IDENTIFICATION OF AN AUTOSOMAL LOCUS AFFECTING STEROID SULFATASE ACTIVITY AMONG INBRED STRAINS OF MICE

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ABSTRACT

We have found an activity variant for testicular and liver steroid sulfatase among inbred strains of mice that is not *X*-linked. C57BL/6J, SM/J and SWR/J testicular extracts hydrolyze ^{3}H-dehydroepiandrosterone sulfate twice as rapidly as do A/J extracts. The C3H/HeJ and DBA/2J strains were intermediate. The K_m values for C57BL/6J and A/J are 2.29 ± 0.10 and 1.01 ± 0.02 μM, respectively. The F_1 values in both directions were intermediate, which argues against *X*-linkage of this trait. F_2 values show scattered high-intermediate-low values compatible with assay variation superimposed on the segregation of codominant alleles. When assayed for both testicular and liver steroid sulfatase, nine recombinant inbred lines between A/J and C57BL/6J segregate to near the parental strain values. Thus, this activity variation for steroid sulfatase appears to be determined by a single gene, which is not *X*-linked. Sex and steroidal hormone differences in liver steroid sulfatase activity were not present in the A/J strain, but females of the C57BL/6J and some recombinant inbred lines had higher levels. Electrophoretic studies only disclosed a variant in the SM/J strain, which seems to be secondary to the well-known neuraminidase variation in SM/J.

STEROID sulfatase (EC 3.1.6.2) is a microsomal enzyme involved in the metabolic activation of a variety of steroidal compounds. In man, a deficiency has been associated with delayed labor (FLIEGNER, SCHINDLER and BROWN 1972) and *X*-linked ichthyosis (SHAPIRO *et al.* 1978). Great interest has been attached to this observation, because the enzymatic deficiency maps to the short arm of the *X* near the *Xg* locus. Like *Xg*, this locus seems not to be inactivated during Lyonization (MOHANDAS *et al.* 1979; MOHANDAS *et al.* 1980). However, the biochemical and genetic nature of this *X*-linked steroid sulfatase locus has many ambiguities. In terms of biochemistry, it is not at all clear that the *X*-linked locus is the structural locus for the enzyme. There is evidence that the microsomal phospholipid composition, and not the apoenzyme, is abnormal in the *X*-linked mutation (MCNAUGHT and FRANCE 1980; MCKEE, ABEYSEKERA and FRANCE 1981) and this changed composition also affects another microsomal enzyme, $^3\beta$-hydroxysteroid dehydrogenase-isomerase (MOR-

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TON and OAKEY 1980). In regards to the genetics, somewhat reasonable dosages for steroid sulfatase are found between normal females and *XX* steroid sulfatase deficiency carriers, but the values for *2X*, normal females compared with *XY* individuals have varied considerably; these values range from 1.3 to 1.7, instead of the expected 2, which is probably explained by partial inactivation of the steroid sulfatase locus (MIGEON *et al.* 1982). Furthermore, it has been reported that, in situations with more than two *X* chromosomes, dosage compensation occurs so that the ratio does not become much greater than 2:1 (ROPERS *et al.* 1981; CHANCE and GARTLER 1983).

An animal model of steroid sulfatase deficiency could help resolve these issues. Although conservation of *X*-chromosomal linkage among mammals is well established, the loci previously tested have been on the long arm of the human *X*. It is not clear whether conservation of loci concerned with sex determination on the short arm of the human *X* are equally conserved: *XO* mice are fertile, but *XO* humans are sterile. Evidence for *X*-linkage and noninactivation of steroid sulfatase has been presented for one rodent, the wood lemming, but the quantitative aspects of the dosage were no better than those found in man (ROPERS and WIBERG 1982). GARTLER and RIVEST (1983) have reported evidence for *X*-linkage of steroid sulfatase in mice on the basis of the ratio of activity between *XX* and *XO* oocytes. Two aspects of their results deserve comment: (1) they found 3600–7200 times greater activity of steroid sulfatase in oocytes than in kidney (an oocyte contains about 28 ng of protein; BRINSTER 1967), and (2) there was no difference in kidney steroid sulfatase activity between *XX* and *XO* mice, *i.e.*, dosage compensation was found.

We have found an activity variant of steroid sulfatase in *Mus musculus*, which is controlled by an autosomal locus. Strain and tissue variation in activity levels suggest that the locus may be the same as that controlling hydrophobic arylsulfatase C reported recently (NELSON, KEINANEN and DANIEL, unpublished data; KEINANEN *et al.* 1983).

MATERIALS AND METHODS

Mice: Inbred mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and were used immediately or maintained by brother-sister mating until use. Recombinant inbred lines between the A/J and C57BL/6J strains were provided by MURIEL NESBITT, University of California, San Diego. Shavings (⅓ cedar) were used as bedding, and the mice were kept in a room with controlled lighting (14-hr light/10-hr dark).

Steroid sulfatase assay: Mouse testes or livers were homogenized in a 4× volume of 0.01 M Tris, 0.005 M $MgCl_2$, 0.001 M DDT buffered to pH 7.4. The homogenate was spun at 800 × *g* for 5 min at 34°, and 50 μl of the supernatant were incubated with 950 μl of 2×10^{-6} M dehydroepiandrosterone sulfate (DHEAS) in 0.05 M imidazole buffer, pH 6.2, that contained 2×10^{-8} Ci of ^{3}H-DHEAS (50–60 mCi/mmol). The mixture was incubated at 37° for 45 min, and the reaction was stopped by the addition of 3 ml of 0.1 M NaOH. Free steroid was extracted with 15 ml of PPO-POPOP toluene-based scintillation fluid. This solution was frozen and then centrifuged at 800 × *g* for 3 min, and 10 ml of scintillation fluid were removed for counting. Results were expressed in picomoles per minute per milligram of protein as determined by the Lowry assay.

Hormone treatments: Testosterone propionate was injected intraperitoneally on day 0 and day 7. Mice were sacrificed and livers removed for assay on day 12. Each animal received 20 μg/g of body weight of the crystalline hormone (Sigma) in a micromolecular suspension prepared by first dissolving it in 95% ethanol and then diluting it approximately 30-fold with doubly distilled water.

Diethylstilbesterol was administered through a subcutaneous implant on day 0. Animals were again used on day 12. To test the effect of the absence of endogenous testosterone, males were castrated on day 0 and then used on day 12.

Microsomal extracts: Testes were homogenized in 8 volumes of 0.01 M Tris, pH 7.4, with 1 mM DTT, 1 mM EDTA, 1 mM phenylmethyl-sulfonyl fluoride (PMSF). They were spun for 5 min at 550 × *g*, and the supernatants were spun for 60 min at 42,000 × *g*. The pellet was rehomogenized in this buffer for enzyme assays or taken up in 200–300 μl of 2% Triton, 1 mM EDTA, 1 mM NADP in electrophoresis buffer (5.6 g/liter of Tris-HCl, 0.133 g/liter of calcium lactate, 0.02 g/liter of DTT, pH 8.3, with NaOH) for electrophoresis.

Electrophoresis: Samples of extract (10 μl) were electrophoresed in 1% acrylamide, 1% agarose gels containing 1% Miramol H2M using the above electrophoresis buffer for about 30 min at 60 mA of constant current, then about 3 hr at constant wattage (LKB apparatus) increasing in steps from 32–60 watts.

Staining for steroid sulfatase activity: The gel was washed in 0.1 M imidazole-HCl, pH 6.2, for several minutes and then flooded with a solution of 12 mg of 4-methylumbelliferyl sulfate in 3 ml of 0.05 M imidazole-HCl, pH 6.2. The gel was incubated for 30 min at room temperature and visualized or photographed with UV.

Neuraminidase treatment: Testes were homogenized in 4 volumes of 1.2 mM PMSF and centrifuged at 42,000 × *g* for 60 min. The pellets were resuspended by homogenization in H_2O and incubated with neuraminidase (Sigma type VI, 6.5 μm/mg [NAN-lactose], lot 18C-8020-1) in 0.025 M sodium acetate, pH 5.0, at 37° for 210 min. The treated samples were diluted with a 2.5-fold excess of 0.1 M Tris-HCl, pH 8.0, and electrophoresed as before.

RESULTS AND DISCUSSION

We initially studied testicular steroid sulfatase because of our interest in expression of *X*-linked enzymes during spermatogenesis. A strain survey of testicular levels of steroid sulfatase revealed significant activity variation among several inbred strains (Table 1). Of six relatively unrelated inbred strains (A/J, C3H/HeJ, C57BL/6J, DBA/2J, SM/J and SWR/J), A/J was the lowest, and C57BL background strains (C57BL/6J and B10.A) were the highest. The major histocompatibility locus of mice, *H-2*, has been shown to have major effects on levels of testosterone, a sometimes sulfated steroid, in mice (IVANYI *et al.* 1972). Thus, we sought to determine whether or not *H-2* has an effect on testicular steroid sulfatase. We studied the effect of the $H\text{-}2^a$ allele from A/J on the high activity level of C57BL/6J in the nearly congenic B10.A line and found no effect. Similarly, the $H\text{-}2^b$ allele of C57BL/6J did not influence the testicular steroid sulfatase activity of A/WY (a line related to A/J) in the A.BYSn congenic line. SM/J, with an electrophoretic variant (see following data), had high activity.

Lineweaver-Burke (double reciprocal) plots of enzymatic activity in testicular homogenates at varying substrate concentrations showed that the enzymes of the extreme strains, A/J and C57BL/6J, differed in their K_m and V_{max} values (Figure 1). The calculated values of K_m and V_{max} for the A/J steroid sulfatase were 1.01 ± 0.02 μM and 9.67 ± 0.07 pmol/min/mg, respectively; the values for C57BL/6J were 2.29 ± 0.10 μM and 29.5 ± 0.51 pmol/min/mg. Unexpectedly, the C57BL/6J strain with the high V_{max} also has the higher K_m, *i.e.*, the enzyme is apparently more efficient despite the fact that it requires higher substrate concentrations to saturate. These K_m values were little changed in microsomal preparations. When microsomal membranes were rehomogenized

TABLE 1

Strain variation in testicular steroid sulfatase

Strain	Background	*H-2*	pmol/min/mg protein
A/J	A	a	(12) 7.44 ± 0.32[a]
A.BY/Sn	A	b	(6) 6.92 ± 0.64
B10.A	B	a	(6) 19.08 ± 1.04
C57BL/6J	B	b	(11) 16.64 ± 0.80
DBA/2J			(4) 12.02 ± 1.04
C3H/HeJ			(4) 10.80 ± 0.60
SM/J			(4) 15.91 ± 1.02
SWR/J			(3) 16.39 ± 0.745

[a] (*n*) Mean ± S.E.

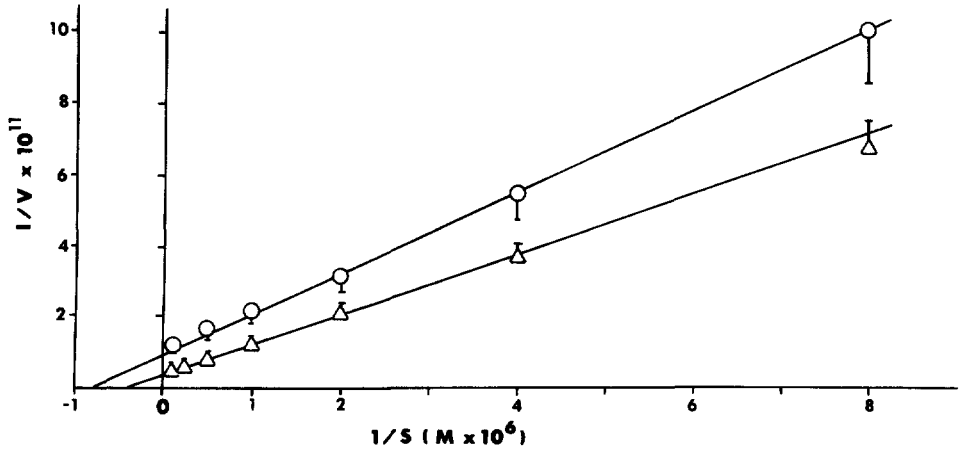

FIGURE 1.—Lineweaver-Burke (double reciprocal plot) of steroid sulfatase activity in crude homogenates of A/J (○) and C57BL/6J (△) testes. The means and S.E. values of quadruplicate determinations are depicted.

and assayed by the method described, A/J steroid sulfatase had a K_m of 0.7 ± 0.03, whereas the value for C57BL/6J was 1.91 ± 0.54. Hence, the K_m difference may reflect some property of the enzyme other than the conformation of the active site, possibly a differential solubility of steroid sulfates in microsomal membranes dependent on differential lipid composition.

Of major interest was whether or not this variation was sex linked. To pursue this, we studied F_1 males with either strain as mother. We also found the variation in liver, a tissue available in both sexes (Table 2). F_1 values were intermediate, although there were slight differences, but in opposite directions, for testes and liver. We conclude that the variation is autosomal, although there might be some influence of the *X* chromosome. Because of the possibility of a slight influence of the *X* chromosome, we asked whether there were any sex steroid effects on the levels of steroid sulfatase. This was studied in detail on the livers of the A/J strain (Table 3). Although a slightly higher level of steroid sulfatase was found in female livers, the most marked effect of a hor-

TABLE 2

Steroid sulfatase in male parental and F_1 mice

Strain	Source of X in F_1 generations	pmol/min/mg protein			
		Testes		Liver	
C57BL/6J		(4) 18.80 ± 0.56[a]		(7) 13.29 ± 1.7	
B6AF$_1$	B6	(4) 15.72 ± 0.76	$t = 5.69$	(4) 9.11 ± 0.65	$t = 2.25$
AB6F$_1$	A	(4) 13.08 ± 0.32	$P < 0.01$	(4) 11.30 ± 0.72	$P > 0.05$
A/J		(4) 7.80 ± 0.16		(7) 5.44 ± 0.66	

[a] (*n*) Mean ± S.E.

TABLE 3

Effects of hormone treatments on A/J liver steroid sulfatase

	pmol/min/mg protein			
	♂		♀	
Control	(10) 3.29 ± 0.20[a]	$t = 2.42$	(10) 4.20 ± 0.28	$t = 2.01$
Testosterone-treated	(10) 4.37 ± 0.64	$P < 0.05$	(10) 4.47 ± 0.48	$P > 0.05$
Diethylstilbesterol-treated	(9) 4.61 ± 0.53		(10) 5.79 ± 0.74	
Castrated	(10) 3.65 ± 0.21			

[a] (*n*) Mean ± S.E.

mone was that of diethylstilbestrol, which significantly elevated steroid sulfatase in males, but the effect was not significant in females. Testosterone had a slight effect in both males and females, and castration had almost no effect. Thus, we were not able to discern any consistent sex steroid or *X*-dosage effects on liver steroid sulfatase in this strain. However, C57BL/6J females and females of some recombinant inbred lines between this strain and A/J showed higher levels of liver steroid sulfatase than males (see following data).

To study the inheritance of this pattern in greater detail, we studied the segregation of the testicular steroid sulfatase in F_2 mice (Figure 2). The physiological and assay variation are such that there is even a slight overlap between the parentals when many assays are done over a long period of time. However, the F_2 mice segregate in a pattern compatible with a 1:2:1 segregation. Recombinant inbred lines offer a more definitive way of studying the segregation of loci between two inbred strains (SWANK and BAILEY 1973). These are prepared by making an F_1 between two lines and reinbreeding a number of lines from brother/sister pairs. Each such line can be considered to be a chromosomal mosaic of the two parental lines, but, because it is highly inbred, each member of the line is genetically identical. As seen in Table 4, both testicular and male liver steroid sulfatase (and, in a few cases, female liver steroid sulfatase) tended to segregate to parental values. This parental segregation is more clearly seen in a scatter diagram (Figure 3). The composite values for testicular and male liver steroid sulfatase clearly separate into two, approximately equal, groups. Such a segregation to near parental values argues strongly that the major genetic effect on steroid sulfatase is determined by alleles at one locus.

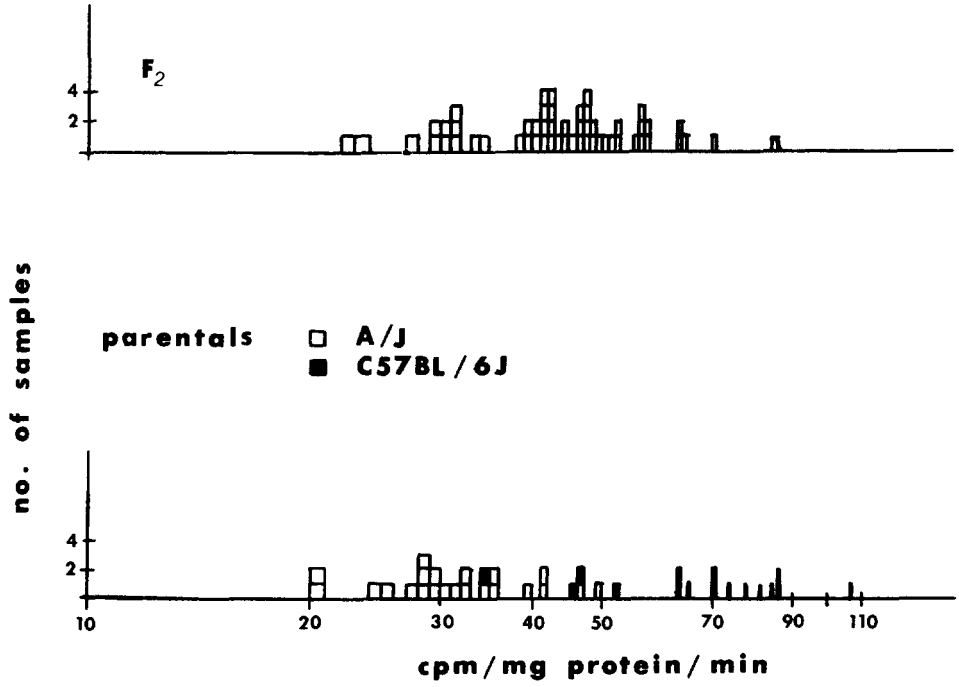

FIGURE 2.—Activity of testicular steroid sulfatase in the parental A/J and C57BL/6J inbred strains (lower panel) and their F_2 progeny (upper panel).

TABLE 4

Inheritance of steroid sulfatase in RI lines

Strain		pmol/min/mg protein		
		Testes	Male liver	Female liver
A/J	parentals	(15) 7.51 ± 0.66	(7) 5.44 ± 0.66	(10) 4.20 ± 0.28
C57BL/6J	parentals	(15) 17.39 ± 0.88	(7) 13.29 ± 1.7	(11) 21.00 ± 2.04
A × B2		(6) 23.01 ± 0.85	(6) 10.85 ± 1.02	(4) 22.76 ± 2.19
A × B13		(9) 16.80 ± 0.68	(6) 13.18 ± 0.93	(4) 16.81 ± 2.0
A × B15		(6) 10.44 ± 0.95	(8) 5.40 ± 0.48	
A × B17		(9) 8.77 ± 0.31	(6) 5.84 ± 0.80	(4) 12.65 ± 1.0
B × A1		(8) 11.04 ± 1.52	(6) 5.57 ± 0.68	
B × A6		(6) 21.19 ± 1.51	(6) 12.12 ± 0.68	
B × A11		(6) 17.23 ± 2.02	(6) 16.04 ± 3.0	
B × A14		(9) 6.84 ± 0.42	(8) 6.00 ± 0.94	(5) 9.36 ± 0.64
B × A15		(7) 11.44 ± 1.26	(6) 8.6 ± 1.42	

It is of interest to note that female liver steroid sulfatase was higher than male liver steroid sulfatase in the C57BL/6J strain and in those recombinant inbred lines in which it was tested. Thus, the estrogen effect suggested by the hormonal induction studies performed in the A/J strain may occur more strongly in the C57BL/6J and some recombinant inbred lines. Alternatively, *X*-related dosage could be postulated for these sex differences. Since this oc-

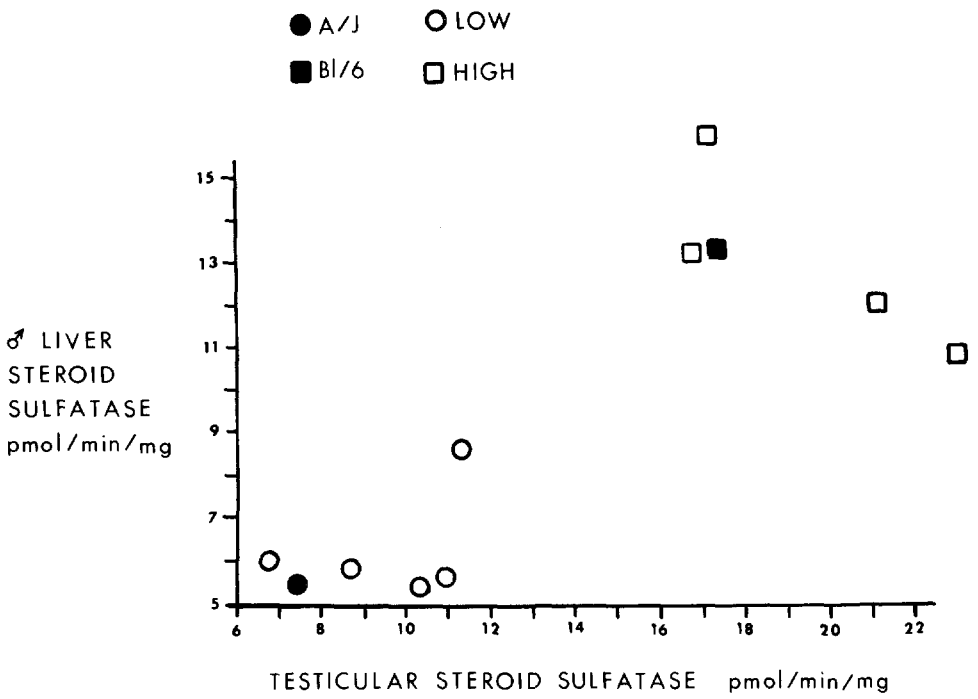

FIGURE 3.—Activity of male liver steroid sulfatase (ordinate) and testicular steroid sulfatase (abscissa) for the two parental strains (filled symbols) and nine recombinant inbred lines (open symbols).

curred in some recombinant inbred lines that were clearly A/J-like for overall activity, the presence or absence of this effect is not linked to the autosomal locus controlling testicular and male liver steroid sulfatase. Thus, it is possible that a second locus influences the female to male liver steroid sulfatase ratio.

In attempts to further study the steroid sulfatase variant, electrophoretic methods were used. These methods determine arylsulfatase C from the microsomal pellet, which is usually considered to be equivalent to steroid sulfatase (SHAPIRO *et al.* 1977; HAMEISTER *et al.* 1979; MEYER *et al.* 1979). Microsomal pellets were solubilized in Triton, electrophoresed on composite acrylamide-agarose gels and stained for activity with 4-methylumbelliferyl sulfate at pH 6.2. A strain survey showed a slight variation in the SM/J strain. The electrophoretically detected steroid sulfatase activity in SM/J testes was slightly faster than that found in the other strain's testes (A/HeJ, 129/Sv, C57BL/J, C57BL/6J and A.BY/Sn). A much more rapidly moving band was also found for rat testes. It seemed probable that this electrophoretic variation would be due to SM/J's deficiency of neuraminidase, which affects the posttranslational modification of a number of enzymes, including arylsulfatase B (POTIER, LU SHUN YAN and WOMACH 1979; PETERS *et al.* 1981). Treatment of SM/J extracts with 10 units of neuraminidase partially converted the band of activity to a C57BL/6-like mobility. Thus, the electrophoretic variation in the Sm/J strain seems to be due to the neuraminidase variation well-known for this

strain. The slight variation in electrophoretic mobility does not correspond to a major change in activity (Table 2).

Thus, we find autosomal inheritance of a murine activity variant in steroid sulfatase, which is expressed in several tissues. It has been reported in abstract that a deficiency of steroid sulfatase in the C3H/An strain is inherited as an autosomal recessive trait (BALAZS *et al.* 1982). Our results differ from those of GARTLER and RIVEST (1983) for oocytes. We have used different substrates (GARTLER and RIVEST used estrone sulfate, whereas we used DHEAS), but KEINANEN *et al.* (1983) have demonstrated a strain variation for sulfatases that parallels the one described here. Their data suggest that one catalytic protein (arylsulfatase C) hydrolyzes 4-methylumbelliferyl sulfate, DHEAS and estrone sulfate, but that it can only hydrolyze the steroid sulfates when located in microsomes. Since GARTLER and RIVEST (1983) found levels of steroid sulfatase three orders of magnitude higher in oocytes than in somatic tissues, and estrone sulfate could be a particularly important substrate for oocytes, it may be that oocytes contain a unique steroid sulfatase and that it is *X*-linked.

The autosomally inherited variation in activity of steroid sulfatase that we have found among inbred strains of mice could be homologous to the autosomal locus for multiple sulfatase deficiency in man. The known variant allele at this locus causes a severe debilitating disease with nearly complete deficiency of a number of sulfatases, including steroid sulfatase, by a mechanism other than a general inhibitor (CHANG and DAVIDSON 1980). Alternatively, it is possible that the variant we have found corresponds to the human *X*-linked variant. If so, this would be the first exception known to the conservation of *X*-linkage among mammals.

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