BBA 66432

SYNTHESIS OF D-RIBITOL 5-PHOSPHATE BY AN INORGANIC PYROPHOSPHATE–RIBITOL PHOSPHOTRANSFERASE ACTIVITY OF MICROSOMAL GLUCOSE-6-PHOSPHATASE

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(Received June 14th, 1971)

SUMMARY

1. Liver microsomal preparations have been shown to catalyze the stereospecific synthesis of a ribitol phosphate from ribitol and inorganic pyrophosphate (PP_i).

2. The compound formed has been isolated as a lithium or barium salt and identified as D-ribitol 5-phosphate (L-ribitol 1-phosphate). Thus the enzyme activity is specific for that primary hydroxyl group of ribitol which corresponds, from the viewpoint of the *meso* C atom at C-3, to that hydroxyl group of glycerol which is similarly phosphorylated to give glycerol 1-phosphate (D-α-glycerophosphate).

3. The ribitol phosphate produced from ribitol with ATP and the L-ribulokinase of *Aerobacter aerogenes* has been identified as D-ribitol 5-phosphate.

4. Glucose is a competitive inhibitor for the synthesis of ribitol phosphate by PP_i–ribitol phosphotransferase activity, while ribitol inhibits the synthesis of glucose 6-phosphate non-competitively.

5. The hydrolysis of PP_i, which proceeds simultaneously with synthetic phosphotransferase activity, is accelerated by ribitol, as by glycerol, whereas it is inhibited greatly by glucose and slightly by ribose.

6. The four pentitols, under identical conditions, serve as acceptors for phosphotransferase activity from PP_i at varying rates: D-arabitol > ribitol > L-arabitol > xylitol.

7. In the livers of fasted and alloxan diabetic rats the levels of enzyme activity are greatly elevated over those found in normal fed animals. *In vitro* the enzyme is activated by pretreatment of microsomal preparations by NH_4OH or by detergents. These properties, as well as the kinetics of the enzyme reaction, support the conclusion that PP_i–ribitol phosphotransferase activity is a property of the same particulate, membrane bound enzyme which is responsible for glucose-6-phosphatase and PP_i–glucose (or glycerol) phosphotransferase activities.

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INTRODUCTION

Microsomal fractions of liver and kidney are capable of utilizing PP_i for the phosphorylation of glucose, glycerol and a wide variety of sugars, sugar alcohols and related compounds[1]. The presence of a free primary alcohol group in the molecule is a requirement for an effective acceptor compound in this reaction. In the case of glycerol, with a "meso" carbon atom and having two primary alcohol groups available for phosphorylation, it has been shown that it is specifically the primary alcohol group on C-1 (stereospecific numbering[2]) that is phosphorylated by the microsomal phosphotransferase and PP_i. The product in this case is glycerol 1-phosphate (D-α-glycerophosphate) in contrast to the more commonly occurring glycerol 3-phosphate (L-α-glycerophosphate), which is produced by the action of glycerol kinase (ATP:glycerol phosphotransferase, EC 2.7.1.30). From the point of view of the *meso* carbon atom (C-3) of ribitol the primary alcohol groups at C-1 and C-5 are analogous to those of C-1 and C-3 of glycerol. In this paper we report the results of a study of the enzymatic phosphorylation of ribitol, some of the properties of the enzyme and the isolation and characterization of the product formed.

METHODS

Enzyme preparation and assay

Rat liver microsomes were the source of the enzyme used. Preparation of the animals, the liver microsomal fractions, activation of the enzyme with NH_4OH and assays of glucose-6-phosphatase and PP_i–glucose phosphotransferase were by methods previously described[3].

PP_i–ribitol phosphotransferase activity was determined essentially by the isotope method developed for acceptor specificity studies of phosphotransferase activity[1] and for a study of PP_i–glycerol phosphotransferase activity[4]. Enzymatic reactions run with ribitol and radioactive $^{32}PP_i$ were stopped by the addition of trichloroacetic acid. Aliquots (2 or 4 μl) of deproteinized reaction mixture were spotted on chromatographic paper and developed by descending chromatography at 3° with methanol–NH_4OH–water (6:1:3, by vol.) to separate the organic phosphate compound formed from radioactive P_i and PP_i. It was found that ribitol phosphate migrated to a distance intermediate between glucose 6-phosphate and glycerol 1-phosphate, *i.e.* 26 cm *vs.* 24 and 28 cm, respectively, in 18 h. The radioactivity in the clearly defined product area, located by means of a paper strip scanner, was measured by liquid-scintillation counting. The quantity of ribitol phosphate formed was calculated by comparison with the quantity of glucose 6-phosphate, measured both by incorporation from the same sample of radioactive PP_i and by reduction of $NADP^+$ in the presence of glucose 6-phosphate dehydrogenase.

RESULTS

Conditions for accumulation of synthetic ribitol phosphate prior to isolation

Ribitol phosphate formation under various conditions was studied by following the accumulation of stable, organic-bound phosphorus. The difference between the total phosphorus and the sum of the free and easily hydrolyzable phosphorus of the

enzymatic reaction mixtures was measured. Alternatively radioactive PP_i was used to follow the phosphorylation. Using the favorable substrate concentrations of 2 M ribitol and 0.08 M PP_i suggested by the kinetic studies, it was found that a maximum yield of ribitol phosphate was obtained with NH_4OH-activated microsomal preparations in a 4-h incubation at 30° and pH 5.2. Under these conditions the yield of ribitol phosphate was about 25% based on the quantity of PP_i added. This corresponded to only a 1% yield based upon the high concentration of ribitol (2 M) used but the excess ribitol could be recovered and reused in the preparation of ribitol 5-phosphate.

Glucose-6-phosphatase and its related enzyme activities have been found to be remarkably active at low temperatures[5]. Ribitol phosphate synthesis by the same enzyme fraction was also found to proceed well at 0°. About 4 days at 0° were required to produce approximately the same quantity of ribitol phosphate as was formed in 4 h at 30°. Overall yields were somewhat lower with untreated microsomes than with NH_4OH-pretreated preparations.

Isolation of ribitol phosphate from enzymatic synthesis

A small amount of radioactive ribitol phosphate was prepared and purified for use as a label in the process of isolation of the larger amount of material needed for characterization of the compound. Synthetic radioactive ribitol phosphate, formed enzymatically from $^{32}PP_i$, was separated from excess ribitol, and from $^{32}P_i$ and $^{32}PP_i$ by chromatography on large sheets of Whatman No. 1 paper developed for 28 h at 0° with CH_3OH–NH_4OH–water (6:1:3, by vol.). Radioactive ribitol phosphate was eluted from the appropriate areas of the dried paper by means of very dilute NH_4OH. The eluate, free of P_i, was concentrated *in vacuo* at a temperature below 30°.

In a typical preparation, non-isotopic ribitol phosphate was synthesized on a larger scale by incubation of 12.16 g ribitol (2 M) in 40 ml of 0.08 M sodium pyrophosphate in acetate buffer of pH 5.2 with a liver microsomal preparation which had been activated with NH_4OH. After incubation at 30° for 4 h, the enzymatic reaction was stopped by heating the mixture at 100° for a few minutes, and the coagulated protein was removed by filtration. The estimated yield of ribitol phosphoric acid, based on stable, organic-bound phosphate formed, was about 200 mg.

After the addition of a portion of the solution of radioactive synthetic ribitol phosphate as a marker, the filtrate was passed through a column of Dowex AG 1-X2 anion exchange resin in the acetate form. The labeled compound, located by means of a monitor Geiger counter, was retained in the upper half of the column. The column was washed well with water to remove cations and ribitol and the adsorbed anions were eluted with 1 M ammonium acetate. The eluate was collected in 2-ml aliquots which were analyzed for P_i and for radioactivity to locate the ribitol phosphate. Ribitol phosphate was found concentrated in the earlier tubes, with some overlap with P_i in intermediate tubes. In subsequent preparations, carried out by the same method, the radioactive label compound was omitted.

The contents of those tubes containing the bulk of ribitol phosphate were combined for further purification. An excess of barium acetate was added and the precipitated $Ba_3(PO_4)_2$ was filtered off and washed well with water. Cations (Ba^{2+} and NH_4^+) were removed by passing the solution through a column of Dowex AG 50W-X8 (H^+ form) resin and the solution was cautiously evaporated to dryness several times *in vacuo* below 30° with the addition of water to remove acetic acid. The

lithium salt of the product proved more satisfactory for purification than the barium salt. Therefore the residue, taken up in a few ml of water, was made slightly alkaline with LiOH and 3 vol. of ethanol were added to precipitate the lithium ribitol phosphate which was purified by solution and reprecipitation with ethanol. 165 mg of the dilithium salt of ribitol phosphate were obtained.

Samples of the product were spotted on Whatman No. 1 paper, developed with *n*-propanol–NH_4OH–water (6:3:1, by vol.) by descending chromatography and visualized by the periodate–Schiff reagent[6]. The isolated enzymatically prepared material exhibited a single spot which had an R_F value (0.25) identical with that of synthetic known D-ribitol 5-phosphate (Sigma).

Characterization of enzymatically synthesized ribitol phosphate

Analysis of the isolated anhydrous dilithium salt (Schwarzkopf Microanalytical Laboratory) was as follows: Found: C, 23.7; H, 5.2; P, 12.4; Li, 5.5. $Li_2C_5H_{11}PO_8$ (244) requires C, 24.6; H, 4.5; P, 12.7; Li, 5.7%. Optical rotation, $[\alpha]_D = -4.2°$, observed for a 2.6% solution of lithium salt in water. Values ranging between $-3.9°$ and $-5.2°$ were obtained on different preparations of isolated lithium or barium ribitol phosphate. Optical rotation measurements were carried out on a Perkin–Elmer Polarimeter, Model 141. The only reported specific rotation for D-ribitol 5-phosphate which we were able to find in the literature was that of LEVENE *et al.*[7], who in 1934 analyzed the compound formed by a platinum-catalyzed reduction of their synthetic ribose 5-phosphate. The optical rotation in that case was observed on a 3.7% aqueous solution of sodium salt prepared by the removal of barium from a solution of barium ribitol phosphate by means of Na_2CO_3. They reported $[\alpha]_D^{22} = -8.9°$, calculated as the sodium salt. Other workers, who have described the synthesis or isolation of the compound[6,8–10], have not reported specific rotation values but used enzymatic methods for identification.

In view of the discrepancy between our observed rotation and that of LEVENE *et al.*[7], and because of the known tendency of polyol phosphates to undergo phosphate migration and cyclization when heated in acid solution[8,11,12], it was necessary, for positive identification, to compare the rotation of the isolated compound with that of known D-ribitol 5-phosphate prepared, isolated, and measured in a variety of ways.

D-*Ribitol 5-phosphate prepared by catalytic reduction of* D-*ribose 5-phosphate*

The starting material for catalytic reductions was a disodium salt of D-ribose 5-phosphate (Sigma). $[\alpha]_D = +16.2°$, found, determined using a 2.5% aqueous solution and calculated as the anhydrous sodium salt. Literature values[13]: $[\alpha]_D = +16.09°$ (natural) and $+16.54°$ (synthetic).

Borohydride reduction, isolation and purification of the ribitol phosphate as a barium salt were carried out as described by BADDILEY *et al.*[6]. The rotation, measured on a 2.5% aqueous solution of sodium salt, prepared by removal of barium with Na_2CO_3, was $[\alpha]_D = -4.1°$. The product of a borohydride reduction, isolated as a lithium salt by the procedure which we used for isolation of the enzymatic product, gave $[\alpha]_D = -5.1°$.

Since the isolation methods used involved purification steps in which acidic solutions were cautiously evaporated *in vacuo*, it seemed desirable to prepare a sample by a method which avoided such steps. The barium salt of D-ribose 5-phos-

phate was reduced with H_2 and Adams catalyst and barium ribitol phosphate was isolated exactly as described by LEVENE *et al.*[7]. Found: P, 8.3. Calculated: P, 8.4. $[\alpha]_D = -4.4°$, calculated as sodium salt, in contrast to the value of $-8.9°$ recorded by LEVENE in 1934. In another platinum-catalyzed hydrogenation, using disodium D-ribose 5-phosphate, the product was converted by means of Dowex AG 50W-X8 (Li^+ form) resin to a lithium salt and purified by reprecipitation from water with alcohol. Found: P, 12.5. Calculated: P, 12.7. $[\alpha]_D = -4.9°$, measured on a 3.5% aqueous solution of dilithium salt, pH 10.7.

The specific rotation of our enzymatically synthesized ribitol phosphate agrees well with that found for authentic D-ribitol 5-phosphate.

We are at a loss to explain the discrepancy between our observed rotations and those of LEVENE *et al.* but, in the course of studying it, we noted the appreciable effect of pH on the rotation of ribitol phosphate. For example, a solution of the dilithium salt, exhibiting an $[\alpha]_D = -4.9°$ at pH 10.7, adjusted with HCl, gave $[\alpha]_D = -4.3°$ at pH 7.0 and $[\alpha]_D = +2.1°$ at pH 2.4. This same solution, after being kept at pH 2.4 for several hours, was readjusted to about pH 11 and the negative rotation was restored, $[\alpha]_D = -5.0°$. It thus appears unlikely that cautious exposure to acidic conditions during the isolation procedure would have significantly changed the product.

Thus the product isolated from the microsomal enzyme catalyzed phosphorylation of ribitol with PP_i is identical with known D-ribitol 5-phosphate. The enzyme specifically catalyzes the phosphorylation of one of the two primary alcohol groups of ribitol.

Ribitol phosphate from ribitol, ATP and L-ribulokinase of Aerobacter aerogenes

Since it had been found that the two enzymes, mammalian PP_i–glycerol phosphotransferase and glycerol kinase (ATP–glycerol phosphotransferase), specifically catalyze the phosphorylation of different primary alcohol groups of glycerol, it seemed of interest to determine if this were also the case for phosphorylation of the primary alcohol groups of ribitol. Unfortunately the two corresponding enzymes for ribitol phosphorylation are not available from the same species. SIMPSON AND WOOD[14] observed that ribitol could be phosphorylated by the L-ribulokinase of *A. aerogenes* but, at that time, the configuration of the product was not determined.

We are indebted to Jean D. Deupree of the laboratory of W. A. Wood, Department of Biochemistry, Michigan State University, for preparing and supplying us with a sample of crude barium ribitol phosphate. She used an *A. aerogenes* PRL-R3 mutant, lacking L-ribulose 5-phosphate 4-epimerase, to phosphorylate ribitol with ATP in the presence of $MgCl_2$, NaF, glutathione and EDTA by the procedure of ANDERSON[15]. The product was converted to a barium salt and precipitated, along with the adenine nucleotides and inorganic salts, by means of ethanol. We received 700 mg of the crude barium salt mixture. Preliminary analysis indicated that the sample contained at least 35% adenine nucleotides and an appreciable amount of inorganic material. That portion which was soluble in water was fractionated by differential ethanol precipitation, removing the bulk of the adenine nucleotides. The remainder of the nucleotides were removed by adsorption on charcoal[16] by four successive treatments of a dilute aqueous solution with charcoal until no significant amount of 260 nm absorbing material remained. The barium ribitol phosphate, after

purification by several reprecipitations from water with ethanol, was converted to the lithium salt by means of a cation resin with LiOH. 103 mg of reprecipitated lithium ribitol phosphate were obtained. Found: P, 12.3. Calculated: P, 12.7. $[\alpha]_D = -4.9°$.

Thus the ribitol phosphate formed from ATP and ribitol by L-ribulokinase of *A. aerogenes* is D-ribitol 5-phosphate, the same compound as is formed by the PP_i–ribitol phosphotransferase of liver and by catalytic reduction of known D-ribose 5-phosphate.

Effect of substrate concentration on rate of phosphotransferase reaction

The rate of formation of ribitol phosphate was studied as a function of ribitol

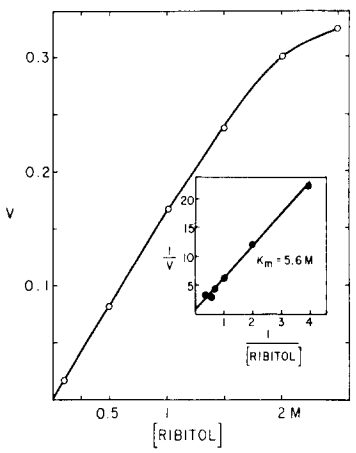

Fig. 1. Effect of ribitol concentration on rate of formation of ribitol phosphate. The $^{32}PP_i$ concentration was constant at 0.05 M, and the ribitol was varied between 0.1 and 2.5 M. The enzymatic reactions were incubated at 30° for 10 min in a mixed acetate and cacodylate buffer at pH 5.2 with liver microsomal enzyme which had been activated by pretreatment with NH_4OH (refs. 17 and 3). The quantity of ribitol phosphate formed was measured by the isotope procedure described under METHODS. v = μmoles of ribitol phosphate formed per min per mg of protein.

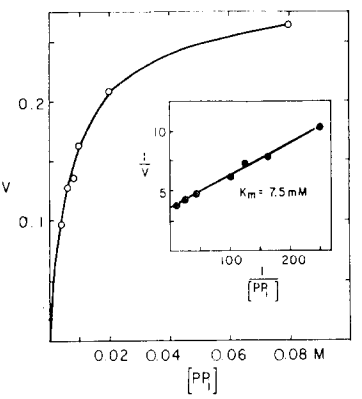

Fig. 2. Effect of PP_i concentration on rate of ribitol phosphate formation. $^{32}PP_i$ was varied between 0.004 and 0.08 M in acetate–cacodylate buffer (pH 5.3) with the ribitol concentration constant at 2 M. The enzyme and assay methods were similar to those of the experiment of Fig. 1. v = μmoles of ribitol phosphate formed per min per mg of protein.

concentration, with PP_i constant at 0.05 M (Fig. 1). Very high concentrations of the sugar alcohol were required to begin to saturate the enzyme site and, as was the case with glycerol, the reaction rate was nearly linearily related to the acceptor concentration up to about 2 M. The apparent K_m obtained from a double-reciprocal plot of the data was about 5.6 M.

When the PP_i concentration was varied, with ribitol constant at 2 M, the results shown in Fig. 2 were obtained. The apparent K_m for PP_i in the PP_i–ribitol phosphotransferase reaction was 7.5 mM, close to the value observed when glucose[5] or glycerol[4] was the acceptor compound.

Effect of acceptor compounds on the hydrolysis of inorganic pyrophosphate

The microsomal enzyme preparation catalyzes the simultaneous occurrence of the transferase and hydrolase reactions of inorganic pyrophosphate:

$$PP_i + \text{acceptor} \rightarrow \text{acceptor–phosphate} + P_i \quad (1)$$

$$PP_i + H_2O \rightarrow 2\ P_i \quad (2)$$

From the quantities of total inorganic phosphate and of acceptor–phosphate formed, the quantities of PP_i used in Reactions 1 and 2 can be calculated.

In earlier studies[4], in which glucose and glycerol were found to be equally effective transferase acceptors (Reaction 1), increasing concentrations of glycerol or glucose had strikingly different effects on the pyrophosphate hydrolysis (Reaction 2). With increasingly higher concentrations of glucose the hydrolysis of PP_i was progressively inhibited so that as much as 90% of the PP_i utilization was in the transferase

TABLE I

EFFECT OF RIBITOL CONCENTRATION ON THE PP_i-PHOSPHOTRANSFERASE AND PP_i-HYDROLASE REACTIONS

Reaction mixtures, with constant $^{32}PP_i$ (0.05 M) and different concentrations of ribitol in cacodylate buffer at pH 5.2, were incubated for 10 min at 30° with NH_4OH-activated microsomal enzyme containing about 1 mg of protein per ml. Total P_i formed was determined by the Fiske–Subbarow method and ribitol phosphate formation by the isotope method.

Ribitol concn. (M)	*P_i (a) (μmoles/min per mg protein)*	*D-Ribitol 5-phosphate (b) (μmoles/min per mg protein)*	*Transfer* (%)*
2.5	1.026	0.324	48
2.0	1.090	0.300	43
1.5	1.030	0.236	37
1.0	0.931	0.164	30
0.5	0.810	0.082	17
0.25	0.750	0.044	10
0.10	0.690	0.017	5
0	0.654	0	0

* Percentage transfer $= \dfrac{\mu\text{moles organic P formed (b)}}{\mu\text{moles } PP_i \text{ reacting } \left(\dfrac{a + b}{2}\right)} \times 100.$

reaction[4,5]. In contrast, increasingly higher concentrations of glycerol had a stimulating effect on PP_i hydrolysis. This different effect of glucose and glycerol was observed also when the reactions were studied as a function of PP_i concentrations, pH, or time as well as of acceptor concentration[4]. In the present experiment ribitol reacted in virtually the same manner as glycerol (Table I). With increasing ribitol concentration the percent transfer progressively increased, but the quantity of total P_i formed indicates that the simultaneous hydrolytic reaction was also stimulated. Ribose was about half as effective an acceptor as ribitol at each concentration level and did not stimulate PP_i hydrolysis. Fig. 3 shows the different effects of the sugars and sugar alcohols on Reaction 2. In this experiment PP_i hydrolysis in the absence of any acceptor other than water was compared with the calculated values obtained in the presence of increasing concentrations of the acceptor compound determined at the same time with the same enzyme preparation. High concentrations of glucose, the most efficient acceptor compound studied, almost completely block the hydrolysis of PP_i by the microsomal enzyme preparation. Ribose, while acting as a fair acceptor compound, had only a slightly inhibitory effect on the pyrophosphatase. The alcohols, glycerol and ribitol, stimulated PP_i hydrolysis.

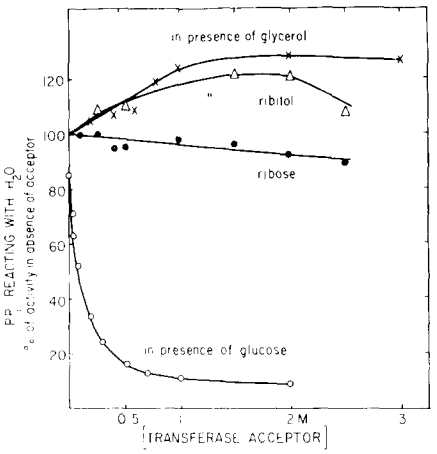

Fig. 3. Effect of transferase acceptor concentration on microsomal inorganic pyrophosphatase reaction. The PP_i reacting with water in the presence of different concentrations of glucose, ribose, ribitol or glycerol was calculated. PP_i reacting with water = total P_i formed (in hydrolase + transferase reaction) *minus* sugar phosphate or pentitol phosphate formed (in transferase reaction) divided by 2. In each case PP_i hydrolysis in the absence of an acceptor other than water was measured with the same enzyme preparation and the results were expressed as percentage of this value.

Inhibition of PP_i–glucose phosphotransferase activity by ribitol and of PP_i–ribitol phosphotransferase activity by glucose

The nature of the inhibition of glucose 6-phosphate formation by ribitol and of ribitol 5-phosphate formation by glucose was studied in a series of experiments carried out with identical $^{32}PP_i$ concentrations, pH, enzyme and assay conditions. Ribitol was constant at 0, 0.5, 1 and 2 M while glucose concentrations were varied between 0 and 0.4 M. The quantities of glucose 6-phosphate and of ribitol 5-phosphate, simultaneously formed, were measured. Each acceptor was considered both as a substrate and an inhibitor. The reaction rates, plotted as a function of substrate concentration at different constant levels of inhibitor, are given in the inset graphs of Figs. 4 and 5. From double-reciprocal plots it can be seen that glucose acts as a competitive inhibitor of ribitol in the formation of ribitol phosphate (Fig. 4) while the ribitol inhibition of glucose 6-phosphate synthesis appears to be noncompetitive (Fig. 5). These as yet unexplained results are similar to those obtained in an experiment in which glucose and glycerol concentrations were simultaneously varied in a microsomal PP_i–phosphotransferase reaction[4]. In that case glucose was a strictly competitive inhibitor of glycerol 1-phosphate synthesis while glycerol affected the formation of glucose 6-phosphate from PP_i in a noncompetitive fashion. From Dixon plots[18] of the ribitol–glucose data approximate K_i values of 0.1 M for glucose and 1.7 M for ribitol were determined.

Comparison of phosphorylation of sugars and corresponding sugar alcohols: effect of configuration

The relative ease with which primary alcohol groups are phosphorylated by PP_i and microsomal enzyme preparations varies greatly with the nature of the acceptor[1]. A series of pentoses, hexoses and their corresponding pentitols and hexitols

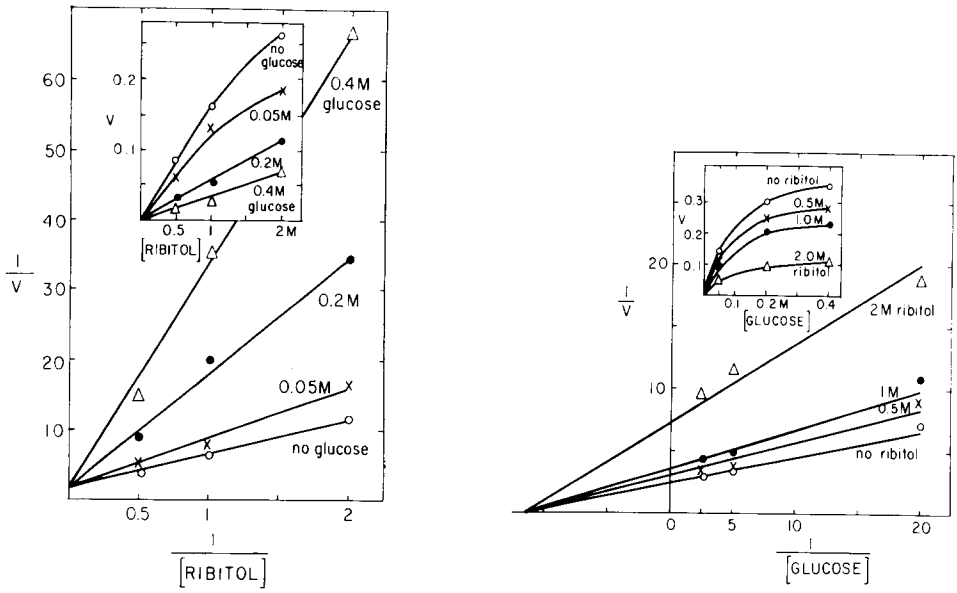

Fig. 4. Inhibition of liver PP_i–ribitol phosphotransferase activity by glucose. $^{32}PP_i$ was constant at 0.05 M while ribitol was varied between 0 and 2 M, and glucose was varied between 0 and 0.4 M at each ribitol concentration. Enzymatic reactions were carried out for 10 min at 30° in acetate buffer at pH 5.2 with an NH_4OH-activated liver microsomal enzyme preparation. Reaction was stopped by heating at 100° for 3 min. The sum of ribitol phosphate and glucose phosphate formed was determined by measuring the incorporation of ^{32}P into the organic phosphate compounds formed. Glucose 6-phosphate formed was measured by reduction of $NADP^+$ with glucose 6-phosphate dehydrogenase. Ribitol phosphate formed was determined by difference. v = μmoles of ribitol phosphate formed per min per mg protein.

Fig. 5. Inhibition of liver PP_i–glucose phosphotransferase activity by ribitol. The experiment is described in the legend to Fig. 4. v = μmoles of glucose 6-phosphate formed per min per mg of protein.

were compared as acceptors using conditions roughly optimal for glucose phosphorylation. Results are given in Table II. Seven of the eight pentose isomers and all of the four corresponding pentitols were available. Pentoses of the ribose or arabinose configuration were phosphorylated by the PP_i–enzyme system but those of the xylose or lyxose configuration were not. In each case the sugar alcohol was a better acceptor than was the corresponding pentose. Reduction of the aldehyde group of D- and L-xylose and of D- and L-lyxose converts these inactive compounds into excellent acceptors. Ribitol was about twice as effective, and D-arabitol and L-arabitol about 7 times as effective, as the corresponding aldoses under the conditions studied. In contrast, for all of the 6-C compounds studied, reduction of the aldehyde group produced an alcohol which was a less effective acceptor than was the corresponding hexose. In all cases both in this and in the earlier study[1], the enzyme preferred the D- to the L-configuration of the acceptor compound.

Since, in the experiment of Table II, no attempt was made to use optimum concentrations of substrate, it was of interest to find that the relative positions of the four pentitols with respect to ease of phosphorylation was unchanged at different acceptor concentrations (Fig. 6). When phosphorylation was studied as a function of

TABLE II

ACCEPTORS IN PP_i-PHOSPHOTRANSFERASE REACTION

Reactions were run for 10 min at 30° with 1.0 M acceptor compound, 0.05 M radioactive pyrophosphate in acetate buffer (pH 5.2) and microsomal enzyme activated with NH_4OH. The quantity of sugar phosphate or sugar alcohol phosphate formed was determined by the isotope procedure described in METHODS. Values are given relative to the quantity of glucose 6-phosphate formed (taken as 100) with the same enzyme at the same time but with an optimum concentration of glucose (0.4 M). The values for D- and L-galactose and galactitol are taken from an earlier study[1].

Values represent phosphorylation relative to glucose 6-phosphate formation.

Pentoses		*Corresponding pentitols*	
D-Ribose	21	Ribitol	44
D-Xylose	1	Xylitol	12
L-Xylose	0		
D-Arabinose	13	D-Arabitol	86
L-Arabinose	2	L-Arabitol	14
D-Lyxose	0		
L-Lyxose	0		
Hexoses		*Corresponding hexitols*	
D-Mannose	136	D-Mannitol	32
D-Galactose	43	Galactitol	10
L-Galactose	23		
D-Glucose (1 M)	92	D-Sorbitol	24
D-Glucose (0.4 M)	100		

acceptor concentration with constant PP_i, pH and enzyme preparation, D-arabitol was a more effective acceptor than ribitol, while L-arabitol and xylitol were much less effective. The apparent K_m for D-arabitol from this data was 1.7 M. The specific activity of the enzyme in the phosphorylation of D-arabitol at nearly optimum substrate concentration (> 2 M) was approximately equal to that for phosphorylation of optimal concentrations of glucose (0.4 M) or glycerol (> 2 M) (ref. 4). v_{max} in all three cases, with activated enzyme preparations, was about 0.4–0.5 μmoles of phosphorylated product formed per min per mg of microsomal protein.

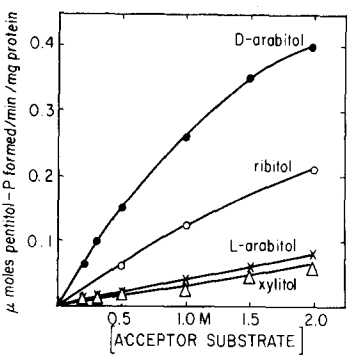

Fig. 6. Pentitol phosphate formation from PP_i by transferase activity, as a function of acceptor concentration. $^{32}PP_i$ was constant at 0.05 M and the reactions were run at the same time with the same microsomal enzyme preparation, activated with NH_4OH, and different concentrations of the sugar alcohols. The quantity of pentitol phosphate formed was determined by the isotope method.

TABLE III

PP_i–RIBITOL PHOSPHOTRANSFERASE ACTIVITY OF LIVER MICROSOMES OF VARIOUSLY TREATED RATS

Enzymatic assays were carried out at 30° using 0.04 M radioactive PP_i, 2 M ribitol, 0.1 M acetate buffer (pH 5.2) and aliquots of microsomal preparations equivalent to approx. 1 mg of protein per ml of reaction mixture. NH_4OH pretreatment consisted of exposure of enzyme preparations containing about 10 mg protein per ml to 0.1 M NH_4OH for 15 min at 30° (pH about 9.8). Values reported are averages of those obtained using three individual rats in each group.

Condition of rats	*Microsome fraction*	*Enzyme activity (μmoles ribitol phosphate formed per min per mg protein)*		*Activation (%)*
		Untreated	*NH_4OH preteated*	
Normal, fed	Total	0.113	0.241	113
Normal, fasted	Total	0.183	0.416	127
Diabetic, fed	Total	0.239	0.834	249
	Rough	0.349		
	Smooth	0.219		

Elevation of PP_i–ribitol phosphotransferase activity in livers of fasted and diabetic animals

The rates of synthesis of ribitol 5-phosphate by liver microsomes of normal fed, normal 24 h fasted and alloxan-diabetic rats were compared (Table III). The specific activity of the enzyme was elevated by about 60% in fasted and by more than 100% in diabetic, compared with normal fed animals. Pretreatment of the microsomes *in vitro* with NH_4OH under optimal conditions resulted in an enzyme activation of more than 100% for fed and fasted and about 250% for the diabetic animals. In these parameters the phosphorylation of ribitol closely paralleled that of glucose[19,3] and of glycerol[3]. When unactivated rough and smooth microsomal subfractions, prepared by differential centrifugation[20], were the source of the enzyme, the specific activity of the PP_i–ribitol phosphotransferase was found to be higher in the rough than in the smooth membranes. This is in accord with the results obtained when the glucose 6-phosphatase and PP_i–glucose phosphotransferase activities of rough and smooth preparations were studied[21].

DISCUSSION

The evidence presented supports the hypothesis that the enzyme responsible for the phosphorylation of ribitol and the other pentitols studied is probably identical with microsomal glucose-6-phosphatase. This membrane-bound, phospholipid-dependent enzyme has an acid pH optimum and no requirement for added cofactors. Once pictured as relatively specific in its catalysis of glucose 6-phosphate hydrolysis, it has been shown to be rather non-specific both with respect to the phosphoryl donor and the phosphoryl acceptor compounds with which it will react. Among the compounds which have been shown to be, in varying degrees, active donors in the phosphorylation of glucose by this crude microsomal membrane enzyme are glucose 6-phosphate[22,23], PP_i (refs. 5,24,26), various nucleoside tri- and diphosphates[27], phosphoramidate[28], and carbamyl phosphate[29].

Whereas the principal acceptor for the transfer of P_i by the enzyme is probably normally water, a large number of other compounds have been found to be adequate as acceptors in the phosphotransferase reaction. In a direct study of the enzymatic synthesis of sugar and sugar alcohol phosphates with radioactive PP_i we found that most hexoses, pentoses and heptoses and their derivatives serve, in varying degrees as acceptors[1]. The present study confirms and reinforces our earlier conclusions[1] as to the reacting groups and configurations favorable for the binding of acceptor substrates to enzyme in the PP_i phosphotransferase reaction. While the principal requirement for an acceptor molecule is the occurrence of a primary alcohol group, the configuration of the remainder of the molecule is of great importance in determining the relative ease of phosphorylation. The phosphotransferase even differentiates between the two seemingly identical primary alcohol groups of glycerol[4] and ribitol. In all cases studied, compounds with the D-configuration on the penultimate carbon atom were more efficient acceptors than their corresponding L-antipodes. This was true for galactose, altrose and arabinose studied earlier[1] and is now seen to apply to the corresponding reduction products, D- and L-arabitol. The primary alcohol group of ribitol which is preferentially phosphorylated by PP_i and the microsomal enzyme is that which is adjacent to the C atom with the D-configuration in the corresponding pentose. The qualitative and roughly quantitative similarity between the relative ease of phosphorylation of specific acceptor compounds and the rates of hydrolysis of the corresponding phosphorylated compounds has been discussed in our earlier paper[1].

ACKNOWLEDGMENT

This work was supported by grant AM-07279 from the National Institutes of Health, U.S. Public Health Service.

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