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THE ENZYMIC SYNTHESIS OF THE SULFATE ESTERS OF ESTRADIOL-17β AND DIETHYLSTILBESTEROL

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SUMMARY

The present study demonstrates the formation of three sulfate esters of estradiol and three of diethylstilbesterol by a microsome-free extract of rat liver.

The sulfate esters were separated by paper chromatography using a potassium phosphate buffer solvent and detected by radioautography using ^{35}S.

Evidence is presented that the three sulfate esters of estradiol are estradiol-3-sulfate, estradiol-17-sulfate and estradiol-3,17-disulfate and that those of diethylstilbesterol are two isomeric monosulfate esters and the disulfate ester of diethylstilbesterol.

INTRODUCTION

DE MEIO *et al.*[1] have presented evidence that two sulfate esters were formed when estradiol-17β (estra-1,3,5(10)-triene-3,17β-diol) was incubated with microsome-free extracts of rat liver. The two products had markedly different electrophoretic mobilities; they were not identified further. Three sulfate esters of estradiol-17β are theoretically possible. The present study demonstrates that three sulfate esters are formed when estradiol-17β is incubated with microsome-free extracts of rat liver. Evidence is presented that they are the three sulfate esters of estradiol, *viz.*, estradiol-3-sulfate, estradiol-17-sulfate and estradiol-3,17-disulfate. Three sulfate esters were also formed from diethylstilbesterol. They appear to be the disulfate and two monosulfate esters of diethylstilbesterol.

MATERIALS AND METHODS

Incubation and isolation procedures

The incubation procedure was that of DE MEIO *et al.*[1]. Microsome-free extracts of rat livers were prepared, lyophilized and stored at — 10° until used. 0.21 μmole of estradiol-17β (Sigma) or 0.21 μmole of diethylstilbesterol (Sigma) in 0.1 ml of absolute ethanol were incubated for 2 h at 37° in a medium containing 150 μmoles of potassium phosphate buffer (pH 7), 15 μmoles of $MgCl_2$, 15 μmoles ATP, 45 μmoles $K_2{}^{35}SO_4$ (0.5 μC/μmole sulfate) and microsome-free liver extract equivalent to 20 mg of protein in a final volume of 3 ml. Control samples containing everything except the estrogen were incubated simultaneously with the experimental samples.

The estrogen sulfates were isolated as described by DE MEIO *et al.*[1]. They were dissolved in 0.1 ml of triple distilled water and 40 μl applied to Whatman No. 1 paper. Samples were subjected to descending chromatography, using as solvents 0.4 M potassium phosphate buffer (pH 6.3) with the estradiol esters and 0.6 M potassium phosphate buffer (pH 6.3) with the diethylstilbesterol esters.

Detection and identification of the estrogen sulfates

Radioautographs were made of the chromatograms by placing them in contact with Kodak No-Screen film for 5–12 days.

To test for the free phenolic group of the estrogens and their monosulfate esters, the chromatograms were sprayed with a mixture of equal volumes of 6–10 % ferric chloride and 1 % potassium ferricyanide[2]. Because of the presence of phosphate ion on the paper, more than the usual amount of ferric chloride was needed. For the detection of 17-ketosteroids, the modified ketosteroid reagent as described by EDWARDS[4] was used.

R_F values of the enzymically synthesized estrogen sulfates were determined from radioautographs, while those of the synthetic estrogens were determined from spots hydrolysed on paper[3] and stained with the ferric chloride–ferricyanide reagent.

Diethylstilbesterol disulfate and estradiol disulfate were prepared as described earlier[5]. Estradiol-17-sulfate was prepared from the estradiol-3,17-disulfate by solvolysis according to DICZFALUSY *et al.*[6]. Estradiol-3-sulfate was obtained from AB Leo, Halsingborg, Sweden*.

Hydrolysis of the estrogen sulfates was carried out on paper as described by SCHNEIDER AND LEWBART[3]. Partial hydrolysis of the disulfate esters was investigated in the following manner. The estrogen sulfates were enzymically synthesized in the usual manner except that 2.5 μC of $^{35}S/\mu$mole of sulfate was used in the medium. Samples of the extracted sulfate esters were chromatographed with the appropriate phosphate buffer and radioautographs were made. The radioactive compound believed to be the disulfate ester was located on the paper chromatograms by reference to the radioautograms. This compound on a series of chromatograms was then hydrolysed as described above for various time intervals. After drying, the paper strips were eluted with 80 % ethanol. The eluates were neutralized with ammonia solution and evaporated to dryness. The residue was dissolved in 3 ml of triple distilled water, and extracted four times with *n*-butanol as described by DE MEIO *et al.*[1]. The butanol extracts were evaporated to dryness. The residue was dissolved in 0.05 ml of triple distilled water, put on Whatman No. 1 paper, and chromatographed with the appropriate phosphate buffer. Radioautographs were made in the usual manner.

Incubation of enzymically synthesized radioactive sulfate esters was carried out as follows: The position of the compound thought to be the monosulfate ester was traced on the chromatogram from the radioautograph. The traced spots were eluted with water. The eluate was evaporated to dryness. The residue was incubated for 2 h in the medium as described above for the incubation of the free estrogen except that non-labeled potassium sulfate was used. The isolation and separation of the resulting sulfate esters was the same as described earlier.

* We are indebted to H. FEX, AB Leo, Halsingborg, for a gift of the estradiol-3-sulfate.

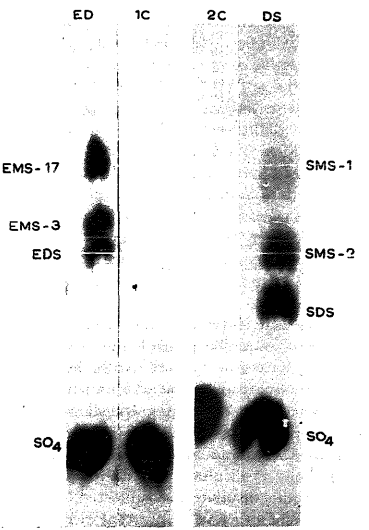
RESULTS

Identification of the sulfate esters formed on incubation of estradiol-17β

Using 0.4 M potassium phosphate buffer (pH 6.3) as a chromatographic solvent for the separation of the estradiol sulfate esters, three distinct radioactive spots (EMS-3, EMS-17 and EDS) were detected (Fig. 1). The R_F values of these spots are very similar to the R_F values of the three synthetic estradiol sulfate esters (Table I).

The following additional experiments were performed to establish that EMS-3 and EMS-17 are the respective monosulfate esters, and that EDS is the disulfate ester of estradiol.

1. The chromatograms were sprayed with the ferric chloride–ferricyanide reagent before and after hydrolysis*. The 17-sulfate ester of estradiol has a free phenolic group



1. Radioautographs of the sulfate esters of estradiol and diethylstilbesterol isolated after incubation of estradiol-17β and diethylstilbesterol with a microsome-free liver extract and $K_2{}^{35}SO_4$ (see MATERIALS AND METHODS). ED, estradiol-17β; C, control; DS, diethylstilbesterol; SO_4, inorganic sulfate. ED and 1C were chromatographed with 0.4 M phosphate buffer (pH 6.3). DS and 2C were chromatographed with 0.6 M phosphate buffer (pH 6.3).

* The ferric chloride–potassium ferricyanide reagent gave a color reaction with a spot on both the experimental chromatographs and the controls, both before and after hydrolysis. The spot had an R_F value of 0.36 and to a small extent overlapped the lower edge of EMS-17 and the upper edge of EMS-3. The color reaction differed somewhat from the reaction obtained with the estradiol esters in that it appeared earlier and was more purple than blue. Preliminary experiments indicate that this spot is due to uric acid.

and should therefore give a blue color reaction before hydrolysis; the 3-sulfate ester and the 3,17-disulfate ester should give a blue color only after hydrolysis. As can be seen in Table I only EMS-17 gave a blue color before hydrolysis, while all three compounds gave a blue color after hydrolysis.

TABLE I

R_F VALUES AND COLOR REACTIONS OF ENZYMICALLY SYNTHESIZED AND SYNTHETIC SULFATE ESTERS OF ESTRADIOL-17β AND DIETHYLSTILBESTEROL CHROMATOGRAPHED WITH PHOSPHATE BUFFER

Compound	R_F values*			Color developed with ferric chloride ferricyanide reagent*		Color developed with ketosteroid reagent*
	Average	Range	No. of samples	Before hydrolysis	After hydrolysis	
EMS-17**	0.28	0.27–0.29	11	Blue	Blue	None
Estradiol-17-sulfate***	0.29	0.28–0.30	3	Blue	Blue	—
EMS-3**	0.40	0.39–0.41	11	None	Blue	None
Estradiol-3-sulfate***	0.40	0.39–0.42	4	None	Blue	—
EDS**	0.46	0.45–0.47	11	None	Blue	None
Estradiol-3,17-disulfate	0.46	0.45–0.48	12	None	Blue	—
Estrone sulfate§	0.47	0.46–0.47	7	None	Blue	Purple
SMS-1**	0.38	0.36–0.41	15	Blue	Blue	—
SMS-2**	0.50	0.46–0.53	17	Blue	Blue	—
SDS**	0.60	0.57–0.62	17	None	Blue	—
Diethylstilbesterol disulfate***	0.60	0.56–0.61	7	None	Blue	—

* Procedure as described in text.
** Enzymically synthesized sulfate esters.
*** Synthetic sulfate esters.
§ Potassium estrone sulfate (Nutritional Biochemicals) chromatographed with 0.4 M potassium phosphate buffer (pH 6.3).

2. EMS-3 and EMS-17 were eluted from the chromatogram and incubated for 2 h in the sulfurylating system containing unlabeled potassium sulfate. All of the radioactivity from each monosulfate now appeared with the R_F value of the EDS spot.

3. EDS was subjected to partial hydrolysis for a period of 60 min and a period of 90 min. In both cases the resulting radioautograph showed a radioactive spot with an R_F value comparable to EMS-17. No radioactive spot comparable to EMS-3 was detected. To ascertain whether the failure to form EMS-3 represents the true nature of the reaction of estradiol-3,17-disulfate under the conditions used in the present experiment, synthetic estradiol disulfate was subjected to hydrolysis for 15, 30, 60, 90 and 120 min. The product had an R_F value like that of EMS-17. It was detected after 15 min of hydrolysis and was still present after 120 min. Some estradiol disulfate was detected up to 90 min. No spot corresponding to EMS-3 was detected. Failure to detect EMS-3 from partially hydrolysed EDS and synthetic estradiol-3,17-disulfate is in agreement with the finding that estradiol-17-sulfate is the only product of solvolysis of estradiol-3,17-disulfate in methanol–5 N HCl (ref. 5) and shows that the 3-sulfate is hydrolysed more readily than the 17-sulfate under these conditions of hydrolysis.

4. DE MEIO *et al.*[1] suggest that part of the estradiol is converted to estrone with microsome-free extract from rat livers. In order to determine whether any of the three radioactive spots detected with estradiol as the substrate was estrone sulfate a test for

17-ketosteroids was carried out on the chromatogram. As is shown in Table I all three spots gave a negative 17-ketosteroid reaction when compared to a synthetic estrone sulfate run in the same chromatographic system. The synthetic estrone sulfate had the same R_F value as EDS in this system. In order to further substantiate that EDS was the disulfate ester of estradiol, EDS, estrone sulfate and estradiol-3,17-disulfate were eluted from a chromatogram. The eluates were chromatographed with the following solvent system[7]; toluene–*n*-butanol–concentrated NH_4OH–H_2O (100:100:20:180). In this system both EDS and synthetic estradiol-3,17-disulfate had an R_F value of 0.08, while estrone sulfate had an R_F value of 0.53.

Identification of the sulfate esters formed on incubation of diethylstilbesterol

Using 0.6 M potassium phosphate buffer (pH 6.3) as a chromatographic solvent for the separation of the diethylstilbesterol sulfate esters, three distinct radioactive spots were detected (Fig. 1). The slowest moving spot SMS-1 was consistently less radioactive than the other two spots. The R_F values of these spots are given in Table I together with the R_F value of synthetic diethylstilbesterol disulfate.

Evidence that both SMS-1 and SMS-2 are monosulfate esters and SDS the disulfate ester of diethylstilbesterol was obtained by the following experiments:

1. The chromatograms were sprayed with the ferric chloride–ferricyanide reagent before and after a 3-h period of hydrolysis*. A monosulfate ester of diethylstilbesterol has a free phenolic group and therefore should give a blue color with the above reagent before hydrolysis while the disulfate should give a blue color only after hydrolysis. As is shown in Table I, SMS-1 and SMS-2 gave a blue color before hydrolysis while all three compounds gave a blue color after hydrolysis.

2. SMS-1 and SMS-2 were each eluted from the chromatogram and incubated for 2 h in the sulfurylating system containing unlabeled potassium sulfate. All of the radioactivity of the extracted sulfate ester now appeared with the same R_F value as the SDS spot.

3. Both synthetic and enzymically synthesized SDS were subjected to partial hydrolysis for various periods of time and the hydrolysis products were chromatographed (see MATERIALS AND METHODS). During the first 15–30 min of hydrolysis both preparations yielded weak spots with R_F values corresponding to SMS-1 and SMS-2. SDS was completely hydrolysed between 45 and 60 min. The recovery of only minute amounts of SMS-1 and SMS-2 from SDS and synthetic diethylstilbesterol disulfate indicates that the hydrolysis of the phenolic sulfate ester of this compound, like that of estradiol, proceeds at a very rapid rate.

Sequence of appearance of the sulfate esters of estradiol and diethylstilbesterol

In a preliminary study the rate of appearance of the sulfate esters of both estradiol and diethylstilbesterol was investigated. The procedure for the conjugation of the estrogens was the same as described earlier except that the volume of all constituents was increased by a factor of five and 3-ml aliquots were removed after 15, 30, 60, 120 and 240 min. The two monosulfate esters of estradiol appeared with approximately equal intensity at 15 min and progressively increased in intensity up to 240 min of incubation. The disulfate ester appeared in trace amounts at 30 min and increased

* The uric acid spot had an R_F value of 0.32 and overlapped part of SMS-1.

in intensity up to 240 min of incubation. The same study with diethylstilbesterol as the substrate demonstrated that the two monosulfate esters appeared prior to the disulfate ester and all three sulfate esters increased progressively in intensity up to 240 min of incubation.

DISCUSSION

The evidence presented in this paper demonstrates the enzymic synthesis of the three possible sulfate esters of estradiol-17β by a microsome-free extract of rat liver. Diethylstilbesterol would be expected to form only two sulfate esters, a monosulfate and a disulfate ester, if one disregards the possibility of cis-trans isomerism. Since three radioactive spots were consistently observed, two of which proved to be monosulfate esters and one a disulfate ester, we must consider the possibility that the cis and trans forms of the monosulfate ester are formed during the incubation or isolation procedures. In support of this possibility, the saturated analog of diethylstilbesterol, hexestrol, yielded only two radioactive spots following incubation with labeled sulfate[8].

In earlier studies from this laboratory, MASON AND GULLEKSON[5] described the inhibitory and protective actions of very low concentrations of the disulfate esters of estradiol-17β and diethylstilbesterol on certain pyridoxal phosphate dependent enzymes. They speculated that some of the metabolic actions of steroids may be regulated by their disulfate esters. At that time there was no evidence that the highly effective disulfate esters are formed in biological systems. The present study demonstrates the presence in rat liver of enzymes for the synthesis of estrogen disulfates. While this study was in progress further evidence has appeared for the biosynthesis of steroid disulfates. WENGLE AND BOSTRÖM[9] have reported the formation of a 3-monosulfate ester and a 3,17-disulfate ester of androst-5-ene-3β,17β-diol when the latter compound was incubated in a sulfurylating system containing a microsome-free extract of rat liver. PASQUALINI AND JAYLE[10] recently reported the isolation of 3β,21-dihydroxy-5-pregnene-20-one disulfate from urine of subjects that had received intramuscular injections of ACTH. Further studies in progress in this laboratory will attempt to demonstrate the synthesis *in vivo* of estrogen disulfates.

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