Biochemical Pharmacology, Vol. 31, No. 13, pp. 2285–2289, 1982.
Printed in Great Britain.

0006-2952/82/132285-05 $03.00/0

ON THE SUBSTRATE SPECIFICITY OF THE DIGITOXIGENIN MONODIGITOXOSIDE CONJUGATING UDP-GLUCURONYLTRANSFERASE IN RAT LIVER

ACHIM SCHMOLDT and JÖRG PROMIES

Pharmakologisches Institut der Universität, Martinistraße 52, 2000 Hamburg 20, Federal Republic of Germany

(*Received* 4 *November* 1981; *accepted* 25 *January* 1982)

Abstract—The aim of the present study was to investigate the specificity of the UDP-glucuronosyltransferase (EC 2.4.1.17) involved in the conjugation of digitoxigenin monodigitoxoside. By *in vitro* assays with detergent activated liver microsomes it was found that (1) digitoxigenin monodigitoxoside is by far the best substrate of all cardenolides and cardenolide digitoxosides tested. (2) In the presence of saturating UDP-glucuronate concentrations an apparent K_m of 5.8 μM was obtained from linear Lineweaver–Burk plots together with a V_{max} of about 150 pmoles/mg microsomal protein/min. (3) Neither phenobarbital nor polycyclic aromatic hydrocarbons caused a considerable induction of the enzyme without change of the apparent K_m, but spironolactone did. (4) The conjugation of the substrate (4 μM) could only be inhibited by the 3′-epi-digitoxoside of digitoxigenin. (5) 25–50 μM substrate inhibited only the conjugation of the 3′-epimer and that of digoxigenin monodigitoxoside. It is suggested that there is a form of glucuronyltransferase which specifically conjugates digitoxigenin monodigitoxoside.

In earlier publications it was reported that the water soluble metabolite of dt-1* in rats consists of the dt-1-glucuronide [1, 2]. The biliar excretion of this metabolite can be enhanced by pretreatment with spironolactone [3]. This led to the conclusion that an induction of glucuronyltransferase(s) is responsible for this effect. In the meantime, we were able to demonstrate that the stepwise cleavage of the sugar chain from dt-3 to dt-1 is a cytochrome P450-dependent reaction in that each of the terminal digitoxosyl groups can only be split off after oxidation of the axial hydroxyl groups to oxo groups [4]. Hence, two monooxygenase reactions are necessary for the formation of dt-1. These reactions are very slow. It can, therefore, be assumed that the monooxygenase reaction is actually the rate limiting step. The increased elimination rate after spironolactone is mainly due to the induction of a relatively specific P450-isocytochrome [5].

In fact there is also an increase in the activity of the glucuronyltransferase(s). Richards and Lage [6] established that after spironolactone pretreatment the glucuronyltransferase activity for the substrate dt-1 in liver homogenates is enhanced. In experiments with liver microsomes Castle [7] could confirm this finding for female rats and after pregnenolone-16a-carbonitrile pretreatment for rats of both sexes.

The aim of this study was to investigate the specificity of this glucuronyltransferase in order to determine whether it is one of the enzyme forms† already known or a particular one.

MATERIALS AND METHODS

Animals. Male Wistar rats (180 ± 20 g body wt) were pretreated with phenobarbital (40 mg/ml 0.9% NaCl, 2 ml/kg i.p. daily for 4 days), benzo[*k*]fluoranthene (40 mg/ml arachis oil, 1 ml/kg i.p. daily for 3 days) or spironolactone (100 mg/2 ml H_2O, 2 ml/kg p.o. daily for 4 days). Control rats received the vehicle only. All animals had free access to laboratory chow and water *ad lib.* until sacrifice (24 hr after the last dose).

Chemicals. 20,22[^{3}H]Digitoxin was a generous gift of B.D.F. Beiersdorf (Hamburg). [^{3}H]dt-1 and [^{3}H]dg-1 were prepared by cleavage of [^{3}H]digitoxin and [^{3}H]digoxin, respectively, according to Satoh and Aoyama [9]. [^{3}H]dt-O was prepared by acidic hydrolysis of [^{3}H]digitoxin. Epimeric cardenolides (epi-dt-1, epi-dt-0) were obtained by reduction of the corresponding dehydro compounds [4]. 1,2[^{3}H]Pregnanediol, 4[^{14}C]testosterone and scintillation cocktail (Biofluor) were obtained from NEN (Dreieich, F.R.G.), carboxy[^{14}C]chenodeoxycholate from Amersham (Braunschweig, F.R.G.) and 6,7[^{3}H]estrone from CEA (Dreieich, F.R.G.). UDP-glucuronate, cardenolides and glucuronidase were from Boehringer (Mannheim, F.R.G.), Brij 58 from Serva (Heidelberg, F.R.G.). All other chemicals and thin-layer plates precoated with silica gel A 60 were obtained from Merck (Darmstadt, F.R.G.).

Incubations. Incubation mixtures contained, in a total volume of 0.25 ml, freshly prepared microsomes

* Abbreviations: dt-3, digitoxin; dt-1, digitoxigenin monodigitoxoside; dg-1, digoxigenin monodigitoxoside; epi-dt-1, digitoxigenin 3′-epi-monodigitoxoside; dt-0, digitoxigenin; epi-dt-0, 3-epi-digitoxigenin.

† The term 'enzyme form(s)' is used in a broad sense according to the nomenclature of multiple forms of enzymes [8].

[10] (0.15–0.75 mg protein) [11], 0.15 M KCl, 50 mM Tris–HCl (pH 7.45, 0.05% w/v) Brij 58, 10 mM $MgCl_2$, indicated substrates (corresponding to 0.05–0.2 μCi in case of labelled substrates). After preincubation (3 min, 37°) the incubations were started by the addition of UDP-glucuronate (final concentration 3 mM). Blank values were obtained by omission of UDP-glucuronate. After 5 or 10 min the incubations were stopped by addition of an equal volume of methanol or $CHCl_3$.

For all substrates the assay conditions were tested for linearity with time and protein concentration.

Analytic procedures. Stopped incubation mixtures containing labelled substrates were diluted with water (1 ml) and, after addition of 50 μg unlabelled substrate, exhaustively extracted with chloroform (3 × 3 ml). Aliquots of the water phase were measured in a liquid scintillation spectrometer [Tricarb 2425 (Packard), Biofluor as scintillator]. The glucuronide fraction was calculated by subtracting the radioactivity left in the water phase after identical extraction of the blanks. Differences between blanks were lower than 6%. In earlier experiments aliquots of the stopped incubation mixtures were spotted on thin-layer plates and developed in a mixture of chloroform/methanol/water (66:31:3, v/v). After scanning the zones of radioactivity (corresponding to glucuronides (R_F 0.25–0.5) and unchanged substrates ($R_F > 0.9$)) were scraped off into scintillation vials, eluted with 1 ml methanol and counted in a liquid scintillation counter after addition of 10 ml scintillator. The recoveries were better than 90% (determined for dt-1 glucuronide, dt-1 and dg-1). Background levels were taken from aliquots of blanks developed on the same thin layer plate, eluted with 1 ml methanol and counted in a liquid scintillation spectrometer. The results obtained by both analytical methods were not significantly different (tested for dt-1, testosterone, and chenodeoxycholate).

Conjugates of bilirubin and *p*-nitrophenol were determined photometrically [12, 13] and that of 4-OH-biphenyl by fluorimetry [14].

Statistics. Statistical significances ($P < 0.05$) were determined by Student's *t*-test for unpaired values of at least 3 separate experiments.

RESULTS

Glucuronidation rates of various substrates

Table 1 shows the glucuronidation rates using microsomes of untreated rats for a number of substrates which are presumably glucuronidated by different enzyme forms (*p*-nitrophenol, 4-hydroxybiphenyl, estrone, testosterone, pregnanediol, bilirubin, chenodeoxycholate) or which are closely related to dt-1 (dg-1, epi-dt-1, dt-0, epi-dt-0). The values for dt-1 are lower by more than a factor of 10^2 for *p*-nitrophenol or 4-hydroxybiphenyl but they are of the same order of magnitude as the values for bilirubin, chenodeoxycholate or estrone (V_{max} values see Table 3). The glucuronidation rat of dt-1 in comparison to that of the other cardenolides is higher by approximately 10–20 fold. The lowest value was found for epi-dt-0.

From the Lineweaver–Burk plot a surprisingly low apparent K_m value of 6 μM is calculated for the dt-1 glucuronidation. This means that a low substrate affinity or access to the active site of the enzyme through the liposomal membrane cannot be the reason for the slow glucuronidation. Similarly, the apparent K_m value for the cofactor UDP-glucuronate was also found to be comparatively low (39 μM). Both apparent K_m values are valid only with saturating concentrations of the respective reaction partner. The rates of the dt-1 conjugation measured with lower UDP-glucuronate concentrations yielded higher apparent K_m values and 1/v (Fig. 1). Apparent K_m values independent of the concentration of the reaction partner, estimated from the plot in Fig. 1 according to Rao *et al.* [15], amount to 52 μM for dt-1 (inset of Fig. 1) and 370 μM for UDP-glucuronate, respectively.

Effect of inducers

Phenobarbital is a known inducer of glucuronyltransferase form '2' which, according to Bock *et al.*, [16] is the enzyme form responsible for the conjugation of 4-hydroxybiphenyl, morphine and many other non-planar exogenous substances. Table 2 shows that this model inducer does not effect the dt-1 glucuronidation. Tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons like

Table 1. Glucuronidation rates of various substrates by liver microsomes of unpretreated rats

Substrate	Concentration (μM)	Glucuronide formation (pmoles/mg protein min)
dt-1	50	138 ± 7
dg-1	50	13 ± 3
epi-dt-1	50	15 ± 3
dt-0	50	11 ± 7
epi-dt-0	50	6 ± 3
p-Nitrophenol	1000	83,000 ± 9000
4-OH-Biphenyl	500	36,000 ± 3000
Bilirubin	200	200
Chenodeoxycholate	250	221 ± 24
Estrone	100	150 ± 40
Testosterone	100	3240 ± 100
Pregnandiol	100	1050 ± 180

Values are means ± S.D. ($n = 3$).

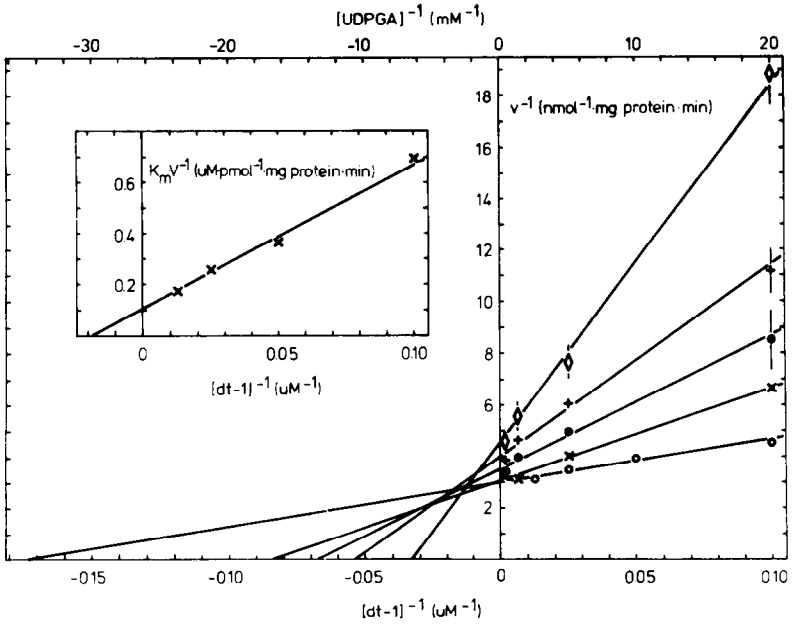

Fig. 1. Lineweaver–Burk plots for dt-1 glucuronidation. 1/dt-1 vs 1/v in the presence of 3 mM UDPGA (○). 1 UDPGA vs 1/v in the presence of 10(◇), 20(+), 40(●), and 80(×) μM dt-1, respectively. Inset: derived plot 1/dt-1 vs $K_m V_{max}$ yielding dt-1-K_m independent of cosubstrate concentration.

Table 2. Effects of inducers on glucuronidation rates of various substrates

Substrate	Relative glucuronidation rate in % of controls after pretreatment with Phenobarbitone	Benzo[*k*]fluoranthene	Spironolactone
dt-1	91 ± 21	108 ± 13	423 ± 46
dg-1			355 ± 30
epi-dt-1	127 ± 24	84 ± 37	316 ± 20
dt-0			166 ± 23
epi-dt-0			231 ± 46
p-Nitrophenol	169 ± 6	345 ± 47	104 ± 14
4-OH-Biphenyl	408 ± 7	116 ± 8	131 ± 14
Bilirubin			104 ± 14
Chenodeoxycholate			125 ± 7
Estrone			121 ± 16
Testosterone			118 ± 12
Pregnanediol			99 ± 26

Values are means ± S.D. of 3 separate experiments with different rats.
* See Table 1 for control values.

Table 3. Kinetic data for the glucuronidation of various substrates by normal and spironolactone pretreated rats

Substrate	K_m (μM) Normal	Pretreated	V_{max} (nmoles/mg microsomal protein min) Normal	Pretreated
p-Nitrophenol	710 ± 57	740	150 ± 24	178
4-OH-Biphenyl	301 ± 73	286 ± 11	65 ± 8	78
Estrone	67 ± 15	63	0.24 ± 0.05	0.22
Testosterone	87	98	5.88	6.64
Pregnanediol	18 ± 5.7	17 ± 1.1	0.95 ± 0.03	0.84
Chenodeoxycholate	29	37	0.25	0.31
Bilirubin	53	52	0.23	0.26
dt-1	5.8 ± 1.6	5.9	0.21 ± 0.03	0.70 ± 0.04

Values were obtained from 1 to 3 Lineweaver–Burk plots with pooled microsomes of 3 rats per plot. Incubations with 0.2–2 mg microsomal protein/ml and 3.2 mM UDP-glucuronate.

benzo[*a*]pyrene, methylcholanthrene or the benzo[*k*]fluoranthene used in this study induce, together with isocytochrome P448, the glucuronyltransferase form '1', which metabolizes *p*-nitrophenol and other planar molecules containing a phenolic hydroxy group and which corresponds to the 'late foetal' group of the transferase's activity [17]. Also this induction has no effect on the dt-1 glucuronidation rate (Table 2).

However, when the aldosterone antagonist spironolactone was used as an inducer a 4–6 fold increase in the dt-1 conjugation rate was observed. The activity of the glucuronyltransferases form '1' and '2' on the other hand remained unchanged. Moreover, in contrast to results of Radzialowski [18] the glucuronidation rate of bilirubin was not enhanced as well.

The conjugation rates for epi-dt-1 and dg-1 increased by approximately the same extent as that of dt-1, whereas the conjugation rates of dt-0 and epi-dt-0 as well as that of the steroids remained almost constant (Table 2). The finding that spironolactone pretreatment does not effect the apparent K_m values (Table 3) indicates an isolated induction of a glucuronyltransferase which specifically conjugates the cardenolide digitoxosides and epidigitoxosides.

Inhibition experiments

If the glucuronyltransferase form, which is induced by spironolactone, is specific for the glycosides used here, then it should not be inhibited by other substrates, provided that more or less unspecific membrane changes [19] do not interfere or remain constant. It was found that for a dt-1 concentration of 4 μM (range of apparent K_m) the substrates (listed in Table 3) of presumable other enzymes had no significant inhibitory effect when assayed in concentrations according to their apparent K_m value. In the presence of 25 μM cardenolides (dg-1, epi-dt-1, or dt-0) only epi-dt-1 inhibited the dt-1 glucuronidation (by 44% with normal and by 40% with spironolactone pretreated rats).

In a reverse set-up, dt-1 itself was tested as an inhibitor or other possible enzymes. In concentrations of 25–50 μM (4- to 8-fold concentration of apparent K_m) dt-1 inhibits solely the conjugation of the cardenolide glycosides and of chenodeoxycholate (Table 4). The conjugation rates of all other substrates remained unchanged.

The thiol reagent *p*-hydroxymercuribenzoate proved to be an extremely effective inhibitor: 50% inhibition by 10 μM, complete inhibition by 20 μM *p*-hydroxymercuribenzoate.

DISCUSSION

The aim of this investigation was to characterize the enzyme form responsible for the dt-1 glucuronidation. The purification of the various glucuronyltransferase forms has been accomplished only in a few cases so far, e.g. for the glucuronyltransferase form '1' and form '2' in rats [16] or the estrone glucuronyltransferase in rabbits [17]. As for the substrates testosterone [15, 21, 22, 23], bilirubin [24, 21], chenodeoxycholate [25] or pregnanediol [22], the existence of a specific enzyme form conjugating the cardenolide derivatives can only be deduced from activity data so far. Apart from the characterization of the kinetic parameters, the determination of the conjugation rates in the presence of possible competitors and the changes in pattern of glucuronyltransferase activity after induction seems particularly suitable to prove the postulated differences. In the case of the dt-1 glucuronidation these two methods of differentiation suggest that the reaction is catalysed by a relatively specific enzyme form.

When the inhibition of the dt-1 glucuronidation by various other substrates was tested it could be demonstrated that this conjugation can only be inhibited by the diastereomer epi-dt-1, even when the enzyme is half saturated; dt-1, on the contrary, inhibits not only the conjugation of epi-dt-1 and dg-1 but also that of chenodeoxycholate. The affinity of the more polar dg-1 to the glucuronyltransferase is presumably very low so that dg-1 can easily be replaced by dt-1; but dg-1, on the other hand, has no effect on the dt-1 glucuronidation.

For the competitive inhibition of the chenodeoxycholate glucuronidation by dt-1 an apparent K_i value of only 7 μM was determined. This corresponds to the apparent K_m value of dt-1. From these results, however, it can not be concluded that the glucuronyltransferase for chenodeoxycholate is identical to the dt-1 conjugating glucuronyltransferase mentioned here. This would be in contrast to the induction experiments with spironolactone where the glucuronidation rate for chenodeoxycholate is increased by a mere factor of 1.2 compared to the 4- to 5-fold increase for dt-1.

Table 4. Inhibition of various glucuronidations by dt-1

Substrate	Concentration (μM) Substrate	dt-1	Inhibition %
dg-1	50	25	88*
epi-dt-1	50	25	42*
dt-0	50	25	5
4-OH-Biphenyl	330	50	−5
Chenodeoxycholate	50	25	47*
Estrone	130	50	−5
Testosterone	90	50	8
Pregnanediol	20	25	12

Values are means of 3 to 4 separate experiments.

High rates of inducibility similar to that observed for dt-1 are found only for the glycosides epi-dt-1 and dg-1, those substrates which inhibit the conjugation of dt-1 (epi-dt-1 only) or whose conjugation in turn is inhibited by dt-1.

The specific inducibility is also a valuable criterion for the discrimination of the dt-1 glucuronyltransferase from the estrone glucuronyltransferase which can also be inhibited by *p*-hydroxymercuribenzoate [26]. An additional difference to the glucuronyltransferase responsible for the conjugation of estrone [21] and pregnanediol [22] consists in the increase of the affinity of dt-1 to the enzyme with increasing UDP-glucuronate concentrations.

The glucuronyltransferase form described here resembles the isocytochrome P450 which is responsible for the digitoxosyl oxidation of dt-1, in that it has a surprisingly high substrate specificity and is selectively inducible by spironolactone [5]. Like the polycyclic aromatic hydrocarbone and tetrachlorodibenzo-*p*-dioxin which induce cytochrome P448 as well as the glucuronyltransferase form '1', spironolactone also causes the combined induction of both a special monooxygenase and a specific glucuronyltransferase.

REFERENCES

1. M. C. Castle and G. L. Lage, *Tox. appl. Pharmac.* **27**, 641 (1974).
2. A. Schmoldt, H. F. Benthe and W. Grote, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **282**, R 85 (1974).
3. H.-F. Vöhringer, L. Weller and N. Rietbrock, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **287**, 129 (1975).
4. A. Schmoldt and C. Rohloff, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **305**, 167 (1978).
5. A. Schmoldt, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **305**, 261 (1978).
6. L. G. Richards and G. L. Lage, *Tox. appl. Pharmac.* **42**, 309 (1977).
7. M. C. Castle, *Biochem. Pharmac.* **29**, 1497 (1980).
8. Nomenclature of multiple forms of enzymes, *J. biol. Chem.* **252**, 5939 (1977).
9. D. Satoh and K. Aoyama, *Chem. pharm. Bull.* **18**, 94 (1970).
10. H. Kutt and J. R. Fouts, *J. Pharmac. exp. Ther.* **176**, 11 (1971).
11. O. H. Lowry, N. J. Rosebrough, A. A. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. A. A. Weber and L. Schalm, *Clinica chim. Acta* **7**, 805 (1962).
13. W. Grote, A. Schmoldt and H. G. Dammann, *Biochem Pharmac.* **24**, 1121 (1975).
14. K. W. Bock, U. C. v.Chlausbruch, R. Kaufmann, W. Lilienblum, F. Oesch, H. Pfeil and K. L. Platt, *Biochem Pharmac.* **29**, 495 (1980).
15. M. L. Rao, G. S. Rao and H. Breuer, *Biochim. biophys. Acta* **452**, 89 (1976).
16. K. W. Bock, D. Josting, L. Lilienblum and H. Pfeil, *Eur. J. Biochem.* **98**, 19 (1979).
17. G. J. Wishart, *Biochem. J.* **174**, 485 (1978).
18. F. M. Radzialowski, *Biochem. Pharmac.* **22**, 1607 (1973).
19. B. Burchell, *Rev. Biochem. Tox.* **3**, 1 (1981).
20. R. H. Tukey, R. E. Billings and T. R. Tephly, *Biochem. J.* **171**, 659 (1978).
21. G. S. Rao, G. Haueter, M. L. Rao and H. Breuer, *Biochem. J.* **162**, 545 (1977).
22. M. Fuchs, G. S. Rao, M. L. Rao and H. Breuer, *J. Steroid Biochem.* **8**, 235 (1977).
23. G. W. Lucier and O. S. McDaniel, *J. Steroid Biochem.* **8**, 867 (1977).
24. G. J. Dutton, in *Glucuronic Acid, Free and Combined* (Ed. G. J. Dutton), p. 185. Academic Press, New York (1966).
25. W. Fröhling, A. Stiehl, P. Czygan and B. Kommerell, *Biochim. biophys. Acta* **444**, 525 (1976).
26. G. S. Rao and H. Breuer, *J. biol. Chem.* **244**, 5521 (1969).