SULFURYLATION OF ESTRADIOL-17β BY EXTRACTS OF OVARY, CORPUS LUTEUM, TESTIS AND ADRENAL CORTEX[1]

Anita H. Payne and M. Mason

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan

Received November 2, 1964

ABSTRACT

Soluble enzyme preparations from bovine ovary, corpus luteum and adrenal cortex and from rat testis sulfurylated estradiol-17β in the presence of ATP and Mg^{++}. Two major products were tentatively identified by chromatographic comparisons as estradiol-3-sulfate and estrone sulfate[2]. In addition, two unidentified, highly-polar derivatives were formed from estradiol-17β in relatively small amounts by ovary, corpus luteum and testis. Estradiol-17-sulfate, which was formed readily in similar extracts of rat liver, was not formed in detectable amounts by the extracts of ovary, corpus luteum, testis, and adrenal cortex under the conditions described.

In earlier experiments in this laboratory[3,4], we demonstrated that the sulfate esters of estradiol-17β, estrone and diethylstilbestrol, in very low concentrations, competitively interfere with the activation of kynurenine transaminase by its coenzyme, pyridoxal 5-phosphate. We postulated[4] that this finding represents a general interaction of conjugated steroids in regulating coenzyme-apoenzyme association and suggested that such interactions may also occur *in vivo*. Additional studies[5-8] have verified that similar relationships occur with other conjugates and other enzymes. To assess the physiological

significance of these observations, information is needed regarding the types, distribution, and metabolism of steroid conjugates in various tissues. In an earlier study[9], we have shown that soluble enzyme preparations from liver form estradiol-3-sulfate, estradiol-17-sulfate, and estradiol disulfate. In the present study, we tested the ability of several steroidogenic tissues to sulfurylate estradiol-17β. Advantage was taken of the previous observation[9] that the use of aqueous salt solutions as chromatographic solvents facilitated the separation of the three sulfate esters of estradiol-17β.

EXPERIMENTAL

Materials-- Estradiol-4-^{14}C (S. A. 30 μc/mg) was purchased from New England Nuclear Corp., estradiol-16-^{14}C (S. A. 63.9 μc/mg) from Nuclear Chicago Corp. A single peak of radioactivity which superimposed added authentic estradiol-17β was observed on scanning chromatograms of each of these products after development in either system O-1 or O-2, described below. Potassium estradiol disulfate was prepared as described earlier[4,10]. Sodium estradiol-3-sulfate was kindly supplied by AB Leo, Halsingborg, Sweden[11]. Potassium estrone sulfate was purchased from California Corporation for Biochemical Research, estradiol-17β and estrone from Sigma Chemical Co.

Bovine tissues, obtained at the time of slaughter, were transported to the laboratory packed in ice and processed immediately. The ovaries and corpus luteum were from a pregnant cow slaughtered during the latter half of the gestation period. Testes were obtained from 79 day old Holtzman rats (260 to 280 g.). Medullar tissue was removed from the adrenal glands and discarded. The corpus luteum was removed from the ovary and processed separately. Soluble extracts of the tissues were prepared, lyophilized, and stored at -10°, as described earlier[9].

Incubation procedure-- Estradiol-4-^{14}C (0.055 μmoles) in 0.03 μl absolute ethanol was incubated for 3 hrs at 37° in a medium containing 50 μmoles potassium phosphate buffer

(pH 7.4), 5 µmoles $MgCl_2$, 5 µmoles ATP, 15 µmoles K_2SO_4, and the tissue extract equivalent to 10.5 mg protein, in a final volume of 1 ml.

Isolation procedure-- The procedure for the isolation of steroid conjugates was essentially that of De Meio, et al.[12] The incubation mixtures were deproteinized with 3 volumes of absolute ethanol. The ethanolic extracts were evaporated under vacuum[13] and the residues dissolved in 6 ml of water. The water was extracted, first with equal volumes of peroxide-free ethyl ether to remove free steroids and lipids, and then with equal volumes of water-saturated n-butanol to obtain the conjugates. After evaporation of the solvents, the ether-soluble fraction was dissolved in 2 ml of absolute ethanol and the butanol-soluble fraction was dissolved in 1 ml of triple distilled water[14]. Duplicate 10 µl aliquots were counted for the determination of radioactivity in the ether-soluble and water-soluble fractions. Radioactivity was determined at "infinite thinness" with a Nuclear Chicago Geiger-Mueller gas-flow counter with a thin end-window. Following removal of aliquots for counting, the aqueous fraction was evaporated to dryness and redissolved in 0.1 ml of water.

Chromatography-- Whatman #1 paper which had been washed with absolute methanol for 72 hours was used in all chromatographic procedures. The aqueous solution was chromatographed in duplicate (40 µl aliquots) with the toluene; butanol: concentrated NH_4OH: water (100:100:20:180) system of Schneider and Lewbart[15], referred to below as system A-1. Radiochromatograms were scanned with a Vanguard Autoscanner 880. The radioactive zones from one A-1 chromatogram were eluted by extraction with 50% ethanol. The residue obtained by evaporation was dissolved in 50 µl of water and chromatographed in 0.4 M $NaHCO_3$ solution (referred to below as system A-2). This system was selected in preference to the 0.4 M potassium phosphate solutions used earlier[9] because it gives better separations of the three sulfate esters of estradiol-17β and because it does not interfere with the staining reactions.

The other A-1 chromatogram was subjected to solvolysis for 90 minutes[16]. The estrogens were subsequently eluted, first with 100% ethanol and then with 50% ethanol. As indicated in the results, 0.020 µmole carrier was added to the ethanol used for elution. The combined ethanol extracts were evaporated to dryness. The residue was dissolved in 6 ml of water and extracted 3 times with 6 ml portions of ethyl ether. The ether extract, which contained the free estrogens liberated by solvolysis, was evaporated to dryness, dissolved in 0.05 ml of absolute ethanol or benzene, and chromatographed in either formamide: chloroform (1:1)[17], designated system 0-1, or formamide: monochlorobenzene

(1:1)[17] designated system O-2. The aqueous phase was also evaporated to dryness, dissolved in 0.05 ml of water, and chromatographed in system A-2. Authentic estrogen sulfates were detected on chromatograms by the technique of Crepy, et al.[18]. Free estrogens were detected with the ferric chloride-potassium ferricyanide reagent[19]. Relative amounts of radioactive free estrogens on the chromatographic strips were determined from the Vanguard scans by weighing the paper cut from the areas under peaks superimposing carrier estradiol and estrone.

RESULTS

Recovery of the added estradiol in the form of water-soluble conjugates from the butanol extracts (see experimental section) varied greatly with the 4 tissues studied (Table I). By subjecting the butanol extracts to chromatography in 4 solvent systems, the radioactivity was separated in most cases into several zones; adrenal cortex extracts, however, gave only one radioactive zone in each of the 4 systems. Extracts of each tissue, except adrenal cortex, resulted in 3 zones in system A-1 (Fig. 2a, 3a, and 4a). The major zone, C, migrated in this system with estradiol-3-sulfate, estradiol-7-sulfate, and estrone sulfate. The amount of estradiol converted by each of the 4 tissues to the conjugates represented by area C was: adrenal cortex, 91%; corpus luteum, 8%; ovary, 1.87%; and testis, 0.11 to 0.45%. Tentative identification of area C was carried out as follows for each tissue extract.

Adrenal cortex-- The section of the chromatogram containing area C was eluted and the eluate chromatographed together with carrier estradiol-3-sulfate in system A-2. A single

TABLE I

RECOVERY OF ADDED RADIOACTIVITY IN ETHER AND BUTANOL EXTRACTS FOLLOWING INCUBATION OF ESTRADIOL-17-4-^{14}C WITH SOLUBLE TISSUE EXTRACTS

Tissue	% Ether-soluble			% Butanol-soluble			Total recovered		
	Experiments			Experiments			Experiments		
	I	II	III	I	II	III	I	II	III
Bovine Adrenal Cortex	6.6	6.2	-	90.0	92.5	-	96.6	98.7	-
Bovine Corpus Luteum	85.2	85.2[a]	91.0[b]	8.6	9.3[a]	8.7[b]	93.8	94.5[a]	98.7[b]
Bovine Ovary	95.5	90.8	93.2	2.3	2.4	2.2	97.8	93.2	95.4
Rat Testis	90.6	88.5	98.5	1.5	1.8	1.5	92.1	90.3	100

[a] K_2SO_4 was not added to the incubation medium.

[b] Incubated with estradiol-17β-16-^{14}C (0.042 μmoles, 0.39 μc).

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radioactive zone was obtained (Fig. 1b) which migrated with authentic estradiol-3-sulfate. Estradiol-17-sulfate (Rf = 0.44) and estradiol-3-sulfate (Rf = 0.55) are well separated in this system; the Rf value of estrone sulfate is 0.61. Radioactive estrone sulfate is not readily detected by the chromatogram scanner when present in mixtures con-

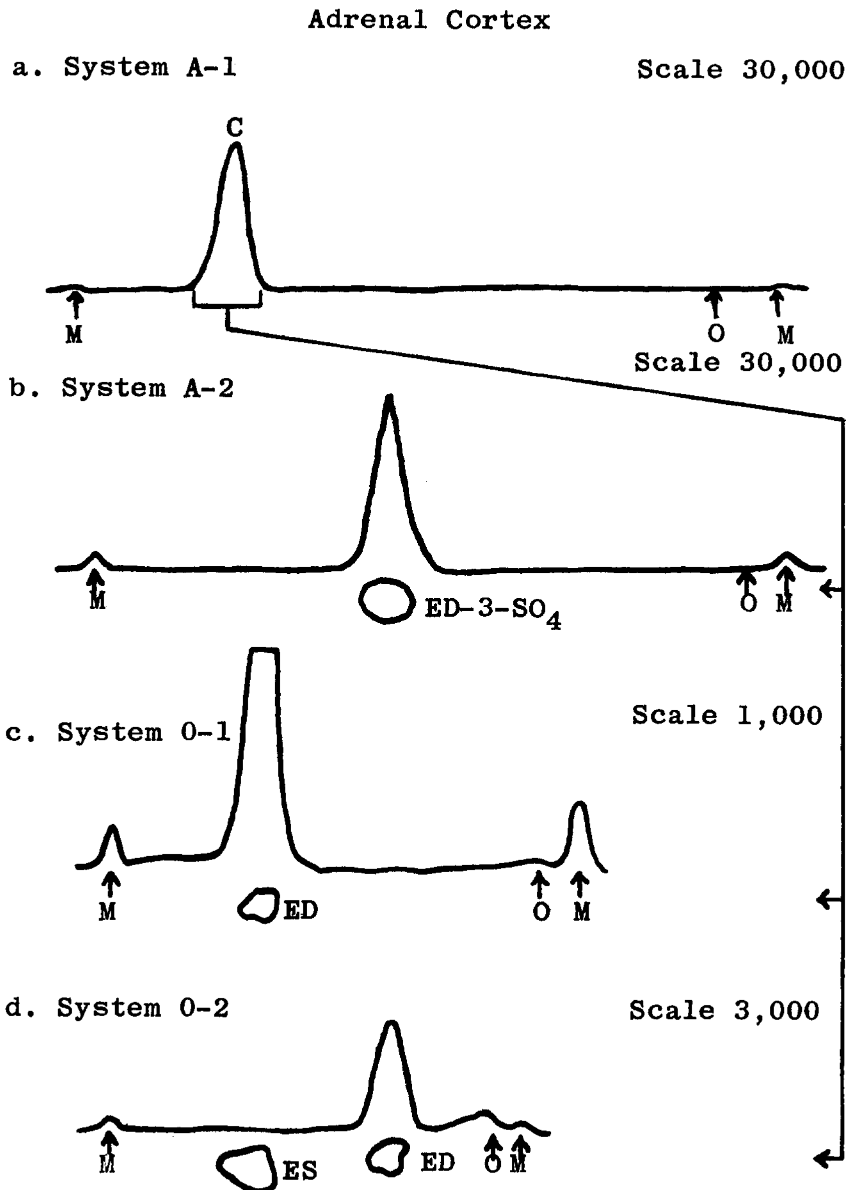

Figure 1. Chromatography of butanol-soluble products formed by incubation of radioactive estradiol-17ß with extracts of adrenal cortex. Peaks designate radioactivity as recorded by the Vanguard scanner; spots below the peaks show the location of carrier estrogens. ED, estradiol-17ß; ED-3-SO_4, estradiol-3-sulfate; ES, estrone; O, origin; M, radioactive marker.

a. Chromatography of butanol extracts in system A-1.
b. Chromatography of eluates of area C in system A-2.
c. and d. Chromatography in systems O-1 and O-2 of products formed by solvolysis of eluates of area C.

taining predominantly estradiol-3-sulfate. The presence of estrone sulfate, however, is easily confirmed by chromatographing the solvolysis products derived from area C in systems 0-1 and 0-2. Ether-soluble radioactive material obtained by solvolysis[20] in the experiments with adrenal cortex migrated in systems 0-1 and 0-2 with carrier estradiol (Fig. 1c, 1d). No evidence for the presence of estrone sulfate was found.

Corpus luteum-- When the products in area C were eluted and chromatographed in the A-2 system (Fig. 2b), a single radioactive zone coincident with carrier estradiol-3-sulfate was obtained. After solvolysis and chromatography of the solvolysis products in systems 0-1 and 0-2 (Fig. 2c and 2d), approximately 87% of the radioactivity was found in one zone superimposing authentic estradiol-17β. A smaller zone with the same mobility as estrone contained approximately 13% of the radioactivity.

Ovary--When the products in area C were eluted and chromatographed (Fig. 3b) together with carrier estradiol-3-sulfate in the A-2 system, one radioactive zone was observed. The zone had two peaks, however, and covered a larger area than the carrier. When both estradiol-3-sulfate and estrone sulfate were added as carriers, the radioactivity superimposed the carriers.

Solvolysis of the material in area C followed by chromatography of the solvolysis products in systems 0-1 and 0-2 (Fig. 3c and 3d) resulted in the appearance of two

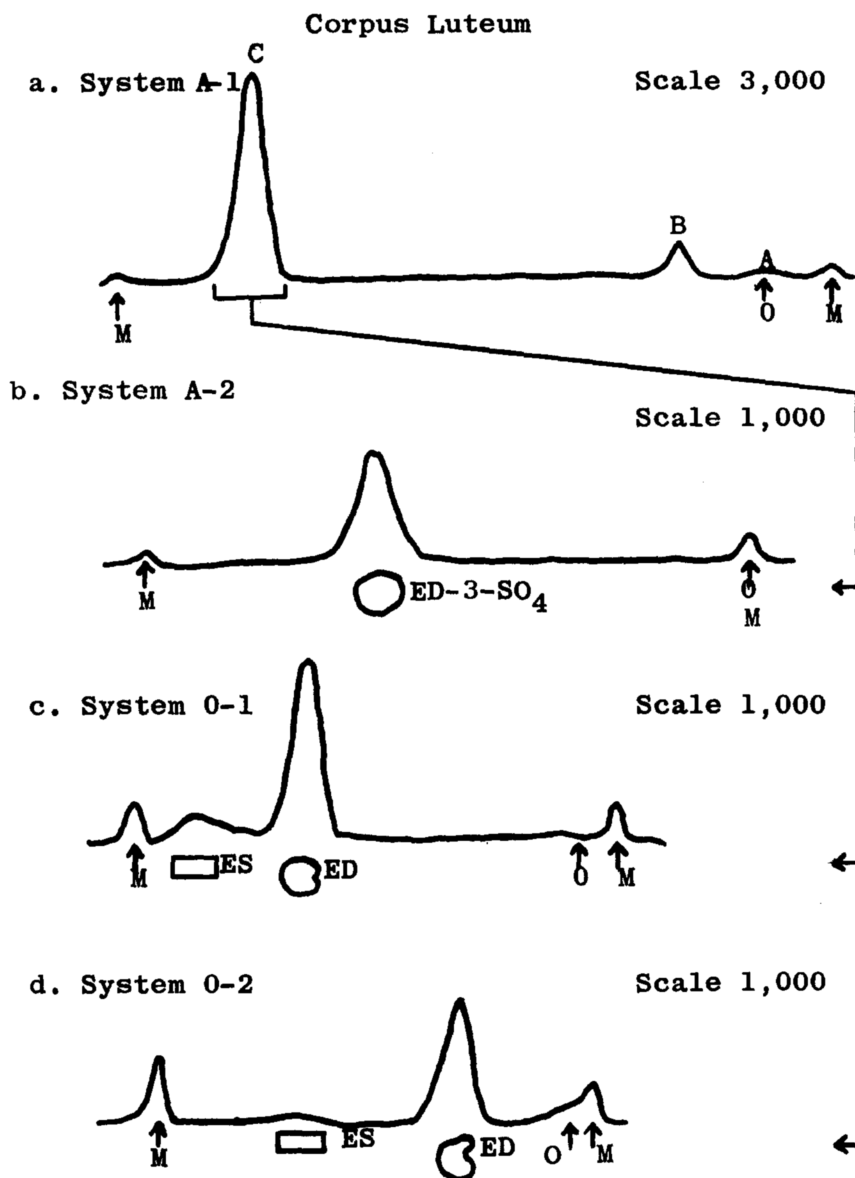

Figure 2. Chromatography of butanol-soluble products formed by incubation of radioactive estradiol-17ß with extracts of corpus luteum. Peaks designate radioactivity as recorded by the Vanguard scanner; spots below the peaks show the location of carrier estrogens; ▭ represents location of estrogens chromatographed separately with the same solvent system. ES, estradiol-17ß; ED-3-SO4, estradiol-3-sulfate; ES, estrone; $ES\text{-}SO_4$, estrone sulfate; O, origin; M, radioactive marker; a, b, c, and d, as defined in Figure 1.

radioactive zones coincident with carrier estrone and estradiol-17β. Approximately 50% of the radioactivity was found in each of the two zones.

<u>Testis</u>--Results obtained with testicular extracts were variable, perhaps because of the low yields of conjugated products. When the products in area C were eluted and chromatographed (Fig. 4b) together with carrier estradiol-3-sulfate in the A-2 system, one broad radioactive zone was obtained. Solvolysis of the material in area C and chromatography of the products in system O-1 and O-2 gave two radioactive zones coincident with carrier estrone and estradiol-17β. Fig. 4c is representative of two experiments in which the relative proportions of estradiol and estrone were 70% and 30%, respectively. Another experiment which showed no conversion of estradiol to estrone is represented in Fig. 4d, in which all of the radioactivity may be seen to be coincident with carrier estradiol-17β. In the same experiment, elution and chromatography in system A-2 of the material in area C yielded a single radioactive zone coincident with carrier estradiol-3-sulfate.

<u>Radioactive zones A and B (Fig. 2a, 3a, and 4a)</u>: The mobility of zone B in system A-1 was very similar to that of estradiol disulfate. The eluted material had an Rf value similar to but slightly greater than that of estradiol disulfate in the A-2 system (0.68 and 0.66, respectively). Both area A and area B were exposed to solvolysis and then separated into ether soluble fractions, which should con-

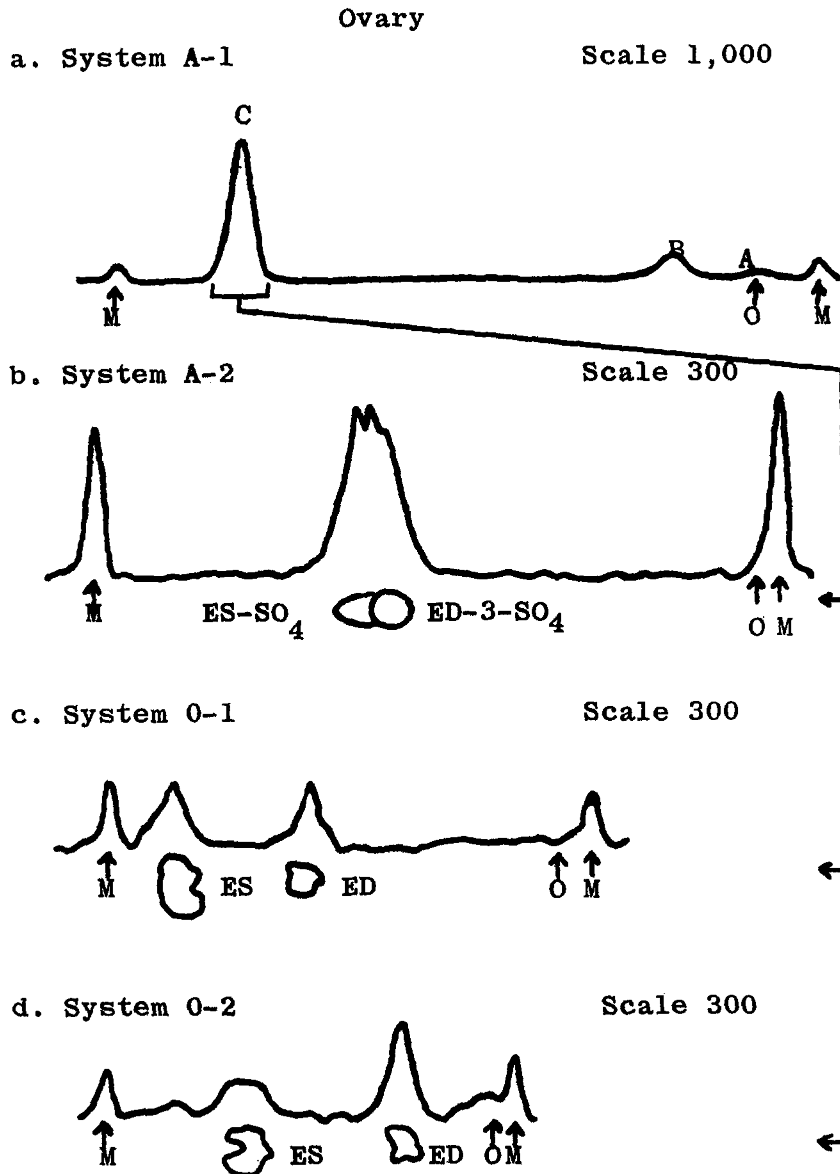

Figure 3. Chromatography of butanol-soluble products formed by incubation of radioactive estradiol-17ß with extracts of ovary. Peaks designate radioactivity as recorded by the Vanguard scanner; spots below the peaks show the location of carrier estrogens. ED, estradiol-17ß; ED-3-SO_4, estradiol-3-sulfate; ES, estrone; ES-SO_4, estrone sulfate; O, origin; M, radioactive marker; a, b, c, and d, as defined in Figure 1.

tain any free steroid liberated by solvolysis, and water-soluble fractions, which should contain any conjugated steroid which was not solvolyzed to the free form. The ether-soluble fractions were chromatographed in system O-1. No radioactive zones were detected on the chromatograms even when the Vanguard scanner was set at maximal sensitivity. The water soluble fractions were chromatographed in system A-2. Radioactive zones were again obtained with the same Rf values as those obtained with the original eluates of zones A and B.

DISCUSSION

The present study indicates that soluble extracts of bovine adrenal cortex, ovary, and corpus luteum, and of rat testis can sulfurylate the 3-hydroxyl group of estradiol-17β but have little if any ability to sulfurylate the 17β-hydroxyl group. These findings differ from results[9] obtained in similar experiments with rat liver extracts in which both the 3- and 17-mono sulfates as well as the disulfate were formed. In a preliminary study, using essentially the same methods as described here, we found that both human and canine adrenal extracts form the 3- and the 17-sulfate esters of estradiol-17β. These variations may reflect species differences in the distribution of specific sulfokinases.

Variation among the four tissues in the amount of estradiol converted to estrone sulfate may be attributable

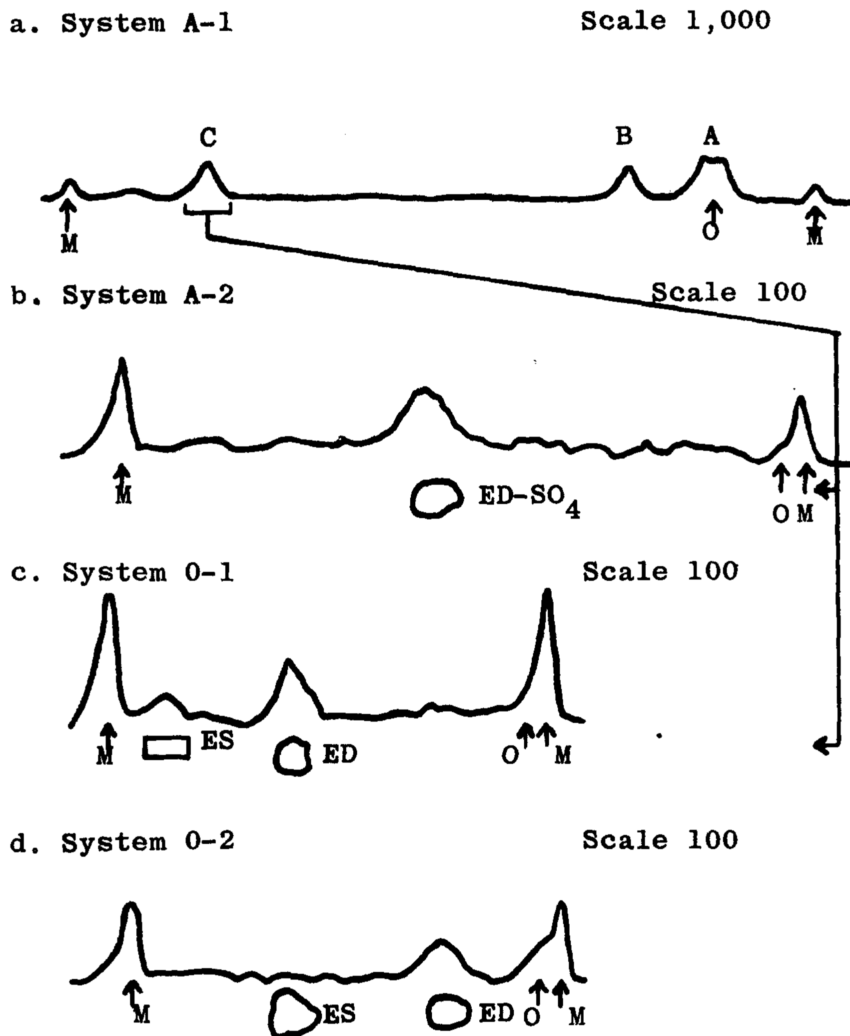

Figure 4. Chromatography of butanol-soluble products formed by incubation of radioactive estradiol-17β with extracts of testis. Peaks designate radioactivity as recorded by the Vanguard scanner; spots below the peaks show the location of carrier estrogens; ▭ represents estrogens chromatographed separately with the same solvent system. ED, estradiol-17β; ED-3-SO_4, estradiol-3-sulfate; ES, estrone; O, origin; M, radioactive marker; a, b, c, and d, as defined in Figure 1. c and d represent two different incubations as outlined in the RESULTS section.

to differences in the levels of 17β-dehydrogenase but is more likely related to the availability of endogenous coenzyme. Whether dehydrogenation occurred before or after sulfurylation remains to be determined.

To date, efforts to identify the two radioactive zones, A and B, which were observed in each experiment except those conducted with adrenal cortex extracts, have not been successful. Although area B migrated with estradiol disulfate in both system A-1 and A-2, the fact that solvolysis did not yield either estradiol-17-sulfate[9] or free estradiol would argue against this product being estradiol disulfate. The low mobility of A and B in system A-1 and their failure to yield free steroid during solvolysis suggest that they may be glucuronide conjugates. Schneider and Lewbart[15] report that steroid glucuronides have relatively low Rf values in alkaline organic systems such as A-1 and that solvolysis in dioxane and HCl, as employed in the present study, does not cleave steroid glucuronides. We found in a preliminary study that the product represented by area B yields free estradiol after incubation with β-glucuronidase (Ketodase, 1000 units, 24 hours). The product represented by area A was apparently unaffected by incubation with β-glucuronidase. Although it migrates in system A-1 as a highly polar compound, no evidence is available at present to indicate that it is either a sulfate or glucuronide conjugate.

The present study demonstrates the ability of extracts

of four different steroidogenic tissues to sulfurylate estradiol-17β. These results, however, provide no measure of the ability of these preparations to sulfurylate other steroid substrates. That other steroids are sulfurylated *in vitro* by adrenal extracts has been reported recently by several authors[21-24]. The sulfurylation of androst-5-ene-3β, 17β-diol by minces and homogenates of normal human ovaries was reported[25] while this manuscript was in preparation. Evidence that corpus luteum of human pregnancy can synthesize estriol sulfates has been presented by Touchstone, Varon and Murawee[26]. They demonstrated that one third of the estriol isolated from a human corpus luteum which has been incubated for 24 hours in the patients plasma was a sulfate ester. These same authors were unable to detect estriol in any form in ovarian tissue of pregnancy.

Recent reports[27-29] on *in vivo* studies show that steroid sulfates may serve as intermediates in steroid metabolism but do not reveal whether all of the various steroidogenic tissues utilize conjugated intermediates. The ability of extracts of adrenal cortex, testis, ovary, and corpus luteum to sulfurylate steroids is at least consistent with that possibility. Other metabolic roles for steroid sulfates are suggested by their effects on enzyme systems *in vitro*[4]. The physiological significance of these latter interactions remains to be demonstrated.

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