'COUPLED TRANSGLUCURONIDATION': A NEW TOOL FOR STUDYING THE LATENCY OF UDP-GLUCURONYL TRANSFERASE

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1. Introduction

The latency of the enzyme UDP-glucuronyl transferase (EC 2.4.1.17) [GTase] has been investigated using conditions in which the glucuronyl donor substrate UDPGA is generated in situ in guinea pig liver microsomes, instead of adding it directly to the assay medium. The theoretical feasibility of this system was postulated by Dutton [1] and was confirmed in these studies using *p*-nitrophenyl-β, D-glucosiduronic acid, Na^+ salt (*p*-nitrophenylglucuronide) and UDP to generate UDPGA (by a GTase-catalysed reverse reaction) and employing *o*-aminophenol as aglycone acceptor in the second step. The effect of Mg^{2+} and detergents on each of these component reactions has been compared with the effect of these agents on the 'coupled transglucuronidation', and additionally on the UDPase (nucleosidediphosphate phosphohydrolase, EC 3.6.1.6) activity of microsomes. These findings are discussed in relation to current theories for explaining GTase and UDPase latency.

2. Materials and methods

2.1. *Animals*

Male guinea pigs, approximate body weight 400 g, were used throughout.
Animals were starved overnight and killed by cervical dislocation.

2.2. *Preparation of microsomes*

Livers were homogenised in 0.25 M sucrose, and microsomes were sedimented in 60 min at 104 000 g_{av} from a supernatant, prepared by centrifuging homogenate for 10 min at 10 000 g_{av}.

3. Results

3.1. *Effect of Mg^{2+} and detergents on the GTase-catalysed reverse reaction between UDP and p-nitrophenylglucuronide.*

When microsomes were incubated with UDP (4 mM) and *p*-nitrophenylglucuronide (2 mM) in the presence of EDTA, *p*-nitrophenol was liberated at the rate of 1.8 nmoles/min/mg protein (table 1A). Virtually no *p*-nitrophenol was formed in blanks without added UDP. These findings confirmed the claim of Vessey and Zakim [2,3] that the GTase-catalysed reaction between *p*-nitrophenol and UDPGA is reversible. When the EDTA in the assay medium was replaced by $MgCl_2$ a 60% inhibition of the reverse reaction was observed (table 1A).

When the microsomes were pre-treated with digitonin at 0.6%, a more than 5-fold increase in the rate of *p*-nitrophenol liberation was observed (table 1A), assayed in the presence of EDTA. This is the first time that latency has been reported for the reverse reaction of GTase. Vessey and Zakim have reported that Triton

Table 1
Effects of EDTA, magnesium chloride and detergents on *p*-nitrophenol formation (nmoles/min/mg protein)

Substrates	Additions to assay			
	EDTA	$MgCl_2$	Digitonin EDTA	Triton EDTA
A. *p*-NPGA UDP	1.8	0.7	9.0	2.9
B. *p*-NPGA UDP *o*-amino-phenol	2.4	0.8	7.7	2.7

(A) Initial rate of *p*-nitrophenol liberation in the GTase-catalysed reverse reaction.
(B) Initial rate of *p*-nitrophenol liberation in coupled transglucuronidation.

In all assays microsomes (1.5 mg in 0.1 ml of 0.25 M sucrose) were added to 0.4 ml of incubation medium to give the following final concentrations. 100 mM sodium maleate buffer, pH 7.1: 3 mM glucaro-1,4-lactone. Final substrate concentrations were 4 mM UDP and 2 mM *p*-nitrophenylglucuronide (*p*-NPGA) for the reverse GTase [3] and coupled reactions, with the addition of 0.4 mM *o*-aminophenol for the latter. When conjugation of *o*-aminophenol was assayed by conventional means, 0.4 mM *o*-aminophenol was incubated with 4 mM UDPGA. $MgCl_2$ or EDTA was present in all assays at 4 mM as indicated. Detergents, when employed, were added as aqueous dispersions to the microsomal suspension on ice, giving final concentrations of 0.6% with digitonin or 0.075% with Triton X-100. Initial rates of *o*-aminophenol conjugation were measured by the method of Dutton and Storey [12]. The same supernatants, made alkaline with a minimum volume of 10 M KOH, were used to measure the UDP-dependent formation of *p*-nitrophenol from *p*-nitrophenylglucuronide. Absorbance at 400 mm (corrected for blanks without UDP), was read immediately since it was found that alkaline hydrolysis of *p*-nitrophenylglucuronide was appreciable at the concentrations employed in the coupled or reverse reactions of GTase. The findings reported were initially observed in 4 experiments using microsomes which had been stored frozen at −10° C for up to 4 weeks. The data in tables 1 and 2, which are concordant with those obtained with frozen microsomes, relates to a typical experiment in which all assays were performed in duplicate on microsomes prepared that day. Duplicates differed by no more than 5%.

produced no activation of the reverse reaction [2]. However, in our experiments Triton at 0.075% produced significant (60%) activation in the presence of EDTA (table 1A), though it appeared much less efficient than digitonin.

3.2. *Coupled transglucuronidation of o-aminophenol with UDP and p-nitrophenylglucuronide.*

When the GTase-catalysed reverse reaction was performed in the presence of EDTA and *o*-aminophenol (0.4 mM), the latter was observed to be conjugated at the rate of 1.1 nmoles/min/mg (table 2A.) The identity of the diazotisable material formed in the coupled reaction with that of authentic *o*-aminophenylglucosiduronate was confirmed by thin layer chromatography.* The liberation of *p*-nitrophenol in this coupled system was at the rate of 2.4 nmoles/min/mg (table 1B) which was approximately 30% greater than in the reverse reaction without added *o*-aminophenol. The figure for *p*-nitrophenol production in the coupled reaction indicated an efficiency of approx. 40% in the conjugation of *o*-aminophenol. When *o*-aminophenol (0.4 mM) was incubated with 4 mM UDPGA (the latter concentration having been shown in preliminary experiments to be approximately saturating) conjugation occurred at the rate of 0.6 nmoles/min/mg in the presence of EDTA (table 2B). Hence conjugation in the coupled system occurred at approximately twice the rate of conjugation in the system in which UDPGA was added directly. These findings confirmed Dutton's postulate that UDPGA, generated in situ by a GTase-catalysed reverse reaction could result in the con-

Table 2
Effects of EDTA, magnesium chloride and detergents on *o*-aminophenol conjugation (nmoles/min/mg protein)

Substrates	Additions to assay			
	EDTA	$MgCl_2$	Digitonin EDTA	Triton EDTA
A. *p*-NPGA UDP *o*-amino-phenol	1.1	0.3	0.5	0.7
B. UDPGA *o*-amino phenol	0.6	0.9	4.3	0.9

Initial rate of *o*-aminophenol conjugation in: (A) coupled transglucuronidation; (B) assay with added UDPGA.

* C. Berry and T. Hallinan. Unpublished data.

jugation of an added aglycone acceptor [1], and showed that ' coupled transglucuronidation' was not only rapid, but efficient also.

3.3. *Effect of Mg^{2+} and detergents on coupled transglucuronidation*

Incubation of *o*-aminophenol (0.4 mM and *p*-nitrophenylglucuronide (2 mM) in the presence of Mg^{2+} produced rates of *o*-aminophenol conjugation which were approximately 30% of those observed in the presence of EDTA (table 2A). The release of *p*-nitrophenol in the coupled system was also inhibited to the same extent (table 1B). *o*-Aminophenol conjugation in the conventional assay system, however, was activated 50% by substituting $MgCl_2$ for EDTA (table 2B). Hence it was unlikely that inhibition of the coupled reaction by Mg^{2+} was due to activation of the enzyme UDPGA-pyrophosphatase, wich is absent or low in guinea pig liver [4–6], since a similar inhibition of the assay with added UDPGA might have been expected, instead of the activation observed.

Digitonin inhibited the rate of coupled transglucuronidation by 57% (table 2A). A smaller inhibition (35%) was observed with Triton (table 2A). Detergents, unlike Mg^{2+} activated both of the component reactions of the coupled system. Conjugation of *o*-aminophenol with added UDPGA was activated more than 7-fold with digitonin but only 50% with Triton (table 2B). Digitonin activation of *o*-aminophenol conjugation in guinea pig liver has also been reported by Winsnes [7]. *p*-Nitrophenol liberation in the coupled reaction was activated 3-fold with digitonin and 13% with Triton (table 1B). This activation was not additive with that of *o*-aminophenol on the reverse reaction, and in fact *o*-aminophenol was inhibitory to the detergent-activated reverse reaction (table 1A and 1B).

4. Discussion

The findings reported here support the suggestion by several authors that in undisrupted microsomes, GTase is separated from its substrates by a permeability barrier [7,8,9]. Restricted access of UDPGA could explain our finding that *o*-aminophenol is conjugated at a faster rate by UDPGA generated in situ comparend with the system to which UDPGA is added at a close to saturating concentration. Since this implies that microsomal vesicles are more permeable to UDP and *p*-nitrophenylglucuronide than to UDPGA, an explanation is provided for the report that in untreated beef liver microsomes V_{max} for the reverse GTase reaction is twice that of the forward reaction [3]. Hänninen and Puukka have proposed that GTase is inside the microsomal vesicle [10]. The location of the enzyme in this compartment would ensure that UDPGA generated in the coupled reaction would be maintained at a high concentration for the conjugation of *o*-aminophenol, accounting perhaps for the surprisingly high efficiency of the coupled reaction (40%) and the observation that detergents inhibit the coupled reaction, while activating both of its component steps. Digitonin in these experiments was more potent in its effects than Triton, and with Triton Vessey and Zakim observed no activation of the reverse GTase reaction [2] and rejected compartmentation as an explanation for the latency of GTase. In its place they suggested that the enzyme is constrained by membrane lipids in a conformation of low catalytic activity [2]. Studies are at present in progress, employing phospholipase C, and the techniques reported here, aimed at discriminating further between compartmentation and lipid constraint models.

The effects of Mg^{2+} and EDTA upon the reverse GTase reaction can be correlated with their effects upon UDPase. Exposure of microsomes to 4 mM EDTA for 5 min inhibited UDPase 97%, while further exposure to EDTA virtually abolished its activity.* It was also confirmed that UDPase, assayed exactly like GTase in 0.1 M maleate buffer, pH 7.1 with 4 mM Mg^{2+} and 4 mM UDP, was highly latent. Digitonin activated it 4–fold.* Since the latency of UDPase is known from immunochemical and other evidence to be due to its location inside microsomal vesicles [11], the relationship between UDPase and GTase activities requires consideration. UDPase hydrolyses one of the reactants in the reverse reaction of GTase. This is very likely to be the explanation for why EDTA, wich virtually abolishes UDPase, activates the reverse GTase reaction. Further investigations are in hand to explore the possibility that in some

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circumstances UDPase may also importantly modulate aglycone conjugation in the forward reaction of UDP-glucuronyl transferase.

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