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Enzymatic Sulfation of Steroids XIII. Hepatic Deoxycorticosterone Sulfotransferases in Rats, Properties and Comparison with Glucocorticoid Sulfotransferases (41026)[1]

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Abstract. A radioisotopic assay for the cytoplasmic deoxycorticosterone sulfotransferase activity (DSA) of rat liver was developed herein. Deoxycorticosterone inhibits the enzyme reaction strongly. For reliable, quantitative results, a complex assay method, using several different deoxycorticosterone concentrations, each studied with four different amounts of enzyme, was necessary. This "mosaic" assay compensated for variations of the enzyme activity observed in male and female rats. The results obtained demonstrated that cytosols from female rats contained five to six times the DSA found in males. The hepatic DSA was examined further by DEAE-Sephadex A-50 fractionation. One peak of enzyme activity was observed in cytosols from males and two peaks of enzyme activity were observed in cytosols from females. Comparison of these DSA peaks with the glucocorticoid sulfotransferases (STI, STII, and STIII) already fractionated in this laboratory, suggested that the single peak of DSA in males and the second peak of DSA eluting from DEAE-Sephadex A-50 columns in females were STIII. The first peak of DSA eluting from the ion-exchange columns in females appeared to be a new enzyme.

The enzymatic sulfation of steroid hormones has been demonstrated in various mammalian tissues. To date, the extensive purification of estrogen (1), 3β-hydroxysteroid (2), glucocorticoid (3–5), and bile acid (6) sulfotransferases has been reported. In addition, sulfated steroids have been detected *in vivo,* not only in numerous organs (7) but also in fetoplacental (8), enterohepatic (9, 10), and systemic (11, 12) circulations, and in the excreta (13, 14) of the species examined. As a result of these observations, roles for sulfated steroids in androgen, estrogen, and corticosteroid metabolism have been proposed (15, 16). Furthermore, glucocorticoid sulfates have been implicated in aging (17) and in both hypertensive (18, 19, 20) and oncogenic (21, 22) pathological processes.

The sulfation of glucocorticoids has long been a primary interest in this laboratory. Specifically, we have isolated and characterized three glucocorticoid sulfotransferases (STI, STII, and STIII) from rat liver (3), shown that they sulfated cortisol and corticosterone (3, 24), and described the endocrine control of their production (23–26). The additional presence in rat liver of deoxycorticosterone sulfotransferase activity has been reported by another laboratory (27). Structural similarities between the glucocorticoids and the mineralocorticoid suggested that corticosteroids might all be sulfated by the same enzymes. However, both the enzymological properties and the endocrine control of the reported deoxycorticosterone sulfotransferase activity (27) differed notably from those we observed with the glucocorticoid sulfotransferases (23, 24).

This study was carried out to clarify the relationship between the rat liver deoxycorticosterone and glucocorticoid sulfotransferase activities. Herein, we have described the properties of the deoxycorticosterone sulfotransferase activity, presented the results of its DEAE Sephadex A-50 fractionation, and compared the indi-

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vidual deoxycorticosterone and glucocorticoid sulfotransferases.

Materials and Methods. *Cytosol preparation.* Male and female CD rats were purchased from Charles River Laboratories (Wilmington, Mass.) at 42–48 days of age and used between 2 and 5 months of age. All procedures were carried out at 0-4°. Livers, taken from rats sacrificed by decapitation, were weighed and homogenized in an equal volume per gram of ice-cold TSM (50 m*M* Tris–250 m*M* sucrose–3.0 m*M* mercaptoethanol, pH 7.5). A 30-min centrifugation was then carried out at 35,000*g* (Sorval RC-2B). The supernatant obtained was recentrifuged at 105,000*g* (Beckman L5-65B) for 60 min. The 50% cytosol, obtained as the final supernatant, was used as the starting material for all studies described.

Assay of deoxycorticosterone sulfation. Hepatic deoxycorticosterone sulfotransferase activity was routinely assayed using a procedure modified from that of Carlstedt-Duke and Gustafsson (27). Primary modifications included the use of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) in the incubation mixture in place of ATP and sulfate, and the use of the extraction procedures we have employed for assay of other steroid sulfotransferases (3). PAPS estimated as 99 ± 2% pure by electrophoretic and chromatographic criteria was prepared as described by the method of Singer (28). The steroid substrate was prepared by adding nonradioactive deoxycorticosterone (Sigma, ST. Louis, Mo.) to [^{3}H]deoxycorticosterone (46.8 Ci/mmole, New England Nuclear, Boston, Mass.) to give a final specific radioactivity of 2.3 mCi/mmole.

Immediately prior to assay, 50% cytosol was diluted, 10-fold from females and 4-fold from males, in Bücher medium (29). One-half-milliliter reaction mixtures were then prepared as follows for use in the standard enzyme assay. Cytosol dilutions were made to 0.10-ml volumes with Bücher medium, then 5–20 nmole of [^{3}H]deoxycorticosterone (2.3 mCi/mmole) in 0.30 ml of Krebs-Ringer bicarbonate buffer (30) was added. Reactions were started by addition of 0.10 ml of 1.2 m*M* PAPS in 1.0 m*M* Tris.

Duplicate reaction mixtures were preincubated for 30 min. Then, one of each pair was boiled for 2 min to terminate the reaction, 0.50 ml of deionized water was added, and the samples were chilled on ice. The remaining reaction mixtures were incubated for an additional 30 min and treated in the same manner. Unreacted deoxycorticosterone was extracted from each reaction mixture with three 2.5-ml aliquots of CH_2Cl_2 as described for assay of glucocorticoid sulfotransferases (3). The aqueous extracts, which contained sulfated deoxycorticosterone (see text), were next counted in 5 ml of Aquasol-2 (New England Nuclear) in an Intertechnique SL-30 liquid scintillation counter. Appropriate quenched standards were used to convert the observed counts per minute to disintegrations per minute by the channels ratio method (3). The deoxycorticosterone sulfotransferase activity was calculated as nanomoles of steroid sulfated 30 min^{-1} ml^{-1}.

DEAE-Sephadex A-50 fractionation of deoxycorticosterone sulfotransferase activity. The fractionation of each 3.0-ml cytosol sample was performed on a 2.0 × 50-cm column of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals), using a linear gradient (32–42 ml hr^{-1}) consisting of 300 ml each of TSM and TSM-300 m*M* KCl. The procedure has been described in more detail elsewhere (3). Eluted fractions were assayed for both cortisol and deoxycorticosterone sulfation in order to compare the deoxycorticosterone sulfotransferase activity with the glucocorticoid sulfotransferases STI, STII, and STIII. Glucocorticoid sulfotransferase activity was assayed using 40 μM [^{3}H]cortisol (New England Nuclear, 47.9 Ci/mmole), as described earlier (3).

Results. *Determination of optimal reaction conditions for assay of the deoxycorticosterone sulfotransferase activity in rat liver cytosol.* The deoxycorticosterone sulfotransferase assay method was developed using liver cytosol samples from both sexes. This included the determination of optimal steroid and PAPS concentrations, the optimum incubation period, and a usable range of cytosol protein concentrations for assay (Fig. 1). Substrate inhibition by

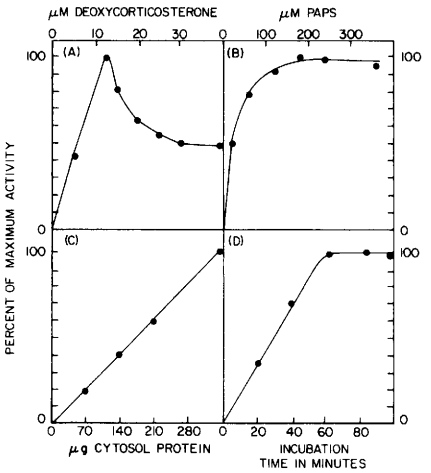


FIG. 1. Determination of optimal conditions for assay of the deoxycorticosterone sulfotransferase activity of cytosol from a female rat. Effects of varying the substrate concentration (A), PAPS concentration (B), enzyme concentration (C), and incubation time (D), are shown. With the exception of the parameter being varied, as indicated, the following were constant in each assay: 12.5 μM deoxycorticosterone, 240 μM PAPS, 350 μg cytosol protein, and preincubation and incubation periods of 30 min each. The mean deoxycorticosterone sulfotransferase activity ± SDM of four cytosols from females was 161.5 ± 34.5 nmole 30 min^{-1} ml^{-1}. Activity in all assays has been expressed as the percentage of maximum activity of the individual cytosol assayed.

deoxycorticosterone was observed in the assay of cytosol samples from both females and males. This complicated the assay method, as will be described later.

The optimum range of deoxycorticosterone concentrations was 12.5–15.0 μM for females (Fig. 1A) and 8.0–15.0 μM for males (not shown). Saturation with PAPS was observed in both sexes when the coenzyme concentrations were greater than 180 μM, as shown for females in Fig. 1B. Routine enzyme assays were therefore performed using 240 μM PAPS and the optimum substrate concentrations, determined individually for each cytosol. Under these conditions the reaction obeyed zero-order kinetics with cytosol samples which contained up to 350 μg cytosol protein from females (Fig. 1C) and up to 870 μg of cytosol protein from males. A 30-min lag in the enzyme reaction was observed, as previously reported by Carlstedt-Duke and Gustafsson (27). Following this lag period, we found the reaction rate to be linear for up to 60 additional min (Fig. 1D). Therefore, preincubation and incubation periods of 30 min each were utilized in standard assays.

Development of a standard deoxycorticosterone sulfotransferase assay. Several factors have been found to affect the deoxycorticosterone sulfotransferase activity of rat liver cytosol. Age and sex effects have been reported by others (27). Furthermore, our studies here have demonstrated extensive substrate inhibition by deoxycorticosterone in both sexes. The collective effects of these factors on the enzyme activity necessitated the determination of an individualized optimum substrate concentration for the quantitative assay of each cytosol sample tested.

To do this a "mosaic" assay, like that described for the substrate-inhibited corticosterone sulfotransferase activity of rat liver cytosol (24) was employed. Using this procedure, several different amounts of cytosol were each assayed simultaneously at three or four deoxycorticosterone concentrations. Cytosols from females were assayed with 10.0, 12.5, 15.0, and 17.5 μM deoxycorticosterone. Under these conditions the enzyme reaction was consistently linear at one or more substrate concentrations. The deoxycorticosterone sulfotransferase activity ± SDM in cytosols from four females was 161.5 ± 34.5 nmole of steroid sulfated 30 min^{-1} ml^{-1}.

In males, the enzyme activity was substantially lower (28.5 ± 15.1 nmole of steroid sulfated 30 min^{-1} ml^{-1}, $n = 10$). Due to the wider range of substrate optima observed, it was necessary to assay each male cytosol twice (by the mosaic method) to ascertain the optimum substrate concentration. First three deoxycorticosterone concentrations between 4.0 and 15.0 μM were used for a preliminary estimate of the enzyme activity. The exact enzyme activity was then ascertained by reassaying within a narrower range of substrate concentrations, determined from the first mosaic assay. The second set of assays of a cytosol from a male rat is shown in Fig. 2. This figure points out the increasing substrate inhibi-

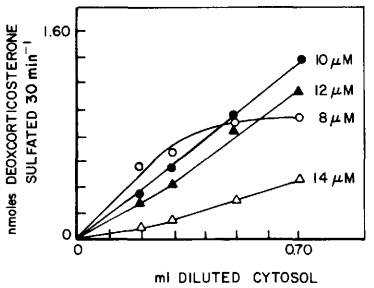


FIG. 2. Mosaic assay of deoxycorticosterone sulfotransferase activity in cytosol from a male rat. Half-milliliter reaction mixtures, described under Materials and Methods, contained indicated amounts of [^{3}H]deoxycorticosterone. Assays were performed using preincubation and incubation periods of 30 min each. The data represent one of eight similar experiments.

tion above the optimum concentration of 10.0 μM deoxycorticosterone.

Identity of the deoxycorticosterone sulfation product. The methodology used for identification of the product of the deoxycorticosterone sulfotransferase reaction (not shown) was similar to that reported earlier in our structure determination of the rat liver cortisol and corticosterone sulfotransferase reaction products (3, 24). The deoxycorticosterone sulfation product was solvolyzable in dioxane–HCl and migrated as a monosulfate on paper electrophoresis. In addition, the solvolysis product was shown to be deoxycorticosterone in several thin-layer chromatography systems. Since deoxycorticosterone contains only a 21-hydroxyl group, it appeared probable that the sulfation product was deoxycorticosterone-21-sulfate.[3]

DEAE-Sephadex A-50 fractionation of deoxycorticosterone sulfotransferase activity. DEAE-Sephadex A-50 fractionation revealed two peaks of deoxycorticosterone sulfotransferase activity in cytosols from females (Fig. 3A) and a single peak of deoxycorticosterone sulfotransferase activity in cytosols from males (Fig. 3B). The first peak of enzyme activity in females eluted at a position between glucocorticoid sulfotransferases STI and STII. The second enzyme peak in females and the single peak in males both appeared to be coincident with STIII. Reproducible fractionation patterns were observed with four cytosols tested from each sex.

Like cytosol, DEAE-Sephadex A-50 fractions exhibited substrate inhibition when assayed for deoxycorticosterone sulfotransferase activity. In fractionated cytosols from females (Fig. 3A) each of the two enzyme peaks exhibited maximum activity when pooled and assayed with 12.5

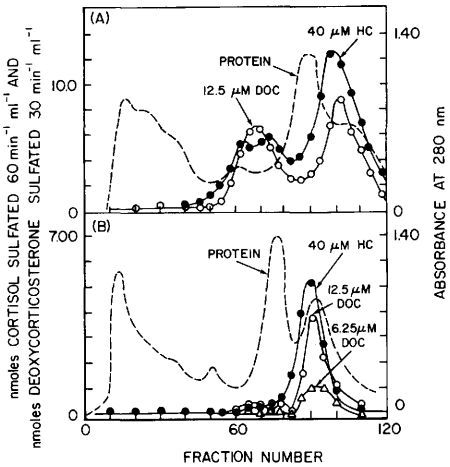
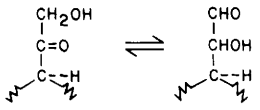


FIG. 3. DEAE-Sephadex A-50 fractionation of deoxycorticosterone and cortisol sulfotransferase activities of rat liver cytosol. A 3-ml sample of 50% cytosol from a female (A) or from a male (B) was loaded on a 2 × 50-cm column and eluted with a linear gradient consisting of 300 ml each of TSM– and TSM–300 mM KCl. Elution was carried out over a 16-hr period at 32–42 ml^{-1} hr^{-1}. Protein was estimated from the absorbence at 280 nm. Eluted fraction from both sexes were assayed at 40 μM cortisol and 12.5 μM deoxycorticosterone. Eluted fractions from the male were also assayed with 6.25 μM deoxycorticosterone. Enzyme activities have been expressed as nmoles cortisol sulfated 60 min^{-1} ml^{-1} and nanomoles of deoxycorticosterone sulfated 30 min^{-1} ml^{-1}.

[3] One cannot absolutely designate the reaction product as deoxycorticosterone-21-sulfate, as the α-ketol side chain of corticosteroids has been shown to rearrange to an α-aldol (31) in some instances. If such a rearrangement occurred here, the reaction product would be deoxycorticosterone-20-sulfate.



μM deoxycorticosterone (Fig. 4A). At this concentration linearity of the reaction with increasing amounts of pooled protein was observed (Fig. 4B). The data showed that the optimum deoxycorticosterone concentration for the assay of DEAE-Sephadex A-50 fractions in four cytosols from females was 12.5 μM.

As already pointed out, the determination of the optimum substrate concentration to be used for assay of the deoxycorticosterone sulfotransferase activity in cytosol was complicated by substrate inhibition and by age and sex factors. The activity in females was approximately five times that observed in males. Furthermore, the results of the mosaic assays demonstrated the great variability of substrate optima in cytosols from males. Therefore, column fractions from the DEAE-Sephadex A-50 chromatography of the four cytosol samples tested were each assayed at a minimum of two deoxycorticosterone concentrations between 3.0 and 15.0 μM. This allowed optimum estimation of the deoxycorticosterone sulfotransferase activity in males. In the chromatogram depicted in Fig. 3B the enzyme assays were performed with 6.25 and 12.5 μM deoxycorticosterone.

Discussion. In this study the deoxycorticosterone sulfotransferase activity of rat liver cytosol was shown to be due to two

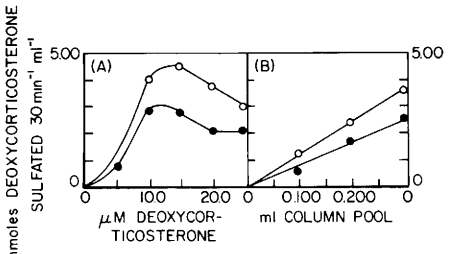


FIG. 4. Assay of deoxycorticosterone sulfotransferase activity in pooled fractions from the DEAE Sephadex A-50 fractionation of cytosol from a female rat. Pool A (●——●) corresponds to the first peak of activity (fractions 60–75) in Fig. 3A, and Pool B (○——○) corresponds to the second peak of activity (fractions 90–115). Effects of varying the substrate concentration (A) and the enzyme concentration (B) on deoxycorticosterone sulfotransferase activity are shown.

enzymes. The first of these was found in both males and females. The second deoxycorticosterone sulfotransferase was found only in females. The enzyme in males cochromatographed with the glucocorticoid sulfotransferase STIII (3) on DEAE-Sephadex A-50 fractionation (Fig. 3B). Approximately half of the deoxycorticosterone sulfotransferase activity in females (Fig. 3A) also cochromatographed with STIII. Until further study allows unequivocal identification of this enzyme, we have tentatively designated it as STIII.

The remainder of the deoxycorticosterone sulfotransferase activity in female rats chromatographed at a position between the glucocorticoid sulfotransferases STI and STII (3) on DEAE-Sephadex A-50 fractionation (Fig. 3A). These results contrasted with our earlier comparison of the enzymes that sulfated cortisol and corticosterone (24), in which identical DEAE-Sephadex A-50 elution profiles indicated that the two glucocorticoids were sulfated by STI, STII, and STIII. We have taken the difference in profiles to indicate that the first peak of deoxycorticosterone sulfotransferase activity is a new steroid sulfotransferase that differs from STI and STII. This enzyme appears to be restricted to female rats.

Further evidence for the existence of this deoxycorticosterone sulfotransferase can be inferred from a comparison of gonadal control of production of the deoxycorticosterone sulfotransferase activity reported by Carlstedt-Duke and Gustafsson (27) and the gonadal control of the glucocorticoid sulfotransferase activity reported by this laboratory (23–26). We have previously described a statistically significant 25–35% decrease of cortisol and corticosterone sulfotransferase activities after ovariectomy of female rats. This was due to decreases of hepatic STI and STII activities. On the other hand, the Scandinavian group reported that ovariectomy had no effect upon hepatic deoxycorticosterone sulfation. This difference of the endocrine regulation of cortisol and deoxycorticosterone sulfation suggests that a deoxycorticosterone sulfotransferase different from STI, STII, and STIII is responsible for ap-

proximately half of the deoxycorticosterone sulfation in females.

The use of the mosaic method of assay demonstrated the effect of substrate inhibition in limiting the range of substrate concentrations useful for quantitation of the deoxycorticosterone sulfotransferase of cytosol or partially purified fractions of enzyme. The similarity of results obtained with both corticosterone (24) and deoxycorticosterone appear to be of further relevance to the assay of other known sulfotransferases, particularly in the use of relative substrate preferences as one means of distinguishing among purified steroid sulfotransferases. The inhibition of activity resulting from substrate inhibition points out the inadequacy of the standard use of equimolar substrate concentrations for determining substrate preferences of purified enzymes. These results thus suggest the advisability of developing an optimum assay for each substrate tested with a particular sulfotransferase before assigning its substrate preference.

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