Sex differences in the hydroxylation of cholecalciferol and of 5 β -cholestane-3 α ,7 α ,12 α -triol in rat liver

Kristin SAAREM and Jan I. PEDERSEN

Institute for Nutrition Research, School of Medicine, University of Oslo, P.O. Box 1046, Blindern N-0316, Oslo 3, Norway

The effect of sex hormones on hydroxylation of cholecalciferol ('vitamin D₃') and of 5 β -cholestane-3 α ,7 α ,12 α -triol has been investigated in female- and male-rat livers. The mitochondrial cholecalciferol 25-hydroxylase and C₂₇-steroid 27-hydroxylase activities were respectively 4.6- and 2.7-fold higher in female- than in male-rat livers. The microsomal 1 α -hydroxycholecalciferol 25-hydroxylase was 2.8-fold higher in male- than in female-rat liver. No significant difference was found in the microsomal 25-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol. Liver microsomes (microsomal fractions) from male, but not from female, rats also catalysed 1-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol. Injection of testosterone into female rats decreased the mitochondrial cholecalciferol 25-hydroxylase and C₂₇-steroid 27-hydroxylase activities, but not to a statistically significant extent. Testosterone treatment had no effect on the microsomal hydroxylases in female-rat liver. Injection of oestradiol valerate to male rats resulted in increased activities of both mitochondrial hydroxylases to the same levels as those of control females, while the microsomal enzyme activities decreased. The present results indicate that sex hormones exert a regulatory control on the mitochondrial cholecalciferol 25-hydroxylase and C₂₇-steroid 27-hydroxylase activities.

INTRODUCTION

Hepatic tissue shows a considerable sexual dimorphism [1], and metabolism of exogenous compounds as well as endogenous steroids shows a pronounced sexual difference in rats [2]. Sex-specific forms of microsomal cytochrome *P*-450 have been isolated [3,4], but there have been no reports regarding sex-specific forms of mitochondrial cytochrome *P*-450 from rat liver.

In rat liver, cholecalciferol ('vitamin D₃') 25-hydroxylase has been shown to be localized both to the mitochondrial [5] and the microsomal [6] fractions. Both hydroxylases are cytochrome *P*-450 enzyme systems [7–9]. The relative physiological importance of these two hydroxylases is not known.

Rat liver mitochondria also catalyse 27-hydroxylation [this enzyme activity has previously been termed 'C₂₇-steroid 26-hydroxylase'; according to a recent investigation on the stereospecificity of the enzyme [9a], the correct name should be 'C₂₇-steroid 27-hydroxylase'] of 5 β -cholestane-3 α ,7 α ,12 α -triol. This reaction is of major importance in the biosynthesis of bile acids [10]. It is not yet known whether 25-hydroxylation of cholecalciferol and 27-hydroxylation of C₂₇ steroids are catalysed by the same, or by different, enzyme(s). The microsomal fraction predominantly catalyses 25-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol [10].

Starvation is known to stimulate enzymes active in fatty acid metabolism, such as ω -oxidation of stearate [11] and β -oxidation of long-chain fatty acids [12]. No effect of starvation on the metabolism of cholecalciferol has been reported.

The purpose of the present work was to study the conversion of cholecalciferol and of 5 β -cholestane-3 α ,7 α ,12 α -triol in male and female rat liver under different physiological conditions, with special emphasis on the mitochondrial hydroxylations of these steroids.

EXPERIMENTAL

Chemicals

ATP, potassium malate, Mops, Hepes and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DPPD (used to prevent peroxidation of cholecalciferol and 1 α -hydroxycholecalciferol [6]) was from Eastman-Kodak Co. (Rochester, NY, U.S.A.). Sep-Pak C₁₈ cartridges were purchased from Waters Associates, Milford, MA, U.S.A.). All chemicals were standard commercial high-purity materials.

Unlabelled steroids

1 α -Hydroxy cholecalciferol was a gift from Løvens Kemiske Fabrik (Copenhagen, Denmark). Cholecalciferol was obtained from Sigma Chemical Co. 1 α -Hydroxycholecalciferol and cholecalciferol were dissolved in ethanol to concentrations of 1 mg/ml and 10 mg/ml respectively just before use. 1,25-Dihydroxycholecalciferol was a gift from Roche Norge A/S (Oslo, Norway), and 25-hydroxycholecalciferol was purchased from Philips-Duphar B.V., Veenendaal, The Netherlands. 5 β -Cholestane-3 α ,7 α ,12 α -triol was generously given by Dr. I. Björkhem (The Research Centre at the Karolinska Institutet, Huddinge University Hospital, Huddinge, Sweden).

Labelled steroids

1 α ,25-Dihydroxy[23,24(n)-³H]cholecalciferol (80–120 Ci/mmol) and 25-hydroxy[23,24(n)-³H]cholecalciferol (91 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.) and purified by h.p.l.c. before use. [7 β -³H]5 β -Cholestane-3 α ,7 α ,12 α -triol (7 Ci/mol), synthesized as described [13], was also generously given by Dr. I. Björkhem.

Abbreviations used: DPPD, diphenylphenylenediamine; DBP, vitamin D-binding protein.

Rat liver material

Male and female rats of the Wistar strain, weighing 150–200 g, were purchased from Møllegaard Breeding Laboratory (Ejby, Denmark). The animals were fed a commercial pellet diet (Ewos R3; Møllesentralen A/S, Oslo, Norway). They were housed in plastic cages (Danish design with galvanized lids), two to four in each cage, and had free access to food and water. The climatic conditions were: 23 °C, 60 % relative humidity and a 12 h light period (07:00–19:00 h). Treatment started 5 days after their arrival at the laboratory. After the animals had been killed by decapitation, the livers were homogenized in 5 vol. (w/v) of 0.25 M-sucrose/15 mM-Hepes (pH 7.4)/0.1 mM-EGTA. Mitochondrial and microsomal fractions were prepared from each liver, essentially as described for subfractionation of human liver material [13]. The mitochondria (8700 g pellet) were washed twice in the homogenization buffer. The microsomal fractions (100 000 g pellet) were used without washing. Mitochondrial and microsomal pellets were resuspended in the incubation buffer to a protein concentration of 10 mg/ml.

Treatment

For starvation experiments the animals were fasted for 48 h before being killed.

For study of the effect of sex hormones, female rats were injected with 5 mg of Triolandrene (slow-release testosterone preparation; CIBA-Geigy AG, Basel, Switzerland) in 100 μ l of castor oil, and male rats were injected with 200 μ g of Primogyn Depot (oestradiol valerate; Schering AG, Berlin, Germany) in 100 μ l of sesame oil. The hormones were administered as one intramuscular injection 14–20 days before the animals were killed. The control animals received the vehicle only.

Incubation, extraction and chromatographic procedures

Liver mitochondria from one animal from each experimental group (total of four animals) were prepared at the same time and immediately incubated. The microsomal fractions were stored at –20 °C until analysed. Incubations were performed in duplicate as described previously [14,15]. The incubation mixture contained the following in 1 ml: 250 μ mol of sucrose, 25 μ mol of Mops buffer, pH 7.4, 10 μ mol of $MgCl_2$, 2 μ g of DPPD, 1 mg of subcellular protein and an NADPH-generating system that was either 2.7 μ mol of ATP and 3 μ mol of potassium malate (incubations with mitochondrial fractions) or 0.7 μ mol of NADP⁺, 0.6 μ mol of glucose 6-phosphate and 0.5 unit of glucose-6-phosphate dehydrogenase (incubations with microsomal fractions). The reaction was started by adding a saturating amount of substrate, which was either 100 μ g of cholecalciferol (mitochondria), 10 μ g of 1 α -hydroxycholecalciferol (microsomes) or 10 μ g of 5 β -cholestane-3 α ,7 α ,12 α -triol containing 100 000 c.p.m. of ³H-labelled steroid (mitochondria and microsomes). After 60 min (cholecalciferol) or 40 min (1 α -hydroxycholecalciferol or 5 β -cholestane-3 α ,7 α ,12 α -triol) the incubations were stopped with 1 ml of acetonitrile. When cholecalciferol or 1 α -hydroxycholecalciferol was used as substrate, about 3000 c.p.m. of [³H]25-hydroxycholecalciferol or [³H]1 α ,25-dihydroxycholecalciferol respectively were added to correct for recovery during extraction and chromatographic procedures.

The precipitated protein was removed by centrifugation and the supernatant transferred to a Sep-Pak C₁₈ cartridge prewashed with 5 ml of methanol and 5 ml of water. The cartridge was washed with 3 ml of water/methanol (3:7, v/v) before elution of the sample with 3 ml of acetonitrile. The acetonitrile was evaporated under N₂ and the residue was dissolved in 100 μ l of methanol. The products from the incubations with cholecalciferol or 1 α -hydroxycholecalciferol were purified by two h.p.l.c. steps [13,14], and product formation was calculated by comparing the peak height at 254 nm with a standard curve obtained with different amounts of authentic 25-hydroxycholecalciferol or 1 α ,25-dihydroxycholecalciferol respectively, after correction for recovery of the added radioactive metabolite. The identity of 25-hydroxycholecalciferol or 1 α ,25-dihydroxycholecalciferol was confirmed by g.c.–m.s. as reported [5,16].

Portions of the 5 β -cholestane-3 α ,7 α ,12 α -triol (containing approx. 20 000 c.p.m.) were separated by h.p.l.c. on a Zorbax ODS column (5 mm $\times$ 250 mm), with water/methanol (3:37, v/v, for mitochondria or 1:9, v/v, for microsomes) as elution solvent at a flow rate of 1 ml/min. The amount of product formed was calculated from the percentage distribution of radioactivity in the collected fractions (each 1 ml). The volume of the fractions between 6 and 12 ml was changed to 0.5 ml in order to obtain a baseline separation of peak I and peak II (cf. below).

5 β -Cholestane-3 α ,7 α ,12 α ,25-tetrol, which was used as standard, was obtained by incubating human liver microsomes with 5 β -cholestane-3 α ,7 α ,12 α -triol as described [14]. The product from the incubations of rat liver mitochondria with 5 β -cholestane-3 α ,7 α ,12 α -triol served as a standard for 5 β -cholestane-3 α ,7 α ,12 α ,27-tetrol [10].

Other methods

Protein was determined by the method of Lowry *et al.* [17], with bovine serum albumin as standard. Vitamin D-binding protein (DBP) was quantified in cholate (0.5 mg/mg of protein)-solubilized subcellular fractions by a single-radial-immunodiffusion technique [18].

Statistical analysis

A two-sample *t* test (MINITAB statistical program; Statistical Department, Pennsylvania State University, Philadelphia, PA 16802, USA) was used to evaluate the significance of difference between the groups. Significance (*P*) values are given in the Tables. A *P* value of greater than 0.05 was taken as not significant. The data were tested for normal distribution before statistical calculations were performed.

RESULTS

Effect of sex on the hydroxylation of cholecalciferol and 5 β -cholestane-3 α ,7 α ,12 α -triol

Mitochondrial cholecalciferol 25-hydroxylase activity was significantly higher (4.6-fold) in fed female rats than in fed male rats (Table 1). The large s.d. for each group reflects the great variation between subcellular fractions from one liver to another, as previously reported [15].

In order to obtain detectable microsomal cholecalciferol 25-hydroxylase activity, 1 α -hydroxychole-

Table 1. 25-Hydroxylation of cholecalciferol by liver mitochondria and of 1 α -hydroxycholecalciferol by liver microsomes from fed and starved female and male rats

The incubations were performed as described in the Experimental section. Results are means $\pm$ s.d.

Rats	Formation (nmol/h per mg) of:	
	25-Hydroxycholecalciferol in mitochondria	1 α ,25-Dihydroxycholecalciferol in microsomes
Females		
Fed	0.372 $\pm$ 0.217* (n = 8)	0.076 $\pm$ 0.033* (n = 9)
Starved	0.183 $\pm$ 0.094 (n = 4)	0.056 $\pm$ 0.031 (n = 5)
Males		
Fed	0.081 $\pm$ 0.049 (n = 10)	0.211 $\pm$ 0.090† (n = 9)
Starved	0.065 $\pm$ 0.049 (n = 6)	0.098 $\pm$ 0.032 (n = 5)

* Significantly different from fed male rats ($P < 0.01$).

† Significantly different from fed female rats ($P < 0.002$).

Table 2. Hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol by mitochondria and microsomes from fed and starved female and male rats

The incubations were performed as described in the Experimental section. Abbreviation: n.d., not detected (detection limit 0.2 nmol/h per mg). Results are means $\pm$ s.d.

Rats	Formation of 5 β - cholestane-3 α ,7 α ,12 α ,27- tetrol in mitochondria (nmol/h per mg)	Formation (nmol/h per mg) in microsomes of:	
		Peak I*	Peak II†
Females			
Fed	17.2 $\pm$ 2.1‡ (n = 6)	6.7 $\pm$ 2.6 (n = 10)	n.d.
Starved	15.0 $\pm$ 5.1 (n = 4)	4.4 $\pm$ 0.5 (n = 4)	n.d.
Males			
Fed	4.8 $\pm$ 1.7 (n = 10)	9.4 $\pm$ 3.7 (n = 9)	5.0 $\pm$ 1.6 (n = 9)
Starved	4.5 $\pm$ 4.7 (n = 6)	5.8 $\pm$ 0.7 (n = 4)	6.3 $\pm$ 0.9 (n = 4)

* Mainly 5 β -cholestane-3 α ,7 α ,12 α ,25-tetrol.

† Characterized as 5 β -cholestane-1,3 α ,7 α ,12 α -tetrol (see the text).

‡ Significantly different from the value for fed males ($P < 0.001$).

calciferol was used as substrate. This compound has previously been shown to be 25-hydroxylated in rat liver in the same way as cholecalciferol, but at higher rate [15]. The microsomal 25-hydroxylase activity was significantly higher in fed male rats than in fed female rats (2.8-fold) (Table 1).

Mitochondrial 27-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol was significantly higher (2.7-fold) in fed female rats than in fed male rats (Table 2). The microsomal fraction from male rats converted 5 β -cholestane-3 α ,7 α ,12 α -triol into two more polar products, indicated as peak I and peak II in the h.p.l.c. profile (Fig. 1a). Only peak I could be detected in the h.p.l.c. profile from incubation of microsomes from female rat liver (Fig. 1b). Peak I co-migrated with 5 β -cholestane-3 α ,7 α ,12 α ,25-tetrol, but was not completely separated from 5 β -cholestane-3 α ,7 α ,12 α ,27-tetrol in the system used. Rechromatography of peak I with water/methanol (1:4, v/v) separated the 25- and 27-tetrols completely (results not shown). Peak I from both sexes contained mainly the 25-hydroxylated tetrol. For the males, 8.4% of the radioactivity in peak I co-migrated with the 27-

hydroxylated tetrol, and for the females, 8.9%. Peaks II from several incubations of microsomes from male rat livers were pooled, converted into their trimethylsilyl derivative and analysed by g.c.-m.s. The mass spectrum characterized this compound as 5 β -cholestane-1,3 α ,7 α ,12 α -tetrol, determined by its ion m/z of 217 [19]. Formation of peak I in male- and female-rat liver microsomes is shown in Table 2. There was a tendency for males to show an increased formation of peak I, but this difference was not statistically significant.

Starvation had no statistically significant influence on any of the hydroxylation reactions studied (Tables 1 and 2). In particular, the sexual difference in mitochondrial hydroxylase activities was not significantly affected by starvation (Tables 1 and 2).

Effect of hormone treatment on hydroxylation of cholecalciferol and 5 β -cholestane-3 α ,7 α ,12 α -triol

In order to verify the involvement of sex hormones in the observed difference between females and males, female rats were injected with testosterone and male rats with oestradiol valerate. Testosterone treatment of

Fig. 1. Reversed-phase h.p.l.c. of incubations with 5β -cholestane- $3\alpha,7\alpha,12\alpha$ -triol and microsomes from male (a) and female (b) rat livers

The elution solvent was water/methanol (1:9, v/v) at 1 ml/ml. The incubations were performed as described in the Experimental section. Peak I was mainly 5β -cholestane- $3\alpha,7\alpha,12\alpha,25$ -tetrol. Peak II was characterized as 5β -cholestane- $1,3\alpha,7\alpha,12\alpha$ -tetrol (see the text). Unconverted substrate was eluted between 22 and 24 ml. Arrows marked 25 and 27 represent retention time of authentic 5β -cholestane- $3\alpha,7\alpha,12\alpha,25$ -tetrol and 5β -cholestane- $3\alpha,7\alpha,12\alpha,27$ -tetrol respectively.

Table 3. 5-Hydroxylation of cholecalciferol by liver mitochondria and of 1α -hydroxycholecalciferol by liver microsomes from untreated and hormone-treated rats

The females were treated with testosterone and the males with oestradiol valerate 14 days before being killed, as described in the Experimental section. The controls received the vehicle only. Incubations were performed as described in the Experimental section; $n = 5$ for all groups. Results are means $\pm$ S.D.

Rats	Formation (nmol/h per mg) of:	
	25-Hydroxy-cholecalciferol in mitochondria	$1\alpha,25$ -Dihydroxy-cholecalciferol in microsomes
Females		
Untreated	$0.435 \pm 0.078^*$	$0.053 \pm 0.020^*$
Hormone-treated	0.283 ± 0.118	0.057 ± 0.008
Males		
Untreated	0.093 ± 0.010	0.152 ± 0.066
Hormone-treated	$0.358 \pm 0.062^*$	$0.062 \pm 0.025^*$

* Significantly different from untreated male value ($P < 0.001$).

female rats tended to decrease the mitochondrial cholecalciferol 25-hydroxylase activity, but the difference from untreated females was not statistically significant (Table 3). Treatment of male rats with oestradiol valerate resulted in a significant increase in mitochondrial

cholecalciferol 25-hydroxylase activity (Table 3), to the same level as that of untreated females.

The microsomal cholecalciferol 25-hydroxylase activity was significantly decreased in hormone-treated males, but no effect was seen in hormone-treated females (Table 3).

The effect of hormone treatment on the hydroxylations of 5β -cholestane- $3\alpha,7\alpha,12\alpha$ -triol was the same, but less pronounced, as that seen for cholecalciferol 25-hydroxylase activities (Table 4). The difference in mitochondrial 27-hydroxylase activity between untreated and hormone-treated males was significant only at $P < 0.06$. The enzyme activity for hormone-treated males, however, reached a level that was not significantly different from that of untreated females (Table 4). The most striking effect was that injection of oestradiol valerate into male rats completely inhibited formation of 5β -cholestane- $1,3\alpha,7\alpha,12\alpha$ -tetrol (Peak II) (detection limit 0.2 nmol/h per mg) in the microsomal fraction. Injection of testosterone to female rats did not induce formation of this metabolite.

DISCUSSION

The results presented show that liver mitochondria from female rats have a higher capacity than those from male rats to 25-hydroxylate cholecalciferol, whereas the opposite appears to be true for the microsomal 25-hydroxylase activity (Table 1). These findings indicate that 25-hydroxylation of cholecalciferol is to some extent subjected to regulatory control by sex hormones.

It may be argued that the presence of DBP in the subcellular fractions might have influenced the cholecalciferol 25-hydroxylase activities. The level of DBP is higher in male than in female rats [20], and DBP has been reported to stimulate cholecalciferol 25-hydroxylase activity [21]. The difference in mitochondrial 25-hydroxylase activity cannot, however, be explained by the presence of DBP, as DBP analysed by a radial-immunodiffusion technique was undetectable in the mitochondrial fractions used (detection limit approx. $0.5 \mu\text{g}/\text{mg}$ of protein). Small amounts of DBP were detected in the microsomal fractions, but no significant difference was found between females and males (0.8 ± 0.1 and $0.7 \pm 0.1 \mu\text{g}$ of DBP/mg of protein respectively). The difference between females and males in respect of microsomal 25-hydroxylase activity cannot therefore be explained by different amounts of DBP in the fractions.

The higher 1α -hydroxycholecalciferol 25-hydroxylase activity in male-rat liver microsomes compared with that of females (Table 1) is in agreement with the documented sex difference in the microsomal metabolism of several drugs and steroid hormones [22]. The microsomal 25-hydroxylase activity seems to be connected with the sex-specific form(s) of cytochrome *P*-450 in the microsomes [22,23].

It has previously been shown that administration of exogenous sex steroids to adult rats can masculinize or feminize the hepatic pattern of steroid metabolism in rat liver microsomes [22]. Tables 3 and 4 clearly show that this also is possible for mitochondrial enzymes like cholecalciferol 25-hydroxylase and C_{27} -steroid 27-hydroxylase activities. From the presented results (Tables 3 and 4) it seems that oestrogen may be more important than testosterone, but further studies are necessary to elucidate this.

Table 4. Hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol by liver mitochondria and microsomes from untreated and hormone-treated rats

The females were treated with testosterone and the males with oestradiol valerate 14 days before being killed, as described in the Experimental section. The controls received the vehicle only. Incubations were performed as described in the Experimental section. $n = 5$ for all groups. Results are means $\pm$ S.D.

Rats	Formation of 5 β -cholestane-3 α ,7 α ,12 α ,27-tetrol in mitochondria (nmol/h per mg)	Formation (nmol/h per mg) in microsomes of:	
		Peak I*	Peak II†
Females			
Untreated	12.1 $\pm$ 6.0‡	2.5 $\pm$ 2.2	n.d.§
Hormone-treated	9.1 $\pm$ 2.2	2.7 $\pm$ 1.2	n.d.
Males			
Untreated	5.0 $\pm$ 2.6	4.3 $\pm$ 1.9	1.7 $\pm$ 0.7
Hormone-treated	9.8 $\pm$ 2.5	1.7 $\pm$ 1.0	n.d.

* Mainly 5 β -cholestane-3 α ,7 α ,12 α ,25-tetrol.

† Characterized as 5 β -cholestane-1,3 α ,7 α ,12 α -tetrol (see the text).

‡ Significantly different from untreated male value ($P < 0.05$).

§ Abbreviation: n.d., not detectable (detection limit 0.2 nmol/h per mg).

|| Different from untreated male value ($P < 0.06$).

In liver microsomes the sex difference in cytochrome *P*-450 is regulated by the hypothalamo-pituitary axis through which the sex hormones exhibit their effects [22]. It is not known whether the liver cholecalciferol 25-hydroxylase activities are regulated through the same system or if there is a direct effect of the sex hormones on the enzyme activities, as is the case for some of the adrenal enzymes [24].

The higher C₂₇-steroid 27-hydroxylase activity in female- compared with male-rat liver mitochondria (Table 2) reflects previous reports of higher oxidation of cholesterol in liver mitochondria from female compared with male rats [25,26]. Monoamine oxidase, a mitochondrial-outer-membrane enzyme, has also been shown to exhibit a higher activity in female than in male rats [27].

The ratio between the enzyme activities in the mitochondria from females and males was 4.6 for cholecalciferol 25-hydroxylase and 2.7 for 5 β -cholestane-3 α ,7 α ,12 α -triol 27-hydroxylase, indicating a similarity between these two enzymes. This does not, however, exclude the possibility of two different enzymes, as discussed previously [7,28].

The minor effect of starvation on the hydroxylation of cholecalciferol and of 5 β -cholestane-3 α ,7 α ,12 α -triol is in agreement with findings by Björkhem & Gustafsson [29], who studied 27-hydroxylation (see the parenthetic note in the Introduction) of various bile-acid intermediates in microsomal and mitochondrial fractions from rat liver [29].

Tetrahydroxylated bile acids with hydroxy groups in position 1,3,7 and 12 have been detected in urine from normal infants [19] and from patients with liver cholestasis [30]. 1 β -Hydroxylation of androgens is known to occur in microsomes from human foetal liver and adrenal glands [31–33]. Formation of 5 β -cholestane-1,3 α ,7 α ,12 α -tetrol in male rat liver microsomes (Table 2) is, however, the first system described for 1-hydroxylation of bile-acid

intermediates in rat liver. The physiological importance of this reaction in the rat is unknown.

In conclusion, we have shown that liver mitochondrial hydroxylating enzymes such as cholecalciferol 25-hydroxylase and C₂₇-steroid 27-hydroxylase (which are both cytochrome *P*-450-dependent) seem to be under the regulatory control exhibited by sex hormones. The mechanism of this regulation requires investigation.

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