Biological Synthesis of Sulphuric Acid Esters of Steroid Hormones or their Metabolites

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The synthesis *in vitro* of the sulphuric acid esters of ordinary phenols by rat-liver preparations has been demonstrated by De Meio & Tkacz (1952), De Meio, Wizerkaniuk & Fabiani (1953), De Meio, Wizerkaniuk & Schreiber (1954) and Bernstein & McGilvery (1952*a, b*). De Meio & Lewycka (1955) were able to observe the formation *in vitro* of dehydroepiandrosterone sulphate by the same type of preparations and Roy (1956) has presented other experimental evidence for the formation of dehydroepiandrosterone sulphate and other steroid sulphates. The requirements for such a synthesis appear to be the same as those found for the formation of phenyl sulphates (De Meio, Wizerkaniuk & Fabiani, 1953; Bernstein & McGilvery, 1952*a*). Direct evidence is presented in this paper for the biosynthesis of the sulphates of dehydroepiandrosterone, testosterone, oestrone and oestradiol-17 β . It is also shown that ox-liver preparations act on dehydroepiandrosterone and oestradiol-17 β , producing other metabolic changes.

EXPERIMENTAL

The techniques used for this work are the same as those described in a previous note (De Meio & Lewycka, 1955), with some additions.

Tissue preparations. The preparations from ox and rabbit livers were obtained in a similar way to those from rat liver. The microsome-free solution was obtained by high-speed centrifuging from a 20% (w/v) liver homogenate (De Meio *et al.* 1953) and the fraction precipitated between 1.2M- and 2.3M-ammonium sulphate concentrations was prepared from this solution. Microsome-free solutions or ammonium sulphate fractions from rat, ox or rabbit liver and containing 15–20 mg. of protein were added to each vessel before incubation.

Substrates. Dehydroepiandrosterone (DHA), testosterone, oestrone and oestradiol-17 β were used in amounts corresponding to 0.21 μ mole and added in 0.2 ml. of 95% ethanol to a final volume of 3 ml./vessel.

Medium. The other components of the medium used for the 2 hr. incubation at 37.5° were the following: 150 μ moles of potassium phosphate buffer (pH 7), 15 μ moles of MgCl₂, 45 μ moles of K₂SO₄, 15 μ moles of adenosine triphosphate (disodium salt, tetrahydrate).

Determination of steroids. After deproteinization of the contents of the vessel by addition of 3 vol. of redistilled

95% ethanol, the free DHA, oestrone and oestradiol-17 β were extracted with peroxide-free ethyl ether, and the bound forms of the same compounds were extracted with butanol, as described previously (De Meio & Lewycka, 1955). After evaporation of the solvent the residue was dissolved in redistilled 95% ethanol and DHA and oestrone were determined colorimetrically by the Zimmermann reaction according to the technique of Pincus & Pearlman (1941), with the Evelyn photoelectric colorimeter and filter no. 515. Oestradiol-17 β was determined on the dry ether or butanol residue by the Kober method as described by Stevenson & Marrian (1947). The average blank values for the ether and butanol extracts corresponded to 6 and 11.5 μ g. of DHA respectively for samples derived from incubation with microsome-free solution and to 4.2 and 8.7 μ g. of DHA for those obtained with the ammonium sulphate fraction. The corresponding values for the Kober reaction were 11.8 and 15.5 μ g. for the microsome-free solution and 4.5 and 4.7 μ g. (expressed as oestradiol) for the ammonium sulphate fraction. Blank values were obtained for each separate determination and subtracted from the values found for the samples. The recovery of steroids and of the available steroid sulphates (DHA and oestrone sulphate) added to the samples was about 75–80%.

Preparation of extracts for paper electrophoresis. Direct proof for the formation of the sulphate was sought by incubating DHA, oestrone, oestradiol-17 β and testosterone with rat- and rabbit-liver preparations, in an amount corresponding to 0.21 μ mole under the conditions indicated above, but with added radioactive sulphate (0.3 μ C/ μ mole of sulphate). After incubation, ethanol was added to each vessel to a concentration of 75% (v/v), the precipitated proteins were separated by centrifuging and the ethanol supernatant was combined with three 2 ml. ethanol washings of the precipitated proteins. The ethanolic solution was neutralized with ammonia solution to prevent hydrolysis of the sulphates, and evaporated to dryness at 30° under reduced pressure. The residue was dissolved in 3 ml. of triple-distilled water and extracted four times with 3 ml. quantities of butanol. The combined butanol extracts, neutralized with ammonia solution, were evaporated to dryness at 30° under reduced pressure; the residue was dissolved in 40–80 μ l. of triple-distilled water and applied in amounts of 25–50 μ l. on paper strips for subsequent electrophoresis.

Paper electrophoresis. Whatman no. 1 paper strips (11 cm. $\times$ 52 cm.) soaked in 0.05M-sodium acetate buffer, pH 4, and lightly blotted were used throughout. The 25 or 50 μ l. of sample was applied as a spot to the paper prepared as indicated above and subjected to electrophoresis. The electrophoresis was carried out at 750 v and a current of 2–30 mA for 2 hr. at room temperature, with an apparatus

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similar to that used by Markham & Smith (1952). At the end of electrophoresis the paper strip was dried and tested for the migration of the steroid and for radioactivity.

Detection of coloured and radioactive spots. DHA, testosterone and oestrone were detected by spraying the paper with the Zimmermann reagent, according to the technique described by Bush & Willoughby (1957), and subsequent drying under an infrared lamp. The presence of the compound was indicated by the appearance of a purplish-pink spot. Testosterone was also detected by its ultraviolet absorption under a Mineralight ultraviolet lamp with maximum emission at 2537 Å. Oestradiol was detected by the technique of Barton, Evans & Gardner (1952), which gives a blue spot when the paper strip is treated with the ferric chloride-potassium ferricyanide reagent. The test is not specific, but the fact that no coloured spot was observed on material obtained by incubating the preparation without added oestradiol-17 β , and that no spot appeared on spraying with the Zimmermann reagent, indicates that it was probably produced by oestradiol.

The radioactive spots were detected by scanning the paper strip with an automatic scanner consisting of a Geiger-Müller end-window counter attached to an automatic recorder.

Incubation with 'active sulphate' intermediate. The formation of DHA sulphate was tested by incubation of 0.21 μ mole of DHA, an amount of transferring enzyme (obtained as described by De Meio, Wizerkaniuk & Schreiber, 1955) corresponding to 10 mg. of protein, 60 μ moles of potassium phosphate buffer, pH 7, and 'active sulphate' intermediate prepared with radioactive sulphate as described by De Meio & Wizerkaniuk (1956) in a final volume of 3 ml. for 2 hr. at 37°. The incubated material was treated as described above and subjected to paper electrophoresis, and the DHA sulphate and radioactivity were detected as already mentioned.

The ^{35}S was supplied as sulphuric acid in dilute hydrochloric acid by the Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A., on allocation from the U.S. Atomic Energy Commission. Adenosine triphosphate was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. and the reduced triphosphopyridine nucleotide from Sigma Chemical Co., St Louis, Mo., U.S.A. The ox liver was kindly supplied by the South Philadelphia Dressed Beef Co.

RESULTS

The results reported in a previous note (De Meio & Lewycka, 1955) indicated that the binding of dehydroepiandrosterone (DHA) was an enzymic process. The binding was decreased by about 66% in the absence of added sulphate and was decreased even more when adenosine triphosphate or Mg^{2+} ions were omitted. De Meio *et al.* (1954) have shown that active ammonium sulphate fractions can be obtained from rat-liver microsome-free solution and that two activities are present in these fractions (De Meio *et al.* 1955). The activating enzyme activity remains after heating the 1.2–2.3 M-ammonium sulphate fraction for 15 min. at 52°, and the transferring enzyme after dialysis against 0.03 M-glycylglycine for 24 hr. at 0–5° or dialysis against distilled water

for 9–13 hr. followed by 4 hr. contact with 0.03 M-glycylglycine at room temperature (24°). Only the combined activating and transferring enzymes show activity, a result similar to that obtained with *p*-nitrophenol as substrate (De Meio *et al.* 1955). In a representative experiment, the activating and transferring enzymes produced the binding of 3.8 and 8.9 $\mu\text{g.}$ of DHA respectively and the combined enzymes changed 24.7 $\mu\text{g.}$ of the DHA to the bound form, out of a total of 60 $\mu\text{g.}$ The preparations that form phenyl and *p*-nitrophenyl sulphate synthesize DHA sulphate. In the presence of DHA less *p*-nitrophenol is changed to the sulphate. A decrease of 16% in the *p*-nitrophenyl sulphate synthesized was observed when 0.72 μmole of *p*-nitrophenol was incubated, under the conditions indicated above for DHA, in the presence of 0.21 μmole of DHA.

The microsome-free solution obtained from ox liver changed 60–70% of the DHA to products giving a negative Zimmermann test. However, the 1.2–2.3 M-ammonium sulphate fraction obtained from this preparation did not show this effect (Table 1). These results indicate that the microsome-free solution obtained from ox liver contains enzymes, other than those involved in the formation of the sulphate, which change DHA into products not reacting in the Zimmermann test. In the rat-liver microsome-free solution this activity seems to be low or absent. The transformation of DHA into products not reacting in the Zimmermann test requires the presence of adenosine triphosphate and does not require oxygen. Adenosine triphosphate can be replaced by reduced diphosphopyridine nucleotide, but not by diphosphopyridine nucleotide.

Table 1 contains representative results of experiments on the binding of oestrone. Rat- and ox-liver preparations bind oestrone, the first doing so to the same extent as for DHA.

Oestradiol-17 β is apparently bound to the same extent as oestrone by rat-liver microsome-free solution, but only about a third as much as is oestrone by the ammonium sulphate fraction. The considerable decrease observed in the absence of sulphate is indirect evidence for the formation of oestradiol sulphate (Table 2). The results contained in Table 2 indicate that part of the oestradiol is changed by ox-liver microsome-free solution to a substance that gives a positive Zimmermann reaction, which is probably oestrone (Pearlman & De Meio, 1949). On the other hand, the ammonium sulphate fraction forms much less of this substance. This may also be the explanation for the difference observed between the microsome-free solution and the ammonium sulphate fraction with rat liver. A lower total recovery of oestradiol was observed with microsome-free solution than with the ammonium sulphate fraction.

Our indirect evidence (De Meio & Lewycka, 1955) indicates that the compound formed by incubation of DHA with rat-liver preparations is DHA sulphate. Direct evidence for the formation of this and other steroid sulphates was obtained through the use of radioactive sulphate and paper electrophoresis as described above.

The material obtained from incubation of DHA, testosterone and oestrone with the liver preparation gave a coloured spot which coincided with the radioactive spot, the radioactivity varying from three to ten times the background count. The coloured spots extended 7–10 cm. from the point of application and the radioactive spots 6–11 cm. The apparent difference in size in the spots is probably due to the difference in sensitivity of the two methods employed for their detection.

Under the conditions for electrophoresis used in our experiments inorganic sulphate labelled with ^{35}S migrated off the paper. The radioactivity on the paper was not due to inorganic sulphate because material obtained by addition of radioactive sulphate after incubation of DHA with unlabelled sulphate gave a coloured spot due to DHA sulphate, but no radioactive spot.

Similar evidence for the coincidence of the coloured and radioactive spots for material obtained on incubation of oestradiol-17 β with the liver preparation is presented here. Coloured spots extending 2–8 cm., and radioactive spots 1–10 cm. from the point of application were obtained. The radioactivity varied from twice to more than ten times the background count. With oestradiol a second spot migrating 18–26 cm.,

Table 1. *Metabolism and synthesis of steroid sulphates by liver preparations*

Medium: 15 μmoles of MgCl_2 , 45 μmoles of K_2SO_4 , 15 μmoles of adenosine triphosphate (ATP), 150 μmoles of potassium phosphate buffer, pH 7, 15–20 mg. of protein as enzyme preparation. Total volume, 3 ml.; incubation for 2 hr. at 37.5°. DPN, Diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Preparation	Steroid	Initial steroid ($\mu\text{g.}$)	Free steroid ($\mu\text{g.}$)	Bound steroid ($\mu\text{g.}$)	Total steroid recovery ($\mu\text{g.}$)	Conditions
Microsome-free solution	DHA	60	8.6	10.0	18.6	In air
Microsome-free solution	DHA	60	10.2	11.1	21.3	In N_2
Microsome-free solution	DHA	60	43.8	8.3	52.1	No ATP
Microsome-free solution	DHA	60	47.4	8.6	56.0	10.8 μmoles of DPN (no ATP)
Microsome-free solution	DHA	60	22.3	0	22.3	10.8 μmoles of DPNH (no ATP)
Ammonium sulphate fraction	DHA	60	26.4	24.2	50.6	—
Rat-liver microsome-free solution	Oestrone	56.2	20.7	26.7	47.4	—
Rat-liver ammonium sulphate fraction	Oestrone	56.2	30	25.9	55.9	—
Ox-liver microsome-free solution	Oestrone	56.2	36.2	9.1	45.3	—
Ox-liver ammonium sulphate fraction	Oestrone	56.2	44.8	11.3	56.1	—

Table 2. *Metabolism of oestradiol-17 β by liver preparations*

Medium was as given in Table 1 with the addition of 56.6 $\mu\text{g.}$ of oestradiol. Dialysis was carried out for 1½ hr. against triple-distilled water.

Preparation	Free oestradiol ($\mu\text{g.}$)	Bound oestradiol ($\mu\text{g.}$)	Observations
Rat-liver microsome-free solution	8.3	27.2	—
Rat-liver microsome-free solution (dialysed)	8.3	28.5	—
	33	1.9	No sulphate
Rat-liver ammonium sulphate fraction	31.4	10.2	—
Rat-liver ammonium sulphate fraction	40.4	5.3	No sulphate
Ox-liver microsome-free solution (dialysed)	29.2	8.2	—
	36.6	0.8	No sulphate
Ox-liver microsome-free solution (dialysed)	24.3	18.3	Determined by Kober reaction
Ox-liver microsome-free solution (dialysed)	7.8*	9.5*	Determined by Zimmermann reaction
Ox-liver ammonium sulphate fraction	31.8	10.7	Determined by Kober reaction
Ox-liver ammonium sulphate fraction	0	2.5*	Determined by Zimmermann reaction

* Probably oestrone.

but less intense than the first, was repeatedly observed.

No migrating coloured or radioactive spots were obtained when the complete preparation was incubated under one of the following conditions: at 0°; with boiled-liver preparation; in the absence of sulphate or of adenosine triphosphate.

The radioactive and coloured spots coincided for DHA sulphate prepared by incubation of DHA with 'active sulphate' intermediate and transferring enzyme as indicated above.

When 10–30 μ g. of DHA, testosterone or oestrone sulphate was added to an extract of a preparation incubated without the addition of the steroid and the product subjected to paper electrophoresis, the coloured spots appeared in the same position as those found for the material obtained by incubation of the preparations with the free steroids.

DISCUSSION

The fact that a threefold increase in binding of dehydroepiandrosterone (DHA) by liver preparations is observed on the addition of sulphate is indirect evidence for the formation of DHA sulphate. The paper-electrophoresis experiments on material obtained after incubation with radioactive sulphate furnish direct evidence for the same process. The radioactive spot must be due to bound sulphate, since inorganic sulphate under identical conditions migrates off the paper. The coincidence of the radioactive and the coloured spots (Zimmermann reagent) indicates that the sulphate and DHA migrate together. As DHA does not migrate by itself, it must be bound to the sulphate. On the other hand, apart from conjugation with sulphate, DHA appears to be unchanged, since the recovery lies within that of the method (about 80 %). Further evidence for the biosynthesis of DHA sulphate is that no migration of DHA is observed if the incubation is carried out in the absence of sulphate. Similar evidence is presented for the formation of the sulphates of testosterone, oestrone and oestradiol-17 β . In the last case there are indications that more than one type of sulphate is formed. Theoretically three possible types of sulphate of oestradiol-17- β can exist, but only further work can determine which of these are involved in the present experiments.

Segal (1955) has presented other evidence for the formation of oestrone sulphate by use of 14 C-labelled oestrone as a substrate, and Roy (1956) for the formation of sulphates of DHA and other steroids.

The requirements for the formation of DHA sulphate and the other sulphates are the same as those found for the synthesis of the sulphuric acid esters of phenols (De Meio *et al.* 1953; Bernstein & McGilvery, 1952a).

The combined activating and transferring enzyme preparations in the presence of SO_4^{2-} and Mg^{2+} ions and adenosine triphosphate synthesize DHA sulphate and, as we have already shown, also *p*-nitrophenyl sulphate (De Meio *et al.* 1955). In the presence of DHA, less *p*-nitrophenol is changed to the sulphate. Further, DHA is changed to the sulphate by incubation with the 'active sulphate' intermediate and transferring enzyme. These observations do not prove that the same system is involved in both processes; it may well be that the two substrates compete for the 'active sulphate' intermediate (Bernstein & McGilvery, 1952b) but that the transferring enzyme may be different. The latter appears to occur, as Gregory & Nose (1957) reported recently the finding of two separate fractions (sulphokinases): one produces the sulphates of steroids containing a β -hydroxyl group at C-3 and the other transfers sulphate to many phenolic compounds including the steroid phenols.

SUMMARY

1. The synthesis *in vitro* of dehydroepiandrosterone, testosterone, oestrone and oestradiol-17 β sulphates with liver preparations has been demonstrated. The system requires Mg^{2+} ions and adenosine triphosphate for activity.

2. Ox-liver microsome-free solution, but not the ammonium sulphate fraction, partially changes dehydroepiandrosterone to a substance or substances reacting negatively to the Zimmermann test. The presence of adenosine triphosphate or of reduced diphosphopyridine nucleotide is required for the process.

3. The microsome-free solution, but not the ammonium sulphate fraction, changes oestradiol into a substance (probably oestrone) giving a positive Zimmermann test.

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Extraction of Proteins other than Myosin from the Isolated Rabbit Myofibril

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Although satisfactory methods now exist for the isolation of myofibrils in appreciable quantities, little systematic combined biochemical and morphological study has yet been made of the organization of the chemical components in these structures. There is now clear evidence that the myofibril is composed mainly of the proteins myosin, actin and tropomyosin, but most investigations (e.g. Hasselbach, 1953; Hanson & Huxley, 1953; Hodge, 1955) concerned with the selective extraction of these proteins have been qualitative in nature. Furthermore, with the notable exception of the work of Hasselbach (1953), few attempts have been made to characterize and clearly identify the proteins extracted by the procedures used. Thus although the studies of Hasselbach (1953), Hanson & Huxley (1953) and Finck, Holtzer & Marshall (1956) indicate that there is a high concentration of myosin in the A band they do not allow us to decide whether this protein is present in the I band as well. Szent-Györgyi, Mazia & Szent-Györgyi (1955) have provided evidence that protein other than myosin is to be found in the A band and draw the conclusion from their results that appreciable amounts of myosin are localized in the I band.

One approach to the problem of the localization of myosin and the other myofibrillar proteins is provided by the observation (Perry, 1953) that a fraction free of myosin could be extracted from

rabbit myofibrils by solutions of low ionic strength. This fraction contained two main components, one of which was shown to be tropomyosin, but the second component could not be identified with myosin or with the F or G form of actin. The present paper is concerned with the identification of the second component as a form of inactive actin and with a further investigation of the selective extraction of proteins other than myosin from the myofibril. It forms the biochemical background to the following paper (Corsi & Perry, 1958) devoted to the study of the localization of myosin, tropomyosin and actin by phase-contrast microscopy of myofibrils extracted under varying conditions of pH and ionic strength.

METHODS

Preparation of myofibrils. In general, myofibrils were prepared from fresh back and leg muscles and from glycerol-treated psoas muscle of the rabbit by the methods already described (Perry & Grey, 1956a). To prevent the occurrence of spontaneous contraction during the homogenization of glycerol-treated muscle in a medium containing 0.1 M-KCl and 0.039 M-borate buffer, pH 7.1 (0.1 M-KCl-borate), it was found necessary to store the psoas-muscle strips in 50% (v/v) aq. glycerol for 2-3 weeks at -10° to deplete the endogenous adenosine triphosphate (ATP). When fresh rabbit muscle was homogenized in 0.1 M-KCl-borate, marked contraction of the myofibrils with reversal of striation usually occurred. This spontaneous contraction was reduced, but not eliminated, by lowering the KCl concentration of this medium to 0.025 M (0.025 M-KCl-borate) and carefully maintaining the temperature at 0° .

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