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STEROID SULFATASE IN THE HUMAN OVARY AND PLACENTA: ENZYME KINETICS AND PHOSPHATE INHIBITION*

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Summary—In 5 placental homogenates the K_m of steroid sulfatase for DHEA sulfate increased from 15.4 in Tris buffer to 26.8 μM in phosphate (both buffers 0.1 M, pH 7.4), $P < 0.05$. In 3 pooled ovarian preparations the K_m increased from 14.3 μM in Tris to 33.0 μM in phosphate, $P < 0.01$. There was no significant difference between the ovarian and placental values for K_m in either Tris or phosphate ($P > 0.5$), and the increase in the K_m produced by phosphate in ovarian tissue was not significantly different from that in the placenta ($P > 0.5$). In the placentas the V_{max} in Tris was 1420 pmol/min/mg protein and this fell to 523 pmol/min/mg protein in phosphate ($P < 0.005$). The V_{max} was 50-fold higher in the placenta than in the ovary in either Tris or phospahte (both $P < 0.001$). In the ovary, the V_{max} was 27.6 pmol/min/mg protein in Tris and 11.0 pmol/min/mg protein in phosphate ($P < 0.05$). The reduction of V_{max} produced by phosphate in the ovary was not significantly different from that in the placenta ($P > 0.5$). The slope of the 1/v vs 1/S plot (K_m/V_{max}) increased 4.7-fold in the placentas and 5.8-fold in the ovaries in phosphate over that in Tris (both $P < 0.001$); the increase in the placentas was not significantly different from that in the ovaries ($P > 0.5$). Phosphate ion acts as a mixed inhibitor of both placental and ovarian steroid sulfatase.

INTRODUCTION

Compartments of the human ovary have significant steroid sulfatase activity [1]. The presence of sulfatase in the ovary supports the hypothesis that DHEA sulfate (DHEAS) may serve as a precursor for ovarian androgen biosynthesis. The apparent K_m of steroid sulfatase for DHEAS as substrate has been most widely studied in placentas with reported values ranging from 3 to 64 μM using a variety of techniques [2–7]. In other tissues, values for K_m were 3.85 μM for testis [8], 7 μM for lung [9], 6 and 10 μM for chorion [10, 11], 5.7 μM for endometrium [12], 6 μM for decidua [11], and 16 μM for brain [13]. To our knowledge, the K_m for steroid sulfatase in the ovary has not been reported. All tissues (placenta, testis, lung, chorion, endometrium, decidua and brain) have had at least one K_m determination in the range of 3 to 16 μM. There has been considerable variation in the buffer salt, concentration and pH used in tissue preparation and incubation between the various determinations of the K_m of placental steroid sulfatase for DHEAS. The variations in the apparent K_m observed between laboratories are so great that little significance can be placed on differences in the apparent K_m observed between tissues when methodological variation is also present.

Phosphate inhibits sulfatase activity [10, 14, 15]. Phosphate is a normal component in extracellular fluid with a concentration of approx. 0.9 to 1.5 μM of which approx. 10% is bound. The phosphorus content of intracellular compartment is approx. 100 μM. However, the majority of intracellular phosphate is in the form of phosphoproteins, phospholipids and phosphosugars, and what effects these various constituents have on the activity of the sulfatase enzyme is unknown. Also, the type of inhibition that phosphate produces had not been determined. Thus, it is not clear whether the values of K_m determined in the presence or absence of phosphate reflect the *in vivo* activity of the enzyme. To determine the K_m of sulfatase for DHEAS in the ovary, the type of inhibition produced by phosphate, and to gain some perspective on the significance of the K_m, steroid sulfatase was examined in both the ovary and

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the placenta using the same method, and the effect of phosphate on the K_m and the V_{max} in both tissues was evaluated.

EXPERIMENTAL

Human tissues

Human tissues were obtained after removal during surgery under a protocol approved by the Human subjects committee of Women and Infants' Hospital. Representative samples of tissue were taken immediately, and frozen at −20°C until processed. Studies performed on both fresh and frozen tissues from the same specimen showed no loss of activity due to freezing. Each placental homogenate was made from a single placenta. Due to the amount of tissue required for the study and the need to preserve sufficient ovarian tissue for the clinical histological evaluation, stromal tissue from 2–3 ovaries was pooled to produce each ovarian homogenate.

Sulfatase assay

The sulfatase assay was performed as described previously [16] with modifications. Preliminary experiments were conducted to validate the conditions of the sulfatase assay with regard to linearlity with time and protein concentration, and with regard to minimal depletion of substrate. After thawing, all tissues were kept on ice until incubation. The tissue was minced in 5 ml/g tissue of either 0.1 M Tris buffer pH 7.4 or 0.1 M potassium phosphate buffer pH 7.4 and homogenized using a Polytron (Brinkman, Westbury, NY 11590). Reaction tubes were prepared by adding varying amounts of [7-^{3}H]DHEAS (sp. act. 0.01 Ci/mmol) in ethanol and taking the tubes to dryness. This produced final concentrations of 2, 5, 10, 20, 30 and 40 μM substrate in the 0.4 ml reaction volume. Either Tris buffer or potassium phosphate buffer (0.3 ml) was added, and the reaction tubes were warmed to 37°C and kept at that temperature until the reaction was stopped. Both zero tubes and homogenate tubes were prepared in triplicate for each homogenate. At time zero 0.4 ml of methanol was added to the zero tubes to stop the sulfatase reaction. The reaction tubes then received 0.1 ml tissue homogenate at 30 s intervals to allow time for subsequent processing. After 20 min, the reaction was stopped by adding 0.4 ml methanol. All reaction tubes were centrifuged at 1500 *g* for 5 min. A 50 μl sample of the supernatant from each tube was added directly to a silica G thin layer plate (Analtech 0.25 mm) along with 0.5 μmol non-isotopic DHEA as carrier. The plates were run 3 times in 98% chloroform 2% methanol for 20 min, and sprayed with primulin stain for localization of the DHEA band under long u.v. light. The DHEA containing band was scraped into a scintillation vial and counted in 5 ml Scint-A XF scintillation fluid (Packard, Downers Grove, IL 60515). The recovery of DHEA following thin-layer chromatography was 92.8 ± 0.63% ($N = 4$). Non-enzymatic hydrolysis of the sulfate was corrected for by subtraction of the mean cpm in the "zero" tubes from that in the assay tubes. The amount of product formed was calculated from the specific activity of the DHEAS after correction for counting efficiency. The protein content of the homogenate was determined using the Bio-Rad Protein Assay method of Bradford (Biorad, Richmond, CA 94804). Individual Lineweaver–Burk plots were prepared for each tissue sample studied, and the values for K_m and V_{max} were determined from the graphs. Sulfatase kinetics were studied at increasing concentrations of substrate (DHEAS: 5, 10, 15 and 20 μM) and increasing concentrations of inhibitor (potassium phosphate: 0, 50, 150 mM) at pH 7.4 in 0.1 M Tris.

Statistical analysis

All data were log-transformed prior to statistical analysis due to log-normal distribution of the data. Differences between the means were compared using a one-way analysis of variance with the Minitab statistical program [17].

RESULTS

The K_m [Fig. 1(a)] and V_{max} [Fig. 1(b)] were determined in 5 placental and 3 ovarian homogenates in both Tris and phosphate buffers. In the 5 placental homogenates the geometric mean K_m increased from 15.4 μM (95% confidence limits 8.0–29.5 μM) in Tris buffer to 26.8 μM (95% confidence limits 13.1–54.6 μM) in potassium phosphate buffer ($P < 0.05$). In the ovarian homogenates the geometric mean K_m increased from 15.7 μM (95% confidence limits 5.79–42.4 μM) in Tris to 47.8 μM (95% confidence limits 5.45–419 μM) in phosphate buffer

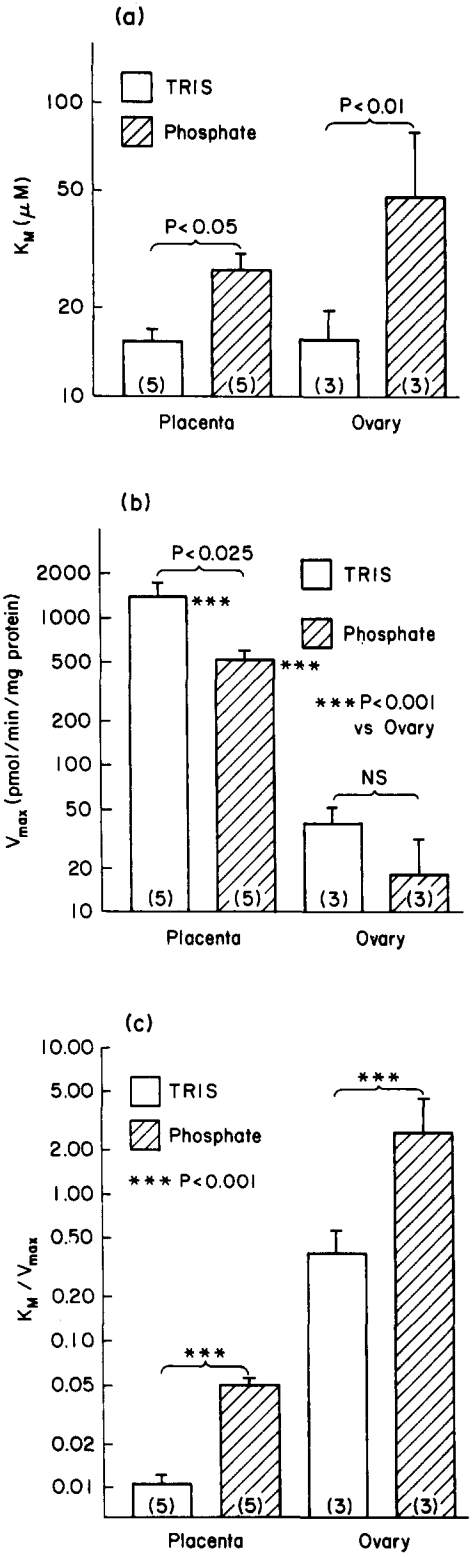

Fig. 1. The K_m (μM) (a), the V_{max} (pmol/min/mg proten) (b), and the ratio of the K_m/V_{max} (c) of steroid sulfatase were determined using DHEAS in placental and ovarian homogenates in 0.1 M pH 7.4 Tris or phosphate buffer (note log scale). The number (N) of different homogenates investigated is shown in parentheses. Each placental homogenate was made from a single placenta while tissue from 2–3 ovaries was pooled to produce each ovarian homogenate.

($P < 0.01$). There was no evidence for a difference between the K_m in the ovary and that in the placenta in either the Tris or the phosphate buffer ($P > 0.2$ based on an overall effect of tissue calculated from the one-way analysis of variance), and the 2.7-fold increase in the K_m produced by phosphate buffer in ovarian tissue was not significantly different from the 1.7-fold increase in the K_m produced by phosphate buffer in the placenta ($P > 0.2$).

In the 5 placental homogenates the geometric mean V_{max} in Tris buffer was 1420 pmol/min/mg protein (95% confidence limits 381–5320 pmol/min/mg protein) and this fell to 523 pmol/min/mg protein (95% confidence limits 246–1110 pmol/min/mg protein) in phosphate buffer ($P < 0.025$). The geometric mean V_{max} was 30-fold higher in the placental

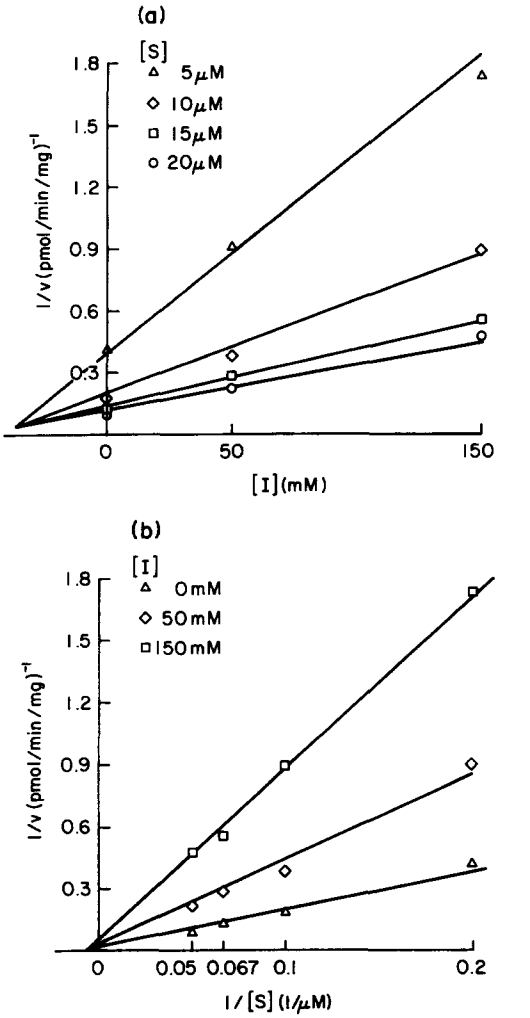

Fig. 2. Placental steroid sulfatase velocity was studied at concentrations of 5, 10, 15 and 20 μM DHEAS (S) in 0, 50 or 150 mM potassium phosphate (I) at pH 7.4 in 0.1 M Tris and plotted (a) according to Dixon (1/v vs I) and (b) Lineweaver–Burk (1/v vs 1/S), where v = pmol of DHEAS hydrolyzed/min/mg protein.

homogenates than in the ovarian homogenates in either Tris or phosphate buffer (both $P < 0.001$). In the ovarian homogenates, the geometric mean V_{max} was 40.7 pmol/min/mg protein (95% confidence limits 7.6–220 pmol/min/mg protein) in Tris and 18.2 pmol/min/mg protein (95% confidence limits 1.7–200 pmol/min/mg protein) in phosphate buffer, but this difference failed to reach statistical significance due to the small N. The reduction to 45% of the Tris V_{max} produced by phosphate in the ovary was not significantly different from the reduction to 37% of the Tris V_{max} produced by phosphate in the placenta ($P > 0.5$). To test the overall effect of phosphate a linear combination of the treatment means was constructed by assigning the λ_i for treatments with Tris [shown as open bars in Fig. 1(b)] values of $+1$ and the λ_i for treatments with phosphate [shown as hatched bars in Fig. 1(b)] values of -1; the $\Sigma\lambda_i = 1 \ -1 \ +1 \ -1 = 0$ [18]. The overall effect of phosphate in both tissues taken together was statistically significant ($P < 0.005$).

The slope of the 1/v vs 1/S plot (K_m/V_{max}) [Fig. 1(c)] increased 4.7-fold in the placentas and 6.8-fold in the ovaries in phosphate over that in Tris (both $P < 0.001$); the increase in the placentas was not significantly different from that in the ovaries ($P > 0.2$). In order to further investigate the type of inhibition, placental steroid sulfatase kinetics were studied at increasing concentrations of substrate (DHEAS: 5, 10, 15 and 20 μM) and increasing concentrations of inhibitor (potassium phosphate: 0, 50, 150 mM) at pH 7.4 in 0.1 M Tris. A plot of the data according to Dixon (1/v vs I) [Fig. 2(a)] and Lineweaver–Burk (1/v vs 1/S) [Fig. 2(b)] confirmed that phosphate ion acted as a mixed inhibitor of steroid sulfatase, i.e. it increased the K_m and decreased the V_{max}.

DISCUSSION

Roy [14] who first reported the 0.55 relative activity of ox liver sulfatase produced by 0.02 M phosphate ion, also reported that 0.001 M Na_2SO_3 resulted in a relative activity of 0.3. He also demonstrated no inhibition with 0.05 M NaCl or KCl, 0.025 M Na_2SO_4 or K_2SO_4, or 0.01 M KCN or NH_4F. The inhibition of steroid sulfatase by steroid sulfates and unconjugated steroids has been reported in the testis [8] and placenta [2]. Androstenediol-3-sulfate, pregnenolone sulfate, and DHEAS were demonstrated to be mutual competitive inhibitors in the testis [8]. Human testicular steroid sulfatase catalyzed hydrolysis of DHEAS was inhibited to the greatest degree by 5-pregnene-3β,20α-diol and 5-pregnene-3β,21-diol-20-one while the most potent inhibitor among the C_{19} steroids was 5α-androstane-3α,17β-diol [8]. The free steroids produced a partial competitive inhibition of testicular steroid sulfatase [8].

Townsley [2] who investigated the inhibitory effects of 129 endogenous and synthetic steroids on DHEAS hydrolysis by human term placental steroid sulfatase, concluded that in all cases the data were compatible with competitive inhibition. After analyzing the effects of structural modifications on inhibition of DHEAS metabolism and reporting a 30% inhibition produced by 0.1 M sulfate ion, Townsley concluded that the data suggested the existence of 3 binding sites in addition to the hydrophobic interactions between the steroid nucleus and the enzyme: (1) the sulfate group; (2) the oxygen function at C3, and (3) the oxygen function at C17. He further concluded that in the case of C21 steroids, the C20 oxygen function was binding to the same enzyme site as that utilized by the C17 oxygen function of the C18 and C19 steroids.

The present investigation has demonstrated that phosphate produced a mixed inhibition of steroid sulfatase activity in both the ovary and placenta since the V_{max} was reduced and the K_m was increased. The size of the phosphate ion is similar to that of the sulfate ion, and both are far smaller than DHEAS or the other steroid sulfate alternative substrates for steroid sulfatase. We feel that our data are most consistent with the phosphate ion binding to the sulfate group binding site and also to a site physically separate from the substrate binding region of the enzyme, producing the finding of mixed inhibition.

Although determinations of the K_m of DHEAS for placental sulfatase have been performed either in phosphate buffer itself [6] or in Tris following dilution of tissue homogenized in phosphate buffer [7], the use of phosphate buffer fails to explain all of the variability in the published determinations of the K_m. There are clearly other methodological differences which may produce changes in the apparent K_m. Two different tissues can only be compared when the same method has been used for both tissues and the difference has been subjected to statistical analysis. We found no statistically significant difference in the K_m between placental and

ovarian steroid sulfatases. The large difference in the V_{max} is explained by the relative enzyme content of the two tissues. We previously measured the relative activities of the steroid sulfatase enzyme in the ovary, placenta, uterus and Fallopian tube and found that the placenta contained far higher steroid sulfatase activity than any of the other tissues [1].

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