THE SYNTHESIS OF GUANOSINE DIPHOSPHATE FUCOSE IN HUMAN ERYTHROCYTES

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THE SYNTHESIS OF GUANOSINE DIPHOSPHATE

FUCOSE IN HUMAN ERYTHROCYTES

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CHAPTER I

INTRODUCTION

The blood group substances are antigenic macromolecules found in various body tissues and fluids. The BGS[1] isolated from the erythrocyte stroma appear to be glycolipids while those isolated from certain body fluids and secretions are glycoproteins (1, 2). The most common BGS in man are the ABO(H) substances which are responsible for ABO(H) blood group serological activity. The carbohydrate moiety of the BGS is responsible for the antigenic properties of the BGS. In the ABO(H) substances the carbohydrate moiety is a polysaccharide composed of D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and L-fucose. In the *in vitro* conversion of A to H, B to H, and H to Le^a substance a single type of sugar is lost for each conversion. This suggests that the reverse of these steps might be responsible for the biosynthesis of the BGS. By analogy with polysaccharide biosynthesis one would expect L-fucose to be incorporated into the BGS polysaccharide via a nucleotide-fucose precursor.

Relatively little has been done in regard to the biosynthesis of BGS. One might expect the erythrocyte or its precursors to contain

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[1]The following abbreviations are used: BGS, blood group substances; PEI, poly(ethyleneimine). All other abbreviations are in accordance with IUPAC-IUB Combined Commission on Biochemical Nomenclature, J. Biol. Chem., 241, 527 (1966).

the enzymes and precursors necessary for BGS biosynthesis in these cells. It is known that human erythrocytes contain UDPG and UDPAG which could be potential precursors to BGS polysaccharides (3). Although GDP-fucose has not been reported in human erythrocytes, GDP-mannose has been isolated from this source (4). GDP-α-D-mannose has been shown to be converted to GDP-β-L-fucose in bacterial (5, 6) and mammalian systems (7). It seems likely that GDP-fucose would be a precursor to the fucose containing BGS polysaccharide. UDPAGal has not been detected in human erythrocytes (3).

The purpose of this research was to attempt to demonstrate the conversion of GDP-mannose to GDP-fucose in human erythrocytes. Initial experiments to detect the conversion by chromatographic methods showed the desirability of having GDP-fucose as a known. Although this was prepared from GDP-mannose using A. aerogenes extracts, the GDP-fucose obtained could not be separated from GDP-mannose by paper chromatography as reported by Ginsburg (5). Thus, it was desirable to chemically synthesize GDP-β-L-fucose and develop a better chromatography system for the separation of GDP-L-fucose from GDP-mannose. The chemical synthesis requires β-L-fucose-1-phosphate as a starting material. The synthesis of this compound had not been previously reported in the literature. Therefore, another goal of this research problem was to synthesize β-L-fucose-1-phosphate. The results of these endeavors are reported in this thesis.

CHAPTER II

LITERATURE REVIEW

BLOOD GROUP SUBSTANCES - CHEMICAL AND SEROLOGICAL CHARACTERIZATION

The ABO blood group was first identified in the red cell by Landsteiner in 1900 (8). Since then, the ABO blood group has been identified in a number of other tissues and secretions from the body as well as in some animals, plants, and bacteria. Kabat (1) gives a summary of the sources of BGS from human as well as others, both plant and animal. Although the blood groups were first identified in red cells, it was found that saliva, gastric juice, seminal fluid, ovarian cyst fluid, and urine were better sources of the BGS. The BGS derived from these secretions are water soluble glycoproteins while the substances isolated from red cells are thought to be glycolipid, although adequate characterization of the aglycone portion of erythrocyte BGS has not yet been done (2). The substances isolated from red cells were isolated by solvent extraction procedures and were generally of low activity (1, 2). For this reason, Kabat (1) suspects that the ABO substances isolated from red cells may consist mainly of serologically inactive glycolipid contaminated with small amounts of antigenic blood group substance which is glycoprotein.

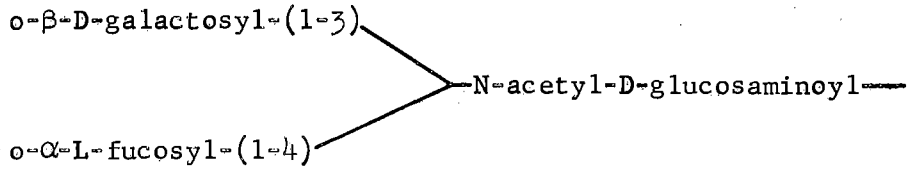
Due to the relative ease with which the soluble BGS can be prepared in large quantities from various tissue fluids, most of the characterization work has been done on them rather than the BGS isolated from erythrocytes. However, Watkins (9) has compared the A and B substances isolated from red cells to those isolated from secretions by a number of different methods. Using both serological and enzymatic inhibition studies Watkins showed that the terminal nonreducing sugars which are important to the antigenic specificity of the BGS appear to be the same for the glycolipid A and B substances from erthrocytes as that shown for the secreted glycoprotein A and B substances. Although the aglycone portion of the BGS from erythrocytes and tissue secretions may be different, it is believed that the carbohydrate portions are similar if not identical. Since most of the work has been done on BGS isolated from tissue secretions, further discussion will be limited primarily to these soluble BGS which are glycoproteins.

Highly purified samples of blood group A, B, H, or Le^a substances were found to have about the same qualitative composition (10). The carbohydrate portion of the glycoprotein contains only the four sugars L-fucose, D-galactose, D-N-acetylgalactosamine, and D-N-acetylglucosamine. The polypeptide portion of the molecule makes up about 15 to 25% of the molecule which has a total particle weight ranging from 2×10^5 to 1×10^6 for substances isolated from ovarian cyst fluid (11). The polypeptide portion of the molecule is composed of 15 amino acids, chiefly serine, threonine, and proline. Although A, B, and H substances are similar in quantitative composition, the

galactose content is highest in B substance and the galactosamine content is highest in A substance (12). The Le^a substance is nearly always lower in fucose content than the A, B, or H substances.

Indirect structural studies on the BGS have been done by a number of methods. Substances which are structurally related to, or identical to, the immunologically determinant group of an antigen combine specifically with antibody and thereby competitively inhibit the reaction between antigen and antibody. This type of experiment has been used to demonstrate that the serological activity of the BGS is associated with the carbohydrate rather than the peptide portion of the molecule. Of the sugars present in the BGS, only L-fucose specifically inhibited the agglutination of human group O(H) cells by a specific anti-H reagent from the eel Anguilla anguilla (13). Inhibition studies with anti-H reagents from other sources also indicated that L-fucose was important in H specificity (14). Similar studies showed that an important part of the chemical structure responsible for A specificity was N-acetyl-galactosamine (14) and that an α-D-galactosyl group has an important role in B specificity (15, 16, 17, 18). Other inhibition studies with oligosaccharide fragments isolated after acid hydrolysis of BGS have shown that H specificity may be due to more than one terminal non-reducing sugar. The most potent inhibitor of certain anti-H reagents was found to be a β-(1-4)-linked di-N-acetylglucosaminide (16). This indicates that N-acetylglucosamine, as well as L-fucose, may be involved in H specificity. Although agglutination inhibition by single sugars can be demonstrated using A, B, or H antibodies, agglutination

of Le^a+ cells is not inhibited by L-fucose alone or by any other sugars found in the BGS. A number of oligosaccharides which contain L-fucose have been isolated from milk (19) and tested for antibody inhibition. It was found that the most probable structure responsible for Le^a specificity is as shown below (16, 20).



Other methods used in studying the BGS have been to determine the changes, both serological and chemical, which occur as a result of incubation of the BGS with enzymes which are known to alter or destroy the serological activity of the substances. A number of different enzymes from several sources have been isolated which will degrade BGS. A mixture of enzymes obtained from Trichomonas foetus preparations has been resolved into several different enzymes, each of which has the capacity to destroy the serological activity of one type of BGS and generate a new serological determinant by liberating a single kind of sugar from the intact BGS substrate. If inactivation of BGS experiments are conducted in the presence of the sugar which would normally be released from the BGS by the BGS inactivating enzyme, product inhibition results and BGS degradation is inhibited. This type of experiment, inhibition of inactivation of BGS, has been used in studying the structure of the BGS. Inhibition of inactivation of A substance by enzyme preparations from Clostridium welchii or Trichomonas foetus was accomplished by N-acetylgalactosamine and not by any of the other sugar components of the BGS. Inactivation of

B substance by purified T. foetus B enzyme was inhibited by D-galactose. Inactivation of H substance by purified T. foetus H enzyme was inhibited by L-fucose. Although Le^a red cell agglutination was not inhibited by fucose (16, 20) Le^a substance inactivation by T. foetus enzyme was inhibited by L-fucose (20). These results implied that the sugars released by enzymatic inactivation of the BGS are the same as those which bring about the inhibition (i.e. product inhibition). These same sugars are thought to be responsible for the serological activity of each of the blood groups (2).

In more direct experiments a preparation of H-decomposing enzyme free from A, B, or Le^a-decomposing enzymes destroyed the H activity of group O red cells and of the water soluble H substance with concomittant development of pneumococcus type XIV specificity and release of L-fucose as the primary sugar (21). This work supports that described above which implicates L-fucose as an important part of the H determinant structure. Watkins (22) has also shown that T. foetus B enzyme destroyed B specificity and at the same time gave enhanced H activity which was accompanied by liberation mainly of galactose and traces of fucose and N-acetylhexosamine. Coffee bean α-galactosidase destroyed B activity and released galactose (23). Watkins (24) showed that the nondiffusable material which remained exhibited H specificity. Purified A-decomposing enzymes from T. foetus destroyed A specificity, enhanced H specificity, and released N-acetylgalactosamine (24, 25). Certain H substances develop considerable Le^a activity upon treatment with T. foetus H-decomposing enzyme. Destruction of H activity by this enzyme is accompanied by release of fucose. It

is assumed that removal of this sugar exposes latent Le^a specific structures. From these studies it is believed that A and B substances contain latent H active structures and that latent Le^a active structures are present in certain H substances. These latent structures are made active by the enzymatic removal of the appropriate sugar from the polysaccharide (2). A summary of the T. foetus enzymatic interconversions of the BGS is shown in Fig. 1 (25).

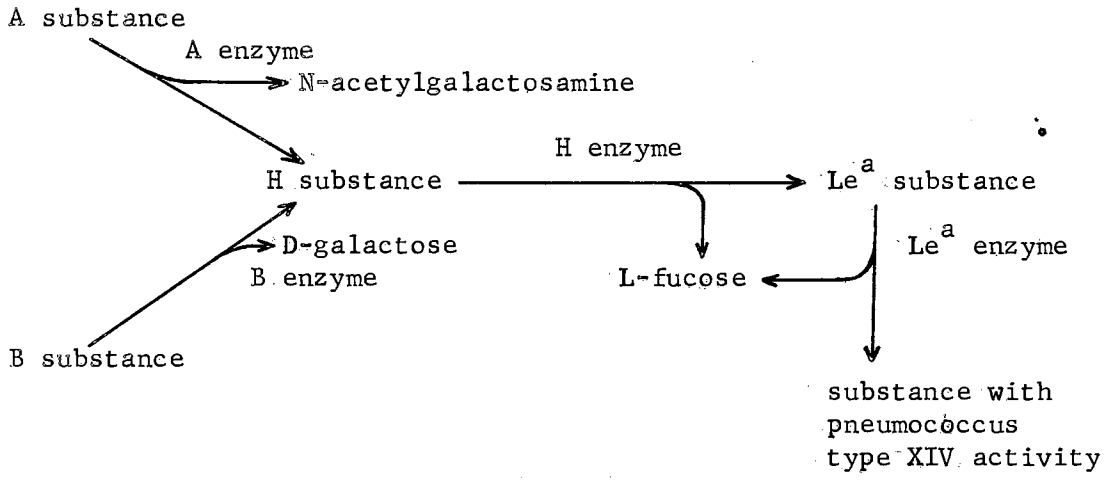


Fig. 1. Interconversions of BGS as effected by T. foetus enzymes.

Presumably the biosynthesis of BGS might take place by a reversal of the reactions shown in Fig. 1. For example, it might be expected that a nucleotide diphosphate sugar such as GDP-fucose would donate the sugar moiety to an acceptor molecule such as pneumococcus type XIV antigen to form Le^a substance. Similar reactions using the appropriate nucleotide diphosphate sugars could account for the formation of H, B, and A substances.

BLOOD GROUP SUBSTANCE BIOSYNTHESIS AND FUCOSE METABOLISM

The only available information on BGS biosynthesis is the work of Kornfeld, Kornfeld, and Ginsburg (26). Using hog stomach, which is known to secrete BGS having the same immunological characteristics as human BGS, maximum incorporation of injected ^{14}C label from glycerol-1,3-^{14}C into GDP-fucose was obtained as early as fifteen minutes. The specific activity of GDP-fucose decreased after this time. After a lag of fifteen minutes, the microsome-bound, fucose-containing polysaccharide reached maximum specific activity, suggesting a precursor-product relationship between GDP-fucose and microsome-bound polysaccharide. At later time periods, the microsome-bound polysaccharide decreased in specific activity while the BGS polysaccharide of the 150,000 x g supernatant fraction increased, again indicating a precursor-product relationship. No fucose-containing oligosaccharides were detected. Only BGS polysaccharide assayed immunologically by a precipitin reaction, contained fucose. This suggested that the polysaccharide portion of BGS is synthesized by the sequential addition of one sugar at a time to the growing polysaccharide chain which is bound at all times to the completed peptide chain on the microsomes. Sarcione (27) has shown rat liver glycoproteins to be synthesized in this manner.

Although nothing is known of the biosynthesis of BGS in the erythrocyte, several possible precursor nucleotide diphosphate sugars have been shown to occur in erythrocytes. Mills (3) has found UDP-glucose and UDP-N-acetylglucosamine in human erythrocytes and Bartlett (28) has found UDP-glucose in both erythrocytes and reticulocytes and

UDP-N-acetyglucosamine only in reticulocytes from rabbits and rats. UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, and probably GDP-mannose have all been found in nucleated pigeon erythrocytes (29). No UDP-N-acetylglucosamine could be demonstrated in non-nucleated erythrocytes (30). Mills (4) has demonstrated the presence of GDP-mannose, a probable precursor of GDP-fucose, in human erythrocytes, however, no GDP-fucose was found in this source. GDP-fucose has been detected in swine erythrocytes (30) as has GDP-mannose.

Ginsburg (5, 6) demonstrated the enzymatic synthesis of GDP-fucose from GDP-mannose by extracts of Aerobacter aerogenes. The overall reaction is shown in equation 1.

1. $\text{GDP-}\alpha\text{-D-man} + \text{TPNH} + H^+ \xrightleftharpoons{\text{DPN}} \text{GDP-}\beta\text{-L-fuc} + \text{TPN} + H_2O$

GDP-fucose formation required stoichiometric amounts of TPNH and the reaction rate was increased by addition of catalytic amounts of DPN. The reaction has been shown to involve GDP-4-keto-6-deoxy-D-mannose as an intermediate.

Ginsburg (7) has also shown the biosynthesis of GDP-fucose from GDP-mannose in rabbit tissues. The specific activity of extracts of large intestine was the highest followed by lung, pancreas, salivary gland, small intestine, and liver. This mammalian system was similar to the bacterial system in its requirement for TPNH and DPN in order to attain the maximum rate of GDP-fucose biosynthesis.

Coffey, et al. (31) has clearly shown rat small intestine to directly incorporate 1-^{14}C-fucose into intestinal glycoprotein which is then secreted into the intestinal lumen. The rat metabolized only one percent of the injected 1-^{14}C-fucose to $^{14}CO_2$ in ten hours. Other

than fucose, no other sugars isolated from glycoprotein were radioactive. These results suggested the existence of an alternate pathway for the activation of fucose prior to incorporation into glycoprotein.

Ishihara, Massaro, and Heath (32) recently reported the occurrence of L-fucose kinase in pig liver which can convert L-fucose to β-L-fucose-1-phosphate via the reaction shown in equation 2.

2. $$\text{L-fuc} + \text{ATP} \xrightleftharpoons{\text{L-fucose kinase}} \beta\text{-L-fuc-1-P} + \text{ADP}$$

In addition the same authors have been able to demonstrate the formation of GDP-fucose from L-fucose-1-phosphate and GTP (equation 3) using a pig liver enzyme.

3. $$\text{GTP} + \text{L-fuc-1-P} \rightleftharpoons \text{GDP-fuc} + \text{PPi}$$

These results provide an alternate route for the formation of GDP-fucose.

The participation of GDP-fucose as a fucosyl donor was implied by the results of Kornfeld, et al. (26), described above. Direct evidence that GDP-fucose is an active fucosyl donor in oligosaccharide biosynthesis has been provided by Grollman, Hall, and Ginsburg (33). The biosynthesis of fucosyllactose in lactating canine mammary tissue was shown to occur by the transfer of the L-fucosyl moiety from GDP-fucose to lactose.

Although the direct incorporation of L-fucose into polysaccharide (31) and the direct synthesis of GDP-fucose from L-fucose (32) suggest that these pathways should be considered in deducing the pathways for erythrocyte GDP-fucose and BGS biosynthesis, the

author feels that the more likely pathway for these biosyntheses in the erythrocyte probably involves GDP-mannose similar to Ginsburg's system (5, 6) since GDP-mannose has been found in human erythrocytes (4) and both GDP-mannose and GDP-fucose have been found in swine erythrocytes (30).

METHODS OF ALDOSE-1-PHOSPHATE SYNTHESIS

Several methods have been used for sugar phosphate synthesis. In 1937, Cori, Colowick, and Cori (34) described the synthesis of α-D-glucose-1-phosphate by the reaction of α-1-bromo-tetraacetyl-glucose with trisilver phosphate in benzene to produce the tertiary ester, tri-(α-D-glucose-1)-phosphate. Removal of two of the sugar residues as well as deacetylation is achieved by mild acid hydrolysis to yield the final product, α-D-glucose-1-phosphate. Poor overall yields were obtained due to the loss of 67% of the starting sugar during acid hydrolysis of the triester. The reaction apparently does not normally give inversion of anomeric configuration, however, attempts to prepare β-D-glucose-1-phosphate from the β-1-chloro-tetraacetylglucose resulted in a mixture of α and β anomers in which the α anomer predominated. Colowick (35) prepared α-D-mannose-1-phosphate and α-D-galactose-1-phosphate by this same method. α-D-galactose-1-phosphate was prepared in a final yield of 33% compared to the calculated theoretical yield. The α-D-mannose-1-phosphate triester was obtained in 96% yield, however, the final yield of α-D-mannose-1-phosphate was not reported. The specific rotation values for both sugar phosphates indicated that they were the α anomers.

Silver diphenyl phosphate has been used by Posternak (36) to prepare aldose-1-phosphates. It was reacted with α-bromoacetylglucose and α-bromoacetylgalactose followed by catalytic hydrogenation to remove phenyl groups to produce α-D-glucose-1-phosphate and α-D-galactose-1-phosphate in 37% and 44% yields, respectively. This reaction does not give inversion. The yields are four to six times greater than with the trisilver phosphate method. Posternak (36) also attempted the synthesis of α-D-glucose-1-phosphate by reacting β-D-2,3,4,6-tetraacetylglucose with diphenyl chlorophosphonate in pyridine. This method gave inversion of configuration, however, the yield (10%) was much lower than in the silver diphenyl phosphate method.

Reithal's "monosilver phosphate" method (37) of preparing β-D-aldose-1-phosphates from α-1-bromo-tetraacetylglucose was used by Putman and Hassid (38) to prepare β-D-glucose-1-phosphate and β-D-galactose-1-phosphate in approximately 10% yields. The recrystallized products were isolated as the cyclohexylammonium salts contaminated with less than 0.1% of the α anomers. This method of preparation of aldose-1-phosphates has usually, but not in all cases, resulted in Walden inversion.

Wolfrom, et al. (39) found that Walden inversion occurred at carbon one when α-bromo-tetraacetylglucose and α-bromo-tetraacetylgalactose were reacted with silver dibenzyl phosphate. An exception to this was α-bromo-tetraacetylmannose which does not give inversion upon reaction with either silver dibenzylphosphate or monosilver phosphate (40). These results show that the hexose phosphate anomer

obtained from the reaction of bromoacetylhexose with silver dibenzyl-phosphate or monosilver phosphate has the C_1C_2-trans configuration regardless of whether the original bromoacetylhexose is C_1C_2-cis or C_1C_2-trans. A strong C_2 neighboring group effect with some sugar acetates (particularly those having C_1C_2-trans diaxial conformation) is thought to influence the steric course of this type of phosphorylation. On the other hand, phosphorylation of the pentose derivative, α-bromoacetylxylose, by silver dibenzyl phosphate gave a mixture of 60% β and 40% α D-xylose-1-phosphate (41). These two anomers have C_1C_2-trans and C_1C_2-cis configurations, respectively. These studies suggested that C_5 neighboring group participation may also influence anomer formation with hexoses and possible explanations were proposed to account for the observed steric control of the reaction product (41). Despite the proposed explanations the influence of neighboring group participation on the steric course of these phosphorylation reactions does not seem to be well understood.

Triethylammonium diphenyl phosphate has been reacted with α-1-bromo-acetylglucosamine to form α-D-glucosamine-1-phosphate after removal of phenyl groups by catalytic hydrogenation and cleavage of acetyl groups with potassium methoxide (42). Retention of configuration was observed in these experiments. The overall yield was approximately 50%. A slight modification of this method has been used for the synthesis of α-D-N-acetyl glucosamine-1-phosphate using conditions which favor acyl migration from oxygen to nitrogen during deacetylation. The starting materials are the same as in the α-D-glucosamine-1-phosphate preparation although yields are much smaller (42).

Wright and Khorana (43) have used triethylammonium dibenzyl phosphate in benzene to phosphorylate β-1-bromo-triacetyl-D-arabinopyranose for synthesis of α-D-arabinopyranose-1-phosphate in 38% yield. The final product has a C_1C_2-*trans* configuration which is attained through Walden inversion during phosphorylation. The advantage of using triethylammonium dibenzyl phosphate instead of the free acid as the phosphorylating agent is that it is more soluble in benzene or toluene and gives higher yields for certain sugars (ribose and arabinose) which usually give very low yields, if any, by other methods. Ribose-1-phosphate has also been synthesized by this method (44). Silver dibenzyl phosphate and monosilver phosphate methods proved unsuitable for the synthesis of β-D-ribose-1-phosphate because of low yields (2 to 5% and none, respectively). However, β-D-ribose-1-phosphate was obtained in 20% yield using the triethylammonium dibenzyl phosphate method showing the superiority of this method.

A new method for glycosyl phosphate synthesis has recently been reported by MacDonald (45). This method involves the direct, phosphorylation of a fully acetylated sugar by warming *in vacuo* in an anhydrous phosphoric acid melt. Evolution of acetic acid occurs in the melt as the acetyl group on carbon one is replaced by a phosphate group. Removal of the remaining acetyl groups is achieved with lithium hydroxide. β-D-glucose pentaacetate and β-D-galactose pentaacetate were each phosphorylated to yield 31% and 35%, respectively, of α-D-glucose-1-phosphate and α-D-galactose-1-phosphate. Sugar acetates such as β-D-glucose and β-D-galactose pentaacetates, having a C_1C_2-*trans* configuration, resulted in a phosphorylated product which was C_1C_2-*cis*.

O'Brien (46), who modified MacDonald's procedure, phosphorylated α-D-pentaacetylglucosamine under a variety of conditions of temperature and ratio of phosphoric acid to acetylated sugar. The best yield (33%) was obtained by increasing the temperature from 50° (as used by MacDonald (45)) to 83° for 45 minutes and increasing the phosphoric acid to sugar acetate molar ratio from 4:1 to 8:1. Although under varying conditions overall yields varied from 7% to 33% the ratio of α and β anomers of N-acetyl-D-glucosamine-1-phosphate was always 2:3. The α and β anomers were separated by column chromatography using Dowex 1 x 4 (Cl^-).

Kim and Davidson had previously reported the synthesis of N-acetyl-α-D-galactosamine-1-phosphate (47) and N-acetyl-α-D-glucosamine-1-phosphate (48) using MacDonald's method. These authors used a 50° reaction temperature and reported that only the α anomers were formed. The yields were 20-35%. O'Brien (46) suggested that Kim and Davidson's failure to find the more labile β anomers may be a result of their destruction by the acidic conditions used.

MacDonald (49) has recently reported several modifications of his original procedure which have allowed him to prepare several phosphorylated sugars in higher yields than before. The molar ratio of phosphoric acid to sugar acetate was increased from 4:1 to an optimum ration of 8:1. α-D-glucose-1-phosphate and α-D-galactose-1-phosphate were prepared in 60% yields from the corresponding β-sugar acetates which have C_1C_2-trans diequatorial configuration by running the reactions in vacuo for two hours at 50°. On the other hand, compounds which had a C_1C_2-cis configuration were found to react

poorly at 50°. For example, the α pentaacetate of glucose heated 2 hours at 50° gave α-glucose-1-phosphate in only 10% yield (49) and the α pentaacetate of glucosamine was reported (46) to give low yields of a mixture of α and β anomers of acetylglucosamine-1-phosphate. The yields from C_1C_2-cis pentaacetates could be increased by increasing the reaction temperature to approximately 85°. For example, the pentaacetates of the α anomers of glucose and galactose gave the corresponding α-sugar-1-phosphate in yields of 40% and 10%, respectively (49). With these two sugars no inversion of anomeric configuration occurred. Similar increases in yield were reported by O'Brien (46) when the C_1C_2-cis pentaacetate of glucosamine was phosphorylated but with the important difference that the product contained a mixture of the α (40%) and β (60%) anomers. Thus, with this particular sugar inversion of configuration occurred to a large degree.

The results from these two laboratories (46, 49) demonstrated that C_1C_2-cis anomers of pentaacetates could be made to react with anhydrous phosphoric acid at elevated temperatures.

MacDonald (49) also reported trying several other interesting modifications of his original procedure. For example, since melted crystalline phosphoric acid is converted to a mixture of ortho and pyrophosphoric acids with concomittant formation of about 6 mole% of water, attempts were made to attain more anhydrous conditions in the melt by using "105% phosphoric acid." This material contains about 2 mole% water and 28 mole% pyrophosphoric acid. By phosphorylating β-D-glucose pentaacetate with "105% phosphoric acid," α-D-glucose-1-

phosphate was isolated in 46% yield. The yield and nature of the product were about the same as that obtained when 100% phosphoric acid was used as the phosphorylating reagent.

Attempts were also made to prepare glycosyl phosphates by direct phosphorylation (with 99% phosphoric acid) of the methyl glycosyl pentaacetate. These attempts, however, were generally less successful. The yields were only 0.4% to 9% when the reaction was conducted at 50°. During attempts to synthesize fructose-2-phosphate (49) it was observed that phosphorylation of β-D-fructopyranose pentaacetate at 50° for 1.5 hours gave D-fructopyranose-2-phosphate in 25 to 40% yield. By contrast, attempts to phosphorylate D-fructofuranose pentaacetate under a variety of conditions always gave low yield (3-5%). An improved yield (54%) of β-D-fructopyranose-2-phosphate was obtained by the phosphorylation at 50° of β-D-fructopyranose pentaacetate in a nonpolar solvent, tetrahydrofuran, rather than a melt composed of the two reactants. However, under the same conditions, β-D-glucopyranose pentaacetate and D-fructofuranose pentaacetate gave very low yields.

It appears that of the methods for phosphorylating sugars described above the MacDonald procedure offers the most promise. Yields are high and the procedure is simple and straightforward. The 50° reaction temperature seems to give the phosphorylated anomer having C_1C_2-*cis* configuration in good yield from sugar acetates with C_1C_2-*trans* configuration, however, good yields were also obtained from sugar acetates with C_1C_2-*cis* configuration by elevating the reaction temperature (83°), O'Brien (46) was able to show that the product

from α-pentaacetyl glucosamine (C_1C_2-cis) contained both the trans and the cis C_1C_2 configuration. Since the configuration of α-L-fucose tetraacetate is also C_1C_2-cis one might expect to find a mixture of α and α L-fucose-1-phosphate (C_1C_2-cis and C_1C_2-trans, respectively) from the phosphorylation of α-L-fucose tetraacetate using the MacDonald synthesis as modified by O'Brien.

NUCLEOTIDE DIPHOSPHATE SUGAR SYNTHESIS

Early attempts at nucleotide anhydride synthesis involved a number of now obsolete methods. No attempt will be made here to mention these as they have been thoroughly reviewed by Michelson (50). At present, there are two general routes in common use by which one can achieve nucleotide anhydride synthesis in good yields. The first is known as the "Michelson synthesis" and has been found to be highly efficient and relatively simple for millimolar scale preparations (51, 52). This synthesis essentially involves the conversion of the nucleotide moiety to the diphenyl nucleoside pyrophosphate by reaction with diphenyl phosphochloridate and tri-n-butylamine in dioxane. The diphenyl nucleoside pyrophosphate does not undergo further reaction or decomposition in dioxane if pyridine is absent. The final anhydride is obtained in very high yields (60-90%) by adding a pyridine solution of the tri-n-octylammonium salt of the second phosphorylated compound, such as a sugar phosphate, to the diphenyl nucleoside pyrophosphate. The ensuing anion exchange reaction occurs rapidly at room temperature. Rigorously maintained anhydrous conditions are not essential; however, decreased yields occur when wet

pyridine (5% water) is used for the anion exchange reaction (52). High yield and ease of purification of the product seem to make this synthesis a very good route.

Although Michelson's method seems to be highly efficient and relatively straightforward, some researchers prefer the phosphoromorpholidate method of Moffat and Khorana (53) for its reliability despite the lower yields. The phosphoromorpholidate method was found to be superior to the phosphoramidate method used previously by these workers in that it is more reactive, has fewer solubility problems, and the nucleotide morpholidate is easier to prepare (53). GDP-mannose has been prepared in 63% yield by the phosphoromorpholidate method as have other nucleotide diphosphate sugars (54). The method works well for all nucleotides although solubility problems have been occasionally noted with guanosine-5'-phosphoromorpholidate in pyridine. The preparation of the phosphoromorpholidate is accomplished by reaction of the nucleoside monophosphate with dicyclohexyl carbodiimide and morpholine in t-butanol and water as solvent. Yields are nearly quantitative. The nucleoside-5'-phosphoromorpholidate is then reacted with the tri-n-octylammonium salt of a sugar phosphate dissolved in pyridine to produce 50-70% yields of nucleotide diphosphate sugar (54).

Although this method works quite well on large scale preparations (0.2-0.5 mmoles), the yield drops to 3% or less when attempted on a micromolar scale. Nordin, et al. (55) found that traces of moisture were responsible for the greatly decreased yield. They have devised an apparatus which permits anhydrous reaction conditions with

greatly increased yields (15-80%), depending on which nucleotide is used and other reaction conditions. An inverted Y-shaped reaction vessel, containing 1-100 μmoles of nucleoside-5'-phosphoromorpholidate in one arm and the tri-n-octylammonium salt of a sugar phosphate in the other, is attached to a vacuum manifold containing a source of dry pyridine. The system is evacuated and pyridine is condensed in the arms of the reaction tube and then allowed to evaporate. Repetition of this procedure dries the compounds completely. Finally, when both are dry, pyridine is allowed to condense in the arms of the tube and the contents of the arms are mixed. The reaction vessel is sealed and set aside to react for 1-10 days at temperatures ranging from 25-40°. The product is isolated by paper chromatography. Yields appear to be somewhat dependent on the temperature at which the reaction is carried out, ratio of phosphoromorpholidate to sugar phosphate, and reaction time, although no definite conclusion as to the optimum conditions can be drawn from the data presented. Using this method, GDP-glucose was prepared in 25% yield from 12.5 μmoles of glucose-1-phosphate. Approximately 7 μmoles of UDP-D-fucose was prepared in a 43% yield from D-fucose-1-phosphate and UMP morpholidate. CMP and IMP morpholidates did not form homogenous solutions in pyridine but this did not seem to have any significant effect on yields obtained with these compounds as compared to yields using the morpholidates which were completely soluble. Due to the small scale and relatively good yields of this procedure, it appeared to be a promising method for GDP-fucose synthesis, particularly since the Michelson synthesis is not suited for micro scale synthesis and re-

quires an excess of the sugar phosphate which in this case would be the least available of the two starting materials.

CHAPTER III

EXPERIMENTAL PROCEDURES

Materials

Anhydrous phosphoric acid (99%) was obtained from Matheson, Coleman, and Bell, "105% Phosphoric Acid" from Dr. K. D. Berlin. α-L-Fucose was obtained from Sigma; DPN, TPN, TPNH, GDP, GMP, and GDP-glucose from P-L Biochemicals or Sigma; and GDP-mannose from Calbiochem. PEI was purchased from Chemirad Corporation and MN-300 cellulose was purchased from Brinkman Instruments. Dowex 50 x 8 cation exchange resin (20-50 mesh) was obtained from J. T. Baker Chemical Co. Aerobacter aerogenes 12658 was obtained from the American Type Culture Collection. Blood was obtained from the University of Oklahoma Medical School Hospital Blood Bank. The blood was drawn from anonymous donors of the desired blood type into Acid-Citrate-Dextrose Solution B anticoagulant. The barium salt of α-L-fucose-1-phosphate was a gift from Dr. Saul Roseman. All other chemicals used were of reagent quality.

Methods

Inorganic phosphate assays were performed according to the Lowry-Lopez method (56). Acid labile (ten minutes in 1 N sulfuric

acid at 100°) and total phosphate were determined by a modified Fiske-Subbarow assay (57). Nelson's assay was used to determine the amount of reducing sugar (58). Fucose assays were performed according to Dische and Shettles (59), using the ten minute hydrolysis period and measuring absorbance at 400 mu with a Beckman Model DU spectrophotometer. This assay was scaled down to one-fifth scale in some cases to increase the sensitivity of the assay. Biuret assays were performed using the method of Gornall, et al. (60). Absorption spectra were obtained using a Beckman Model DB spectrophotometer with an attached Sargent Model SRL recorder. The concentration of solutions of guanosine derivatives was determined assuming a molar absorption coefficient of 11.6×10^3 at 260 mu (this value was calculated from data given in reference (61).).

PEI-cellulose thin layer chromatography was performed according to Randerath and Randerath (62). Fifteen grams of MN-300 cellulose were blended for about thirty seconds in a Waring blender with 100 ml of dialyzed one percent PEI solution (pH 6.0) and spread to a thickness of 0.5 mm on 10 x 20 cm glass plates. The plates were allowed to dry overnight and were then washed once by ascending chromatography with water. The plates were then allowed to dry overnight prior to use. All plates were developed in an ascending manner using one molar lithium chloride as solvent. Nucleotides were located on the developed plates by means of a 253.7 mu ultraviolet lamp. They appeared as light absorbing spots.

NMR spectra were courteously run by Varian Associates on a 100 megacycle instrument with an external capillary tetramethyl silane

standard and D_2O as the solvent. Elemental analysis was performed by Galbraith Laboratories.

α-L-Fucose tetraacetate was prepared according to the method of Levvy and McAllan (63). Five grams of α-L-fucose were stirred with acetic anhydride (46.2 ml) and pyridine (60 ml) at 4° for two days. Following chloroform extraction and evaporation, α-L-fucose tetraacetate was crystallized from 95% ethanol in a 68% yield (6.87 gm or 20.7 mmoles). The crystals, after drying in vacuo, had a melting point range of 91°-93° which agrees closely with the literature value of 92°-93° (63).

CHAPTER IV

RESULTS

Preparation and Properties of Fucose-1-Phosphate

Initial attempts to prepare fucose-1-phosphate were conducted on a small scale using a slightly modified MacDonald's procedure (45) as described below. Two mmoles of α-L-fucose tetraacetate (0.6 gm) were placed in the side arm of a Thunberg tube which contained magnesium perchlorate descicant in the lower portion of the tube. Twenty mmoles of 99% phosphoric acid (2 gm) were weighed into the lower part of another Thunberg tube and magnesium perchlorate was placed in the upper part of the tube. Both Thunberg tubes were dried in vacuo for two days. The upper part of the Thunberg tube containing the fucose tetraacetate was quickly joined to the lower part of the tube containing the phosphoric acid. The tube was then evacuated with an oil pump and the lower portion of the tube was placed in a 50° water bath until the phosphoric acid melted. The fucose tetraacetate was added slowly to the phosphoric acid melt (50°) and stirred by swirling to dissolve all of the fucose tetraacetate. The reaction was carried out at 50° for eight hours. Ten ml of tetrahydrofuran were used to dissolve the reaction mixture and

to transfer it to 72.6 ml of ice cold 1 N lithium hydroxide. After allowing the mixture to stir overnight at room temperature it was filtered to remove precipitated lithium phosphate. The filtrate was passed through a Dowex 50 x 8 acid form resin column (65 ml of resin) in the cold room (4°) and eluted with water. Cyclohexylamine (4 ml) was added to the effluent to make it basic. After lyophylization and 2-propanol extraction, the residue was treated with 0.15 gm of magnesium acetate (containing four moles of water of hydration) dissolved in 6.0 ml of 1.5 N ammonium hydroxide to remove last traces of inorganic phosphate. The precipitate of magnesium ammonium phosphate was filtered off and the filtrate was passed through another Dowex 50 x 8 (acid form) resin column (bed volume = 60 ml) in the cold room. The column was eluted with water and concentrated to a syrup by use of a flash evaporator at room temperature. The fucose-1-phosphate was crystallized twice from ethanol. The final product (237 μmoles estimated as acid labile phosphate) represented an 11.8% yield based on fucose tetraacetate. It was contaminated by 2.9% inorganic phosphate. During this preparation of fucose-1-phosphate it was learned that the conditions used in the Fiske-Subbarow inorganic phosphate assay were too acidic and resulted in a considerable amount of hydrolysis of the fucose phosphate during the assay. In order to prevent hydrolysis of the product during inorganic phosphate assay, the Lowry-Lopez inorganic phosphate assay (56), which is conducted at pH 4.0, was used. No significant hydrolysis of fucose-1-phosphate occurred under Lowry-Lopez assay conditions. Comparison of the results of total phosphate and acid labile phos-

phate assays (57) showed that complete hydrolysis of the fucose-1-phosphate occurred in 1 N sulfuric acid at 100° for ten minutes (the conditions of the acid labile phosphate assays). It was not possible to determine the anomeric configuration of the product obtained from this small scale reaction due to the small amount of product.

An attempt was made to synthesize fucose-1-phosphate on a larger scale. The procedure was essentially that used previously for the reaction on a two mmole scale with the exception of some modifications which are described below. The reaction mixture contained 16 mmoles (4.8 gm) of fucose tetraacetate and 64 mmoles (6.4 gm) of phosphoric acid. The reaction was run at 50° for eight hours to produce a crude yield of 30%. After purification according to MacDonald (45) the twice crystallized product was obtained in 20.2% yield (1.4709 gm). It contained 2.24% inorganic phosphate. Due to the relatively high inorganic phosphate content further purification was necessary before satisfactory characterization of the product could be achieved. A portion of the crystals (0.66 gm) was further purified by again precipitating inorganic phosphate with magnesium ammonium acetate and subsequently passing the clear filtrate obtained from this step through a Dowex 50 x 8 resin column (bed volume = 130 ml) which previously had been equilibrated with cyclohexylamine. The effluent was evaporated to dryness and subsequently crystallized twice from ethanol and water. The final product was obtained as 0.2745 gm of white needle-like crystals (41.6% recovery based on the 0.66 gm of crystals used for further purification). This preparation contained 0.123% inorganic phosphate.

The highly purified fucose-1-phosphate was used for further analyses. Elemental analysis of the crystals were as follows: C, 48.69; H, 9.08; N, 6.13; P, 6.8; O (by difference), 29.3. The values calculated for the dicyclohexylammonium salt of fucose-1-phosphate ($C_{18}H_{39}N_2O_8P$) and corrected for the small amount of inorganic phosphate present in the preparation were: C, 48.87; H, 8.86; N, 6.32; P, 7.00; O, 28.88. The results from the elemental analysis agree quite well with the expected values. The ratio of fucose to acid labile phosphate to reducing sugar was found to be 1.02:1.00:0.93. The $[\alpha]_D^{25}$ value was determined on a Rudolph Model 80 Polarimeter using a solution which contained 39.92 mg fucose-1-phosphate per ml of water and was found to be -74.93°. The large negative molar rotation of -33.119° suggested that the sample was the α anomer of fucose-1-phosphate and compares well with the value (-34,400) reported by Leaback, Heath, and Roseman (64) for the α anomer. NMR spectra of the lithium salts of the synthesized α-L-fucose-1-phosphate and a sample of α-L-fucose-1-phosphate obtained from Dr. Roseman were identical. When the samples were dissolved in D_2O each exhibited the anomeric proton absorption band at 5.89 ppm relative to external tetramethylsilane standard. The polarimetry and NMR data have identified the final product of this preparation as α-L-fucose-1-phosphate.

Using a modified MacDonald procedure (49), further attempts at phosphorylation of fucose tetraacetate were made. Fucose tetraacetate (3 mmoles) was reacted in vacuo with 24 mmoles of "105% phosphoric acid" for twenty-one minutes at 85°. Considerable charring

and evolution of acetic acid was noted as the reaction progressed. The purification was carried out as described by MacDonald (49), using Dowex 50 x 8 (cyclohexylammonium form) resin columns in the cold room instead of the acid form Dowex 50 x 8 (acid form) columns he used previously (45). The final yield of an as yet unidentified product was 20.2%. The product was heavily contaminated with 22.5% inorganic phosphate. Crystallization of this product failed to remove the brown coloring. Treatment with charcoal removed most of the color but large amounts of the product were absorbed as well. Elution of the charcoal with 1 N cyclohexylamine solution and then with 15% pyridine solution failed to give full recovery of the product.

Preparation and Properties of GDP-Fucose

GMP phosphomorpholidate was prepared according to Moffat and Khorana (53). GDP-α-L-fucose was prepared using a vacuum system and reaction vessel similar to that described by Nordin et al. (55), and the condensation procedure of Roseman et al. (54), as modified by Nordin et al. (55), for the micro scale synthesis of nucleotide sugars. GMP morpholidate (139 μmoles) was dried in one arm of the reaction vessel (55) and 65 μmoles of α-L-fucose-1-phosphate (as the tri-n-butylammonium salt) were dried in the other. Dry pyridine was condensed in each arm of the reaction vessel and then evaporated in vacuo five times to thoroughly dry the reaction vessel and starting materials. About two ml of pyridine was condensed in each arm of the reaction vessel. The GMP morpholidate could not be completely dissolved in the pyridine so the insoluble material was suspended. The

entire suspension was transferred to the arm of the vessel which contained the completely dissolved fucose phosphate salt. After thorough mixing, the volume of pyridine was reduced to approximately 2 ml by evaporation in vacuo. The reaction vessel was sealed and allowed to incubate at 37° for three days. The vessel was then opened and its contents were suspended in water and filtered. The filtrate was streaked on Whatman 3MM chromatography paper and chromatographed in ethanol: 1.0 M ammonium acetate, pH 7.5, (7:3) solvent (54) which separated GDP-fucose from the faster moving GMP morpholidate and the slower moving GMP in the reaction mixture. The GDP-fucose band was cut from the paper and eluted with water. The amount of GDP-α-L-fucose recovered was 16.8 μmoles (determined spectrophotometrically). This represented a 25.9% yield based on α-L-fucose-1-phosphate.

The ratio of guanosine: acid labile phosphate: total phosphate: fucose for the synthetic GDP-α-L-fucose was 1.05 : 0.87: 1.92: 1.00. The low acid labile phosphorus data suggested that GMP might be present as a contaminant. Chromatography on PEI-cellulose thin layer plates did show a small amount of GMP in the GDP-fucose preparation.

Ultraviolet spectra were made of the GDP-fucose solution at pH 1, pH 7, and at pH 11. These spectra clearly showed the nucleotide moiety to contain guanosine (Fig. 2). Attempts to show the release of GMP by hydrolysis in 0.1 N HCl for ten minutes in a boiling water bath and the release of GDP by hydrolysis in 0.01 N HCl for ten minutes in a boiling water bath were not entirely successful. Chromatography of GDP-fucose on Whatman No. 1 paper using ethanol: 1 M

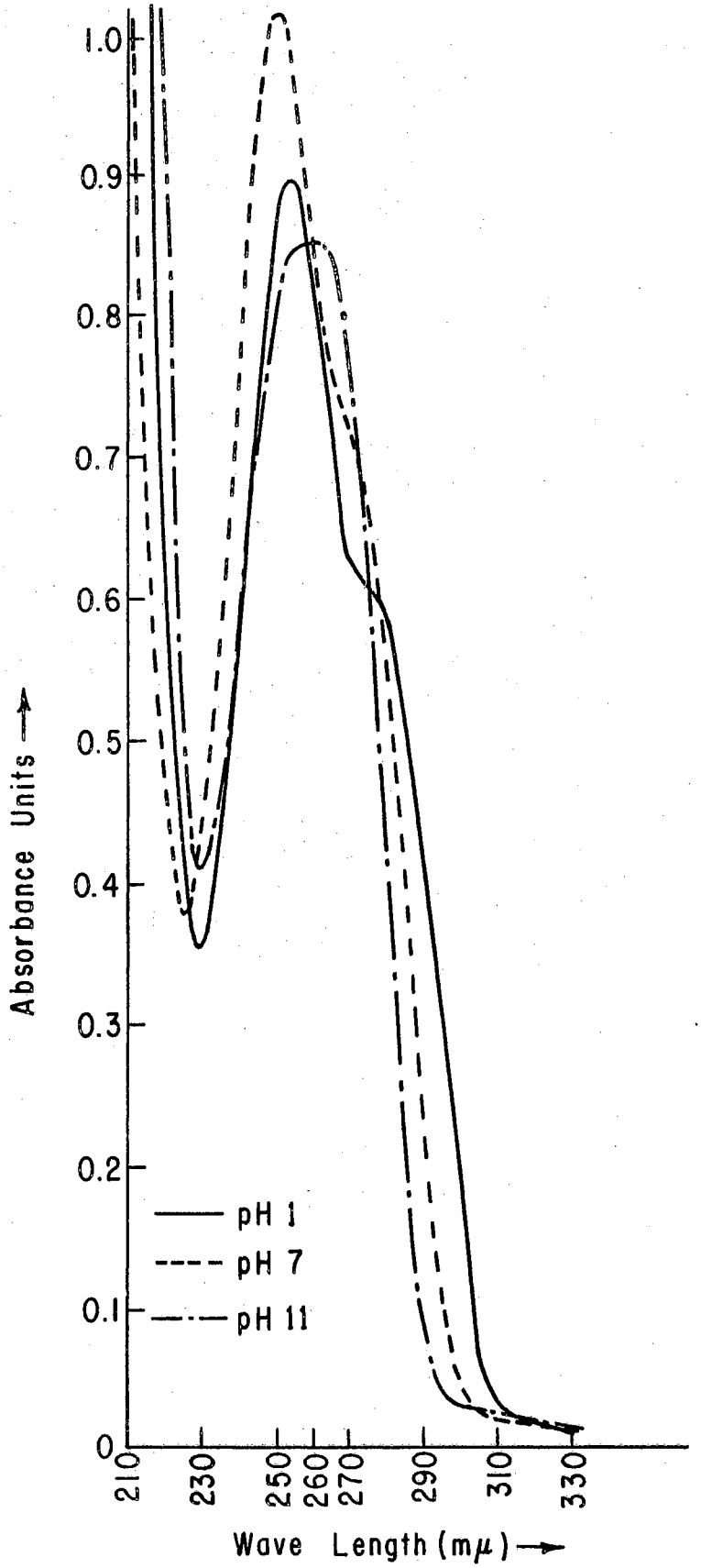
Figure 2

Ultraviolet Spectra of GDP-α-L-Fucose Dissolved

in Buffers of pH 1, pH 7, and pH 11

Buffers were made according to Pabst Circular OR-10 (61).

Spectra were made on Beckman Model DB Recording Spectrophotometer.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance Units
pH 1
pH 7
pH 11
210
230
250
260
270
290
310
330
Wave Length (mμ)

ammonium acetate, pH 7.5, (7:3) gave the following R_{GMP} values: GDP-α-L-fucose, 1.46; GDP-mannose, 1.28; GDP-glucose, 1.32. In spite of the R_{GMP} values found it did not appear that these compounds could be completely separated from a mixture by suing this chromatography system. Attempts to separate GDP-mannose, GDP-fucose and GMP using paper electrophoresis were not successful. The compounds were electrophoresed for 15 minutes at 3000 volts while on Whatman 3MM paper. Migration for GDP-fucose, GDP-mannose, and GMP was 8.7, 8.7 and 5.3 cm, respectively when pyridine buffer, pH 7.4, was used. When run in formic acid buffer, pH 2.0, for 15 minutes at 3000 volts the distances migrated were: GDP-fucose, 5.3 cm; GDP-mannose, 5.3 cm; and GMP, 0.0 cm.

GDP-fucose Biosynthesis

Aerobacter Aerogenes Experiments: Attempts to prepare GDP-β-L-fucose biochemically using crude extracts of A. aerogenes were conducted as described by Ginsburg (5). Attempts were made to measure the rate of the enzymatic synthesis of GDP-fucose, from GDP-mannose and TPNH, by the spectrophotometric determination of TPNH oxidation at 340 mμ. The endogenous rate of TPNH oxidation in these experiments was high. TPNH oxidation was increased only slightly by the addition of GDP-mannose in the reaction mixture. The results appeared to indicate that GDP-fucose was being synthesized enzymatically at the rate of 1.45 mμmoles per minute per mg of protein. Fucose assays were conducted on nucleotides from a one hour incubation mixture, after adsorption and elution from charcoal. The values obtained

with samples run in triplicate were eratic, therefore no adequate quantitative estimation of the amount of fucose in the samples was possible. In the author's hands variation of replicates generally has been the case when materials of biological origin were assayed for fucose. In spite of the variability of the results from the fucose assays, the assays served as a qualitative test for fucose. It was concluded that fucose was present in the nucleotide preparation recovered from the incubation mixture. Samples of the nucleotides eluted from charcoal were chromatographed by thin layer chromatography on PEI-cellulose. Only a single ultraviolet absorbing spot, not present in an unincubated control, was present in the separated nucleotides from a mixture which had been incubated for one hour. A reproduction of the PEI-cellulose plate is shown in Fig. 3. It was presumed that this spot was GDP-β-L-fucose. In a similar experiment, PEI-cellulose thin layer chromatography of the nucleotides isolated from a 14 hour incubation mixture using another Aerobacter aerogenes preparation yielded similar results. Later, after GDP-α-L-fucose (prepared as described earlier) became available for use as a standard PEI-cellulose chromatography of the nucleotides from the incubation mixture was repeated. The results are shown in Fig. 4. It can be seen that the chemically synthesized GDP-α-L-fucose and one of the new products formed in the incubation mixture had the same mobility. Chemically synthesized GDP-α-L-fucose and the product from incubation with Aerobacter aerogenes preparations migrated slightly slower than GDP-mannose (Fig. 3 and Fig. 4). The chromatographic results strongly suggested that GDP-β-L-fucose was

Figure 3

Results from PEI Thin Layer Chromatography of Nucleotides from A. aerogenes One Hour Incubation

GDP-Mannose was incubated with A. aerogenes crude preparations for one hour at room temperature. Nucleotides were adsorbed and eluted from charcoal and then separated by thin layer chromatography on PEI plates as shown. Lane 1, GMP and GDP-mannose; Lane 2, GDP-mannose incubated one hour with A. aerogenes preparation; Lane 4, DPN; Lane 5, TPN; Lane 6, GDP and GMP. Note the new spot appearing after GDP-mannose incubation with A. aerogenes preparation for one hour (Lane 2). This is suspected to be GDP-fucose.

S = Solvent Front

O = Origin

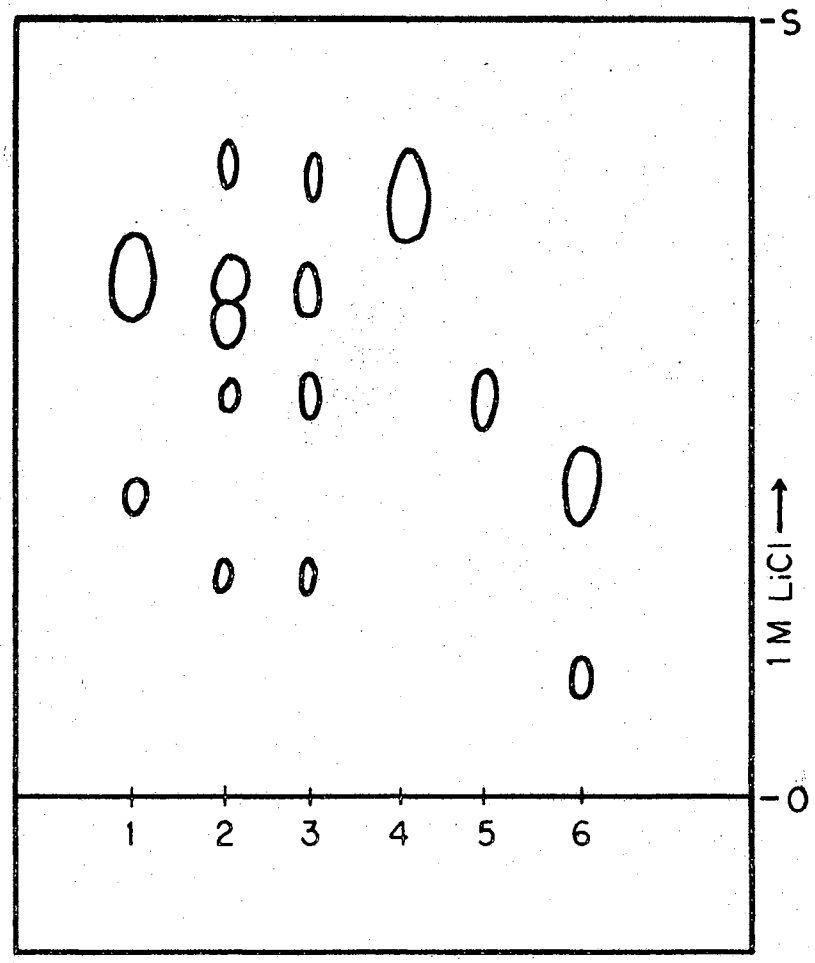

-S
1 M LiCl
-O
1
2
3
4
5
6

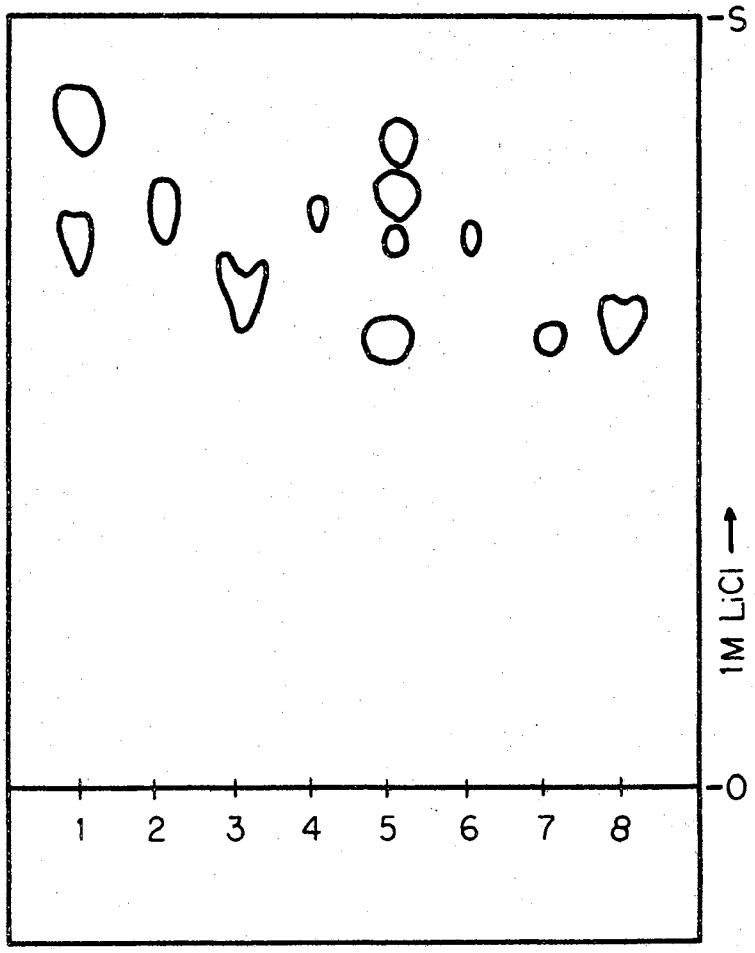
Figure 4

Demonstrations of GDP-Fucose Biosynthesis from GDP-Mannose by A. aerogenes Preparations

GDP-Mannose was incubated for 14 hours at room temperature with A. aerogenes preparations. Nucleotides were adsorbed and eluted from charcoal and separated by PEI thin layer chromatography as shown in the tracing. Lane 1, DPN and TPN; Lane 2, GDP-mannose; Lane 3, GDP-fucose; Lane 4, charcoal adsorbed nucleotides from 0 hour incubation of GDP-mannose with A. aerogenes crude preparation; Lane 5, charcoal adsorbed nucleotides from 14 hour incubation of GDP-mannose with A. aerogenes crude preparation; Lane 6, 0 hour A. aerogenes incubation mixture containing GDP-mannose; Lane 7, 14 hour A. aerogenes incubation mixture containing GDP-mannose; Lane 8, GDP-fucose. Aliquots of the crude incubation mixture without charcoal treatment were spotted in Lanes 6 and 7. Note the appearance of a new spot in Lane 5 having the same mobility as GDP-fucose in Lane 8.

S = Solvent Front

O = Origin


S
O
1M LiCl
1
2
3
4
5
6
7
8

synthesized from GDPM by the Aerobacter aerogenes extracts as previously reported by Ginsburg (5). It was attempted to conclusively demonstrate this conclusion to be true by isolating GDP-fucose by preparative chromatography prior to further characterization of the product. Unfortunately, this was not successful since in the author's hands the solvent used by Ginsburg (5) failed to completely separate unreacted GDP-mannose from the GDP-fucose which formed.

Erythrocyte Experiments. Whole human blood, (type A+) drawn in Acid-Citrate-Dextrose solution, was obtained fresh for use in studies of GDP-fucose biosynthesis by erythrocyte hemolysates. All steps were carried out at 4° unless otherwise stated. The whole blood (150 ml) was centrifuged at 4,000 x g for 20 minutes. The serum was discarded and the buffy coat of white cells was removed from the top of the packed erythrocytes (about 74 ml) by means of a cotton swab. The packed erythrocytes were then washed twice with five volumes of isotonic Tris-HCl buffer, pH 8.0 (0.164 M). Hemolysis of the erythrocytes was accomplished by the addition of three volumes of 1 mM Tris-HCl buffer, pH 7.4, to the packed erythrocytes followed by continuous stirring at 0° for twenty minutes (or until hemolysis was complete as determined with a microscope). The hemolyzed erythrocyte suspension was then centrifuged at 13,000 x g for 30 minutes to separate the stroma. The clear red hemolysate (approximately 100 ml) was carefully decanted from the packed stroma (about 35 ml). The protein concentration in the hemolysate was 152 mg per ml as determined by Biuret assay. Five ml incubation mixtures were prepared in 0.01 M Tris-HCl buffer, pH 8.0, to contain 2.24 μmoles of GDP-mannose, 1.3

μmoles of TPNH, 0.5 μmoles of DPN, 20 μmoles of glucose-6-phosphate, 25 μmoles of $MgCl_2$, 3.75 IUB units of glucose-6-phosphate dehydrogenase (to regenerate TPNH), and 1.5 ml of hemolysate. Incubation was conducted at 23°. Aliquots (1.0 ml) were taken at zero, two, four, six, and eight hours after initiation of the reaction by the addition of hemolysate. As a reagent control, a similar incubation mixture was prepared but which contained no GDP-mannose. Aliquots (1.0 ml) were withdrawn from the reagent control at the same time intervals as for the complete incubation mixture. The reaction was stopped in the aliquots from both reaction mixtures by heating for one minute in a boiling water bath. Denatured protein was removed by centrifugation. Acid-washed Norite A (40 mg) was added to the clear supernatant solutions to adsorb nucleotides. The aliquots containing charcoal were allowed to stand 15 minutes at 0° with occasional shaking and were then centrifuged 30 minutes. The charcoal was washed twice with 5 ml aliquots of water. These washings were discarded. The charcoal was then eluted three times with 2 ml aliquots of ethanol: H_2O: NH_4OH (50:50:0.1). The combined eluants were evaporated to dryness and then resuspended in 1.0 ml of fresh eluant. Last traces of charcoal were removed by centrifugation and the supernatant solution was evaporated to dryness. The residue was dissolved in 0.5 ml water. Fucose assays were performed on 0.2 ml aliquots. Results of these assays were inconclusive. However, PEI-cellulose thin layer chromatography of the nucleotides isolated from the complete incubation mixture clearly showed that a product with an R_f similar to that of GDP-fucose from Aerobacter aerogenes was present. The intensity of

the ultraviolet absorption of this product (determined by visual observation) progressively increased with each time aliquot and was absent in the zero time aliquot (Fig. 5). Attempts to show GDP-fucose formation from GDP-mannose by the same A+ hemolysate, which had been stored frozen (-15°) for two weeks, were unsuccessful. Later attempts to show GDP-fucose biosynthesis by hemolysates of A- and O+ erythrocytes from other donors were not successful.

Another attempt to show GDP-fucose biosynthesis in A+ erythrocytes was made using whole blood obtained from another donor (female). The whole blood was stored overnight at 4° before making the hemolysate. Centrifugation of 20 ml of whole blood at 5,000 x g for 20 minutes gave 9 ml of lightly packed erythrocytes. These were washed twice with two volumes of 0.164 M Tris-HCl buffer, pH 7.4. The washed erythrocytes (about 5 ml of packed cells) were suspended in an equal volume of 0.164 M Tris-HCl buffer, pH 7.4, and ruptured by sonication, using a Bransson Sonifier Model S125 (power setting of 4), for 8-10 seconds. The beaker containing the washed erythrocytes was packed in ice during sonication. Following sonication, the ruptured cell suspension was centrifuged at 30,000 x g for 30 minutes. The precipitate was discarded and the supernatant solution (slightly less than 10 ml) was sued in incubation mixtures similar to those described previously. One 5 ml reaction mixture was prepared to contain GDP-mannose and another 2 ml reaction mixture was prepared without GDP-mannose. Both reaction mixtures contained 0.3 ml of sonicate per ml. Zero time aliquots (1.0 ml) were taken from both reaction mixtures and the reactions were then allowed to incubate for 4 hours at 23°.

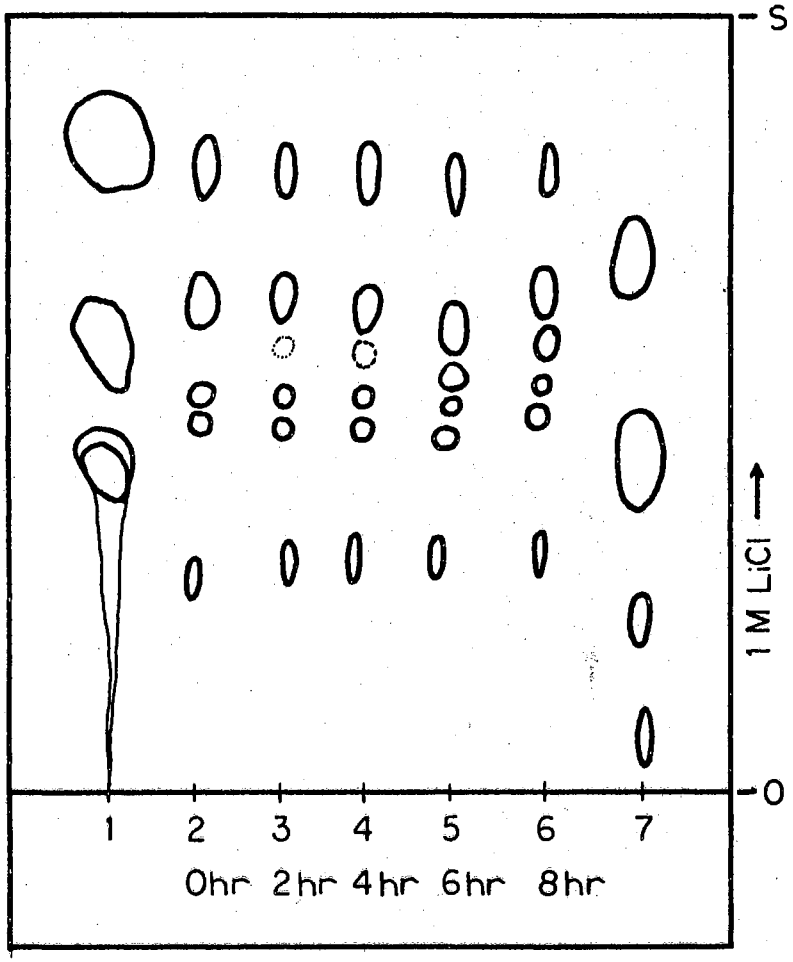
Figure 5

PEI Thin Layer Chromatography of Nucleotides from Serial Two Hour Aliquots of Type A+ Erythrocyte Hemolysate Incubation

GDP-Mannose was incubated with type A+ erythrocyte hemolysate as described in the text. Aliquots were taken every two hours. The nucleotides from each aliquot were adsorbed and eluted from charcoal and then chromatographed by PEI thin layer chromatography as shown. Lane 1, TPNH, TPN, DPN; Lane 2, nucleotides from 0 hour incubation aliquot; Lane 3, nucleotides from 2 hour incubation aliquot; Lane 4, nucleotides from 4 hour incubation aliquot; Lane 6, nucleotides from 8 hour incubation aliquot; Lane 7, GTP, GDP, GMP, GDP-mannose. Incubation of GDP-mannose with erythrocyte hemolysate resulted in the formation of a compound (GDP-fucose) having a mobility slightly less than GDP-mannose and becoming a more prominent spot with each aliquot, indicating that the synthesis of this compound (GDP-fucose) is a function of time.

S = Solvent Front

O = Origin


S
1 M LiCl
O
1
2
3
4
5
6
7
Ohr
2hr
4hr
6hr
8hr

The reaction in all aliquots was stopped by heating in boiling water for one minute. Precipitated protein was removed by centrifugation. Nucleotides were isolated and eluted from charcoal as before. After PEI-cellulose thin layer chromatography of the nucleotide fraction using the previously synthesized GDP-α-L-fucose as a standard, a GDP-fucose spot was detected in the separated nucleotides from the 4 hour incubation mixture which contained GDP-mannose. This spot was not detected in the sample from the reaction mixture which had been incubated in the absence of GDP-mannose. A reproduction of the PEI plate is shown in Fig. 6. The GDP-fucose spot appeared quite strong indicating that a considerable amount had been synthesized during the reaction.

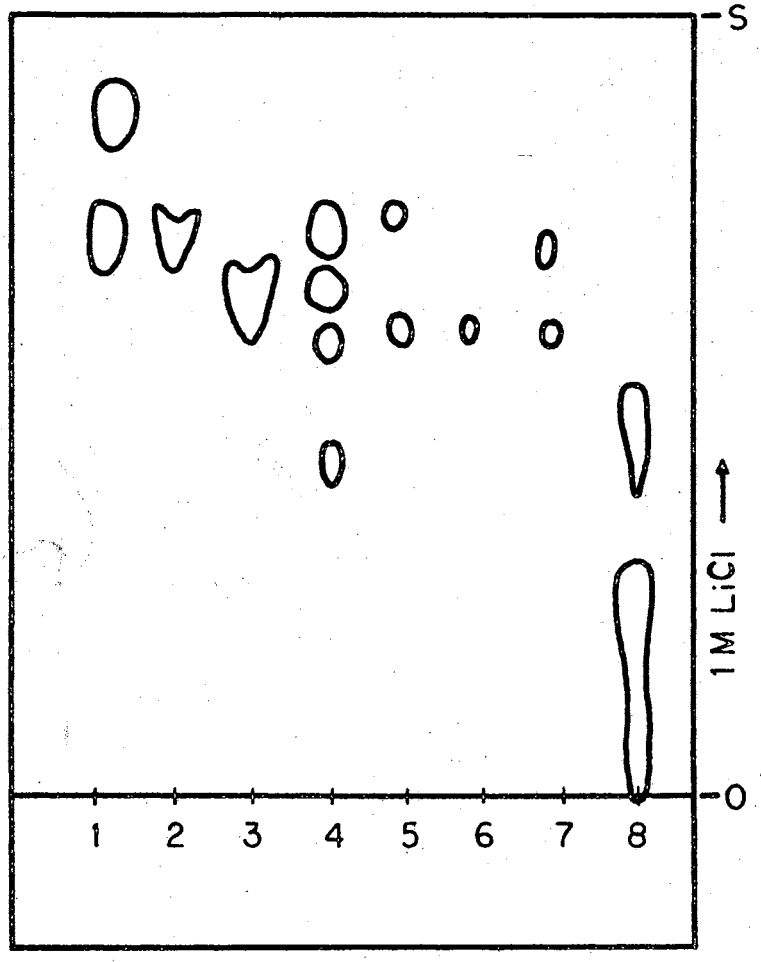
Figure 6

Demonstration of GDP-Fucose Biosynthesis from GDP-Mannose by Type A+ Erythrocyte Proteins

GDP-Mannose was incubated with Type A+ erythrocyte sonicate as described in the text. The nucleotides were adsorbed and eluted from charcoal and then separated by thin layer chromatography on PEI plates as shown. Lane 1, DPN and TPN; Lane 2, GDP-mannose; Lane 3, GDP-fucose; Lane 4, GDP-mannose incubated 4 hours with erythrocyte sonicate; Lane 5, GDP-mannose added to erythrocyte sonicate but not incubated (i.e. 0 time control); Lane 6, erythrocyte sonicate incubated 4 hours in the absence of GDP-mannose; Lane 7, erythrocyte hemolysate not incubated; and Lane 8, GMP and GDP. Incubation of GDPM for 4 hours with erythrocyte sonicate resulted in the formation of a new nucleotide having the mobility of GDP-fucose (Lane 4).

S = Solvent Front

O = Origin


S
1 M LiCl
O
1
2
3
4
5
6
7
8

CHAPTER V

DISCUSSION

One of the goals of the research reported in this thesis was the synthesis of β-L-fucose-1-phosphate which would then be used for the synthesis of GDP-β-L-fucose. In order to synthesize the β anomer of the fucose-1-phosphate the reaction used for the phosphorylation of α-L-fucose tetraacetate would have to give inversion of anomeric configuration. MacDonald's original procedure using a reaction temperature of 50° (45) at first appeared to be the simplest and most direct route for the synthesis of β-L-fucose-1-P since the reaction gave inversion of anomeric configuration for the two sugar acetates used by him. However, characterization of the fucose phosphate obtained by this phosphorylation procedure showed that inversion did not occur when α-L-fucose tetraacetate was phosphorylated. The final product was α-L-fucose-1-phosphate. MacDonald (49) later reported the results of some experiments in which inversion did or did not occur, depending on the sugar acetate used. With the sugars that he studied it was apparent that the product formed by the phosphorylation reaction had the C_1C_2-*cis* configuration regardless of whether the starting material had a C_1C_2-*cis* or C_1C_2-*trans* configuration. Thus the formation of α-L-fucose-1-phosphate, which has a C_1C_2-*cis* configuration, would be expected on the basis of results reported in

MacDonald original procedure employing a reaction temperature of 50° cannot be used for the synthesis of β-L-fucose-1-P which has a C_1C_2-trans configuration.

Another interesting aspect of the phosphorylation reaction at 50° was the finding that it was difficult to phosphorylate sugar acetates having a C_1C_2-cis configuration as do α-D-glucose pentaacetate and α-D-galactose pentaacetate (49). However, α-L-fucose tetraacetate (C_1C_2-cis configuration) did not seem to be particularly difficult to phosphorylate at 50° since a 30% crude yield was obtained. O'Brien (46) reported that the phosphorylation of α-D-glucosamine pentaacetate at a reaction temperature of 83° gave a product which was 40% α (C_1C_2-cis) and 60% β anomer (C_1C_2-trans). Thus, it was felt that the phosphorylation of α-L-fucose tetraacetate at an elevated temperature might give complete inversion of configuration or at least result in a mixture of anomers in the product. However, the product from a recent attempt to phosphorylate α-fucose tetraacetate at 85° could not be purified enough to allow polarimetry or NMR studies which would characterize its anomeric configuration. These results would be interesting indeed if available. Additional attempts to synthesize β-L-fucose-1-phosphate using the elevated reaction temperature would certainly seem worthwhile.

The synthesis of GDP-fucose reported in this thesis was accomplished in a slightly higher yield than reported in the literature for GDP-glucose (55) using a similar micro-scale synthesis. It was presumed that the GDP-fucose synthesized was GDP-α-L-fucose since the

Roseman synthesis (54) used for the condensation of GMP Morpholidate and α-L-fucose-1-P has not been known to give inversion of anomeric configuration.

The chromatographic assays for the biosynthesis of GDP-fucose reported in this thesis were conducted on the assumption that synthetic GDP-α-L-fucose and naturally occurring GDP-β-L-fucose would have the same mobilities in the chromatography system used. This assumption appears to be true since the Aerobacter aerogenes system, which is known to synthesize GDP-β-L-fucose, produced a single new spot after incubation with GDP-mannose. This spot had the same mobility as synthetic GDP-α-L-fucose. These studies justified the use of GDP-α-L-fucose as a standard in the erythrocyte studies.

The PEI-cellulose chromatography of the nucleotides derived from erythrocyte preparations incubated with GDP-mannose showed a single new ultraviolet absorbing spot. This spot had the same mobility as the synthetic GDP-α-L-fucose. The author feels that the GDP-fucose synthesized by erythrocyte preparations was probably GDP-β-L-fucose (as it was for Aerobacter aerogenes) since GDP-α-L-mannose was the substrate for both the erythrocyte and bacterial systems.

The significance of GDP-fucose biosynthesis by erythrocytes seems worthy of further investigation, particularly since fucose is an important determinant of the antigenic activity of BGS. To demonstrate the incorporation of the fucosyl moiety of biosynthesized GDP-fucose into erythrocyte BGS polysaccharide would indeed by interesting.

SUMMARY

The phosphorylation of α-L-fucose tetraacetate with fused anhydrous phosphoric acid at 50° for eight hours gave a 20% yield of purified fucose-1-phosphate. Polarimetry studies showed the product to have a molar rotation of -33,100°. NMR spectra showed a peak at 5.89 ppm which is characteristic of the α-anomer. It was concluded from these data that the product was α-L-fucose-1-phosphate.

GDP-α-L-fucose was synthesized by the condensation of GMP morpholidate with the tri-n-butylammonium salt of α-L-fucose-1-phosphate. The yield was 25.9% based on fucose phosphate.

Biosynthesis of GDP-fucose from GDP-α-D-mannose by type A+ erythrocyte preparations was shown using the synthetic GDP-α-L-fucose as a chromatography standard. PEI-cellulose thin layer anion exchange chromatography was used as the assay technique.

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