Inhibition of lipid-linked oligosaccharide synthesis by aryl phosphates

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(Received 15 September 1980/Accepted 24 November 1980)

Our previous work has shown that phenyl phosphate acts as an exogenous substrate for GDP-mannose:dolichyl phosphate mannosyltransferase in rat liver microsomal fractions to give rise to phenyl phosphate β -D-mannose, a compound which, unlike Dol-P-Man (dolichyl phosphate β -D-mannose), cannot act as mannose donor for further mannose-adding reactions in microsomal fractions. The study has now been extended to the action of various aryl phosphates and structurally related compounds on several other glycosyltransferase systems in the microsomal fractions. (1) Examination of the ability of these compounds to accept sugars from various sugar nucleotides indicated that the individual compounds have specificity as sugar acceptors. Thus phenyl phosphate acted as an effective acceptor for both mannose and glucose, whereas benzenephosphonic acid was active only in accepting mannose. *p*-Nitrophenyl phosphate was a more effective glucose acceptor than phenyl phosphate, but had only 8% of the mannose-accepting activity of phenyl phosphate. (2) Phenyl phosphate had an inhibitory effect on the transfer of mannose from GDP-mannose to lipid-linked oligosaccharides and to glycoproteins in rat liver microsomal fractions. The inhibition depended on the concentration of phenyl phosphate and on the extent of inhibition of Dol-P-Man synthesis. It is proposed that phenyl phosphate has a direct effect on the synthesis of Dol-P-Man and that its inhibition of synthesis of lipid-linked oligosaccharides and glycoproteins could be a consequence of this effect.

Phenyl phosphate has been shown (Kato *et al.*, 1980*a,b*) to inhibit the transfer of mannose from GDP-mannose to endogenous Dol-P in rat liver microsomal fractions. This inhibition is due to the ability of exogenous phenyl phosphate to act as an artificial acceptor for microsomal GDP-mannose:dolichyl phosphate mannosyltransferase. The microsomal biosynthetic apparatus for Dol-P-Man synthesis diverts a large proportion of synthetic activity from the endogenous Dol-P to this exogenous substrate. The product thus formed, phenyl phosphate β -D-mannose, is quickly discharged from microsomal fractions and, in this sense, differs from Dol-P-Man that is retained in the microsomal fractions to act as mannose donor for further synthetic reactions. These observations suggest that phenyl phosphate can be used as a specific exogenous probe to perturb the normal pathway of

lipid-linked oligosaccharide and glycoprotein synthesis involving Dol-P-Man as an intermediate.

We have now investigated systematically the influence of various aryl phosphates and phosphonates on the synthesis of Dol-P-linked monosaccharides and oligosaccharides and glycoproteins, by using rat liver microsomal fractions as a standard multienzyme system.

Experimental

Materials

Rat liver microsomal fractions were prepared as described previously (Kato *et al.*, 1980*a*). GDP-[U- 14 C]mannose (210 mCi/mmol), GDP-[U- 14 C]fucose (174 mCi/mmol), UDP-[U- 14 C]galactose (192 mCi/mmol), UDP-[U- 14 C]glucuronic acid (231 mCi/mmol), UDP-[U- 14 C]xylose (160 mCi/mmol) and [U- 14 C]mannose (213 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A.; CMP-*N*-acetyl[4,5,6,7,8,9- 14 C]neuraminic acid (259 mCi/mmol), UDP-*N*-acetyl[U- 14 C]glucosamine (300 mCi/mmol), UDP-

Abbreviations used: Dol-P-Man and -Glc, dolichyl phosphate β -D-mannose and β -D-glucose.

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N-acetyl[1-¹⁴C]galactosamine (61 mCi/mmol) and UDP-[U-¹⁴C]glucose (312 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Phenyl phosphate, *p*-nitrophenyl phosphate, benzenephosphonic acid, α -naphthyl phosphate, β -naphthyl phosphate, Triton X-100 and Univer-Gel scintillation fluid were purchased from Nakarai Chemicals, Kyoto, Japan; 4-methylumbelliferyl phosphate and Dol-*P* were from Sigma Chemical Co., St. Louis, MO, U.S.A.; *p*-nitrobenzenesulphonic acid and phenyl sulphate were from Tokyo Kasei Kogyo Co., Tokyo, Japan; Sephadex G-25 (Superfine grade) was from Pharmacia Japan, Tokyo, Japan.

Chromatography

Paper chromatography was performed on Toyo no. 50 paper (48 cm long) by the descending technique in the following solvent systems: A, *n*-butyric acid/0.5 M-NH₃ (5:3, v/v); B, butanol/ethanol/water (13:8:4, by vol.).

Gel chromatography was performed on a column (48.5 cm $\times$ 0.5 cm diam.) of Sephadex G-25, equilibrated with 0.1 M-NaCl. The sample solution (about 130 μ l, containing labelled sugars, see below) was applied to the column and was then eluted with 0.1 M-NaCl. Effluent fractions (0.2 ml) were collected at a rate of 3.5 ml/h and their radioactivities determined in a liquid-scintillation spectrometer.

Assay for transfer of sugar from sugar nucleotide to endogenous or exogenous Dol-*P*

The assay procedures were the same as those used for measuring mannose transfer from GDP-[¹⁴C]-mannose to endogenous Dol-*P* (Kato *et al.*, 1980a), except that various other sugar nucleotides were used as donor substrates. The incubation mixtures contained 0.2 mg [as protein, assayed by the method of Lowry *et al.* (1951)] of microsomal fraction, 0.04 μ Ci of ¹⁴C-labelled sugar nucleotide, 1.2 μ mol of MnCl₂, 0.3 μ mol of NaF, 0.3 μ mol of 5'-AMP (sodium salt) and 2.5 μ mol of Tris/maleate, pH 6.8, in a total volume of 100 μ l. Controls contained heat-killed microsomal fractions. Where indicated, 6 nmol of exogenous Dol-*P* was added to the incubation mixtures by the procedure described previously (Kato *et al.*, 1980a). The incubation was carried out at 37°C for 30 min and stopped by heating at 100°C for 1 min. The heated samples were applied to Toyo no. 50 paper and subjected to paper chromatography in solvent A for 24 h. The regions containing Dol-*P*-Glc, detected by radioactivity scanning, were cut out and their radioactivities determined in a liquid-scintillation spectrometer. With this solvent system, the glycosylation products from Dol-*P* listed in Table 2 (below) had the same mobility as that of the Dol-*P*-Man standard (R_F = 0.95; see Kato *et al.*, 1980a).

Assay for transfer of sugar from sugar nucleotide to exogenous aryl phosphates and related compounds

The assay procedures are the same as those used for measuring mannose transfer from GDP-[¹⁴C]-mannose to phenyl phosphate (Kato *et al.*, 1980a), except that various other donor (0.04 μ Ci) and acceptor compounds (2.5 μ mol) were used in a total volume of 100 μ l. Controls contained heat-killed microsomal fraction. The incubation was performed at 37°C for 30 min and stopped by heating at 100°C for 1 min. The heated samples were applied to Toyo no. 50 paper and subjected to paper chromatography in solvent A for 24 h. The regions of aryl phosphate (or phosphonate) sugars, detected by radioactivity scanning, were cut out and their radioactivities determined in a liquid-scintillation spectrometer. With this solvent system, the glycosylation products from added acceptors listed in Tables 1–3 below had essentially identical R_F values, namely 0.58. Where indicated, the [¹⁴C]mannosylated products from a mixture of phenyl phosphate and benzenephosphonic acid were separately measured as follows. The products obtained by the solvent A system (see above) were eluted from the paper strip with water and subjected to a second paper chromatography in solvent B, whereby phenyl phosphate mannose (R_{mannose} = 0.74) was separated from the product from benzenephosphonic acid (R_{mannose} = 0.60). The regions of these products were cut out and their radioactivities determined as described above.

Assay for incorporation of mannose from GDP-[¹⁴C]mannose into phenyl phosphate mannose, Dol-*P*-Man, lipid-linked oligosaccharides and glycoproteins

The incubation mixtures were prepared as described for Dol-*P*-Man or Dol-*P*-Glc synthesis (see above), except that 1.5 mg (as protein measured by the Lowry method) of microsomal fraction, were used as enzyme. Where indicated, exogenous phenyl phosphate was added to the reaction mixture. Controls contained heat-killed microsomal fractions. The incubation was performed for 30 min at 37°C and stopped by the addition of 1 ml of ice-cold 5% trichloroacetic acid. The subsequent procedure for isolation of phenyl phosphate [¹⁴C]mannose, Dol-*P*-[¹⁴C]Man, lipid-linked [¹⁴C]oligosaccharides and [¹⁴C]glycoproteins was similar to that described by Waechter *et al.* (1973). A graphic summary of the procedure is presented in Scheme 1. After these separation procedures, the radioactivities of the separated compounds were determined as follows. (1) The region of phenyl phosphate [¹⁴C]-mannose (R_{mannose} = 0.58) on the paper was cut out, placed in scintillation fluid and counted for radioactivity. (2) The chloroform/methanol extract con-

Scheme 2. *Method of release of mono- and oligo-saccharides from dolichyl derivatives by mild acid hydrolysis*
All centrifugations were at 10000 g for 30 min. Neutralization of acidic solutions was achieved with 5 M-NaOH.
Abbreviation used: 5% TCA, ice-cold 5% (w/v) trichloroacetic acid.

37°C for 30 min, the reaction was stopped by the addition of 2.4 ml of ice-cold 5% (w/v) trichloroacetic acid. The subsequent procedure for mild acid hydrolysis of the precipitate was similar to that described by Godelaine *et al.* (1977). A graphic summary of the procedure is presented in Scheme 2.

Results

Specificity of phenyl phosphate and related compounds in accepting sugar residues from sugar nucleotides

Our previous work (Kato *et al.*, 1980a) has shown that the microsomal preparation from rat liver, when incubated within a range of microsomal

concentrations from 0.05 to 0.5 mg (as protein)/100 µl of labelling medium, catalyses the incorporation of mannose from GDP-[¹⁴C]mannose into Dol-P-Man with no detectable incorporation into lipid-linked oligosaccharides and glycoproteins. By using this microsomal preparation, a series of specificity studies were carried out, as shown in Tables 1, 2 and 3 and Fig. 1.

Table 1 shows the relative abilities of various aryl phosphates and related compounds to accept mannose from GDP-[¹⁴C]mannose. Apparently, the acceptor activity of phenyl phosphate is a relatively specific one, since all of the other compounds tested, with one exception, exhibited little or no mannose-accepting activity. The exception was ben-

Table 1. *Mannose transfer from GDP-[¹⁴C]mannose to various aryl phosphates and related compounds*

Standard assays were performed as described in the Experimental section, except that acceptors (as their sodium salts) were added to give the final concentrations indicated in parentheses.

Acceptor added		10 ⁻³ × [¹⁴ C]Mannose transferred (c.p.m./30 min per mg of protein)
Phenyl phosphate	(5 mM)	53.1
	(25 mM)	72.8
<i>p</i> -Nitrophenyl phosphate	(5 mM, 25 mM)	5.9
α -Naphthyl phosphate	(5 mM, 25 mM)	2.0
β -Naphthyl phosphate	(5 mM)	3.0
	(25 mM)	0
4-Methylumbelliferyl phosphate	(5 mM)	0.6
	(25 mM)	0
Benzenephosphonic acid	(5 mM)	17.0
	(25 mM)	11.2
Phenyl sulphate	(5 mM, 25 mM)	0
<i>p</i> -Nitrobenzenesulphonic acid	(5 mM, 25 mM)	0

Table 2. *Transfer of sugar from various [¹⁴C]sugar nucleotides to added dolichyl phosphate and phenyl phosphate*
Standard assays were performed as described in the Experimental section with 6 nmol of exogenous dolichyl phosphate or 2.5 μ mol of exogenous phenyl phosphate. No transfer occurred with UDP-xylose, UDP-galactose, UDP-glucuronic acid, UDP-*N*-acetylglucosamine, GDP-fucose or CMP-*N*-acetylneuraminic acid.

[¹⁴ C]Sugar nucleotide added	10 ⁻³ × [¹⁴ C]Sugar transferred (c.p.m./30 min per mg of protein) to:	
	Dolichyl phosphate	Phenyl phosphate
GDP-mannose	76.1	72.8
UDP-glucose	16.4	56.5
UDP- <i>N</i> -acetylglucosamine	22.7	0

Table 3. *Comparison of acceptor specificities of glucose transfer from UDP-glucose and mannose transfer from GDP-mannose*

Standard assays were performed as described in the Experimental section. The data for mannose transfer are from Table 1.

[¹⁴ C]Sugar nucleotide	10 ⁻³ × [¹⁴ C]Sugar transferred (c.p.m./30 min per mg of protein) to:			
	Phenyl phosphate	Benzenephosphonic acid	<i>p</i> -Nitrophenyl phosphate	4-Methylumbelliferyl phosphate
UDP-glucose	56.5	0	67.8	7.1
GDP-mannose	72.8	11.2	5.9	0

zenephosphonic acid, which showed about one-third the activity of 5 mM-phenyl phosphate.

Benzenephosphonic acid acted as a competitive inhibitor of the mannose transfer to phenyl phosphate (Fig. 1a), and phenyl phosphate behaved similarly towards the mannose transfer to benzenephosphonic acid (Fig. 1b), suggesting that the same enzyme was involved in both reactions.

Table 2 shows the abilities of added Dol-*P* and phenyl phosphate to accept sugar residues from various sugar nucleotides. The microsomal preparation catalysed the transfer of sugar into phenyl phosphate only from GDP-mannose and UDP-

glucose. Although UDP-*N*-acetylglucosamine served as an effective donor towards added Dol-*P*, it showed no activity towards phenyl phosphate, suggesting that there are differences in acceptor specificity between the enzyme responsible for the synthesis of lipid-linked *N*-acetylglucosamine [predicted to be dolichyl pyrophosphate *N*-acetylglucosamine on the basis of previous studies (Molnar *et al.*, 1971; Leloir *et al.*, 1973; Ghalambor *et al.*, 1974)] and those responsible for Dol-*P*-Man and Dol-*P*-Glc synthesis.

Table 3 shows the abilities of several aryl phosphates and benzenephosphonic acid to accept

Fig. 1. (a) Effect of phenyl phosphate concentration on the rate of mannose transfer to phenyl phosphate in the absence (●) and presence (○) of 13 mM-benzenephosphonic acid, and (b) effect of benzenephosphonic acid concentration on the rate of mannose transfer to benzenephosphonic acid in the absence (●) and presence (○) of 5 mM-phenyl phosphate

Standard assays were performed as described in the Experimental section, except that substrate (acceptor) and inhibitor were added as indicated. The [^{14}C]mannosylated products from phenyl phosphate and benzenephosphonic acid were separated by paper chromatography and their radioactivities were determined. v = radioactivity (c.p.m.) of [^{14}C]mannose transferred/30 min per 0.2 mg of microsomal protein.

glucose from UDP-[^{14}C]glucose. The data concerning their abilities to accept mannose (taken from Table 1) are also shown for comparison. As Table 3 shows, there are some striking differences

between glucosyltransferase and mannosyltransferase in their acceptor specificities. For example, benzenephosphonic acid, which served as an effective acceptor towards mannosyltransferase, did not act on glucosyltransferase. *p*-Nitrophenyl phosphate, in contrast, acted much more effectively on glucosyltransferase than on mannosyltransferase.

Inhibition of lipid-linked oligosaccharide synthesis by phenyl phosphate and p-nitrophenyl phosphate

The above observations, together with those described in our previous papers (Kato *et al.*, 1980a,b), indicate that both phenyl phosphate and *p*-nitrophenyl phosphate interfere directly with the synthesis of Dol-*P*-Man and Dol-*P*-Glc, and might thus also affect the subsequent glycosylation reactions. To test this possibility, rat liver microsomal fractions were incubated with GDP-[^{14}C]mannose or UDP-[^{14}C]glucose at a microsomal concentration of 1.5 mg of protein/100 μl of labelling medium, much higher than that used in the assay for lipid-linked monosaccharide synthesis. As mentioned above, no detectable incorporation of [^{14}C]mannose and [^{14}C]glucose into lipid-linked oligosaccharides and glycoproteins was observed at microsomal concentrations lower than 0.5 mg of protein/100 μl of labelling medium. When, however, incubations were performed at the higher microsomal concentration, the incorporation of mannose and glucose into lipid-linked oligosaccharides and into glycoproteins could be demonstrated; under the standard assay conditions, about 3 and 2 pmol of [^{14}C]mannose and [^{14}C]glucose respectively were incorporated into chloroform/methanol-insoluble fractions. The addition of an excess of microsomal fraction might relieve a deficiency of endogenous precursors or a lability of some enzymes, thereby accelerating the synthesis of lipid-linked oligosaccharides and glycoproteins that could not otherwise be detected. Further extraction of the [^{14}C]mannose-labelled material with chloroform/methanol/water (see Scheme 1) yielded a considerable portion of the label in the extract, suggesting that the chloroform/methanol-insoluble component contained lipid-linked oligosaccharides (see below for further evidence). The label remaining in the chloroform/methanol/water-insoluble residue has not been characterized, but it probably represents glycoproteins on the basis of previous studies (see Waechter *et al.*, 1973).

In one series of experiments, rat liver microsomal fractions (1.5 mg of protein/100 μl) were incubated with GDP-[^{14}C]mannose or UDP-[^{14}C]glucose for 30 min at 37°C. The lipid-linked saccharides and glycoproteins were isolated by precipitation with trichloroacetic acid and the saccharides were released from the precipitate by mild acid hydrolysis, as outlined in the Experimental section

Fig. 2. Chromatography on Sephadex G-25 of the saccharide released by mild acid hydrolysis of microsomal lipids that had been labelled with GDP-[^{14}C]mannose (a) or with UDP-[^{14}C]glucose (b) in the absence (○) and presence (●) of aryl phosphate

The aryl phosphates added were 10mM-phenyl phosphate (a) and 10mM-*p*-nitrophenyl phosphate (b). V_0 = Blue Dextran; the standard, [^{14}C]mannose, was recovered in fraction 33.

(Scheme 2). Gel filtration of the hydrolysates on a column of Sephadex G-25 (Fig. 2) yielded in each case two radioactive peaks, one appearing in the position expected for monosaccharides and the other in the area for oligosaccharides. The [^{14}C]glucose-labelled oligosaccharide chains appeared larger than the [^{14}C]mannose-labelled oligosaccharide chains and showed a faster elution from the gel. When the products of phenyl phosphate- or *p*-nitrophenyl phosphate-treated microsomal fractions were examined in the same way (Fig. 2), in each case a significant inhibition of incorporation of ^{14}C into oligosaccharides was observed. However, the elu-

Fig. 3. Effect of phenyl phosphate concentration on the formation of phenyl phosphate mannose (Phe-P-[^{14}C]Man) (Δ), Dol-P-[^{14}C]Man (●), lipid-linked [^{14}C]oligosaccharides (▲), and [^{14}C]glycoproteins (○) from GDP-[^{14}C]mannose

Standard assays were performed as described in the Experimental section, except that the phenyl phosphate concentration was varied as indicated.

tion position of oligosaccharide peak was essentially the same for control and aryl phosphate-treated microsomal fractions.

Fig. 3 shows the effect of phenyl phosphate concentration on the formation of lipid-linked oligosaccharides and glycoproteins from GDP-[^{14}C]mannose. For the separate determination of lipid-linked oligosaccharides and glycoproteins, the insoluble material remaining after extraction with chloroform/methanol (2:1, v/v) (cf. Scheme 1) was extracted several times with chloroform/methanol-water (10:10:3, by vol.). The radioactivity in the pooled extracts was taken as a measure of lipid-linked oligosaccharides and that in the insoluble fraction as a measure of glycoproteins. As Fig. 3 shows, mannose incorporation into lipid-linked oligosaccharides and into glycoproteins was as sensitive to phenyl phosphate as was the synthesis of Dol-P-Man.

It should be noted that the inhibition of Dol-P-Man synthesis by phenyl phosphate reached a plateau at 40% of the control value at 20mM-inhibitor and remained fairly constant over the range

Fig. 4. Effect of *p*-nitrophenyl phosphate concentration on the formation of *p*-nitrophenyl phosphate [^{14}C]glucose (PNP- P -[^{14}C]Glc) (Δ), Dol- P -[^{14}C]Glc ($\bullet$) and lipid-linked [^{14}C]oligosaccharides plus [^{14}C]glycoproteins ($\circ$), from UDP-[^{14}C]glucose

Standard assays were performed as described in the Experimental section, except that the *p*-nitrophenyl phosphate concentration was varied as indicated.

20–40 mM (see the Discussion section for further comments on the biphasic response).

Fig. 4 shows the effect of *p*-nitrophenyl phosphate concentration on the incorporation of glucose into a chloroform/methanol-insoluble material (containing both lipid-linked oligosaccharides and glycoproteins; for some unknown reason, attempts to separate these two components quantitatively from each other by chloroform/methanol/water extraction have been unsuccessful). The synthesis of Dol- P -Glc appeared to be more sensitive to inhibitor than was the synthesis of Dol- P -Man (cf. Fig. 3). Nevertheless, the incorporation of glucose into the chloroform/methanol-insoluble material was much less sensitive to inhibitor than was the incorporation of mannose into lipid-linked oligosaccharides and glycoproteins. This phenomenon was demonstrated consistently by several different microsomal preparations. One possible explanation for the low sensitivity towards Dol- P -Glc concentration is that the microsomal enzyme catalysing glucosyl transfer to lipid-linked oligosaccharides has a very high affinity for Dol- P -Glc, so that the reaction may proceed at an only slightly decreased rate, even if the synthesis of Dol- P -Glc is inhibited to a large extent.

Discussion

The acceptors tested in previous studies on mannosyl transferase have been confined to isoprenologues ranging from C_{15} farnesyl phosphate to C_{100} dolichyl phosphates (Richards & Hemming, 1972; Wedgwood *et al.*, 1974; Tkacz *et al.*, 1974; Mankowski *et al.*, 1975). It is somewhat surprising, therefore, that such chemically distant compounds as phenyl phosphate and benzenephosphonic acid are effective as sugar acceptors. This may be taken as a reflection of a low specificity of the mannosyltransferase for the organic moiety adjacent to the phosphate group. If, however, the relative activities for various aryl phosphates (Table 1) are compared with one another or with those obtained with different donor substrates (Tables 2 and 3), it is apparent that each transferase system exhibits a fairly high degree of specificity towards the organic moiety of aryl phosphates. Thus, for GDP-[^{14}C]mannose as donor, the aryl portion in the acceptor must be an unsubstituted benzene ring, and only very slight activity can be detected with phosphate esters bearing substituted benzene rings. For UDP-[^{14}C]glucose as donor, however, the substitution of -H for - NO_2 on the benzene ring causes a large enhancement in the acceptor activity. Although a definite conclusion cannot be drawn yet as to the nature of primary structural requirements for individual glycosyltransferases, the results encourage our expectation that other aryl phosphates or phosphonates with much higher specificity may be synthesized for further investigations.

Mention must be made of the similarity between the observations reported here (Figs. 3 and 4) and by previous investigators (Kerr & Hemming, 1978) regarding the biphasic response of glycosyltransferases to inhibitors. The previous investigators showed that GDP-mannose and UDP-mannose markedly inhibited glucosyl transfer from UDP-glucose to Dol- P in liver microsomal preparations and that the inhibition reached a plateau at 20–50% of control values at an inhibitor concentration of 1–2 μM . They suggested that the microsomal preparations must contain two isoenzymes or two conformational states of glucosyltransferase, one of which is sensitive to inhibition by GDP-mannose or UDP-mannose, the other being insensitive. The data plotted in Figs. 3 and 4 in the present paper are in good agreement with this hypothesis and further suggest that aryl phosphates may be potentially useful as agents for investigating the mechanism of assembly of the oligosaccharide chains of glycoproteins.

This research was supported in part by grants-in-aid from the Ministry of Education, Science and Culture, from the Ministry of Health and Welfare, and from the Takeda Science Foundation, Japan. Part of the work was

done while S. K. was supported by a fellowship from the Japan Society for the Promotion of Science.

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