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PURIFICATION OF A RAT LIVER CYTOSOLIC SULFOTRANSFERASE RESPONSIBLE FOR THE CONJUGATION OF DIGITOXIGENIN*

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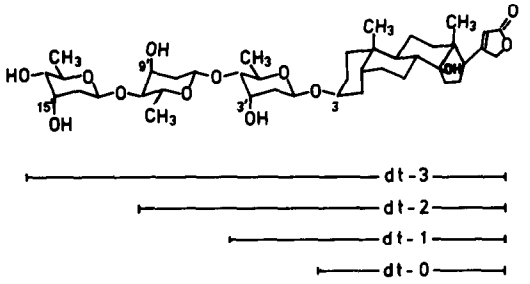
Abstract—Previous investigations on the digitoxin metabolism hardly considered the role of the sulfate ester conjugation. Therefore, this study examined whether digitoxin (dt-3) or one of its cleavage products might be sulfated *in vitro*. It was proven that digitoxigenin (dt-0) is by far the best substrate for the cytosolic sulfotransferases (ST). Digitoxigenin-monodigitoxoside (dt-1) and digitoxigenin-bisdigitoxoside (dt-2) are sulfated in trace amounts whereas dt-3 is not sulfated at all. The purification of the responsible enzyme was performed by liquid chromatography on Q-Sepharose and hydroxyapatite. During the purification procedure this enzymatic activity corresponded exactly to that towards dehydroepiandrosterone (DHEA). The 134-fold purified and gel electrophoretically homogeneous enzyme protein (M_r 33,000) showed a V_{max} of 12.5 nmoles dt-0 sulfate/min mg protein and a K_M of 37 μmol/L. The purified enzyme conjugated dt-1 and dt-2 in trace amounts only and was inhibited competitively by DHEA. It can be concluded that in the rat a 3β-hydroxy-steroid sulfotransferase is responsible for the sulfation of dt-0. The purified enzyme reacts with dt-1, dt-2 and digoxigenin (dg-0) in traces only, a sulfation of dt-3 is not detectable.

The metabolism of digitoxin (digitoxigenin-trisdigitoxoside, dt-3‖) has been studied extensively during recent years. The metabolism takes place by a sequential and exclusively oxidative cleavage of the sugar side-chain. This reaction is catalysed by a specific cytochrome P-450 form. The cleavage of dt-3 was detected in rats and in humans only [1–3], whereas digitoxigenin-bisdigitoxoside (dt-2) can also be cleaved in mice, rabbits and guinea-pigs [4]. These cleavage reactions mainly terminate after the formation of digitoxigenin-monodigitoxoside (dt-1) which is preferably glucuronidated [5]. The glucuronidation of dt-1 is catalysed by a specific UDP-glucuronosyltransferase, which was purified from rat liver recently [5, 6]. For both the cytochrome P-450 as well as the UDP-glucuronosyltransferase no other endogenous or exogenous substrates are known so far. Both enzymes are induced by spironolactone and pregnenolone-16α-carbonitrile treatment of the rat [7, 8].

DIGITOXIN

(digitoxigenin - trisdigitoxoside)



Only little is known about the sulfoconjugation of digitalis glycosides and their cleavage products. To our knowledge no results of *in vitro* experiments have been published so far. *In vivo* results from humans and rats showed only indirect evidence of sulfoconjugates. Following administration of [^{3}H]dt-3 metabolites of the water soluble fraction in urine and bile were designated to be sulfoconjugates when radioactivity was found in the chloroform phase after sulfatase incubation [9]. Sulfate esters of dt-0 formed by guinea-pig liver samples were analysed by hydrolysis of the conjugate in dioxane/trichloroacetic acid [10].

The mainly cytosolic sulfotransferases (ST) were investigated extensively during recent years. Some phenol sulfotransferases (EC 2.8.2.1) play an important role in the metabolism of a variety of phenols, the catechol drugs and some neurotransmitters. These enzymes were purified and characterized from various organs of humans and rats [11–15]. Additionally, investigations were published on 3β-hydroxysteroid-ST (EC 2.8.2.2) [16–18], bile acid-ST [19,

* This paper is dedicated to Prof. Dr K. J. Netter on the occasion of his 60th birthday.

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‖ Abbreviations: dg-0, digoxigenin; dg-3, digoxin; DHEA, dihydroepiandrosterone; dt-0, digitoxigenin; dt-1, digitoxigenin-monodigitoxoside; dt-2, digitoxigenin-bisdigitoxoside; dt-3, digitoxigenin-trisdigitoxoside, digitoxin; epi-dt-0, 3-epi-digitoxigenin; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; ST, sulfotransferase.

Table 1. Specific activities of cytosolic rat liver sulfotransferases with different substrates

Compound	Specific activity (pmol/min mg)
Dehydroepiandrosterone	380.0
Digitoxigenin	56.0
Digitoxigenin-monodigitoxoside	1.8
Digitoxigenin-bisdigitoxoside	0.5
Digitoxin	nd
Digitoxigenin	17.5
Digoxigenin-monodigitoxoside	2.6
p-Nitrophenol	105.0
Androsterone	38.0
Testosterone	6.8
Estrone	7.3

Each value represents the mean of two experiments (differences ≤ 10%).
nd, not detectable (<0.02 pmol/min mg).

20], estrone-ST (EC 2.8.2.4) [21], glucocorticoid-ST [22] and amine-conjugating sulfotransferases [23].

The aim of the present study was to investigate whether digitoxin, digoxin or one of the metabolites are conjugated by sulfotransferases of rat liver and whether an individual sulfotransferase is responsible for this sulfoconjugation.

MATERIALS AND METHODS

Animals and materials

Female Wistar rats (160–180 g body wt) were from Wiga-Charles River (Sulzfeld, F.R.G.). The animals were kept under usual animal husbandry conditions with a standard chow and water *ad lib.*

3′-Phosphoadenosine-5′-phosphosulfate (PAPS), dehydroepiandrosterone (DHEA), estrone, androsterone, testosterone and 4-nitrophenol were purchased from the Sigma Chemical Co. (Deisenhofen, F.R.G.). Dt-3, dt-2, dt-1, digitoxigenin (dt-0), digoxigenin (dg-0) and digoxigenin-monodigitoxoside (dg-1) were obtained from Boehringer Mannheim (Mannheim, F.R.G.). 2-Mercaptoethanol, diethylether, dichloromethane, Tris and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, F.R.G.). Sucrose was from Serva (Heidelberg, F.R.G.). Q-Sepharose fast flow was obtained from Pharmacia-LKB (Freiburg, F.R.G.), which also was the source of all liquid column chromatography equipment used. Hydroxyapatite was purchased from Calbiochem (Frankfurt, F.R.G.). Gel electrophoresis materials and equipment were from Biorad (Munich, F.R.G.).

[1-^{14}C]4-Nitrophenol was purchased from Amersham (Braunschweig, F.R.G.). [1,2-^{3}H]Dehydroepiandrosterone, [9,11-^{3}H]androsterone, [1,2-^{3}H]-testosterone, [U-^{3}H]dt-3 and Biofluor were from NEN (Dreieich, F.R.G.), [6,7-^{3}H]estrone was from CEA (Dreieich, F.R.G.).

[^{3}H]Dt-2, [^{3}H]dt-1 and [^{3}H]dg-1 were obtained by cleavage of [^{3}H]dt-3 or [^{3}H]dg-3, respectively, according to the method of Satoh and Aoyama [24]. [^{3}H]Dt-0 and [^{3}H]dg-0 were produced by acid hydrolysis of [^{3}H]dt-3 and [^{3}H]dg-3 [25].

Methods

Preparation of liver cytosol. Female Wistar rats (150–170 g body wt) were killed by cervical dislocation and the livers were removed and stored on ice immediately. The liver tissue was cut into small pieces, washed extensively to remove remaining blood and was homogenized in buffer A (10 mmol/L Tris, 250 mmol/L sucrose, 3 mmol/L 2-mercaptoethanol, pH 7.4) in a Potter–Elvehjem homogenizer for 30 sec. All purification steps were performed at 4°. The homogenate was subjected to a centrifugation procedure consisting of 10 min at 10,000 *g* followed by ultracentrifugation of the remaining supernatant for 1 hr at 100,000 *g* as described by Marcus *et al.* [16]. The clear supernatant

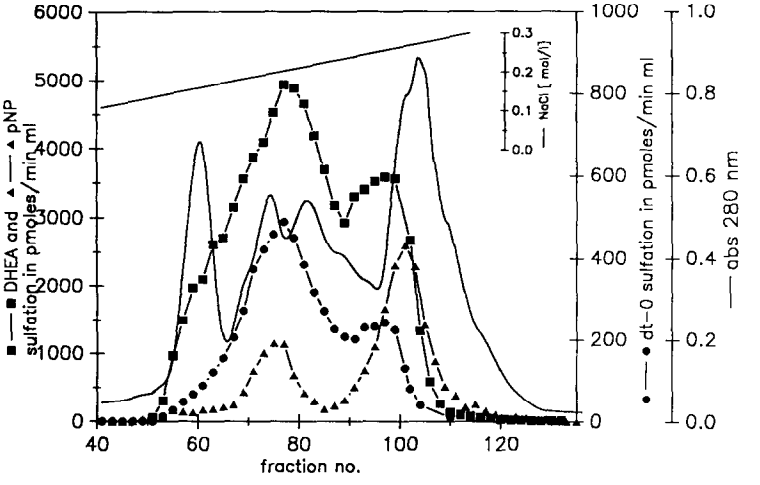

Fig. 1. Ion exchange chromatography liver dt-0 of sulfotransferase activity performed with 100,000 *g* supernatant fractions. Enzyme activities were measured with 250 μM *p*-nitrophenol, 50 μM DHEA, and 50 μM dt-0 as substrates.

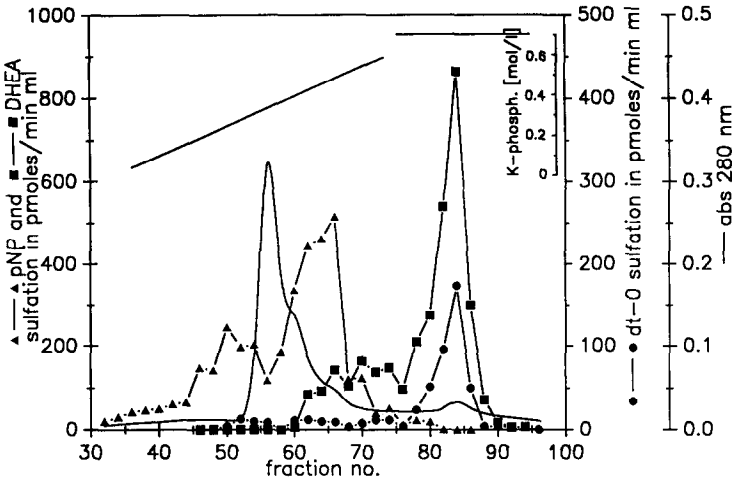

Fig. 2. Elution profile of the dt-0 sulfotransferase activity on hydroxyapatite after ammonium sulfate precipitation. Linear potassium phosphate gradient from fraction 34 to 75; thereafter, elution was performed by 700 mmol/L potassium phosphate buffer. Enzyme activity was measured with 250 μM *p*-nitrophenol, 50 μM DHEA and 50 μM dt-0.

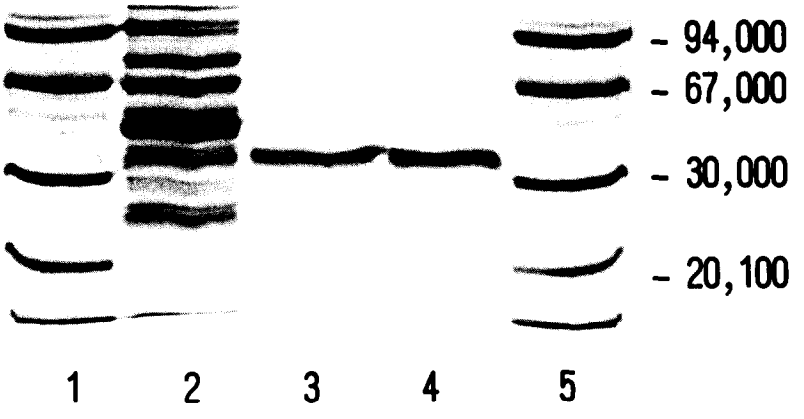

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified sulfotransferase. The electrophoresis was carried out as described in Materials and Methods. Marker proteins (lane 1 and lane 5): phosphorylase b (94,000), serum albumin (67,000), carbonic anhydrase (30,000) and trypsin inhibitor (20, 100). Samples consisted of 40 μg enzyme protein eluted from Q-Sepharose (lane 2) and 10 μg of purified enzyme after hydroxyapatite chromatography (lane 3 and 4).

Table 2. Purification of rat liver dt-0 sulfotransferase

Purification step	Specific activity (pmol/min mg)	Purification (-fold)	Recovery (%)
100,000 *g* Supernatant	47.9	1	100
Q-Sepharose fast flow	768.5	16.1	80.6
$(NH_4)_2SO_4$-precipitation	753.1	15.7	72.6
Hydroxyapatite	6451.3	134.7	27.8

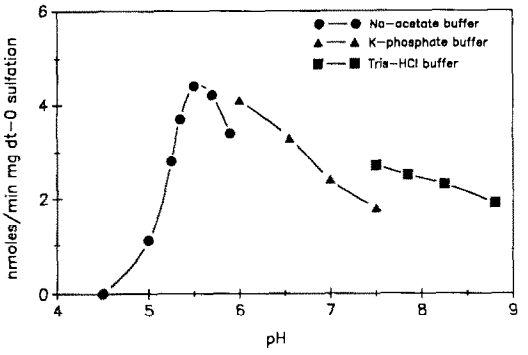

Fig. 4. Dt-0 sulfation in dependence on pH. Assays were performed as described in Materials and Methods with the given buffer modifications (100 mmol/L).

Table 3. Specific activities of purified 3β-hydroxysteroid-ST using various substrates

Compound	Specific activity (pmol/min mg)
Dehydroepiandrosterone	66,275 ± 8960
Digitoxigenin	9220 ± 161
Digitoxigenin-monodigitoxoside	76 ± 15
Digitoxigenin-bisdigitoxoside	80 ± 12
Digitoxin	nd
Digoxigenin	trace†
p-Nitrophenol	nd
Androsterone*	2677 ± 102
Testosterone*	216 ± 43
Estrone*	43 ± 10

Each value represents the mean of three determinations ± SD.
* Determination carried out before dialysis.
† Trace, <50 pmol/min mg.
nd, not detectable.

contained the cytosolic sulfotransferases and was used for chromatographic procedures.

Enzyme assays. For the determination of the sulfation rate of various substrates microassays with volumes of 50 to 60 μL were performed. All substrates were either [^{3}H]- or [^{14}C]-labeled and dissolved in propyleneglycol. The incubation solutions consisted of one of the following substrate concentrations: 50 μmol/L (DHEA, dt-0, dt-1, dt-2, dt-3), 100 μmol/L (androsterone, testosterone, estrone) and 250 μmol/L (4-nitrophenol) together with 200 μmol/L PAPS, 20–30 μL of the diluted protein solution, 5 mmol/L 2-mercaptoethanol and 100 mmol/L buffer for establishing the optimum pH range. Na-acetate buffer (pH 5.5) was used for DHEA, dt-0, dt-1, dt-2 and dt-3; potassium phosphate buffer (pH 6.5) was used for 4-nitrophenol and Tris–HCl buffer (pH 8.0) was used for the determination of androsterone, testosterone and estrone (all pH adjustments for these buffers were made at 37°). The amount of radioactivity was 3.8 mCi/mmol androsterone, 19 mCi/mmol testosterone, 14 mCi/mmol estrone, 48 mCi/mmol DHEA, 52 mCi/mmol dt-0, 62 mCi/mmol dt-1, 30 mCi/mmol dt-2 and 60 mCi/mmol dt-3.

The reactions were started by the addition of PAPS except for the incubations under acidic conditions where the reactions were started by adding protein in order to prevent protein inactivation. Blank values were obtained by omitting the cosubstrate. Following incubation for 10 min (estrone incubation 30 min) at

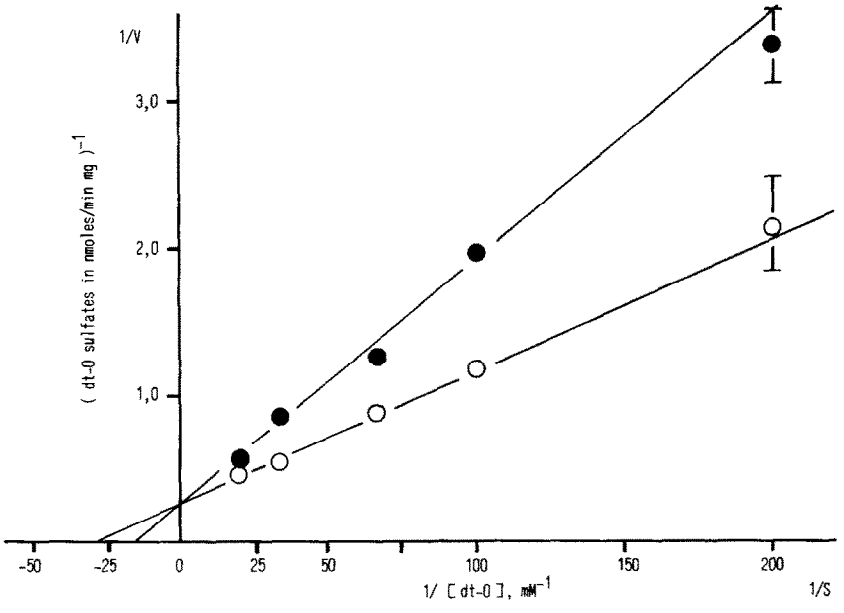

Fig. 5. Lineweaver–Burk plot of sulfotransferase activity of the purified protein towards dt-0. Competitive inhibition by 5 μM DHEA (—●—). Values are means of 2–3 separate measurements (SD or range mostly smaller than symbols).

37° the reaction was stopped by the addition of extraction solvent. After addition of further 300 μL water to facilitate phase separation and two extractions with 3 mL of diethylether (DHEA and 4-nitrophenol) or dichloromethane (all other substrates) each, 200 μL samples of the water phase were used for liquid scintillation counting of the radioactivity with Biofluor as scintillation cocktail in a liquid scintillation counter (2423, Packard-Canberra, Frankfurt, F.R.G.).

Gel electrophoresis. SDS–polyacrylamide gel electrophoresis as well as the sample preparation were performed according to the method of Laemmli [26] in a Mini Protean II cell (Biorad) on a 12% running gel with the modifications described by the manufacturer. Calibration was obtained with molecular weight standards ("low molecular weight standards") of Pharmacia, which were pretreated with the same procedure as the samples were. Staining was obtained with Coomassie blue according to the guidelines of the manufacturer of the electrophoresis cell.

Ion-exchange chromatography. The supernatant obtained from ultracentrifugation was pooled (90 mL; 3.83 g protein) and applied to a 2.6 × 40 cm Q-Sepharose fast flow column, which had been equilibrated with buffer A previously. The column was further washed with 450 mL of the equilibration buffer and was eluted with a linear gradient of 350 mL of buffer A and 350 mL 0.3 mol/L NaCl in buffer A. The flow rate was approximately 30 mL/hr and fractions of 6 mL were collected. Every second fraction was tested for enzymatic activity. The main peak fractions containing activity towards dt-0 were pooled (fractions 70–82). This and all subsequent purification steps were done at 4° or on ice.

Ammonium sulfate precipitation. The pooled fractions were subjected to the slow addition of ammonium sulfate (51.6 g/100 mL, 75% saturation) under gentle stirring for 1 hr followed by centrifugation at 10,000 *g*. The resulting pellet was redissolved in 100 mmol/L potassium phosphate, 250 mmol/L sucrose, 3 mmol/L 2-mercaptoethanol (pH 6.8) and was dialysed twice against 2L of buffer B (10 mmol/L potassium phosphate, 250 mmol/L sucrose, 3 mmol/L 2-mercaptoethanol, pH 6.8).

Hydroxyapatite. The dialysed sample of the previous step was applied to a hydroxyapatite column (1.6 × 40 cm), previously equilibrated with buffer B. After sample application (60 mL; 165 mg protein) the column was washed with 200 mL buffer B followed by a linear gradient of 100mL buffer B and 100 mL of 600 mmol/L potassium phosphate with sucrose and 2-mercaptoethanol as above. The flow rate was approximately 18 mL/hr and fractions of 6 mL were collected. Thereafter, the phosphate content was increased to 700 mmol/L. The eluted enzyme was dialysed against buffer B prior to the determination of the enzymatic activity.

RESULTS

To test the hypothesis that digitoxin and its cleavage products are sulfated by rat liver cytosolic enzymes, the glycosides and the free genin were incubated with rat liver cytosol in the presence of PAPS. As shown in Table 1, dt-0 was the best substrate for the cytosolic sulfation activity. The sulfation rate ranging up to 50 pmol/min mg cytosolic protein was in the same order of magnitude as the androsterone sulfation rate. In contrast, dt-1 and dt-2 were sulfated in trace amounts only whereas no sulfation rate was detectable with dt-3.

To purify the activity of the enzyme, the cytosol was separated on Q-Sepharose fast flow and individual fractions eluted by a linear NaCl-gradient were tested for enzymatic activity with dt-0 as substrate. The main activity was found in fraction Nos 70–82 corresponding to 0.18–0.22 mol NaCl as shown in Fig. 1. When the substrates 4-nitrophenol and DHEA were tested additionally it was possible to separate enzyme activities for either dt-0 and 4-nitrophenol sulfation, whereas the activity towards dt-0 and DHEA coeluted in parallel. For further purification fraction Nos 66–85 were pooled and ammonium sulfate precipitated prior to hydroxyapatite chromatography. The precipitation procedure did not result in an increase of specific activity.

The application of a linear gradient of potassium phosphate from 0–600 mmol/L to the hydroxyapatite column resulted in a complete separation of activities towards 4-nitrophenol, whereas activity towards dt-0 and DHEA was eluted with a stepwise gradient at 700 mmol/L potassium phosphate. Figure 2 shows that the elution profiles for dt-0 and DHEA are exactly parallel, with elution of the main activity in fraction No. 84.

SDS–PAGE for the detection of a homogeneous preparation showed a single band at a molecular weight of M_r 33,000 (Fig. 3). In total, a 134-fold purification was obtained as outlined in Table 2.

All of the aforementioned results support the assumption that dt-0 is sulfated by the DHEA- or 3β-OH-steroid-sulfotransferase. To further substantiate this hypothesis and to characterize the purified enzyme, enzyme kinetic determinations were performed.

Figure 4 shows a pH optimum for dt-0 sulfation at about 5.5. The Lineweaver–Burk plot resulted in a K_M of 37 μmol/L for the dt-0 sulfation, measured at pH 8.0 as well as at pH 5.5, whereas the V_{max} rose from 3.7 nmol/min mg protein (pH 8.0) to 12.5 nmol/min mg protein (pH 5.5). Additionally, the Lineweaver–Burk plot (Fig. 5) shows that the addition of 5 μmol/L DHEA resulted in a competitive inhibition of dt-0 sulfation.

Table 3 shows enzymatic constants and sulfation rates of the purified enzyme for other substrates. With regard to the digitoxosides of dt-0 the data correspond to the results obtained with cytosol as enzyme source in so far as dt-1 and dt-2 are only poor or no substrates (dt-3) for sulfation by the purified enzyme as well.

DISCUSSION

Chromatography of cytosol as described in this investigation resulted in a suitable purification procedure for sulfotransferases from rat liver cytosol. The major contaminating proteins could be easily removed on Q-Sepharose fast flow. The ammonium

sulfate precipitation did not improve the specific activity of the enzyme but served as a simple step to facilitate a change of the buffer system for further hydroxyapatite chromatography. The last step removed the remaining phenosulfotransferase activity.

Coelution of the activity towards dt-0 and DHEA and comparisons with the elution characteristics of the 3β-hydroxy-steroid-ST(s) [27] further indicate that the purified enzyme is a 3β-hydroxy-steroid-ST. Additionally, the competitive inhibition of dt-0 sulfation by DHEA, the pH-optimum and the molecular weight of the purified enzyme strongly support the proposition that this enzyme is the "isoenzyme 2" [27], which is responsible for the sulfoconjugation of dt-0.

Even the molecular structure of the substrate dt-0 allows the assumption that dt-0 is a substrate for a 3β-hydroxy-steroid-sulfotransferase, because dt-0 contains a 3β-hydroxy-group as well. Surprisingly, when dg-0 was used as a substrate, the additional 12β-hydroxy-group resulted in a nearly complete loss of sulfation under the given assay conditions. Therefore, sulfoconjugation to a large extent depends on steric effects regardless of whether the same acceptor groups are present as shown for the substrates dt-0 and dg-0 or not. The question of which other group may be sulfated in dt-1 and dt-2 remains unanswered, because in these molecules the 3β-hydroxy-group is blocked by the glycosylation with digitoxose(s).

The results strengthen the hypothesis from previous *in vitro* studies that the sulfation of digitoxin does not play an important role when compared to glucuronidation. As shown in the introductory part of this study, the dt-1 is mainly glucuronidated before oxidative reactions under formation of the free genin are possible. The so formed small amounts of dt-0 will probably be sulfated because dt-0 cannot be glucuronidated by the dt-1 glucuronosyltransferase [6]. Whether epi-dt-0, a product formed very fast from dt-0 [28], is a better substrate for the sulfation remains to be investigated. This, however, is improbable because 3β-hydroxysteroids have a higher affinity towards sulfotransferases when compared to their stereoisomers [16, 18].

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