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ACCEPTOR-SPECIFIC GLUCURONYL TRANSFER CATALYZED BY β-GLUCURONIDASE

REINHARD NIEMANN and ECKHART BUDDECKE

Institute of Physiological Chemistry, University of Münster, D-4400 Münster (F.R.G.)

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Summary

Highly purified rat liver microsomal or lysosomal β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) catalyzes the specific transfer of glucuronyl residues from phenyl-β-D-[U-^{14}C]glucuronide to acceptor sugars. Specificity requirements of acceptor sugars are found to be: pyranose structure, $^4C_1$-conformation and equatorial position of C2 and C3 hydroxyl groups or pyranose structure, $^1C_4$-conformation and equatorial position of C3 and C4 hydroxyl groups. The acceptor capacities of 30 monosaccharides and glycosides including di- and tri- saccharides conform to this principle.

The specificity of the β-glucuronidase catalyzed glucuronyl transfer is proved by the exclusive formation of β-glucuronyl (1—3)glycosidic linkages.

Glucuronyl transfer rates increase with increasing donor substrate and increasing acceptor sugar concentration. In the presence of 1 M acceptor sugar the ratio of the transfer rate to the rate of enzymatic hydrolysis is about 2 : 1.

An 'acceptor substrate binding site' on the surface of the β-glucuronidase molecule which brings the C3 hydroxyl function of the acceptor sugar close enough to the C1 atom of the glucuronyl residue, is postulated.

Introduction

Liver β-glucuronidase (β-D-Glucuronide glucuronosohydrolase, EC 3.2.1.31) is known to display dual localization in endoplasmic reticulum and lysosomes [1,2]. Both enzymes have been separated and purified to homogeneity by immunoaffinity chromatography [3,4]. In mice, the enzyme at both sites is derived from the same structural gene [5], the microsomal β-glucuronidase being associated with endoplasmic reticulum by the anchor protein, egasyn [6—8].

Although β-glucuronidase preparations from these two subcellular fractions have the same pH optimum and the same or quite similar glycosidase activity [5,7], the physiological function of the microsomal β-glucuronidase remains obscure. From a consideration of physiological data it was suggested that microsomal β-glucuronidase is essentially inactive and may serve as a structural protein of the endoplasmic reticulum [2].

In this paper it will be shown that under appropriate conditions both microsomal and lysosomal β-glucuronidase of rat liver have the ability to catalyze the transfer of β-glycosidically linked glucuronyl residues to stereospecific sites of acceptor sugars resulting in the exclusive formation of β-glucuronyl(1—3)-glycosidic linkages. Although a number of glycosidases have been reported to transfer glycosyl residues to carbohydrates [10—12] or alcolhols [13—16], such a sterospecific transfer reaction has not yet been described.

Materials and Methods

Materials

All chemicals were of the highest purity available, sugars were obtained from the following sources: D-xylose, D-mannose, D-glucose, D-galactose, D-glucosamine, D-fructose, *p*-nitrophenyl-β-D-glucopyranosiduronic acid, maltose, and sucrose from Merck (Darmstadt), methyl-α-D-glucopyranoside and methyl-β-D-glucopyranoside from Baker (London), D-fucose and phenyl-β-D-glucopyranoside from Schuchardt (Munich), L-arabinose, L-glucose, cellobiose, D-arabinose, and D-saccharic acid-1,4-lactone from Sigma (Frankfurt), 2-deoxy-D-glucose, lactose, and *N*-acetylglucosamine from Serva (Heidelberg), phenyl-β-D-glucuronide monohydrate from Koch-Light (Colnbrook, Bucks, UK). Allose, 2-*O*-methyl-glucopyranoside, and 3-*O*-methyl-glucopyranoside were a gift from D. Balkau (Institute of Organic Chemistry, University of Münster), abequose was a gift from Prof. Lüderitz (Max-Planck-Institute for Immunology, Freiburg). Methyl-2,4,6-tri-*O*-methyl-D-glucopyranoside and methyl-3,4,6,-tri-*O*-methyl-D-glucopyranoside were prepared according to refs. 9 and 17; chitotriose according to Rupley [18]; and βGlcNAc(1—4)βGlcUA(1—3)βGlcNAc and βGalNAc-4-sulfate(1—4)β-GlcUA(1—3)-GalNAc-4-sulfate were prepared from hyaluronic acid and chondroitin 4-sulfate according to [19—23].

Sephadex G-15, G-25, G-200, DEAE-Sephadex, DEAE-Sephacel and CNBr-activated Sepharose were products of Pharmacia Fine Chemicals (Uppsala).

Synthesis of sugars

The synthesis of phenyl-β-D-[U-^{14}C]glucopyranosiduronic acid (Phe-[^{14}C]-GlcUA) included the following steps: (1) preparation of β-D-[U-^{14}C]glucopyranose pentaacetate from D-[U-^{14}C]glucose (Amersham, Buchler, Braunschweig), specific activity 108 μCi/mmol, and acetic anhydride with sodium acetate as catalyst according to ref. 24, (2) preparation of tetraacetyl-β-phenyl-D-[U-^{14}C]glucopyranoside according to ref. 25, (3) deacetylation of the above compound [26] to phenyl-β-D-[U-^{14}C]glucopyranoside, (4) catalytic oxidation of phenyl-β-D-[U-^{14}C]glucopyranoside by gaseous oxygen in the presence of freshly reduced platinum catalyst [27,28] to yield phenyl-β-D-[U-^{14}C]glucopyranosiduronic acid.

D-Saccharic acid-1,4-lactone-benzidine-Sepharose was synthesized by the method of Warburton and al. [29].

Preparations of enzymes

Lysosomal and microsomal β-glucuronidase. The lysosomal and microsomal fractions of the rat liver were the same as described by Himeno et al. [7]. The lysosomal β-glucuronidase was purified to a specific activity of 16.8 U/mg protein by the following procedures: (1) gel filtration on Sephadex G-200, the β-glucuronidase being eluted with 0.1 M Tris-HCl buffer (pH 8.0), 1 M NaCl, 0.1 M methyl-α-D-glucoside, 0.2% Triton X 100, (2) ion exchange chromatography on DEAE-Sephacel, using a linear gradient from 0.0—0.6 M NaCl containing 0.1 M methyl-α-D-glucoside and buffered with 0.01 M Tris/HCl (pH 8.0); (3) hydrophobic affinity chromatography on D-saccharic acid-1,4-lactone-benzidine-Sepharose according to Warburton et al. [29]; β-glucuronidase was eluted as a single symmetric peak by 0.1 M borate buffer (pH 8.0), 0.1 M α-methyl-α-D-glucoside, 0.1 M glucuronolactone, 0.1% Triton X 100.

The microsomal β-glucuronidase was solubilized [7] and purified to an activity of 9.4 U/mg protein by ion-exchange and affinity chromatography as described for the lysosomal β-glucuronidase.

Uronate dehydrogenase (EC 1.2.1.35). Cultivation of *Pseudomonas syringae* and purification of uronate dehydrogenase were performed by the method of Wagner and Hollmann [30], but with supplementary affinity chromatography on 5'-AMP-Sepharose which resulted in a highly purified enzyme preparation (specific activity 106 U/mg protein).

Enzyme assays

Hydrolase and transfer reaction rates were calculated on the same enzyme activity basis. One unit of β-glucuronidase activity hydrolysed or transferred 1 μmol substrate/min under the conditions specified. Highly purified enzyme solutions were diluted for assay with a solution of 0.1% bovine serum albumin in 0.01 M Tris-HCl buffer (pH 8.0) to minimize losses owing to surface adsorption [31].

Hydrolase activity of β-glucuronidase was determined with 10 mM *p*-nitrophenyl-β-D-glucuronide, 100 mM sodium acetate buffer (pH 5.0), 0.075 M NaCl in 0.5 ml. Incubations were at 37°C for up to 30 min and were arranged so that less than 5% of the substrate was hydrolyzed. The reaction was terminated by the addition of 1 ml 0.2 M glycine/NaOH buffer (pH 10.4). The developed color was measured at 405 nm.

For the determination of the transferase activity of β-glucuronidase the standard incubation mixture consisted of 10 mM phenyl-β-[U-^{14}C]glucuronide (donor substrate), 100 mM acceptor sugar, 100 mU β-glucuronidase and 0.05 M phosphate buffer (pH 7.4), 0.1 M NaCl in a final volume of 0.5 ml. The incubation at 37°C was stopped by the addition of 100 μl 15% trichloroacetic acid before 5% of the donor substrate was used. The ratio of glucuronyl transfer (formation of di- or oligosaccharide) to substrate hydrolysis was assayed after gel filtration on Sephadex G-15 of the supernatant of the trichloroacetic acid precipitate. The Sephadex G-15 column (240 × 1 cm) was calibrated with [^{14}C]GlcUA and eluted with 0.02 M HCl, 0.1 M NaCl. Radioactivity analysis

of the 3-ml fractions by scintillation spectrophotometry revealed 3 radioactivity peaks (Fig. 1), corresponding to the product of glucuronyl transfer, to free glucuronic acid and the phenyl-β-D-[U-^{14}C]glucuronide. The percentage of transfer rates are expressed as [product of transfer reaction] X 100/[product of transfer reaction] + [free glucuronic acid].

Large scale preparation of β-glucopyranosiduronyl-glucoside was performed as described in Fig. 1. with the exception that the incubation mixture contained 0.2 M methyl-D-glucoside and 5.7 U β-glucuronidase in 5 ml buffer and that the incubation time was prolonged to 3 h.

Analysis of sugars

The identification of the products of transfer reactions was accomplished after large scale production of glucuronyl-methyl-glucoside (see Fig. 1). The transfer product was isolated and desalted by gel filtration, lyophilized and permethylated [32]. The permethylated product was hydrolyzed with 2 M HCl under nitrogen for 4 h at 110°C, desiccated over P_2O_5 and prepared for gas liquid chromatography by treating with trimethylchlorosilane and hexamethyldisilazan in pyridine. D-Mannitol served as internal standard, methyl-2,4,6-tri-*O*-methyl glucoside and methyl-3,4,6-tri-*O*-methylglucoside after hydrolysis as references. A Packard 7400 series gas chromatograph with a column of 0.2 cm diameter (3 m length) packed with SE-30 (3% on chromosorb W (HP) 80—100 mesh) and a nitrogen flow rate of 20 ml/min were used.

Free glucuronic acid was identified and quantitated by enzymatic conversion to glucaric acid by means of glucuronic acid dehydrogenase from *Pseudomonas syringae* [24].

Determination of radioactivity

Radioactivity was determined by addition of 2 ml of aqueous solutions to 5 ml Unisolve I (Zinsser, Frankfurt, Germany) and counting in a Packard Tricarb 2450 B Spectrophotometer.

Results

β-Glucuronidase catalyzed glucuronyl transfer

Both microsomal and lysosomal β-glucuronidase preparations were found to catalyze both the hydrolysis of β-glucuronides substrates and the transfer of β-glucuronyl residues to appropriate acceptor sugars.

Incubation of highly purified β-glucuronidase, in the presence of Phe-[U-^{14}C]GlcUA as donor substrate and D-glucose as acceptor substrate at neutral pH results in the formation of β-[U-^{14}C]glucopyranosiduronyl-glucose, free D-[U-^{14}C]glucuronic acid and phenol. The reaction products were resolved by gel chromatography (Fig. 1). The [^{14}C]GlcUA-Glc disaccharide formed was submitted to an enzymatic hydrolysis by β-glucuronidase at pH 5.0 and the quantitatively liberated glucuronic acid was identified as glucaric acid after enzymatic conversion by microbial glucuronic acid dehydrogenase. The released glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase method.

The ratio of the rate of hydrolysis of Phe-[^{14}C]GlcUA to the rate of transfer

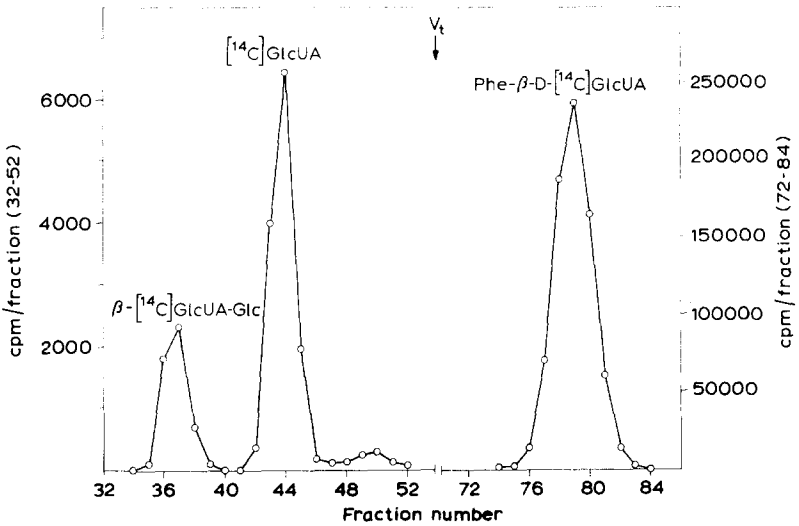

Fig. 1. β-Glucuronidase-catalyzed transfer of glucuronic acid from Phe-[^{14}C]GlcUA to glucose. Separation of the transfer product by gel filtration. The reaction mixture contained 0.01 M Phe-[^{14}C]GlcUA, 0.1 M D-glucose, 0.1 M NaCl, and 0.16 U lysosomal β-glucuronidase in 0.5 ml 0.05 M phosphate buffer pH 7.4. After 30 min the incubation was stopped by addition of 1 ml 15% trichloroacetic acid and the precipitate formed was discarded. The supernatant was submitted to gel filtration on a Sephadex G-15 column (240 X 1 cm) which was equilibrated and eluted with 0.02 M HCl containing 0.1 M NaCl. The reaction products ([^{14}C]GlcUA-Glc and [^{14}C]GlcUA) were clearly separated. Note the retardation of Phe-[^{14}C]GlcUA by the dextran gel. The void volume of the column was 70 ml (fraction 24).

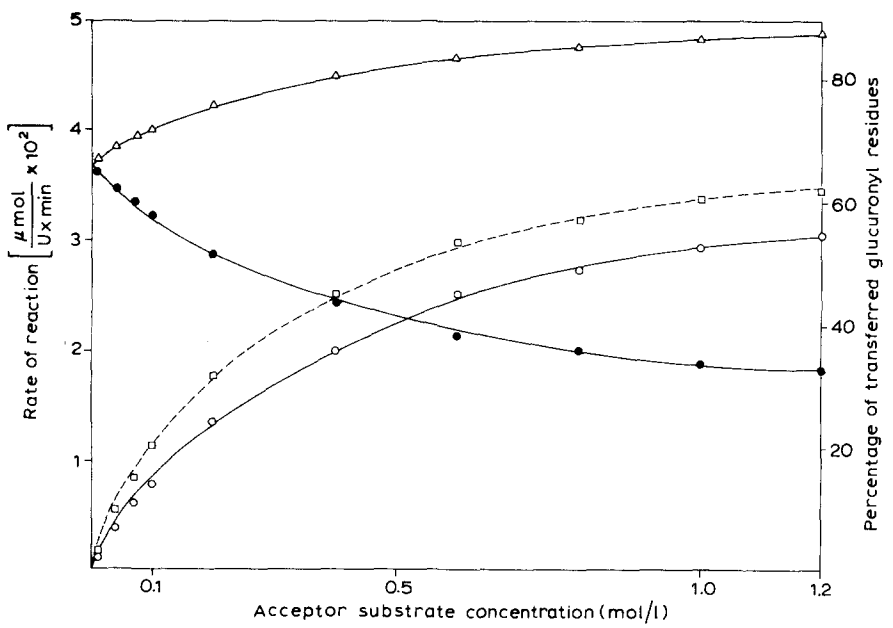

Fig. 2. Effect of varying acceptor substrate concentration on the transferase and hydrolase activity of β-glucuronidase. The reaction mixture contained 0.01 M Phe-[^{14}C]GlcUA, the specified concentration of methyl-α-D-glucoside, 0.1 M NaCl and 0.16 U lysosomal β-glucuronidase in 0.5 ml 0.05 M phosphate buffer pH 7.4. The reaction products were separated after a 30 min incubation period according to the procedure described in Fig. 1. The rates of reaction are expressed as $\mu mol \cdot U^{-1} \cdot min^{-1}$. ●———●, hydrolysis; ○———○, glucuronyl transfer; △———△, sum of hydrolysis and transfer; □———□, percent of transferred glucuronyl residues as referred to the total amount consumed Phe-[^{14}C]GlcUA during the incubation.

of glucuronyl residues depends on the concentration and structure of the acceptor sugar (Fig. 2, Table I), on the concentration of the donor substrate (Fig. 3) and on the pH of the reaction mixture (Fig. 4).

The data of Fig. 2 reveal that at increasing acceptor substrate concentration, the rate of hydrolysis is depressed, while the glucuronyl transfer continuously increased. At 1.2 M methyl-α-D-glucoside the rate of transfer exceeds that of hydrolysis, the ratio being 1.6/1.0. The glucuronyl transfers follows saturation kinetics. The K_m value of methyl-α-D-glucopyranoside was determined as 0.4 M.

The influence of the donor substrate concentration on the rate of hydrolysis and transfer is shown in Fig. 3. Both reactions increase with increasing molarity of Phe[^{14}C]GlcUA, but the rate of hydrolysis increases more rapidly so that the

TABLE I

ACCEPTOR CAPACITY OF VARIOUS SUGARS FOR β-GLUCURONIDASE CATALYZED GLUCURONYL TRANSFER

Phe-[^{14}C]GlcUA served as donor substrate. Transfer products were isolated by gel filtration. Transfer rates are expressed as percent of transfered glucuronyl residues as refered to the total amount of Phe-[^{14}C]GlcUA used by the enzyme for hydrolysis and transfer.

Acceptor sugar (0.1 M)	Free GlcUA (hydrolysis) (cpm)	Glucuronide formed (transfer) (cpm)	Transfer rate (%)
Monosaccharides			
D-Glucose	7490	1860	19.9
L-Glucose	7360	1650	18.4
D-Galactose	7470	1850	19.9
D-Fucose	7920	3220	28.9
3,6-Dideoxy-D-galactose	9110	90	<1
D-Mannose	8870	480	5.2
2-Deoxy-D-glucose	8700	190	2.2
D-Allose	8530	790	8.5
D-Fructose	8680	250	2.8
D-Xylose	7520	1720	18.6
L-Arabinose	7000	1560	18.2
Glucosamine	8840	170	1.9
N-Acetyl-glucosamine	8760	60	<1
Monosaccharide derivatives			
2-*O*-Methyl-D-glucose	8850	250	2.8
3-*O*-Methyl-D-glucose	8540	340	3.8
Methyl-α-D-glucopyranoside	7540	2020	21.1
Methyl-β-D-glucopyranoside	7520	1930	20.4
Phenyl-β-D-glucopyranoside	7200	2430	23.7
Di- and trisaccharides			
Maltose	7700	1250	14.0
Cellobiose	7660	1260	14.1
Lactose	7180	2520	25.9
Saccharose	7380	1710	18.8
β-GlcNAc (1—4) β-GlcUA (1—3) β-GlcNAc	9120	20	<1
β-GlcNAc (1—4) β-GlcNAc (1—4) β-GlcNAc	8810	50	<1
β-GalNAc-4-sulfate (1—4) β-GlcUA (1—3) β-GalNAc-4-sulfate	9020	30	<1

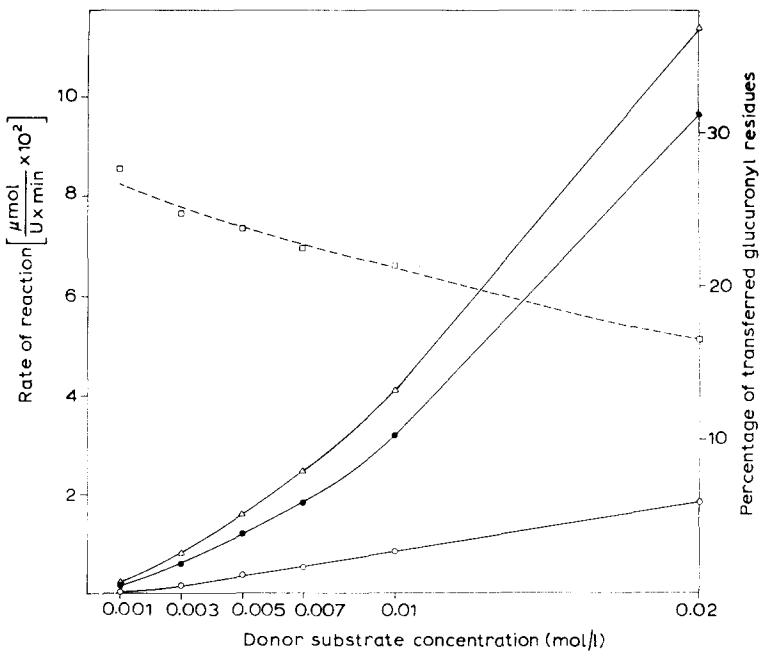

Fig. 3. Effect of varying donor substrate concentrations on the transferase and hydrolase activity of β-glucuronidase. The incubation mixture contained the specified concentration of Phe-[^{14}C]GlcUA, 0.1 M methyl-α-D-glucoside, 0.1 M NaCl and 0.16 U lysosomal β-glucuronidase in 0.5 ml 0.05 M phosphate buffer pH 7.4. Separation of [^{14}C]GlcUA-methylglucoside and free glucuronic acid by gel filtration, other conditions and symbols as in Fig. 2.

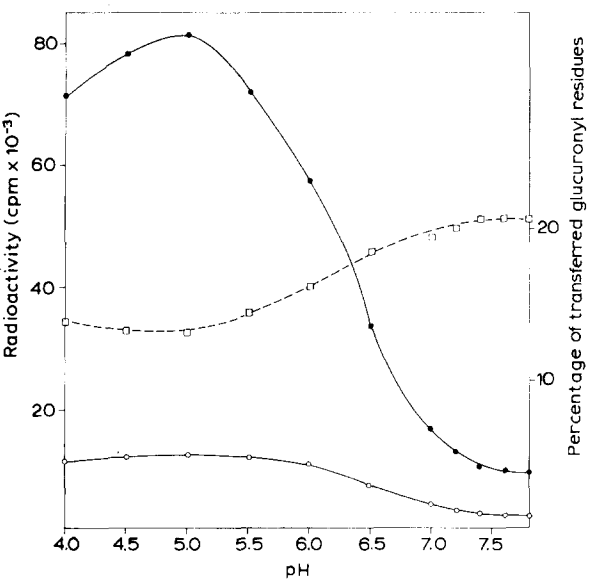

Fig. 4. pH dependent rate of hydrolysis of Phe-[^{14}C]GlcUA and glucuronyl transfer from Phe-[^{14}C]-GlcUA to methyl-α-D-glucoside. 0.01 M Phe-[^{14}C]GlcUA, 0.1 M methyl-α-D-glucoside, 0.1 M NaCl and 90 mU lysosomal β-glucuronidase were incubated in 0.5 ml 0.1 M acetate (pH 4.0—6.5) or 0.05 M phosphate (pH 7.0—7.8) buffer at 37°C for a limited time, so that not more than 5% of the added Phe-[^{14}C]-GlcUA was consumed for hydrolysis and transfer reactions. All values were corrected for a 30 min incubation period. Symbols as in Fig. 2.

percentage of transferred glucuronyl residues becomes smaller.

The maximal rate of Phe-GlcUA hydrolysis and glucuronyl transfer are observed about pH 5. However, in contrast to the sharp pH optimum of the β-glucuronidase-catalyzed hydrolysis of Phe-[^{14}C]GlcUA, the pH dependence of glucuronyl transfer was minor. At 0.1 M methyl-α-D-glucoside, the transfer rates between pH 4.0 and 8.0 were in a range of 15—21% of the rate of hydrolysis (Fig. 4).

All preceding experiments were performed with microsomal as well as with lysosomal β-glucuronidase. When equal activities of lysosomal and microsomal β-glucuronidase were compared for their hydrolase and transferase capacity, virtually no differences between the two enzyme preparations could be detected (Table II). Thermal denaturation of the enzyme preparations (boiling for 10 min) abolishs hydrolase and transferase activity completely.

Acceptor specificity of glucuronyl transfer

The glucuronyl transfer exhibits acceptor specificity. When a series of mono-, di- and trisaccharides was tested for their acceptor properties, differences in the transfer rates were found.

The transfer rates (see Methods) determined for the various acceptor sugars are given in Table I. Two groups of sugars, one possessing reasonable acceptor activity and the other having poor or no acceptor properties may be distinguished. At 0.1 M acceptor sugar concentration, the maximum transfer rate was observed with D-fucose (Table I) and transfer rates up to 70% were reached at D-fucose concentrations of 1.0 M.

Considering the configuration and structure of acceptor sugars, the specific requirements of acceptor sugars for a glucuronyl transfer may be suggested as follows: (a) pyranose structure and (b) equatorial position of the C2 and C3 hydroxyl group with $^4C_1$ conformation of the pyranose ring or equatorial position of C3 and C4 hydroxyl group with $^1C_4$ conformation of the pyranose ring (Fig. 5).

TABLE II

COMPARISON OF THE HYDROLASE AND TRANSFERASE ACTIVITY OF HIGHLY PURIFIED β-GLUCURONIDASE PREPARATIONS

All incubation mixtures contained 0.01 M Phe-[^{14}C]GlcUA, 0.1 M methyl-α-D-glucoside, and 0.16 U of the specified β-glucuronidase preparation. Incubation conditions, separation and determination of the reaction products as in Fig. 1.

β-Glucuronidase purified from	Specific activity * (U/mg protein)	Activity (μmol · U^{-1} · min^{-1}) × 10^2	
		Hydrolase	Transferase
Liver homogenate	5.7	3.23	0.72
Microsomal fraction	9.5	3.13	0.74
Lysosomal fraction	16.8	3.19	0.78

* Expressed as μmol glucuronic acid hydrolysed/min at pH 5.0 using unlabelled *p*-nitrophenyl-β-D-glucuronide as substrate (see Methods). Cell free supernatant of liver homogenate has a specific activity of 0.03 U/mg protein.

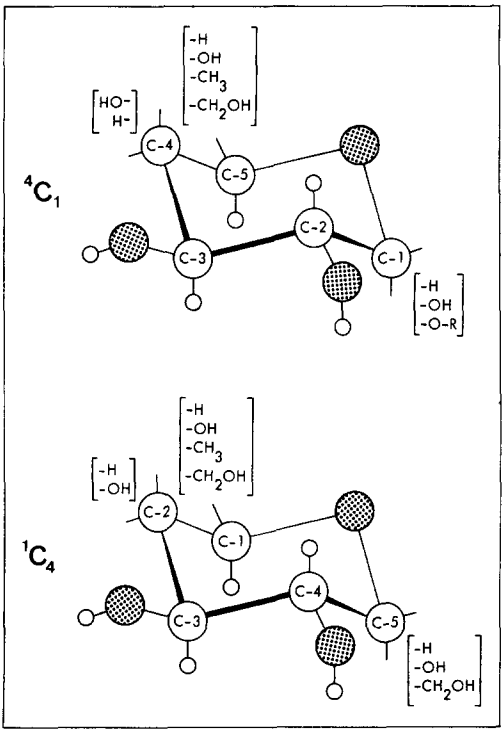

Fig. 5. Specific requirements of acceptor sugars for β-glucuronidase catalyzed glucuronyl transfer. Pyranoses in the $^4C_1$ or $^1C_4$ ($^2C_5$) conformation with an adequate hydroxyl group configuration serve as glucuronyl acceptors to form β-glucuronyl(1—3)glycosides. Sugars meeting requirements for the acceptor specificity are listed in Table I.

Type of linkage between glucuronyl residue and acceptor sugar

The susceptibility of the isolated transfer products to the hydrolytic action of β-glucuronidase establishes that their glycosidic linkages have the β-configuration. More detailed information about the type of glycosidic linkage formed by the glucuronyl transfer to methyl-α-D-glucoside was obtained by analyses of the transfer product. After the transfer product had been isolated, permethylated and hydrolysed, 2,4,6-tri-*O*-methylglucose was identified by gas chromatography, using authentic 2,4,6-tri-*O*-methylglucose and 3,4,6-tri-*O*-methylglucose as references. These results prove that the product of the transfer reaction is a 3-*O*-(β-D-glucopyranosiduronyl)methylglucopyranoside.

Discussion

In the present study highly purified β-glucuronidase is shown to transfer glucuronyl residues from low energy glucuronides selectively to C3 hydroxyl groups of specific acceptor sugars. This observation is not unexpected, since it is known for a number of glycosidases that the reaction catalyzed by these enzymes proceed via a glycosyl-enzyme intermediate which reacts either with water to yield the hydrolysis product (monosaccharide) or, in the presence of added nucleophiles (e.g. sugars or alcohols) to yield the transfer product [10—

16]. These transfer reactions have been explained by the greater nucleophilicity of sugars or alcohols than water towards the glycosyl enzyme intermediate. However, the results presented here make necessary a modification of this interpretation. With the assumption that the glucuronyl transfer to an acceptor sugar is a mere nucleophilic competition of the hydroxyl group of the acceptor monosaccharide with water, the exclusive formation of 1—3 glycosidic bond would be not expected, nor could the pronounced differences in the acceptor capacities of the sugars tested be explained. These findings rather suggest a specific kind of non-covalent binding between the glucuronyl enzyme complex and the acceptor sugar molecule. This binding could be mediated by an 'acceptor-binding site' on the surface of the enzyme molecule, which brings the C3 hydroxyl function of the $^1C_4$-pyranose ring close enough to the C1 atom of the glucuronyl residue. The observation that the transfer rate is enhanced when D-fructose (Table II, Fig. 5) or phenyl-β-D-glucupyranoside is used as acceptor instead of glucose, suggests that hydrophobic forces are involved in the stereospecific binding of the acceptor sugar.

The retention of the β-configuration of the D-glucuronyl residue during the enzyme catalyzed transglycosylation suggests that the reaction proceeds rather by a two step mechanism involving the intermediate formation of a D-glucuronyl-enzyme complex than by a single S_N2 displacement reaction where an inversion of the anomeric configuration would have been expected.

In mammalian liver the biosynthesis of glucuronides proceeds by the action of acceptor specific and acceptor unspecific UDP-glucuronate β-glucuronyl transferases (EC 2.4.1) which transfer residues from the high energy UDP-glucuronate to a wide range of acceptors. It is difficult to speculate on whether or not the di- or trisaccharide formation in the course of the glucuronyl transfer reaction is a physiologically important process, since the physiological concentration of potential acceptor sugars and the pH value in the vicinity of the microsomal enzyme are really not known.

Moreover the high stereospecificity of the β-glucuronidase catalyzed glycosyl transfer and the reasonable recovery of transfer products suggest an application to the chemical synthesis of β-glucuronyl-(1-3) sugars.

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