SHORT COMMUNICATIONS

BBA 23556

Uridine diphosphate β-glucuronic acid. A new substrate for β-glucuronidase

The glucosiduronic acids which are isolated from urine are always of the β-configuration with reference to the oside linkage. Yet the accepted glucuronyl donor, uridine diphosphate glucuronic acid (UDPGlcUA) is of the opposite, α-configuration. It is tacitly, therefore, assumed that glucuronyltransferase produces inversion of the oside linkage as the glucuronic acid combines with aglycone acceptor[1]. The latter mechanism has never been clarified.

One need not postulate that the same enzyme was catalyzing both an inversion and a group transfer at the same time if the UDPGlcUA was of the β-oside configuration to begin with. Such a compound, uridine diphosphate β-glucuronic acid (β-UDPGlcUA), has so far not been isolated from natural sources but has been synthesized[2] and was found in a preliminary experiment to be unsatisfactory as a glucuronyl donor using a microsomal suspension and *p*-nitrophenol as the acceptor.

On the other hand, β-UDPGlcUA could be a substrate for β-glucuronidase and would then serve as a glucuronyl donor in a transfer reaction.

In order to examine these possibilities, it was first necessary to prepare β-UDPGlcUA of high purity, to characterize it, to devise conditions for separating it from uridine diphosphate α-glucuronic acid (α-UDPGlcUA) and to carry out hydrolytic reactions with β-glucuronidase. This paper describes the results of these experiments.

Materials. Starting materials for the synthesis of β-UDPGlcUA included 1,3-dicyclohexyl guanidinium uridine 5'-phosphoramidate (graciously supplied by Takeda Chemical Industries, Osaka, Japan) and the triethyl ammonium salt of β-glucuronic acid 1-phosphate (β-GlcUA-1-*P*) prepared in this laboratory, α-UDPGlcUA, UDP, UMP used as standards were obtained from Sigma Chemical Co., U.S.A. Silica gel (F_{254}, 10 cm × 20 cm) plates used for thin-layer chromatography were supplied by E. Merck, Germany. Other reagents were obtained from commercial sources and were of reagent grade.

Mouse urinary β-glucuronidase purified according to PETTENGILL AND FISHMAN[4] of specific activity 50000, was the enzyme source.

Synthesis of β-UDPGlcUA. The method of HONJO *et al.*[2], was employed to synthesize the β-UDPGlcUA with modifications. Thus, 1 g of the cyclohexylamine salt of β-GlcUA-1-*P*[5,6] was converted to its triethylammonium salt and 1,3-dicyclohexyl guanidinium uridine 5'-phosphoramidate (1.6 g) in pyridine (200 ml) was added to it. The pyridine mixture was incubated at 37° for 8 days with occasional stirring. Product formation was checked periodically on thin-layer chromatographic plates with an aliquot of the reaction mixture by developing them in the solvent 0.5 M ammonium acetate buffer (pH 3.8)–ethanol (2:5, v/v) with α-UDPGlcUA as standard. The spots

Abbreviations: β-GlcUA-1-*P*, β-glucuronic acid 1-phosphate; α-UDPGlcUA, uridine diphosphate α-glucuronic acid; β-UDPGlcUA, uridine diphosphate β-glucuronic acid.

were identified under ultraviolet light and developed with a conc. H_2SO_4–ethanol (1:1, v/v) spray with heating. To enhance the purity of β-UDPGlcUA the product was passed through a second charcoal column and the fractions of β-UDPGlcUA were pooled, evaporated to dryness at 20°, redissolved in small amounts of water, and analysed.

Observations. The R_F values obtained in descending paper chromatography and thin-layer chromatographic plates are presented in Table I which shows that there is a good separation of the uridine phosphates and 1,3-dicyclohexyl guanidinium uridine 5′-phosphoramidate which are the probable impurities of the synthesized β-UDPGlcUA. However, thin-layer chromatography and not paper chromatography was able to separate α-UDPGlcUA and β-UDPGlcUA.

TABLE I

R_F VALUES FOR β-UDPGlcUA, α-UDPGlcUA AND THEIR PRECURSORS

Solvent for paper chromatography: ammonium acetate–ethanol (3:7.5, v/v)[6], 8 h. Solvent for thin-layer chromatography: 0.5 M ammonium acetate buffer (pH 3.8)–ethanol (2:5, v/v).

Compound	*R_F values*	
	Paper chromatography	*Thin-layer chromatography*
UMP	0.31	0.35
UDP	0.40	0.06
α-UDPGlcUA	0.28	0.10
β-UDPGlcUA	0.30	0.25
UMP-NH_2*	0.45	0.50

* 1,3-dicyclohexyl guanidinium uridine 5′-phosphoramidate.

The uridine absorption in ultraviolet light was compared with the standard α-UDPGlcUA in solution at pH 1.0 (1 M HCl) and was read in a DU-spectrophotometer from 248 to 280 nm. The products were also developed and compared by descending paper chromatography with the solvent ammonium acetate–ethanol (3:7.5, v/v)[2] with the standards of α-UDPGlcUA, UDP, 1,3-dicyclohexyl guanidinium uridine 5′-phosphoramidate, UMP.

Conditions for β-UDPGlcUA as substrate for β-glucuronidase (Table II). The enzyme used was purified mouse urinary β-glucuronidase[4]. The substrates were standard α-UDPGlcUA and synthesized β-UDPGlcUA in 0.2 M acetate buffer (pH 4.5), saccharolactone (0.67 mM) was used as an inhibitor. The reaction mixture was incubated at 37° for 24 h, chilled to stop the reaction and the FISHMAN AND GREEN[7] method was applied directly.

The FISHMAN AND GREEN[7] test for bound glucuronic acid of the synthesized compound recovers the amount of glucuronic acid expected from the companion measurements of the ultraviolet absorption at 260 nm, made on β-UDPGlcUA.

The results of hydrolysis of β-UDPGlcUA was presented in Table II which shows that β-UDPGlcUA is a substrate for β-glucuronidase since 12 out of 13 μg of its glucuronic acid were liberated in the enzyme digest. Saccharolactone inhibited hydrolysis by 75%. On the other hand, no glucuronic acid was released from α-UDPGlcUA by β-glucuronidase.

The ultraviolet absorption spectra of β-UDPGlcUA at 262 nm is indistinguishable from that of α-UDPGlcUA. It has been found (Table I) that β-UDPGlcUA travels close to α-UDPGlcUA on paper but can be separated from it by thin-layer chromatography. The above results indicate that the β-UDPGlcUA synthesized was sufficiently pure to be used as a substrate for β-glucuronidase. Indeed, it is a valid substrate (Table II) for β-glucuronidase which was also verified by the well known saccharolactone inhibition of β-glucuronidase.

TABLE II

HYDROLYSIS OF α-UDPGlcUA AND β-UDPGlcUA BY β-GLUCURONIDASE

Test substrate	*Enzyme*	*Substrate*	*Saccharo-lactone*	*Glucuronic acid in the incubation mixture (μg)*		
				Total	*Bound*	*Free*
α-UDPGlcUA	+	+	−	10.0	10.0	0.0
	−	+	−	10.0	10.0	0.0
	+	+	+	10.0	10.0	0.0
	+	−	−	0.0	0.0	0.0
β-UDPGlcUA	+	+	−	13.0	1.0	12.0
	−	+	−	13.0	13.0	0.0
	+	+	+	13.0	10.0	3.0
	+	−	−	0.0	0.0	0.0

One wonders whether β-UDPGlcUA may be a constituent of normal mammalian cells. In this regard, attention may be directed to the endogenous glucuronide synthesis of homogenates[8] unrelated to UDPGlcUA glucuronyltransferase as a likely location of β-UDPGlcUA.

This study was aided in part by U.S. Public Health Service Research Career Award No. 5-K6-CA-18, 453-05 to William H. Fishman.

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Received September 8th, 1969

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