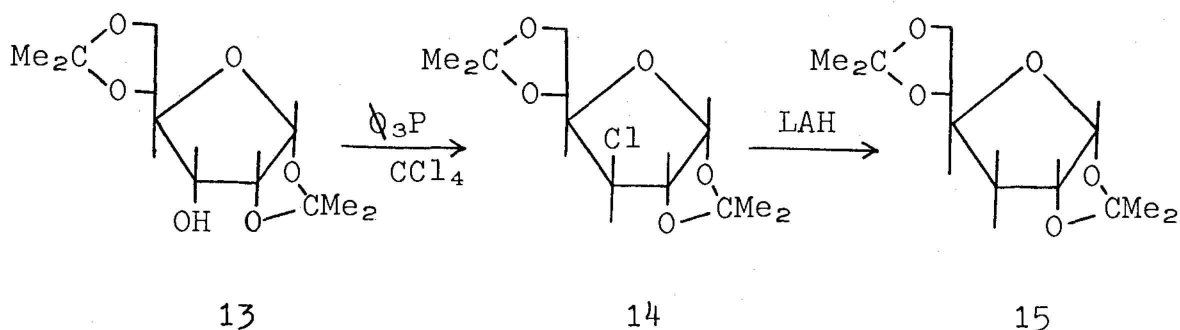
The synthesis of 3-deoxy-D-ribose 5-phosphate (21) from α-D-glucose is possible only through a suitably blocked intermediate. Acetone reacts with α-D-glucose leaving the C-3 hydroxyl unprotected and gives 1,2;5,6-di-O-isopropylidene-D-glucofuranose (11) by the method of Schmidt[47].

A variety of diverse routes exist for the formation of 3-deoxy-D-ribose 5-phosphate (21) with most of them converging through the intermediate 3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (15). Freundenberg and Wolf[48] first described the sequence of reactions from the readily accessible 1,2;5,6-di-O-isopropylidene-D-glucofuranose (11) to 3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (15) and finally to 3-deoxy-D-glucose. 1,2;5,6-Di-O-isopropylidene-D-glucofuranose was converted to 1,2;5,6-di-O-isopropylidene-3-O-[(methylthio)thiocarbonyl]-D-glucofuranose and thermal rearrangement of the above compound afforded the 3-thio-derivative. Černý and Pacák[14] have improved the rearrangement step as have Hedgley, Overend and Rennie[13]. Subsequent desulphurization of the rearranged product gave 3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose, however the yield was low. Other methods of synthesis of the 3 deoxy



derivative have been accomplished[49,50], some of which proceed through the 3 iodo derivative[49,51], but overall yields are also generally low.

Synthesis of 3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (15) and hence 3-deoxy-D-ribose 5-phosphate (21) has been accomplished in a slightly different manner from those previously discussed. The principal intermediate in the total synthesis is the chloro sugar, 3-chloro-3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (14). Formation of the 3 halo derivative directly from protected 1,2;5,6-di-O-isopropylidene-D-glucofuranose (11) has been unsuccessful. Reaction of 11 with triphenyl phosphite methiodide and triphenyl phosphite dibromide yields the 6 iodo and 6 bromo derivatives respectively[52]. Also reaction with chlorinating reagents leads to the rearranged 6 chloro product[53,54,55]. Halogen attack by a S_N2 mechanism is hindered by the 1,2 isopropylidene residue. Instead the 3 epimer, 1,2;5,6-di-O-isopropylidene-D-allofuranose (13), prepared by the methods of Slessor[56] and Theander[57] by oxidation of 1,2;5,6-di-O-isopropylidene-D-glucofuranose (11) followed by stereospecific borohydride reduction, was used in which the 3-OH group is endo. The 3-tosyl derivative of 13 is known to undergo nucleophilic displacement even with chloride as nucleophile[38]. Substitution of the 3-OH by chlorine from the unhindered side, by using triphenylphosphine in carbon tetrachloride, proceeded in very high yield

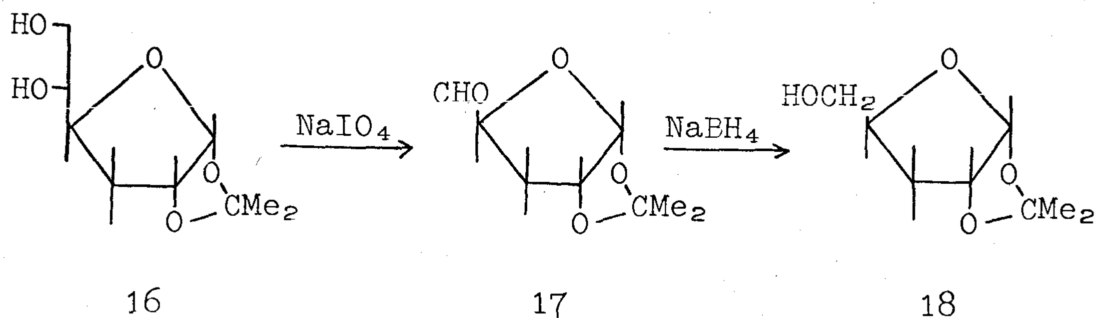


and 3-chloro-3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (14) was isolated as a liquid in 85% yield after distillation.

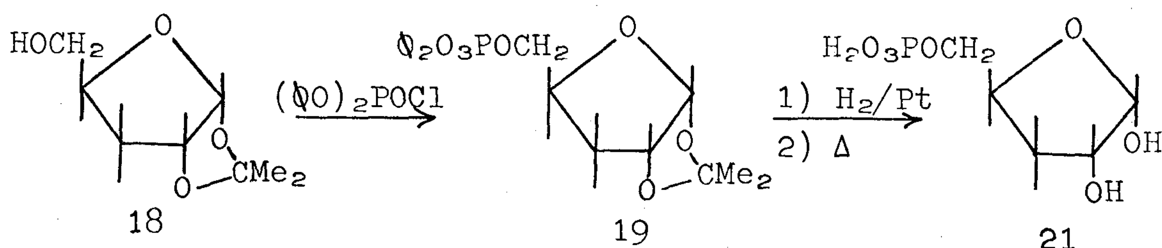
Reduction of 3-chloro-3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (14) was carried out in tetrahydrofuran with lithium aluminum hydride. The reduction of the gluco derivative represents a convenient route to 3-deoxy- 1,2;5,6-di-O-isopropylidene-D-glucofuranose (15) from 1,2;5,6-di-O-isopropylidene-D-glucose (11). The deoxy derivative 15 is reported crystalline for the first time, although its melting point is low (10-12°).

Now that 3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (15) has been synthesized the steps necessary for phosphorylation can be considered. Partial hydrolysis of 3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose (15) in dilute acid by the method of Hedgley, Overend and Rennie[13] gave 3-deoxy-1,2-O-isopropylidene-D-glucofuranose (16). An extremely efficient synthesis from this compound to 3-deoxy-D-ribose 5-phosphate has been devised by the Szabós[7].

By this method 3-deoxy-1,2-O-isopropylidene-D-glucofuranose (16) was treated with one mole of sodium metaperiodate which liberated formaldehyde and the dialdehyde (17). Subsequent reduction of the 5-aldehydo group with sodium borohydride, whereas Szabó used Raney nickel, yielded 3-deoxy-1,2-O-isopropylidene-D-*erythro*pentose (18).



Phosphorylation can now occur exclusively on the hydroxymethyl group since the other hydroxyls are blocked. The phosphorylating agent was diphenylphosphorochloridate. 3-Deoxy-1,2-O-isopropylidene-D-*erythro*pentose 5-diphenyl phosphate (19) was produced, which upon hydrogenation gave 3-deoxy-1,2-O-isopropylidene-D-*erythro*pentose 5-(dihydrogen phosphate) (20), isolated as the barium salt. Removal of the barium ions was accomplished with H^+ ion exchange resin, after which the phosphoric acid group of the sugar was used to cleave off the 1,2-isopropylidene residue leaving 3-deoxy-D-*erythro*pentose 5-phosphate (3-deoxy-D-ribose 5-phosphate) (21).



Attempted Synthesis of 3-Deoxy-D-ribulose 5-phosphate (22)

Several possible synthetic routes exist for the isomerization of the α-hydroxyaldehyde, 3-deoxy-D-ribose 5-phosphate (21), to the α-hydroxyketone, 3-deoxy-D-ribulose 5-phosphate (22). The enzyme ribosephosphate isomerase could mediate this interconversion, but low yields would be expected because of the reversible nature of the reaction. Furthermore, recent investigations by various physical means of isomerase action on D-ribose 5-phosphate led to the conclusion that D-ribulose 5-phosphate was not formed, but instead the primary product was the β-diketone phosphate (Figure 7)[58]. Rehydration of the β-diketone phosphate by

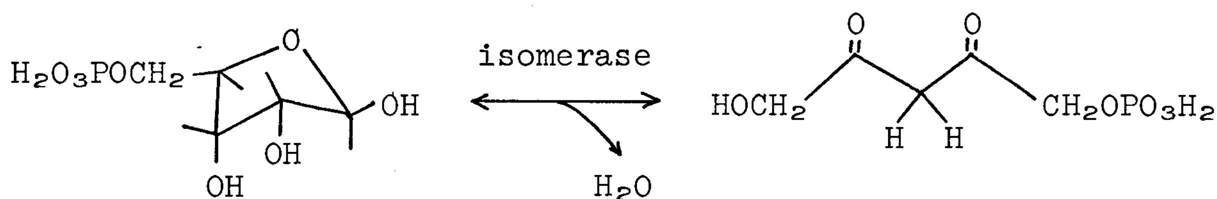


Figure 7. Action of ribosephosphate isomerase on ribose 5-phosphate

another enzyme is thought to generate D-ribulose 5-phosphate. This is not compatible with isomerization of 3-deoxy-D-ribose 5-phosphate since the 3 position no longer has a hydroxyl group that can be lost as water, nevertheless the enzymatic conversion was attempted.

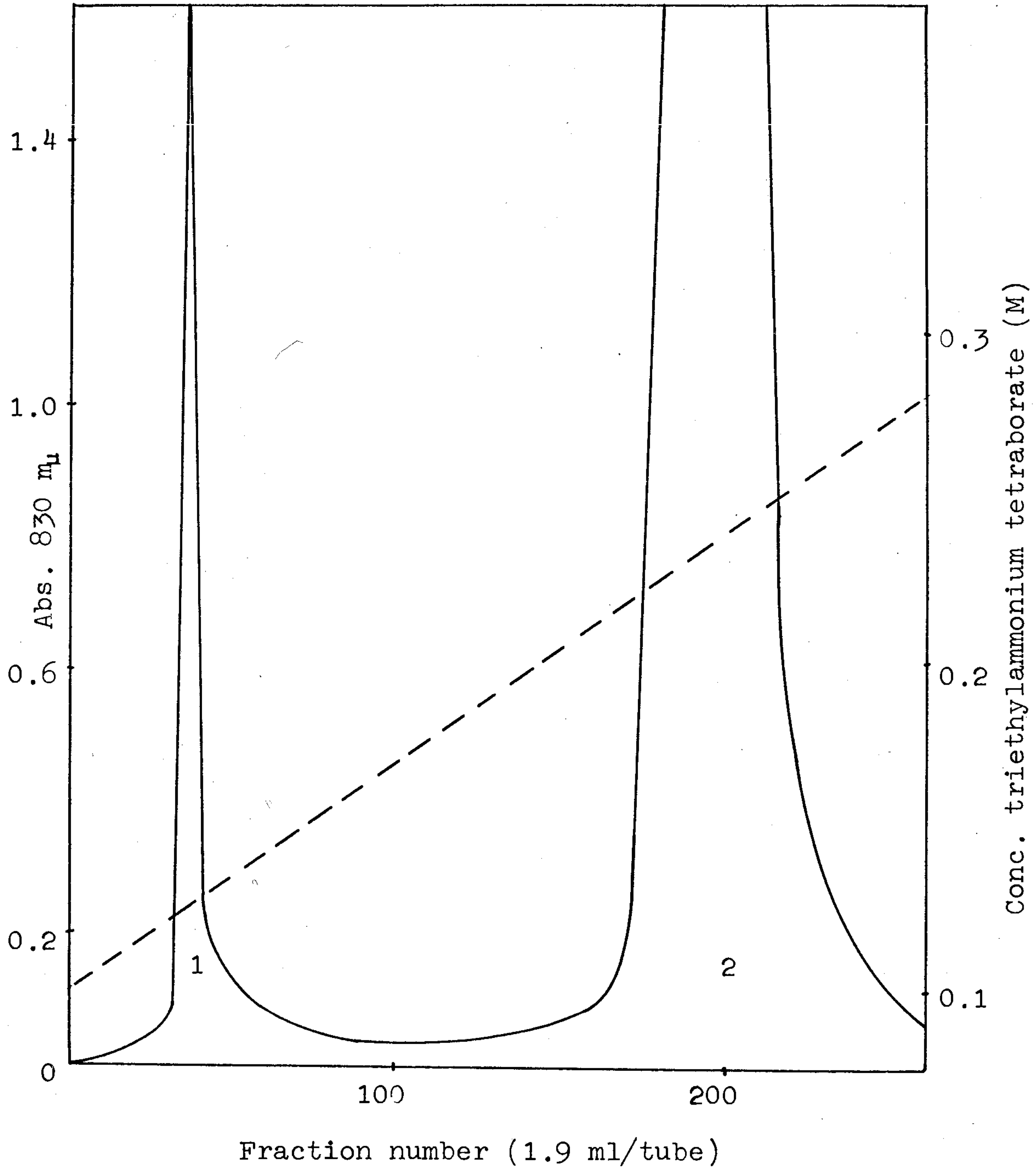
Separation of the sugar phosphates produced can be

effected on an anion-exchange resin column as described by Lefebre et al[45]. This procedure is based on the use of linear gradient elution of the sugar phosphates with triethylammonium tetraborate followed by freeze-drying and removal of the salts by methanol distillation.

After ribosephosphate isomerase treatment of 3-deoxy-D-ribose 5-phosphate and dialysis, the resulting residue adjusted to pH 8.0 was applied to the anion-exchange column. Elution of the sugar phosphates with a linear gradient from 0.1M to 0.4M triethylammonium tetraborate and phosphate determination by Bartlett's procedure[59] gave the result shown in Figure 8. The compound contained in peak 1 was shown to be inorganic phosphate by paper chromatography. Optical rotation and paper chromatography confirmed the sugar phosphate present in peak 2 was the starting material, 3-deoxy-D-ribose 5-phosphate (21) and hence enzymatic isomerization was not a viable route.

Base catalyzed isomerization of 3-deoxy-D-ribose 5-phosphate is possible. Glucose 6-phosphate in aqueous alkaline solutions has been shown to form fructose 6-phosphate and 6-phosphoglucometasaccharinic acid through the 1,2-enediol intermediate[60]. Furthermore Khym et al.[61] has illustrated that the order of stability of ribose phosphates in alkali is ribose 2-phosphate, ribose 3-phosphate and ribose 5-phosphate. This is consistent with the increasing number

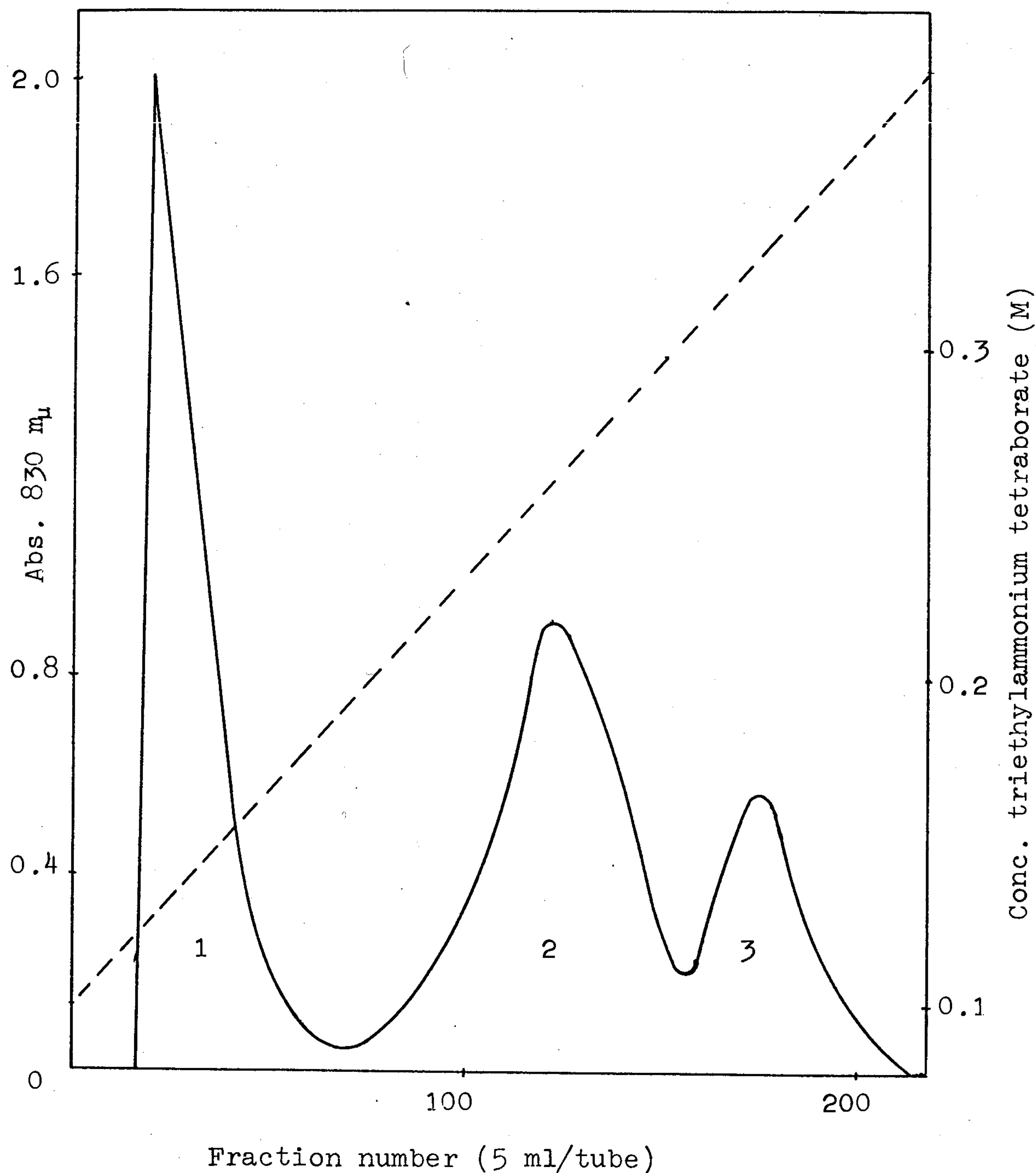
Figure 8. Ion exchange chromatography of the isomerase product with the broken line representing the gradient from 0.1M to 0.4M triethylammonium tetraborate (800 ml total volume).



of enediols that can exist. However, 3-deoxy-D-ribose 5-phosphate can only form the 1,2-enediol and upon base treatment a mixture of 3-deoxy-D-glyceropentulose 5-phosphate and 3-deoxy-D-threopentose 5-phosphate would be expected.

Base treatment of 3-deoxy-D-ribose 5-phosphate followed by anion-exchange chromatography of the resulting sugar phosphates with triethylammonium tetraborate and Bartlett's phosphate analysis[59] gave the results shown in Figure 9. The compound present in peak 1 had an identical R_{PO_4} as inorganic phosphate on paper chromatography. Optical rotation and paper chromatography showed peak 2 contained 3-deoxy-D-ribose 5-phosphate (21). Unfortunately, it could not be confirmed that the compound in peak 3 was 3-deoxy-D-ribulose 5-phosphate since spectroscopic methods showed it had less free carbonyl present than the parent compound, 3-deoxy-D-ribose 5-phosphate. Furthermore phosphoribulokinase treatment of the peak 3 component gave no indication of diphosphate formation. Therefore, it is most likely that 3-deoxy-D-threopentose 5-phosphate was isolated instead of 3-deoxy-D-glyceropentulose 5-phosphate.

Figure 9. Ion exchange chromatography of the base treated product with the broken line representing the gradient from 0.1M to 0.4M triethylammonium tetraborate (1090 ml total volume).



CONCLUSIONS

The only previously unknown deoxy fructose, 4-deoxy-D-fructose, has been successfully prepared. Kinetic studies using this compound as a sustrate for yeast hexokinase have confirmed that the 4-OH group is a binding group in the enzyme-substrate complex. Enzymatic synthesis of unknown 4-deoxy-D-fructose 6-phosphate and 4-deoxy-D-fructose 1,6-diphosphate has been achieved in low yield from 4-deoxy-D-fructose.

A new improved method of synthesis of 3-deoxy-1,2;5,6-di-O-isopropylidene-D-glucofuranose through the 3 chloro derivative has been accomplished by application of the triphenylphosphine-carbon tetrachloride chlorination procedure. 3-Deoxy-D-ribose 5-phosphate, produced from the above deoxy derivative, was not successfully converted to 3-deoxy-D-ribulose 5-phosphate by ribosephosphate isomerase. Base treatment of 3-deoxy-D-ribose 5-phosphate most likely affords 3-deoxy-D-threopentose 5-phosphate rather than 3-deoxy-D-ribulose 5-phosphate, however the identity could not be confirmed. The inability of the enzyme ribosephosphate isomerase to convert the deoxy α-hydroxyaldehyde to the deoxy α-hydroxyketone illustrates the extreme specificity of this enzyme and agrees with the primary product of the normal reaction being the β-diketone phosphate.

EXPERIMENTAL

General Methods

Melting points were determined on Fischer-Johns apparatus and are uncorrected. Evaporations were carried out on Buchi rotary evaporators at bath temperatures not exceeding 45°. Freeze-drying was done at -40°/0.3 torr for 24 h. Bovine serum albumin was purchased from Nutritional Biochemicals, adenosine triphosphate, yeast hexokinase (Type 5), potato acid phosphatase (Type 2), rabbit muscle phosphofructokinase (Type 1), spinach ribosephosphate isomerase (Type 1) and spinach phosphoribulokinase (Type 2) from Sigma Chemical Company.

Thin-layer chromatography (t.l.c.) was effected on Silica gel G with solvents: a, 2:1 ethyl ether-toluene, b, 14:3:3 butanone-methanol-water, c, 9:1 butanone-water. Paper chromatography was performed on Whatman No. 3MM paper with the following solvent systems: (ascending) A[43], 8:4:5:5:3 1-butanol-1-propanol-acetone-80% (w/v) formic acid-30% (w/v) trichloroacetic acid [tetrasodium EDTA (0.06 g) added to solvent (125 ml) and solvent run twice in the same direction], (ascending) B[43], 16:3:1 methanol-90% formic acid-water [tetrasodium EDTA (0.05 g) added to solvent (100 ml) and solvent run once], (descending) C[62], 6:3:1 1-propanol-conc. ammonia-water, (descending) D, 10:4:3 ethyl acetate-pyridine-water, and (descending) E, 5:4:3 ethyl acetate-pyridine-water. Sugars were detected on t.l.c. by using 10% sulfuric acid and heat for development. Reducing sugars

and simple polyhydric alcohols were detected on paper chromatograms with the silver nitrate-sodium hydroxide reagent[63]. Phosphorylated compounds were chromatographed on paper as their sodium salts and reacted with the ammonium molybdate reagent[44] upon irradiation with U.V. light to give bright blue spots. The sugar phosphate R_f values were measured relative to inorganic phosphate (R_{PO_4}) which gave yellow spots with the ammonium molybdate reagent. The phosphate esters were isolated as their barium salts and converted to the more soluble potassium of sodium salts by passing through Dowex-50 (H^+) ion exchange resin in the potassium or sodium form respectively.

Optical rotations were measured on a Perkin-Elmer Model P22 spectropolarimeter at 24°. N.m.r. spectra were obtained with a Varian A 56-60 spectrometer with tetramethylsilane as an internal reference and deuterium oxide as the solvent. Infrared spectra were recorded on a Unicam SP 200 infrared spectrophotometer. Samples were dissolved in deuterium oxide, placed in a 0.1 mm calcium fluoride cell and run against deuterium oxide in the region 1600-1900 cm^{-1}. Mass spectroscopy of compound 8 was conducted on the pertrimethylsilyl (TMS) derivative prepared by a modified method of Sweeley et al.[64]. 4-Deoxy-D-threohexulose (6 mg) was dissolved in pyridine (1 ml), hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml) were then added. The mixture was shaken vigorously and kept for 1 h at 40°.

The solid material was filtered off and the filtrate evaporated to a colorless syrup. The mass spectrum of the freshly prepared derivative was then recorded with a Hitachi Perkin-Elmer RMU-7 mass spectrometer at an ionizing potential of 80 ev and an inlet temperature of 50°.

1,2;5,6-Di-O-isopropylidene-D-mannitol (1) -Compound 1 was prepared from D-mannitol by the method of Tipson[11]. M.p. 119-120° (lit.[11] 120-121°).

1,2;5,6-Di-O-isopropylidene-3-O-p-toluenesulfonyl-D-mannitol (2) -Compound 2 was prepared from 1,2;5,6-di-O-isopropylidene-D-mannitol essentially by the method of Bladen and Owen[12]. The same amount of reagents were used and identical reaction conditions were maintained, however the reaction was found not to be complete after the recommended 20 h reaction time. T.l.c. (solvent a) showed that 2 days were needed for a maximum yield to be obtained. The crude 1,2;5,6-di-O-isopropylidene-3-O-p-toluenesulfonyl-D-mannitol produced, containing small amounts of the starting material and 1,2;5,6-di-O-isopropylidene-3,4-di-O-p-toluenesulfonyl-D-mannitol was then used for preparation of the 3,4-epoxide.

3,4-Anhydro-1,2;5,6-di-O-isopropylidene-D-talitol (3) -The procedure outlined by Bladen and Owen[12] was also used to produce crystalline 3,4-anhydro-1,2;5,6-di-O-isopropylidene-D-talitol, m.p. 53-55°, $[\alpha]_D$ -15.6° (*c* 1.0, chloroform) [lit.[12], m.p.54-56°, $[\alpha]_D^{22}$ -16.2° (*c* 1.5, chloroform)]

4-Deoxy-D-threohexulose (8) -Compound 3 (15 g) was dissolved in ether (200 ml) and lithium aluminum hydride (3 g) was added carefully. The reaction was complete after 1 h at room temperature. 10% aqueous ammonium chloride (50 ml) was added to destroy the excess hydride, followed by acetone (100 ml) and ethyl acetate (300 ml). The suspension was

filtered through a Celite pad, the filtrate extracted and the residue washed thoroughly with ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness. The product was dissolved in chloroform, washed with water, dried over calcium chloride, filtered and evaporated to give a chromatographically pure syrup (4,5) (15 g).

Hydrolysis of the epimeric diisopropylidene deoxy hexitol mixture (4,5) (11.5 g) was effected in 0.05N hydrochloric acid (100 ml) for 30 min. at 100°. The solution was deionized with Dowex-3 (OH^-) and then evaporated to dryness. The syrupy mixture of the two epimeric deoxy hexitols (6,7) (7.9 g) obtained was not separated on paper chromatography and had, $[\alpha]_D$ -18.2° (c 0.77, water).

Acetobacter suboxydans (American Type Culture Collection 621) was used to produce compound 8. Asceptic conditions were employed in all subsequent procedures involving the bacteria. A stock culture was maintained on agar slants by the procedure outlined by Lockwood[26]. A broth containing a mixture of the two deoxy hexitols (6,7) (7.5 g), yeast extract powder (0.32 g) and water (63 ml) was made in a 500 ml erlenmeyer. The solution was sterilized in an autoclave for 20 minutes at 15 lb. steam pressure. After cooling to room temperature, the solution was inoculated with a 48 hours' culture of Acetobacter suboxydans grown on sorbitol. At intervals, a loopful of the reaction mixture on a sterile wire loop was taken and the reaction progress

was followed on t.l.c. (solvent b). Agitation was avoided in the seven day incubation period at 30°. Bacteria were removed by shaking with activated carbon (0.75 g) followed by filtration through Celite. Evaporation to dryness yields a syrupy mixture of the deoxy hexitol (7) and 4-deoxy-D-threohexulose (8).

Separation of the mixture was effected on bisulphite resin. The material (3 g) was dissolved in water (10 ml) and applied to the top of a jacketed column (5 cm x 30 cm) of Dowex-1 (HSO_3^-; 200-400 mesh) maintained at 25°. Water was used as eluant at a flow rate of 0.5 ml/min, and fractions (10 ml) were collected. Fractions exhibiting positive spot-tests on paper (Ag^+/EtO^- reagent) were chromatographed (solvent E, 10 h). Two compounds were eluted in fractions 20-27 and 29-42. These were pooled, passed through Dowex-3 (OH^-) and evaporated to dryness. Two syrupy products were obtained, fraction 20-27 the deoxy hexitol (7) (0.61g) and fraction 29-42, chromatographically pure (solvent E, 10 h) 4-deoxy-D-threohexulose (8) (1.88 g) $[\alpha]_D$ -3.9° (c 1.12, water). Isopropylidene derivatives of compound 8 were prepared by adding 4-deoxy-D-threohexulose (8) (0.8 g) to acetone (100 ml) and conc. sulfuric acid (1 ml). After 2 h at room temperature the reaction was neutralized with 50% sodium hydroxide, filtered and evaporated to dryness. The residue was dissolved in chloroform and the resulting solution washed with aqueous

sodium bicarbonate. The sodium bicarbonate solution was extracted with chloroform and the combined chloroform extracts washed with a little water. The organic phase was then dried over calcium chloride, filtered and evaporated to give a syrup. T.l.c. (solvent a) indicated three isopropylidene derivatives of 4-deoxy-D-*threo*hexulose were present (R_f 0.21, 0.09 and 0.06).

N.m.r. spectroscopy of compound 8 in deuterium oxide showed no low field anomeric proton. The mass spectrum of the pertrimethylsilyl derivative of compound 8 showed the molecular ion present at mass 452. A signal of high intensity was visible at m/e 349 amounting to 25% of the base peak at m/e 73. This chemical and spectroscopic evidence substantiates the hexulose structure of this preparation.

Assay System for Yeast Hexokinase -The reaction system (6.0 ml) adjusted to pH 8.5 with sodium hydroxide contained final concentrations of 4.3×10^{-3}M ATP, 1.6×10^{-2}M $MgCl_2$ and various concentrations of the hexoses. The actual volume before addition of the yeast hexokinase enzyme was 5.9 ml. Yeast hexokinase (6.2 mg) and 0.2% bovine serum albumin (2 ml) adjusted to pH 8.5 (0.1 ml) was added to the reaction mixture. A pH-stat containing 0.002M sodium hydroxide was used to maintain constant pH 8.5. For determining initial velocities the blank titration value was subtracted from the actual values obtained.

4-Deoxy-D-*threo*hexulose 6-phosphate (9) -Compound 8

(0.85 g), adenosine triphosphate (0.46 g) and magnesium chloride (0.57 g) were dissolved in water (180 ml) and the solution was adjusted to pH 8.0 with 0.1N sodium hydroxide. The reaction was initiated by the addition of hexokinase (50 mg) dissolved in 0.2% bovine serum albumin pH 8.0 (8 ml). A pH-stat containing 0.02M sodium hydroxide was used to maintain constant pH 8.0. The reaction was stopped when base consumption equals the blank titration value obtained in the absence of enzyme. The aqueous solution was placed in presoaked dialysis tubing and the tubing immersed in a column containing water. Water was then passed through the column at a flow rate of 0.5 ml/min. The eluant (1 liter) was concentrated and acid washed charcoal (30 g) was added. The resulting suspension was filtered through Celite and the residue washed thoroughly with water. The filtrate and washings were adjusted to pH 6.5. 1M barium acetate (10 ml) was added to ensure an excess of barium ions. The barium salt of 4-deoxy-D-threohexulose 6-phosphate (9) was precipitated at 0° overnight with 5 volumes of ethanol, collected by centrifugation and washed with 80% ethanol. The pH was adjusted to 7.0 and the solution freeze dried. Paper chromatography of the freeze-dried residue (0.185 g) (solvent A, R_{PO_4} 0.56 and solvent B) showed small amounts of another phosphorylated compound (solvent A, R_{PO_4} 0.42). N.m.r. spectroscopy of the sodium salt of 4-deoxy-D-fructose 6-phosphate (9) in deuterium oxide showed a low field

anomeric proton present in small amounts, characteristic of an anomeric C-1 hydrogen. Hydrolysis with acid phosphatase was carried out on the sodium salt of 4-deoxy-D-threohexulose 6-phosphate (9) essentially by the method of Wood[43]. The sugar phosphate (9) (2 mg) was added to 0.05M acetate buffer pH 4.9 containing 5mM Mg (1 ml). To initiate the reaction, acid phosphatase (0.2 mg) was introduced and the mixture incubated for 8h at 37°. Deproteinization was accomplished with an equal volume of 10% trichloroacetic acid, followed by filtration and neutralization to pH 7.0. The solution was deionized with Dowex-50 (H^+), followed by Dowex-3 (OH^-). Paper chromatography (solvent D, 12 h) showed the free sugar had an identical mobility to 4-deoxy-D-threohexulose (8).

4-Deoxy-D-threohexulose 1,6-diphosphate (10) -The potassium salt of compound 9 (0.12 g), adenosine triphosphate (0.47 g), magnesium chloride (0.15 g) and potassium chloride (0.91 g) were dissolved in water (150 ml) and the pH adjusted to 8.5 with 0.1N sodium hydroxide. Phosphofructokinase (50 µl) was diluted with 0.05M cysteine hydrochloride pH 8.5 (1 ml) and left for 5 minutes at room temperature to ensure maximal activity. Addition of the phosphofructokinase solution initiated the reaction and a constant pH of 8.5 was maintained by a pH-stat containing 0.002M sodium hydroxide. Completion of the reaction and workup were as described for compound 9. The barium

salt of 4-deoxy-D-*threo*hexulose 1,6-diphosphate (10) was precipitated by cooling the reaction mixture on ice, collected by centrifugation and washed with 30% ethanol. The residue was dissolved in acetic acid pH 5 and the precipitation procedure repeated again. The product (10) (37 mg) was obtained chromatographically pure (solvent A, R_{PO_4} 0.44 and solvent C). Hydrolysis with acid phosphatase was carried out on the sodium salt of 4-deoxy-D-*threo*hexulose 1,6-diphosphate (10) by the method outlined for compound 9. Paper chromatography (solvent D, 12 h) showed the free sugar was 4-deoxy-D-*threo*hexulose (8).

1,2;5,6-Di-O-isopropylidene-α-D-glucofuranose (11) -Compound 11 was prepared from D-glucose by the method outlined by Schmidt[47], m.p. 110-111°, $[\alpha]_D$ -17.5° (*c* 0.4, water) [lit.[47] m.p. 110-111°, $[\alpha]_D$ -18.5° (*c* 5.0, water).

1,2;5,6-Di-O-isopropylidene-α-D-ribohexofuranos-3-ulose (12)-1,2;5,6-Di-O-isopropylidene-α-D-glucofuranose (11) was oxidized by the IO_4^-/RuO_2 method as described by Slessor and Tracey[56] m.p. 109-110°, $[\alpha]_D$ 37.5° (*c* 0.4, water), [lit.[57] m.p. 108-110°, $[\alpha]_D$ 40.2° (*c* 0.5, water)].

1,2;5,6-Di-O-isopropylidene-α-D-allofuranose (13) -Compound 13 was prepared by the sodium borohydride reduction of 1,2;5,6-di-O-isopropylidene-α-D-ribohexofuranos-3-ulose as outlined by Theander[57], m.p. 74-75°, $[\alpha]_D$ 34° (*c* 0.4, water), [lit.[57] m.p. 76-77°, $[\alpha]_D$ 37.7° (*c* 0.5, water)].

3-Chloro-3-deoxy-1,2;5,6-di-O-isopropylidene-α-D-glucose (14) -1,2;5,6-Di-O-isopropylidene-α-D-allose (13) (5 g) and triphenylphosphine (10 g) were dissolved in carbon tetrachloride (400 ml) and the solution was refluxed for 96 h during which time white crystals precipitated. The solution was cooled and diluted with petroleum ether (30-60°) (1 liter). The resulting mixture was kept at -20° overnight, filtered and evaporated to give a syrup, which was dissolved in petroleum ether (30-60°) (200 ml). This solution was kept for 3 h at 5°, filtered, and evaporated to give a syrup (4.56 g) after vacuum distillation; $b_{0.004}$ 76°,$[\alpha]_D$ -16.5° (c 0.4, chloroform) (lit.[38], $b_{0.05}$ 84-88°). Hydrolysis[38] gave 3-chloro-3-deoxy-D-glucose, m.p. 147-149, $[\alpha]_D^{22}$ 66.5→60.9 (c 0.6,water,24 h) [lit.[38], m.p. 150-152°, $[\alpha]_D^{22}$ 66° (c 0.18, water).

Anal. Calc. for $C_{12}H_{19}ClO_5$: C, 51.71; H, 6.82; Cl,12.75. Found: C, 51.86; H, 6.87; Cl, 12.94.

3-Deoxy-1,2;5,6-di-O-isopropylidene-α-D-ribohexose (15) -Compound 14 (10 g) and lithium aluminum hydride (5 g) were dissolved in dry tetrahydrofuran (40 ml) and the mixture was refluxed for 24 h. The excess hydride was destroyed with 10% aqueous ammonium chloride, the suspension filtered through a Celite pad, and the residue thoroughly washed with acetone. The combined filtrate and washings were evaporated to a residual liquid and distillation ($b_{0.005}$ 70-72°) gave a syrup (5.71 g) that crystallized at low temperature;

m.p. 10-12°, $[\alpha]_D$ -2.75° (c 0.4, chloroform) [lit.[13], $b_{0.3}$ 74-78°, $[\alpha]_D$ -5.78 (c 4.2, ethanol)].

3-Deoxy-1,2-O-isopropylidene-D-ribohexofuranose (16) -Compound 16 was prepared by mild acid hydrolysis of 15 as described by Hedgley, Overend and Rennie[13], except reaction progress followed by t.l.c. (solvent c). M.p. 82°, $[\alpha]_D$ -15.0° (c 1.3, water) (lit.[13], m.p. 84°).

3-Deoxy-1,2-O-isopropylidene-D-erythropentose (18) -Compound 18 was prepared according to the method of Szabó and Szabó[7] with the following change. Reduction of the dialdehyde residue (17) obtained from sodium metaperiodate treatment of 16 was effected with sodium borohydride. The residue (14.5 g) was dissolved in water (145 ml) and sodium borohydride (2.9 g) was added. After standing for 15 min at room temperature the aqueous solution was then neutralized with solid carbon dioxide. Extraction with chloroform (4 x 100 ml) gave a solution which was dried with calcium chloride, filtered and evaporated to a crystalline residue. Recrystallization from hexane gave 3-deoxy-1,2-O-isopropylidene-D-erythropentose (10.3 g), m.p. 75.5-77°, $[\alpha]_D$ -11.0° (c 0.106, chloroform) [lit.[7], m.p. 74-76°, $[\alpha]_D$ -3.0° (c 0.106, chloroform)].

3-Deoxy-D-erythropentose 5-phosphate (21) -Compound 21 was prepared from 3-deoxy-1,2-O-isopropylidene-D-erythropentose (18) by the procedure of Szabó and Szabó[7]. Compound 18 was treated with diphenyl phosphorochloridate to

give crystalline 3-deoxy-1,2-O-isopropylidene-D-*erythro*-pentose 5-diphenyl phosphate (19), m.p. 74.0-74.5°, $[\alpha]_D$ -10.0° (*c* 0.14, ethanol) [lit.[7], m.p. 74° $[\alpha]_D^{25}$ -13.6° (*c* 0.14, ethanol)]. The diphenyl phosphate (19) was hydrogenated to yield 3-deoxy-1,2-O-isopropylidene-D-*erythro*pentose 5-phosphate (20) isolated as its crystalline barium salt $[\alpha]_D$ -28.0° (*c* 0.05, water) [lit.[7], $[\alpha]_D^{25}$ -24.8° (*c* 0.05, water)]. 3-Deoxy-D-*erythro*pentose 5-phosphate (21) was obtained by mild acid hydrolysis of 10 and isolated as the barium salt, $[\alpha]_D$ -4.9° (*c* 0.122, water) [lit.[7], $[\alpha]_D^{25}$ -10.65 (*c* 0.122, water)].

Attempted Preparation of 3-Deoxy-D-glyceropentulose 5-phosphate (22) -Method A- The sodium salt of 3-deoxy-D-*erythro*pentose 5-phosphate (21) (0.15 g) was dissolved in TRIS buffer pH 7.0 (100 ml). Ribosephosphate isomerase (6 mg) was added and the reaction mixture incubated at 37° for 5 days. It was then introduced into presoaked dialysis tubing and dialyzed against water in a column. Water was passed through the column at a flow rate of 0.5 ml/min. The eluant (500 ml) was concentrated to a small volume, adjusted to pH 8.0 with ammonium hydroxide and applied to a column (1.5 cm x 40 cm) of Dowex-1 (borate form) resin. After washing to neutrality with water, the phosphate esters were eluted with a linear gradient from 0.1M to 0.4M triethylammonium tetraborate (800 ml total volume) at a flow rate of 0.25 ml/min. Fractions (1.9 ml) were

collected and samples (0.5 ml) of every fourth fraction were analyzed for phosphate by Bartlett's[59] method. The fractions contained in the two peaks (Figure 8) were pooled, freezedried and evaporated to dryness five times with methanol. Paper chromatography (solvent A, R_{PO_4} 1.00 and solvent B, R_{PO_4} 1.00) showed peak 1 (Figure 8) was inorganic phosphate. The residue from peak 2 was dissolved in a minimum volume of water, adjusted to pH 6.5 with 1N potassium hydroxide and 25% (W/V) barium acetate (0.3 ml) was added. After 30 minutes at 0° the precipitate was centrifuged down, 5 volumes of ethanol were added to the aqueous solution followed by 25% (W/V) barium acetate (0.2 ml). The solution was left at 0° overnight, the precipitate collected by centrifugation, washed with 80% ethanol and centrifuged again. This procedure was repeated once more and the final precipitate was adjusted to pH 6.5-7.0 and then freeze dried. Optical rotation $[\alpha]_D$ -6.2 (c 0.42, water) and paper chromatography showed peak 2 (Figure 8) was the starting material, compound 21.

Method B- Compound 21 (200 mg) was dissolved in 0.05M sodium bicarbonate -0.021M sodium hydroxide (240 ml) and refluxed for 50 minutes on a steam bath. To stop the reaction it was cooled to 0° and then applied to a column (1.5 cm x 40 cm) of Dowex-1 (borate form) resin. After washing to neutrality with water, the phosphate esters were eluted with a linear gradient from 0.1M to 0.4M tri-

ethylammonium tetraborate (1090 ml total volume) at a flow rate of 0.25 ml/min. Fractions (5 ml) were collected and samples of every second fraction were analyzed for phosphate by Bartlett's[59] method. The fractions contained in the three peaks (Figure 9) were pooled and worked up in a similar manner to that used in Method A. Paper chromatography (solvent A, R_{PO_4} 1.00 and solvent B, R_{PO_4} 1.00) showed peak 1 (Figure 9) was inorganic phosphate. Optical rotation $[\alpha]_D$ -5.6° (c 0.354, water) and paper chromatography (solvent A, R_{PO_4} 0.66 and solvent B) showed peak 2 (Figure 9) was 3-deoxy-D-*erythro*pentose 5-phosphate. Infrared spectroscopy in the 1600-1900 cm^{-1} region for the above compound (21) (6 mg/0.15 ml D_2O) gave an ϵmax of 22 at 1730 cm^{-1}. Paper chromatography (solvent A, R_{PO_4} 0.76 and solvent B) showed peak 3 (10 mg) (Figure 9) was pure and had an optical rotation $[\alpha]_D$ 7.1° (c 0.35, water). Infrared spectroscopy of the compound contained in peak 3 (Figure 9) (6.2 mg/0.15 ml D_2O) gave an ϵmax of 14.3 at 1720 cm^{-1}.

Attempted Preparation of 3-Deoxy-D-*glycero*pentulose 1,5-diphosphate (23) -A solution (0.5 ml) was made containing 0.01M $MgCl_2$, 0.011M ATP, 0.001M glutathione and the sodium salt of the compound from peak 3 Method B (2 mg). The pH was adjusted to 7.9 with 1N NaOH and phosphoribulokinase (0.1 ml of a solution 0.9 mg enzyme/2 ml water adjuated to pH 7.9) was added. The rate of pH decrease was

identical to the blank containing no substrate. A rapid pH decrease was evident with the normal substrate, D-glyceropentulose 5-phosphate.

REFERENCES

1. M. Dixon and E.C. Webb, "Enzymes", 2nd ed., Academic Press, New York, 1964, p. 215.
2. N.R. Lazarus, A.H. Ramel, Y.M. Rustum, and E.A. Barnard, Biochem., 5, 4003 (1966).
3. A. Ginsburg, J. Biol. Chem., 234, 481 (1959).
4. P.C. Huang and O. Neal Miller, J. Biol. Chem., 231, 201 (1958).
5. G. Avigad and S. Englard, J. Biol. Chem., 243, 1511 (1968).
6. H.A. Lardy, The Enzymes, 6, 67 (1962).
7. P. Szabó and L. Szabó, J. Chem. Soc., 2944 (1965).
8. J.B. Lee and T.J. Nolan, Can. J. Chem., 44, 1331 (1966).
9. S. Hanessian, Advan. Carbohyd. Chem., 21, 143 (1966).
10. W.G. Overend, Advances in Chemistry Series, No. 74, American Chemical Society, Washington, D.C., 1968, p.141.
11. R.S. Tipson, Carbohyd. Res., 7, 232 (1968).
12. P. Bladen, and L.N. Owen, J. Chem. Soc., 604 (1950).
13. E.J. Hedgley, W.G. Overend, and R.A.C. Rennie, J. Chem. Soc., 4701 (1963).
14. M. Černý and J. Pacák, Chem. Listy, 49, 1848 (1955), Chem. Abstr., 50, 9298a (1956).
15. S. Hanessian, Advances in Chemistry Series, No. 74, American Chemical Society, Washington, D.C., 1968, p.159.
16. M. Černý, J. Pacák and J. Staněk, Chem. and Indust., 945 (1961).

17. P.A.J. Gorin, L. Hough and J.K.N. Jones, J. Chem. Soc., 4700 (1954).

18. D.H. Ball and F.W. Parrish, Advan. Carbohyd. Chem., 24, 139 (1969).

19. C. Fouquey, J. Polonsky and E. Lederer, Bull. Soc. Chim. Fr., 803 (1959).

20. G. Bertrand, Ann. Chim., 3, 209, 287 (1904).

21. R.M. Hann, E.B. Tilden and C.S. Hudson, J. Amer. Chem. Soc., 60, 1201 (1938).

22. N.K. Richtmyer, L.C. Stewart and C.S. Hudson, J. Amer. Chem. Soc., 72, 4934 (1950).

23. P.P. Regna, J. Amer. Chem. Soc., 69, 246 (1947).

24. L. Hough, J.K.N. Jones and D.L. Mitchell, Can. J. Chem., 37, 725 (1959).

25. H. Kaufmann and T. Reichstein, Helv. Chim. Acta, 50, 2280 (1967).

26. L.B. Lockwood, "Methods in Carbohydrate Chemistry", Vol. 1, R.L. Whistler and M.L. Wolfrom, Ed., Academic Press, New York, 1963, p. 151.

27. B. Lindberg and K.N. Slessor, Carbohyd. Res., 5, 286 (1967).

28. H.-CH. Curtius, M. Muller and J.A. Vollmin, J. Chromatog., 37, 216 (1968).

29. G.R. Waller, S.D. Sastry and K. Kinneberg, J. Chromatog., Sci., 7, 577 (1969).

30. A. Sols, G. de la Fuente, C. Villar-Palasi and

C. Asensio, Biochim. Biophys. Acta, 30, 92 (1958).

31. J.R. Burt, Biochem. J., 77, 16p (1960).
32. R.A. Darrow and S.P. Colowick, Methods in Enzymology, 5, 226 (1962).
33. T.A. Mahowald, E.A. Noltmann and S.A. Kuby, J. Biol. Chem., 237, 1535 (1962).
34. J.E. Dyson and E.A. Noltmann, Anal. Biochem., 11, 362 (1965).
35. G.G. Hammes and D. Kochavi, J. Amer. Chem. Soc., 84, 2069 (1962).
36. R.A. Alberty, R.M. Smith and R.M. Bock, J. Biol. Chem., 193, 425 (1951).
37. V. Zewe, H.J. Fromm and R. Fabiano, J. Biol. Chem., 239, 1625 (1964).
38. J.E.G. Barnett, A. Ralph and K.A. Munday, Biochem. J., 114, 569 (1969).
39. F. Trigalo, P. Szabó and L. Szabó, J. Chem. Soc. C, 901 (1968).
40. L. Szabó, Advances in Chemistry Series, No. 74, American Chemical Society, Washington, D.C., 1968, p. 70.
41. T.H. Chiu and D.S. Feingold, Biochim. Biophys. Acta, 92, 489 (1964).
42. C.A. Swenson and R. Barker, Biochemistry, 10, 3151 (1971).
43. T. Wood, J. Chromatog., 35, 352 (1968).
44. T. Wood, J. Chromatog., 6, 142 (1961).
45. M.J. Lefebvre, N.S. Gonzalez and H.G. Pontis,

J. Chromatog., 15, 495 (1964).

46. I. Mandl and C. Neuberg, Methods in Enzymology, 3, 162 (1957).

47. O.T. Schmidt, "Methods in Carbohydrate Chemistry", Vol. 2, R.L. Whistler and M.L. Wolfrom, Ed., Academic Press, New York, 1963, p. 320.

48. K. Freudenberg and A. Wolf, Ber., 60, 232 (1927).

49. D.M. Brown and G.H. Jones, J. Chem. Soc. C, 252 (1967).

50. J. Defaye and J. Hildesheim, Carbohyd. Res., 4, 145 (1967).

51. D.M. Brown and G.H. Jones, Chem. Commun., 561 (1965).

52. N.K. Kochetkov and A.I. Usov, Tetrahedron, 19, 973 (1963).

53. D.C.C. Smith, J. Chem. Soc., 1244 (1956).

54. S. Hanessian and N.R. Plessas, Chem. Commun., 1152 (1967); J. Org. Chem., 34, 2163 (1969).

55. C.R. Haylock, L.D. Melton, K.N. Slessor and A.S. Tracey, Carbohyd. Res., 16, 375 (1971).

56. K.N. Slessor and A.S. Tracey, Can. J. Chem., 47, 3989 (1969).

57. O. Theander, Acta Chem. Scand., 18, 2209 (1964).

58. F.C. Knowles and N.G. Pon, J. Amer. Chem. Soc., 90, 6536 (1968).

59. G.R. Bartlett, J. Biol. Chem., 234, 466 (1959).

60. C. Degani and M. Halmann, J. Amer. Chem. Soc., 90, 1313 (1968).

61. J.X. Khym, D.G. Doherty and W.E. Cohn, _J. Amer. Chem. Soc._, _76_, 5523 (1954).

62. C.S. Hanes and F.A. Isherwood, _Nature_, _164_, 1107 (1949).

63. L. Hough and J.K.N. Jones, "Methods in Carbohydrate Chemistry", Vol. 1, R.L. Whistler and M.L. Wolfrom, Ed., Academic Press, New York, 1962, p. 21.

64. C.C. Sweeley, R. Bently, M. Makita and W.W. Wells, _J. Amer. Chem. Soc._, _85_, 2497 (1963).