Studies on Ester Sulphates

14. The *in vitro* Formation of Steroid Disulphates in Rat Liver Extracts *

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1. The *in vitro* synthesis of disulphates of certain dihydroxy steroids in an unfractinated cell-free rat liver enzyme system containing 35S-labelled sulphate has been demonstrated.

2. Studies on the influence of various substitutions at the 17α position on sulphurylation of the 17β hydroxyl group of various steroids have also been undertaken.

3. In general, it is found that the ratio of disulphate to monosulphate formed is greatest at low substrate levels.

4. The following observations are made with respect to the influence of steroid structure on the degree of sulphurylation:

   (a) Of the 3,17- and 3,20-dihydroxy steroids studied, those having a 3β,17β and 3β,20β configuration of the hydroxyl groups are sulphurylated to the greatest extent.

   (b) An ethyl or methyl group in the 17α position inhibits sulphurylation of the 17β hydroxyl group. An ethynyl group in the corresponding position has no inhibitory effect on the sulphurylation of such a hydroxyl group.

   (c) A double bond between the fourth and fifth carbon atoms inhibits sulphurylation of the 3β hydroxyl group.

Several papers on the enzymic synthesis of steroid sulphates *in vitro* have appeared in the literature1-5. One problem of interest in this field is the specificity of the steroid sulphokinases. In a recent paper, Nose and Lipmann reported the separation of a rabbit liver extract into at least two fractions, differing with respect to steroid sulphokinase activity 5. One of these fractions had the ability to sulphurylate oestrone, and the other to sulphurylate dehydroepiandrosterone, androsterone and pregnenolone. Results were also obtained indicating the existence of a still greater variety of individually specific steroid sulphokinases.

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On the other hand, no results with bearing on the enzymic in vitro synthesis of steroid disulphates have so far been presented, except for one observation by de Meio et al. (1958), i.e., that incubation of oestradiol in a sulphurylating system gave rise to two conjugated compounds separable on electrophoretic strips. This was in contrast to the view earlier expressed by Schneider and Lewbart, namely, that enzymic formation of steroid disulphates is unlikely to occur in vitro.

A survey study was previously made of the steroid-sulphurylating capacity of particle-free rat liver supernatant fluid. It was then found that incubation of certain dihydroxy steroids with an unfractionated cell-free rat liver system and $^{35}$S-labelled sulphate often resulted in formation of two labelled, conjugated compounds, detectable on autoradiograms of two-dimensional paper chromatograms and electrophoretic strips. Since monohydroxy steroids, when sulphurylated, give rise to only one type of sulphate conjugate, it was tentatively suggested that this observation was, in fact, an indication of steroid disulphate formation. Evidence in support of this view is given in the present paper. A short report of some of the relevant observations has recently been given in this journal.

**EXPERIMENTAL**

*Enzyme preparation.* The enzyme solution was prepared from the livers of adult Wistar female rats, weighing 180—220 g. Homogenization was performed in 3 volumes of ice-cold 0.15 M KCl, containing 0.001 M EDTA * (pH 7.0), in a Potter-Elvehjem homogenizer. The homogenate was centrifuged in a Spinco Model L ultracentrifuge at an average 105000.g for 60 min. The clear supernatant was diluted to contain 10 mg/ml of protein, as determined by the Lowry method. The supernatant fluid was kept at —20°C until used, and did not lose any appreciable amount of activity when stored at this temperature for one month.

*Assay system.* Enzymic sulphurylation of steroids was performed by incubation of mixtures containing the following constituents in a final volume of 120 μl: (a) 2, 4, 10, or 50 μmoles of substrate **; the steroid was added in ethanolic solution, the ethanol being evaporated in vacuo before addition of other components; (b) 0.1 μC of carrier-free $^{35}$S-labelled sodium sulphate ***; (c) 50 μl of a buffer solution containing equal parts

* The following abbreviations are used: EDTA, ethylenediamine tetraacetic acid; PAPS, 3’-phosphoadenosine-5’-phosphosulphate; ATP, adenosine triphosphate.

** The following compounds were kindly supplied by Schering AG, Berlin: Androstane-3α,17β-diol; androstane-3β,17β-diol; A¹-androstane-3β,17α-diol; A²-androstane-3β,17β-diol; 17α-ethyl-A¹-androstane-3β,17β-diol; 17α-methyl-A¹-androstane-3β,17β-diol; 17α-methyl-androstane-3β,17β-diol; A¹,3,17α(10) oestratriene-3,17α-diol (17α-oestradiol); A¹,3,17β(10) oestratriene-3,17β-diol (17β-oestradiol); pregnane-3α,20α-diol; A¹-pregnene-3β,20α-diol; 17α-hydroxy-A¹-pregnene-3,20-dione (17α-hydroxy-progesterone); 17β-hydroxy-19-nor-A¹-androstene-3-one (19-nortestosterone); 17α-hydroxy-A¹-androstene-3-one (cis-testosterone).

17β-Hydroxy-A¹-androstene-3-one (testosterone) and 17α-methyl-17β-hydroxy-A¹-androstene-3-one (17α-methyl-testosterone) were kindly supplied by Pharmacia, Uppsala, Sweden.

17α-Ethynyl-17β-hydroxy-19-nor-A¹-androstene-3-one (17α-ethynyl-19-nortestosterone) and 17α-ethyl-17β-hydroxy-19-nor-A¹-androstene-3-one (17α-ethyl-19-nortestosterone) were kindly supplied by AB Astra, Södertälje, and Ercro, Stockholm, Sweden, respectively.

The following compounds were obtained from L. Light & Co. Ltd., Coinbrook, England: A¹-androstene-3β,17β-diol; allopregnane-3β,20α-diol; allopregnane-3β,20β-diol; pregnane-3α,20β-diol; A¹-pregnene-3β,20β-diol; A¹-pregnene-3β,20α-diol; 17β-3β,20β-diol; 17β-hydroxy-androstene-3-one; 17β-hydroxy-etiocholan-3-one.

*** Obtained from the Radiochemical Centre, Amersham, England.

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of 0.3 M KH₂PO₄ (pH 6.8), 0.03 M K₂SO₄ and 0.005 M MgCl₂; (d) 50 μl of rat liver supernatant fluid prepared as described above; (e) 20 μl of 0.02 M ATP, disodium salt†.

Unless otherwise stated, incubation was carried out for 120 min at 37.5°C in open test tubes.

5 μl aliquots of the incubation mixtures were subjected to paper chromatography and electrophoresis. Two-dimensional ascending paper chromatography was performed, using phenol-water (4/1 by weight) as first solvent (solvent I), and either butanol-acetic acid-water (12/3/5 by vol.), solvent II, or the upper phase of butanol-2 N ammonia, (1/1 by vol.), solvent III, as second solvent.

High-voltage paper electrophoresis was carried out in 0.075 M acetate buffer (pH 5.5) for 1 h at 1500 V (30 V/cm).

Radioactive areas on paper chromatograms and electrophoretic strips were located by autoradiography, as previously described⁹.

The autoradiograms were studied in a viewing box with a capacity of 120 autoradiograms, thereby facilitating comparison of both strength and localization of the produced spots.

Quantitative determinations of steroid sulphate formation were made by cutting out the autoradiographically localized compounds from paper chromatograms, and eluting the piece of equal size with 5 ml of 50% ethanol by mechanical shaking for 15 min. Aliquots of the eluates were plated in duplicate on frosted aluminium plates (infinite thinness), and counted in a Geiger-Müller counter (mica end-window tube, 1.9 mg/cm²).

The recovery of steroid sulphates as determined by this procedure was tested by use of ³⁵S-labelled monosulphate of Δ⁴-androstene-3β,17β-diol. The radioactive steroid ester was applied in aqueous solution to paper strips. The dried strips were eluted as described above. In four experiments of this type the recovery was 95, 96, 100, and 102%, i.e., an average of 98%.

Elution of inorganic ³⁵S-labelled sulphate was performed according to the same principle, except that 25 ml of deionized water were used as eluent.

Sulphate determinations on the incubation mixtures showed that the sulphate content did not differ significantly from the amount added as K₂SO₄. It could therefore be concluded that each incubation tube contained 500 μmole of sulphate. Consequently, the amount of ester sulphate formed could be calculated from the ratio of ester sulphate to total sulphate activity on the chromatograms.

The standard deviation (s) of a single determination of steroid sulphate formed in the assay system was estimated from differences (d) between duplicate analyses by the following formula

\[ s = \sqrt{\frac{\sum d^2}{2n}} \]

where n = the number of duplicate estimations.

A standard deviation of ±0.10 μmole was calculated from nine duplicate assays of 17-hydroxy steroids forming steroid sulphates in the range of 0.07–1.57 μmole/2 h.

In all determinations, reasonable agreement existed between the obtained values and the strength of the spots on the autoradiograms.

**Preparative separation of steroid sulphates.** For the separation of steroid mono- and disulphates from inorganic sulphate, the Sephadex gel filtration method described by Beling was used¹¹.

**Hydrolitic desulphation.** Hydrolysis of steroid sulphate was performed essentially according to Bitman and Cohen¹⁵. Tracer amounts of ³⁵S-labelled steroid sulphates were dissolved in 100 μl of 0.075 M acetate buffer, pH 5.5, and the test tubes sealed and heated in a boiling water bath for 4 h. Aliquots of these mixtures were subjected to paper chromatography and autoradiography as described above. In cases where quantitative determinations were performed, the elution procedure mentioned above was used.

**Enzymic desulphation.** Enzymic desulphation of steroid sulphates was performed in 100 μl of 0.5 M triethanolamine-acetate buffer, pH 7.0, containing 5 mg of commercial steroid sulphatase prepared from ox liver¹⁶ (Schering AG, Berlin), by shaking in open test tubes at 37.5°C for 120 min. Blanks without the enzymic preparation were run simultaneously. No desulphation could be observed in these tubes. Aliquots of these incubation

† Obtained from Sigma Chemical Company, Saint Louis, Mo., U.S.A.

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mixtures were subjected to paper chromatography and autoradiography as described above.

Preparation of sulphurylated conjugates of $A^4$-androstene-3$\beta$,17$\beta$-diol. Sulphurylated conjugates of $A^4$-androstene-3$\beta$,17$\beta$-diol were prepared chemically according to Roy,$^{14}$ and used as reference substances for the paper-chromatographic and paper-electrophoretic comparison of these compounds with the corresponding conjugates formed biosynthetically.

Chemically detectable amounts of the latter were prepared as follows. The assay system previously described was magnified 20 times, with the exception of $^{35}$S-labelled sulphate, which was kept constant. Thus, 500 mmoles of $A^4$-androstene-3$\beta$,17$\beta$-diol was used as substrate in the incubation mixture, which had a total volume of 2.4 ml. After incubation for 4 h at 37.5°C, proteins were precipitated with 20 ml of methanol. The methanol was evaporated in vacuo after centrifugation, and the residue taken up in a small volume of ethanol. It was then assayed by two-dimensional paper chromatography and subsequent autoradiography as already described.

Location reagent. A 70% solution of phosphoric acid$^{15}$ was used as location reagent for conjugates of $A^4$-androstene-3$\beta$,17$\beta$-diol prepared as stated. The papers were dipped in the solution and spread on a glass disc, after which they were heated at 100—110°C for 15 min. The steroid sulphates exhibited bluish-pink fluorescence in UV light (366 m$\mu$).

RESULTS

Mono- and disulphate formation of $A^4$-androstene-3$\beta$,17$\beta$-diol. Incubation of $A^4$-androstene-3$\beta$,17$\beta$-diol in the sulphurylating system gave rise to two different $^{35}$S-labelled compounds (MS and DS), separable on paper chromatograms (Fig. 1) and electrophoretic strips (Fig. 2). One of these products (MS), believed to be the monosulphurylated conjugate, had the same properties in our chromatographic and electrophoretic systems as sulphurylated conjugates of certain monohydroxy steroids$^6$. The other product (DS), inferred to be

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Fig. 1. Autoradiogram of a two-dimensional paper chromatogram, illustrating the separation of monosulphate (MS) and disulphate (DS) of $A^4$-androstene-3$\beta$,17$\beta$-diol formed by incubation of the free steroid alcohol in the sulphurylating system described in the text. The area within dotted lines contains inorganic sulphate and 3'-phosphoadenosine-5'-phosphosulphate (PAPS). Solvent I: Phenol-water (4/1 by weight); solvent II: Butanol-acetic acid-water (12/3/5 by vol.).
Fig. 2. Autoradiogram of an electrophoretic strip, illustrating the separation of monosulphate (MS) and disulphate (DS) of \( A^4 \)-androstene-3\( \beta \),17\( \beta \)-diol formed by incubation of the free steroid alcohol in the sulphurylating system described in the text. The positions of inorganic sulphate (S) and 3'-phosphoadenosine-5'-phosphosulphate (PAPS) are also indicated. Starting point to the left in the figure. Electrophoresis was performed in 0.075 M acetate buffer, pH 5.5 (30 V/cm) for 1 h.

The disulphurylated conjugate, appeared on the chromatograms in such a position that it was easily distinguished from the monosulphate.

In the alkaline solvent III no significant movement of the disulphate was observed. The disulphate of \( A^4 \)-androstene-3\( \beta \),17\( \beta \)-diol moved approximately twice as fast as the corresponding monosulphate on the electrophoretic strips. The sulphate esters of all dihydroxy steroids studied behaved similarly to the conjugates of \( A^4 \)-androstene-3\( \beta \),17\( \beta \)-diol on both paper chromatography and paper electrophoresis.

The influence of \( A^4 \)-androstene-3\( \beta \),17\( \beta \)-diol concentration on the relative formation of the two conjugates is illustrated in Fig. 3. Optimal disulphate formation was found at low substrate levels, where accumulation of the monosulphate was weak or lacking. Higher substrate levels facilitated formation of the monosulphate, and slightly reduced that of the disulphate.

The formation of both conjugates as a function of time was also studied (Fig. 4).

Fig. 3. Effect of varying \( A^4 \)-androstene-3\( \beta \),17\( \beta \)-diol additions on the formation of monosulphate (MS) and disulphate (DS) of this steroid alcohol. Assays were performed as described in the text. The amounts of steroid sulphates formed are expressed in terms of the sulphate content of the esters.

Fig. 4. Rate of formation of monosulphate (MS) and disulphate (DS) of \( A^4 \)-androstene-3\( \beta \),17\( \beta \)-diol during incubation of 10 \( \mu \)moles of the free steroid alcohol in the assay system described in the text. Steroid sulphates are expressed in terms of radioactivity in 5 \( \mu \)l aliquots of the incubation mixtures.

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When tracer amounts of the partially purified $^{35}$S-labelled monosulphate of $^{A^-}$-androstene-3$\beta$,17$\beta$-diol were incubated for 120 min in the sulphurylating system in the absence of labelled inorganic sulphate, the results of two experiments showed that 86 and 93%, respectively, of added monosulphate was transformed into disulphate.

Hydrolysis of the labelled monosulphate of $^{A^-}$-androstene-3$\beta$,17$\beta$-diol in the acetate buffer under the aforementioned conditions split the conjugate. After 4 h, 92% of all radioactivity was found to be due to inorganic sulphate. A large amount of $^{35}$S-labelled inorganic sulphate was also found when enzymic desulphation with steroid sulphatase was performed as described.

The labelled disulphate was also hydrolyzed in the acetate buffer (Fig. 5). Splitting of the conjugate was observed, and after 4 h hydrolysis, 41% of the original disulphate activity was revealed as a monosulphate, and 48% as inorganic sulphate. About 6% of the disulphate remained unchanged.

During hydrolysis, formation of a third product could also be demonstrated. It amounted to 4—6% of the total radioactivity added, and in our chromatograms had a position close to that of the monosulphate.

The steroid sulphatase preparation brought about some degree of desulphation of the $^{A^-}$-androstene-3$\beta$,17$\beta$-diol disulphate, presumably to the free steroid, since no detectable monosulphate appeared.

A comparison was made between the paper-chromatographic and paper-electrophoretic features of MS and DS and those of the pyridine salts of the two types of sulphate esters of $^{A^-}$-androstene-3$\beta$,17$\beta$-diol formed by organic synthesis. MS was found to behave like one type, and DS like the other.

Larger amounts of $^{35}$S-labelled mono- and disulphates of $^{A^-}$-androstene-3$\beta$,17$\beta$-diol were also prepared enzymically and isolated (cf. EXPERIMENTAL). After paper chromatography of these conjugates, the labelled spots were localized by autoradiography. Subsequent treatment of the papers with phosphoric acid showed distinct bluish-pink fluorescence in the centre of both the mono- and disulphate spots. This fluorescence was not observed in chromatograms from control tubes to which the substrate had been added after incubation.

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Fig. 6. Effect of varying substrate additions on the formation of monosulphate (MS) and disulphate (DS) of the following substrates: A. Androstane-3α,17β-diol; B. Androstane-3β,17β-diol; C. Δ^4-Androstene-3β,17β-diol; D. Δ^5-Androstene-3β,17α-diol. The amounts of steroid sulphates formed are expressed in terms of the sulphate content of the esters.

Sulphurylation of non-substituted androstane- and androstene-diols. The ability of the rat liver supernatant fluid to sulphurylate androstane-3α,17β-diol, androstane-3β,17β-diol, Δ^4-androstene-3β,17β-diol and Δ^5-androstene-3β,17α-diol was also tested. The results obtained are summarized in Fig. 6. Of the two androstane 3,17-diol epimers tested, that having the 3β,17β configuration of the hydroxyl groups was more extensively esterified than the epimer having 3α,17β hydroxyl groups. Androstane-3β,17β-diol was sulphurylated to a considerable degree, as was the case with the corresponding Δ^5 unsaturated steroid.

The sulphurylation of Δ^5-androstene-3β,17α-diol was found to diverge from that of the corresponding 17β epimer. This was particularly marked with respect to disulphurylation, which decreased greatly when the substrate level was raised. On the other hand, the monosulphate formation of the unsaturated 17α epimer exceeded that of Δ^5-androstene-3β,17β-diol. The degree of sulphurylation of Δ^4-androstene-3β,17β-diol differed greatly from that obtained with the corresponding Δ^5 isomer. Thus, no disulphate formation was ever observed, and the monosulphate formation was always extremely low.

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No splitting of androstane-3,17-diol conjugates could be demonstrated after treatment in the acetate buffer. The monosulphate of 4α-androstene-3β,17α-diol was, on the contrary, completely desulphated in this hydrolytic procedure. Although the disulphate was also split, insignificant amounts of monosulphate were formed during the process.

**Sulphurylation of substituted androstane- and androstene-diols.** Of the substituted androstane- and androstene-diols, 17α-methyl-androstane-3β,17β-diol, 17α-ethinyl-Δ4-androstene-3β,17β-diol and 17α-methyl-Δ4-androstene-3β,17β-diol were tested in the assay system. The results obtained are summarized in Fig. 7.

In contrast to androstane-3β,17β-diol, 17α-methyl-androstane-3β,17β-diol was not disulphurated to any significant extent although considerable monosulphate formation occurred.

17α-Ethinyl-Δ4-androstene-3β,17β-diol was both mono- and disulphurated to a degree comparable to that of Δ4-androstene-3β,17β-diol.

17α-Methyl-Δ4-androstene-3β,17β-diol exhibited a high degree of mono-sulphurylation, whereas formation of the disulphate did not occur to any significant extent.

When testing these conjugates by the hydrolytic procedure, it was found that the monosulphate of 17α-methyl-androstane-3β,17β-diol was not split. Both the mono- and disulphate of 17α-ethinyl-Δ4-androstene-3β,17β-diol were desulphated. The disulphate of the latter did not, however, yield any detectable amounts of monosulphate during this process. The monosulphate of 17α-methyl-Δ4-androstene-3β,17β-diol seemed to be completely split by hydrolysis.

**Sulphurylation of 17-monohydroxy steroids.** The ability of the rat liver supernatant fluid to sulphurylate certain 17-monohydroxy steroids is summarized in Table 1.

As can be seen from these data, 19-nortestosterone was sulphurylated to approximately the same extent as testosterone. Introduction of an ethyl group into the 17α position of 19-nortestosterone or of a methyl group into the 17α position of testosterone greatly decreased sulphurylation of the 17β hydroxyl

**Table 1.** Steroid sulphate formation of some 17-hydroxy steroids. 10 μmole of the steroid substrates were incubated for 120 min in the assay system described in the text. The amounts of steroid sulphates formed are expressed in terms of the sulphate content of the esters.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Steroid sulphate formed (μmole of sulphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>0.80</td>
</tr>
<tr>
<td>17α-Methyl-testosterone</td>
<td>0.07</td>
</tr>
<tr>
<td>19-Nortestosterone</td>
<td>0.83</td>
</tr>
<tr>
<td>17α-Ethinyl-19-nortestosterone</td>
<td>0.76</td>
</tr>
<tr>
<td>17α-Ethyl-19-nortestosterone</td>
<td>0.13</td>
</tr>
<tr>
<td>Δ5-testosterone</td>
<td>1.58</td>
</tr>
<tr>
<td>17α-Hydroxy-progesterone</td>
<td>0.30</td>
</tr>
<tr>
<td>17β-Hydroxy-androstane-3-one</td>
<td>0.95</td>
</tr>
<tr>
<td>17β-Hydroxy-etiocholane-3-one</td>
<td>0.53</td>
</tr>
</tbody>
</table>

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Fig. 7. Effect of varying substrate additions on the formation of monosulphate (MS) and disulphate (DS) of the following substrates: A. 17α-Methyl-androstan-3β,17β-diol; B. 17α-Ethynyl-Δ4-androstene-3β,17β-diol; C. 17α-Methyl-Δ4-androstene-3β,17β-diol.

The amounts of steroid sulphates formed are expressed in terms of the sulphate content of the esters.

group. 17α-Ethynyl-19-nortestosterone was, on the contrary, sulphurylated to the same extent as testosterone and 19-nortestosterone. 17α-Hydroxyprogesterone, having an acetyl group in the 17β position, showed a low degree

Fig. 8. Effect of varying substrate additions on the formation of monosulphate (MS) and disulphate (DS) of the following substrates: A. Pregnane-3α,20α-diol; B. Pregnane-3α,20β-diol; C. Allopregnane-3β,20α-diol; D. Allopregnane-3β,20β-diol.

The amounts of steroid sulphates formed are expressed in terms of the sulphate content of the esters.

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Fig. 9. Effect of varying substrate additions on the formation of monosulphate (MS) and disulphate (DS) of the following substrates: A. $A^1$-Pregnen-3β,20β-diol; B. $A^4$-Pregnen-3β,20α-diol; C. $A^5$-Pregnen-3β,20β-diol.

The amounts of steroid sulphates formed are expressed in terms of the sulphate content of the esters.

of sulphurylation when compared to cis-testosterone. Table 1 also includes two androstane-17β-dols. One of them, which had the hydrogen atom at the 5-position in the α position, was found to be more extensively esterified than that having the corresponding hydrogen atom in the β position.

**Sulphurylation of allopregnone-, pregnane- and pregnene-diols.** The capacity of the rat liver supernatant fluid to sulphurylate certain allopregnone-, pregnane- and pregnene-3,20-diols was tested by use of the following substrates: pregnane-3α,20α-diol, pregnane-3α,20β-diol, allopregnone-3β,20α-diol, allopregnone-3β,20β-diol, $A^4$-pregnene-3β,20β-diol, $A^5$-pregnene-3β,20α-diol and $A^5$-pregnene-3β,20β-diol (Figs. 8 and 9).

Pregnan-3α,20α-diol and pregnane-3α,20β-diol were sulphurylated to a low extent. This was found to apply to both mono- and disulphurylation. No great difference was observed between the sulphate esterification of these two epimers.

Allopregnone-3β,20α-diol was sulphurylated to a much greater extent, although mainly monosulphate was formed. The epimer — allopregnone-3β,20β-diol — was, on the other hand, disulphurylated to a high degree.

Sulphurylation of $A^4$-pregnene-3β,20β-diol in the assay system never yielded detectable amounts of disulphate, and only small amounts of a monosulphate.

$A^5$-Pregnene-3β,20α-diol exhibited the same kind of sulphurylation as allopregnene-3β,20α-diol, i.e., formation chiefly of monosulphate and only small amounts of disulphate.

In contrast to $A^4$-pregnene-3β,20β-diol, $A^5$-pregnene-3β,20β-diol was mainly disulphurylated.

The conjugates of the $A^5$ unsaturated pregnene-diols were also tested with the hydrolytic procedure. The monosulphates of $A^4$-pregnene-3β,20α-diol and $A^5$-pregnene-3β,20β-diol were both completely desulphated after treatment with acetate buffer. The disulphates of the corresponding steroids were also split by this hydrolytic process, forming monosulphate and inorganic sulphate.

**Sulphurylation of oestradiols.** Both 17α- and 17β-oestradiol were sulphurylated in the assay system, giving rise to detectable amounts of the monosul-
phates, although 17α-oestradiol was sulphurylated to a greater extent than the 17β epimer. Disulphate formation of both compounds was always extremely weak (Fig. 10).

**DISCUSSION**

Many of the results described in the previous section show that sulphurylation of certain dihydroxy steroids occurred in the assay system used. The general observation that, on electrophoretic strips, disulphurylated compounds moved approximately twice as fast towards the anode as the corresponding monosulphates indicated that the disulphate had a greater negative charge than the monosulphate. These findings are in good agreement with the data on the electrophoretic behaviour of mono- and disulphurylated steroids, formed by organic synthesis, that have been reported by other workers 16,17. In our chromatographic systems, the disulphates seemed to exhibit greater polarity than the corresponding monosulphates, a fact that further emphasized the presumed structure of the conjugates 16,17.

For a more detailed study of the two kinds of conjugate, the formation and degradation of the sulphates of Δ⁵-androstene-3β,17β-diol were chosen as objects.

The formation of both the mono- and disulphate of this steroid as a function of time appeared repeatedly, as illustrated in Fig. 4. The decreasing appearance of isolated monosulphate, as well as the increase in rate of formation of the corresponding disulphate during incubation, suggest that the monosulphate of this steroid might have been a precursor of the disulphate.

Further evidence of this supposition was the fact that the isolated radioactive monosulphate of Δ⁵-androstene-3β,17β-diol was transformed into radioactive disulphate during incubation in the assay system in the absence of labelled inorganic sulphate.

Hydrolysis of the conjugates of Δ⁵-androstene-3β,17β-diol in acetate buffer also seemed to give some information about the structure of the esters. Accord-

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ing to Bitman and Cohen,\textsuperscript{12} steroid sulphates having a β configuration of the sulphate and a β-γ unsaturation to this grouping are split when heated at 100°C in an acetate buffer (pH 4.7) for 4 h. Since splitting of this kind of conjugate has been observed even at pH 7.0,\textsuperscript{14} it seems probable that the isolated monosulphate of \textit{A}\textsuperscript{4}-androstene-3β,17β-diol split by our hydrolytic process at pH 5.5 consisted mainly of the 3β sulphate of the steroid. The nature of the disulphate of \textit{A}\textsuperscript{4}-androstene-3β,17β-diol was further established by this conjugate being split into approximately equivalent amounts of inorganic sulphate and a substance which appeared on the chromatograms to be a monosulphate. It therefore seems reasonable to suggest that the monosulphate formed during hydrolysis was the 17β sulphate of \textit{A}\textsuperscript{4}-androstene-3β,17β-diol.

Although a steroid sulphatase preparation was found to desulphate conjugates of \textit{A}\textsuperscript{4}-androstene-3β,17β-diol, no further conclusions could be drawn about the structure of the conjugates.

The proposed structure of the mono- and disulphate of \textit{A}\textsuperscript{4}-androstene-3β,17β-diol was also substantiated as follows. With respect to localization and chemical reactivity on paper chromatograms and paper electrophoretic strips, the monosulphate of \textit{A}\textsuperscript{4}-androstene-3β,17β-diol behaved as one of the two types of sulphate esters of \textit{A}\textsuperscript{4}-androstene-3β,17β-diol formed by organic synthesis, while the disulphate behaved as the other.

When discussing the amounts of ester sulphate formed in relation to the amounts of substrate added, it was necessary to consider the solubility of the substrate in water. The reason why we did not want to increase the solubility of the steroids by addition of alcoholic solvents, as done by other authors,\textsuperscript{1–5} was that alcohols were also sulphurylated in our assay system.\textsuperscript{18,19} Moreover, the conditions we used seemed more appropriate from the physiological point of view. Low solubility of added steroids evidently affected the appearance of several substrate concentration curves. It was considered, however, that as long as changes in the formation of ester sulphates were seen when increasing the amount of substrate added, saturation of the medium had not been reached. Although such observations showed considerable variations in the solubility of dihydroxy steroids, comparisons could usually be made at substrate additions of 2 or 4 μmoles.

\textit{Cis}-testosterone, having a hydroxyl group in the 17α position, has previously been shown to be sulphurylated to a higher degree than testosterone.\textsuperscript{2} The low degree of disulphurylation obtained when incubating \textit{A}\textsuperscript{4}-androstene-3β,17α-diol was therefore somewhat surprising, if compared to results obtained with the corresponding 3β,17β epimer. These findings might, however, be accounted for in several ways. One is that the same sulphokinase was needed for sulphurylation of both the 3β and the 17α positions. Another possible explanation is that a hydroxyl or a sulphate-esterified hydroxyl group in the 3β or 17α position of \textit{A}\textsuperscript{4}-androstene-3β,17α-diol inhibited sulphurylation of the other hydroxyl group. Competition between the free steroid alcohol and the monosulphate for PAPS might, however, also account for the results.

In comparison to \textit{A}\textsuperscript{4}-androstene-3β,17β-diol, \textit{A}\textsuperscript{4}-androstene-3β,17β-diol behaved in a surprising manner. The low degree of sulphurylation of the latter compound could have been a result of low solubility of the steroid, but since only monosulphate was formed, it seems reasonable to suggest that sulphuryla-

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tion of one of the hydroxyl groups was inhibited. A conceivable explanation of this finding is that the double bond between the fourth and fifth carbon atoms influenced sulphurylation of the 3β hydroxyl group.

The differences between the degree of sulphurylation of androstane-3α,17β-diol and androstane-3β,17β-diol were to be expected, since 3α hydroxyl groups are not sulphurylated as well as the 3β epimer.

The extremely low degree of disulphurylation found when incubating 17α-methyl-androstane-3β,17β-diol and 17α-methyl-Δ⁵-androstene-3β,17β-diol may imply that the methyl group in the 17α position inhibited sulphurylation of the 17β hydroxyl group. Hydrolysis of the monosulphate of 17α-methyl-Δ⁵-androstene-3β,17β-diol in acetate buffer also indicated that the sulphate group was bound in the 3β position. An ethynyl group in the 17α position evidently did not inhibit sulphate esterification of the 17β hydroxyl group. The structure of the isolated monosulphate of this steroid could not, however, be elucidated by the methods used.

The previous observations on the effect of substitutions upon the sulphurylation of 17β hydroxyl groups were strongly supported by data obtained from the studies on 17-monohydropx steroids, although the latter experiments gave no evidence that the substrates were present in the medium in optimal concentrations. Thus, it is probable that a methyl or ethyl group in the 17α position inhibits 17β sulphurylation, whereas an ethynyl group in the corresponding position does not have this effect.

Other changes in the testosterone molecule, such as removal of a methyl group in the 10 position or reduction of the double bond, evidently did not change the degree of sulphurylation to any great extent. In comparing the degree of sulphurylation of the 17α hydroxyl group of cis-testosterone and 17α-hydroxy-progesterone, differences in water solubility of the steroids could explain the results obtained, although it does not appear unlikely that the acetyl group in the 17β position may inhibit sulphurylation in the 17α position.

It may also be pointed out that the two alkyl-substituted steroids, 17α-methyl-testosterone and 17α-ethyl-19-nortestosterone, which are sulphurylated at the slowest rate of all 17-monohydropx steroids tested, are both known to produce liver dysfunction.

The supposition that the sulphurylating activity of rat liver supernatant fluid is also exerted on hydroxyl groups in the 20 position was confirmed by the finding that disulphates of allopregnane-3β,20α-diol and 3β,20α-diol detected in the assay system.

The low degree of sulphurylation of pregnane-3α,20α-diol and the 20β epimer might well be explained by low water solubility of both steroids.

Disulphurylation of allopregnane and Δ⁵-pregnene-3β,20α-diols was evidently greatly favoured when the hydroxyl group in the 20 position was in the β position and when the 3 hydroxyl group had a β configuration.

Evidence was obtained that the monosulphate of the two epimeric Δ⁵-pregnene-3β,20α-diols isolated from the incubation mixtures consisted mainly of the 3β sulphate. This makes it clear that the only sulphurylation in the 20 position observed in these cases did, in fact, occur in connexion with disulphurylation. The low degree of 20α sulphurylation could have been a result of competition between the 3β and 20α hydroxyl groups for PAPS or for the same

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sulphokinase, as discussed in connexion with $A^8$-androstene-3$\beta$,17$\alpha$-dihol sulphurylation. Nothing is, however, so far known about the ability of rat liver supernatant fluid to sulphurylate 20-monohydroxy steroids. It cannot, therefore, be stated whether the low 20$\alpha$ sulphurylation found was a result of inhibition, or of low activity of the sulphokinase responsible.

$A^4$-Pregnene-3$\beta$,20$\beta$-dihol deviated markedly from the corresponding $A^8$ isomer as regards its sulphurylation. Since no disulphate of this $A^4$ unsaturated steroid was obtained in the assay system, it might be concluded that sulphurylation of one of the hydroxyl groups was inhibited. Since $A^4$-androstene-3$\beta$,17$\beta$-dihol behaved similarly in this respect, it is conceivable that the double bond between the fourth and fifth carbon atoms inhibited sulphurylation of the 3$\beta$ hydroxyl group.

The two oestradiols studied mainly formed monosulphate during incubation. Since 17$\alpha$-oestradiol was sulphurylated to a greater degree than the natural epimer, it might be suggested that the 17$\alpha$ hydroxyl group was sulphurylated to a greater extent than the epimeric hydroxyl group. This should be compatible with the observed high sulphurylation rate of cis-testosterone in comparison to that of the natural epimer, testosterone.

The enzyme system used seemed to have a sulphurylating action on a number of dihydroxy steroids. As postulated by other authors, the sulphurylation of steroids having different kinds of hydroxyl groups might be brought about by different enzymes. This question is, of course, also of interest in the present study, in which the sulphurylation of two hydroxyl groups was studied simultaneously. The results do not, however, permit any definite conclusions about the specificity of the sulphokinases of the rat liver supernatant fluid.

The disulphates of the dihydroxy steroids studied were probably formed as a result of two successive sulphurylations. Although some evidence of this probability has so far been obtained only with respect to $A^8$-androstene-3$\beta$,17$\beta$-dihol, it seems likely that all dihydroxy steroids are disulphurylated in this manner. Since 3$\beta$ sulphate alone but no 17$\beta$ sulphate was present in the monosulphate fraction isolated in assays of $A^8$-androstene-3$\beta$,17$\beta$-dihol, it appears most probable that the 3$\beta$ sulphate of this steroid was the first conjugate formed. The results obtained might also imply that the 17$\beta$ sulphokinase had a higher affinity for the 3$\beta$ sulphate of the latter steroid than for the free steroid alcohol.

On the basis of similar evidence, it might also be suggested that the 3$\beta$ sulphates are the first conjugates formed during sulphurylation of the two epimeric $A^8$-pregnene-3$\beta$,20-diols. At least in the case of $A^8$-pregnene-3$\beta$,20$\beta$-dihol, the 20$\beta$ sulphokinase seemed to have a greater affinity for the conjugate in question than for the free steroid alcohol.

In all dihydroxy steroids tested, the ratio of disulphate to monosulphate isolated from incubation mixtures was always greatest at low substrate levels. This might indicate that many dihydroxy steroids metabolized in vivo can be disulphurylated and possibly excreted as such conjugates, particularly under conditions where the formation and excretion of dihydroxy steroids is increased. It has, in fact, been shown recently that the disulphate of $A^8$-pregnene-3$\beta$,21-diol-20-one may be present in human urine.

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