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THE INHERITED DEFICIENCY OF HEPATIC UDP-GLUCURONYLTRANSFERASE: STRUCTURE–ACTIVITY RELATIONSHIPS OF *IN VITRO* STIMULATORS

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Abstract—More than 18 compounds have been tested for their ability to stimulate defective UDP-glucuronyltransferase activity of Gunn rat liver homogenates towards 2-aminophenol, up to levels of transferase activity in similarly treated Wistar rat liver homogenates. The minimum structural requirements of an effective compound are a combination of the presence of an electron-attracting atom or group and a sparing solubility in water. The activation of defective UDP-glucuronyltransferase towards 2-aminophenol by pentan-3-one is reversible. The possible mechanism of action of alkyl ketone activators is discussed.

Defective glucuronidating ability in humans can result from an inherited genetic deficiency. The hyperbilirubinaemic condition can be merely troublesome, as in Gilbert's syndrome, or lethal, as in Crigler-Najjar syndrome [1]. A mutant strain of rat, Gunn rat, is considered a good animal model for the study of the human Crigler-Najjar syndrome [2]. The biochemical lesion in Gunn rat liver results in the complete absence of bilirubin UDP-glucuronyltransferase activity and poor ability to glucuronidate 2-aminophenol (see ref. 3). Stevenson *et al.* [4] showed that the carcinogen diethylnitrosamine, when added to Gunn rat liver extracts *in vitro*, stimulated the deficient UDP-glucuronyltransferase activity towards 2-aminophenol up to the enhanced activity levels observed in diethylnitrosamine-treated homogenates of Wistar rat liver; the deficiency of UDP-glucuronyltransferase activity towards 2-aminophenol was no longer apparent. This unusual effect has been widely investigated and shown to be limited to a few substrates (see ref. 3). However, recent work from this laboratory has shown that considerably less hazardous alkyl ketones are as effective as diethylnitrosamine in overcoming the apparent deficiency [5].

We have examined further the unexplored potential of these compounds as tools for investigation of this inherited genetic deficiency disease by attempting to answer three further questions concerning their action *in vitro*:

(a) What are the essential structural features of the compound which causes an optimal response?
(b) Could reduced levels of a compound with improved structural properties be used?
(c) Is the effect reversible?

MATERIALS AND METHODS

Chemicals. Butan-2-one, chloroform, carbon tetrachloride, diethylamine, diethylether, diethylformamide, dichloromethane, dimethylformamide, octan-2-ol, methanol, pentan-2-one, pentan-3-one, propan-2-ol, propan-2-one and 2-aminophenol were all obtained from BDH Chemicals, Poole, Dorset, U.K., Analar grade being purchased whenever possible. Decan-2-one and dodecan-2-one were from ICN Pharmaceuticals, Plainsville, NY, U.S.A., dimethylsulphoxide was obtained from Ralph N. Emmanuel, Wembley, U.K. UDP-glucuronic acid and 1-chloro-3-tosylamido-7-amino-L-2-heptanone HCl were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Animals. Gunn and Wistar rats were from the colonies maintained in the Institute's animal unit.

Addition of activators. Liver homogenates (20%, w/v) were prepared in ice cold 0.25 M sucrose by using three strokes of a Teflon–glass homogenizer. Small aliquots of aqueous solution of activators were added to incubation mixtures for UDP-glucuronyltransferase assays (0.2ml–see below) containing approximately 2.5mg of homogenate protein. After mixing, the solutions were maintained at 0–5° for 10 min. The assays were then started by addition of UDP-glucuronic acid.

Enzyme assays. UDP-glucuronyltransferase was assayed with 2-aminophenol as substrate by the method of Winsnes [6] and with bilirubin as substrate by the method of Heirwegh *et al.* [7]. Protein concentrations were measured by the method of Lowry *et al.* [8] and Bradford [9].

Isolation of UDP-glucuronyltransferase. Liver ammonium sulphate extracts were prepared as previously described by Weatherill and Burchell [10].

RESULTS

Structural requirements for optimal activation of Gunn rat liver UDP-glucuronyltransferase in vitro. All Gunn rat liver homogenates were assayed for UDP-glucuronyltransferase activity towards bilirubin. This enzyme activity was completely absent from all the Gunn rat livers used in these studies.

Table 1. *In vitro* stimulation of UDP-glucuronyltransferase activity of Gunn and Wistar rat liver homogenates by various compounds

Activator (10mM)	UDP-glucuronyltransferase activity (pmoles 2-aminophenylglucuronide formed/min/mg protein)					
	Females			Males		
	Gunn	Wistar	Ratio of activities (Gunn/Wistar)	Gunn	Wistar	Ratio of activities (Gunn/Wistar)
Methan-1-ol	11*	33*	0.33			
Propan-2-ol	14*	33*	0.43			
Dimethylsuphoxide	14 ± 1	30 ± 4	0.45			
Dimethylformamide	20 ± 2	41 ± 3	0.49			
None	7 ± 0.5	27 ± 1	0.27	4 ± 1	30 ± 4	0.13
Propan-2-one	32 ± 6	43 ± 12	0.74	27*	39*	0.61
Butan-2-one	68 ± 6	74 ± 14	0.92	49 ± 5	45 ± 6	1.08
Pentan-3-one	79 ± 10	67 ± 14	1.18	53 ± 3	57 ± 4	0.93
Diethylformamide	58 ± 7	60 ± 14	0.96	34 ± 4	45 ± 6	0.76
Diethylether	18 ± 5	37 ± 11	0.49	14 ± 3	29 ± 4	0.49
Diethylamine	12 ± 4	40 ± 10	0.30	3 ± 1	29 ± 3	0.10

Enzyme assay mixtures were prepared using 20% (w/v) liver homogenates. Activities are presented as means of at least four results ± S.E.M., except where indicated by asterisk (*) which shows average of three results.

More than 18 compounds were tested for their ability to activate Gunn rat liver UDP-glucuronyltransferase *in vitro*. The effects of ten of these compounds, which are completely miscible with and soluble in water at the concentrations used are shown in Table 1. These compounds were selected to indicate what structural requirements are essential for optimal activation of Gunn rat liver transferase towards 2-aminophenol. Optimal results were obtained using butan-2-one and pentan-3-one, which, as previously reported [5], completely abolish the apparent deficiency of UDP-glucuronyltransferase towards this substrate. Table 1 also shows that the apparent deficiency is not abolished by diethylamine. However, the gradual introduction of a carbonyl group and extension of the hydrocarbon side chain both increase the activating ability of these compounds. Substitution of a sulphonyl group in dimethylsulphoxide does not appear to be as effective as the carbonyl group in acetone. Octan-2-ol, decan-2-one and dodecan-2-one did not activate the transferase, although it was impossible to obtain suitable aqueous solutions of these compounds. Thus the presence of a carbonyl group and an ethyl side chain group appear to be minimal requirements for optimal activation.

However, Table 2 shows that dichloromethane, trichloromethane and tetrachloromethane also activate the defective UDP-glucuronyltransferase activity towards 2-aminophenol in Gunn rat liver homogenates. The effectiveness of these compounds seemed to increase with the number of chlorine atoms present in the molecule. This combination of an electron-attracting group and a hydrophobic component appears to be the only common structural feature.

Effect of alkyl ketone concentration on UDP-glucuronyltransferase activity in Gunn rat liver. We have investigated the effect of alkyl ketone concentration on Gunn rat liver homogenate transferase activity, to find out whether or not lower concentrations of these stimulators could be employed. Figure 1 shows the results of this experiment using three alkyl ketones. UDP-glucuronyltransferase activity of female Gunn rat liver homogenates was measured in the presence of up to 100 mM alkyl ketone. The results show that the optimal effect could be obtained with as little as 5 mM pentan-2-one, whereas 100 mM propane-2-one was required. Overall, pentan-2-one was slightly more effective than butan-2-one at concentrations below 5 mM, and butan-2-one was more effective than propan-2-one over the whole 0–

Table 2. The effect of chloromethanes on the UDP-glucuronyltransferase activity of female Gunn and Wistar rat liver homogenates *in vitro**

Activator (10 mM)	UDP-glucuronyltransferase activity (units/mg protein)		Ratio of activities
	Gunn	Wistar	Gunn/Wistar
Dichloromethane (4)	12 ± 4	25 ± 5	0.48
Trichloromethane (5)	24 ± 2	32 ± 5	0.75
Tetrachloromethane (4)	27 ± 10	30 ± 7	0.90
Control (5)	5.7 ± 1	22 ± 3	0.26

* Enzyme assay mixtures were prepared using 20% (w/v) liver homogenates. One unit of enzyme activity is expressed as 1 pmole of 2-aminophenylglucuronide formed/min. Activities are presented as means ± S.E.M. Figures in parentheses indicate the number of samples used.

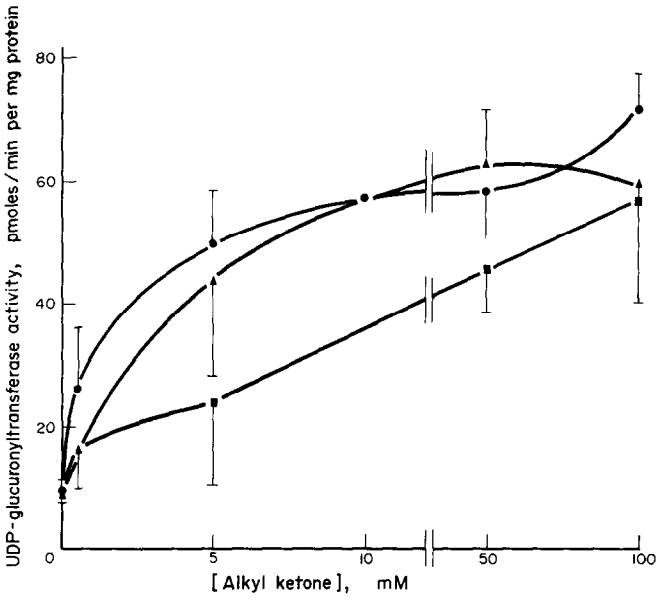

Fig. 1. Effect of alkyl ketone concentration on UDP-glucuronyltransferase activity of female Gunn rat liver homogenates. Enzyme assay mixtures were prepared using 20% (w/v) liver homogenates and 2-aminophenol as substrate. Mean values of three complete experiments are shown. Points with vertical bars show the extent of the variation of each three values obtained: ■———■ propan-2-one; ▲———▲ butan-2-one; ●———● pentan-2-one.

100 mM concentration range. All these three compounds appear to be equally effective when used at 100 mM final concentration.

We have also examined the effect of the detergent Lubrol 12A9 on UDP-glucuronyltransferase activity towards 2-aminophenol in female rat liver homogenates to compare the mechanism of activation by alkyl ketones with that by detergents. Figure 2 shows the results of this experiment. Both Gunn and Wistar rat liver UDP-glucuronyltransferase(s) appear to be optimally stimulated approximately 2-fold by a final detergent concentration of 1 mM. This concentration of detergent is somewhat different to the concentration of alkyl ketone required for activation of deficient Gunn rat liver transferase activity and also does not abolish the apparent enzyme deficiency. Thus the activation of defective Gunn rat liver UDP-glucuronyltransferase towards 2-aminophenol by alkyl

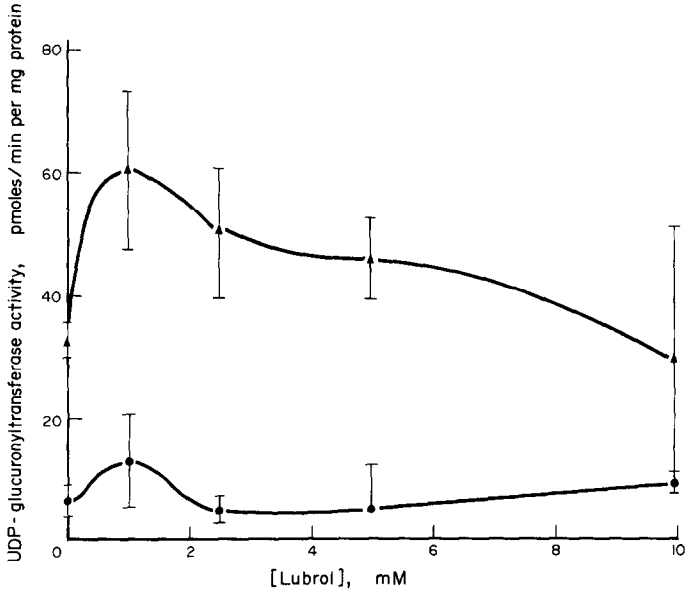

Fig. 2. Effect of Lubrol 12A9 concentration on UDP-glucuronyltransferase activity of female Gunn (●———●) or Wistar (▲———▲) rat liver homogenates. Enzyme assay mixtures were prepared using 20% (w/v) liver homogenates and 2-aminophenol as substrate. Mean values of three complete experiments are shown. Points with vertical bars show the extent of the variation of each three values obtained.

Table 3. Reversible activation of partially purified UDP-glucuronyltransferase by pentan-3-one*

Sample	UDP-glucuronyltransferase activity (nmoles 2-aminophenyl-glucuronide formed/min/mg protein)	
	Female	Male
Ammonium sulphate fraction + buffer	0.00	0.03
Ammonium sulphate fraction + 10 mM pentan-3-one (A)	0.26	0.39
Dialysed (A)	0.01	0.04
Dialysed (A) + 10 mM pentan-3-one	0.34	0.38

* Ammonium sulphate fractions were prepared from 4 female or 4 male Gunn rat livers as previously described [10]. Either pentan-3-one (10 mM in dialysis buffer) or dialysis buffer were added to aliquots of ammonium sulphate fractions. After assay, these test and control experimental mixtures were then dialysed overnight at 4° against 250 vol. of dialysis buffer. The resulting mixtures were then assayed for UDP-glucuronyltransferase activity ± 10 mM pentan-3-one.

ketones appears to occur by a different mechanism to the small activation caused by Lubrol 12A9.

Mechanism of interaction of pentan-3-one with UDP-glucuronyltransferase. We have attempted to examine the nature of the alkyl ketone interaction with UDP-glucuronyltransferase. Neither the detergent Lubrol 12A9 (Fig. 2), phospholipids [10] or UDP-*N*-acetylglucosamine (unpublished work) act in the same manner as alkyl ketone. Diethylnitrosamine has been shown to activate purified defective Gunn rat liver UDP-glucuronyltransferase by apparently directly interacting with the enzyme protein [10]. How does pentan-3-one bind to UDP-glucuronyltransferase?

Crude ammonium sulphate extracts of liver, in which the transferase is stable during dialysis, were employed to answer this question. Results are shown in Table 3. Gunn rat liver extracts (10 mg protein/ml) were treated with 10 mM pentan-3-one, which increased the transferase activity towards 2-aminophenol in Gunn rat liver preparations 13-fold. UDP-glucuronyltransferase activity of Gunn rat liver extracts cannot be 'completely' activated up to the levels exhibited by Wistar rat liver extracts, due to a differential inactivation of the transferase during isolation [10]. However, the significant activation of both liver extracts can be reversed by dialysis of alkyl ketone-treated extracts against 5 l. of 25 mM phosphate buffer/0.05% Lubrol, pH 7.4 for 16 hr. Dialysed ammonium sulphate extracts were also treated with 10 mM pentan-3-one and the transferase assayed to indicate further the reversibility of this phenomenon and the stability of the transferase in the ammonium sulphate extracts. Table 3 shows that ammonium sulphate fractions can be reactivated by further pentan-3-one treatment. Dialysis of other portions of ammonium sulphate fractions as control experiments indicated that the transferase activity towards 2-aminophenol does not change during dialysis. Thus the interaction of pentan-3-one with UDP-glucuronyltransferase is completely reversible.

DISCUSSION

The minimum structural properties of stimulators required to abolish the apparent genetic deficiency of Gunn rat liver UDP-glucuronyltransferase activity towards 2-aminophenol appear to be an electron-attacking group and a hydrophobic characteristic. They are all polar solvents. These are the only properties of the stimulators that can be identified from molecules as diverse in structure as carbon tetrachloride and pentan-2-one. Carbon tetrachloride and pentan-2-one may act in the same manner, for, when used in combination, their effects are not additive. Similarly, diethylnitrosamine and pentan-2-one do not produce an additive effect on the transferase activity. How do these compounds exert this remarkable effect on Gunn rat liver UDP-glucuronyltransferase?

Earlier work has shown that high concentrations (150 mM) of *n*-pentane [13] and high concentrations (0.55–2.7 M) of acetone, chloroform and ethyl-ether [14] activated Wistar rat liver microsomal UDP-glucuronyltransferase up to 5-fold. Kinetic measurements of the transferase activity indicate that this phenomenon is due to release of latent activity, and the same effect can be reproduced using Triton X-100 [13]. Alkyl ketones (10 mM) activate Wistar rat liver UDP-glucuronyltransferase only 1 to 2-fold, and chloromethanes (10 mM) by only 30 per cent. However, these same concentrations of alkyl ketone stimulate Gunn rat liver UDP-glucuronyltransferase some 20-fold, suggesting that this latter effect is not a simple release of latent activity. The alkyl ketone activation cannot be reproduced by addition of the non-ionic detergent Lubrol 12A9, UDP-*N*-acetylglucosamine (unpublished work) or phospholipids [12]. Indeed, if latency of the transferase is abolished by solubilization and purification of the defective enzyme from Gunn rat liver, alkyl ketones are still able to stimulate the purified enzyme [5]. These results stress the unusual nature of this activation phenomenon.

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