*Biochem. J.* (1969) **115**, 489
*Printed in Great Britain*



# Androgen Sulphate Formation in Male and Female Mice

By D. A. LEWIS
*Pharmacology Group, School of Pharmacy, Bath University of Technology, Bath BA2 7AY*

(*Received* 2 *June* 1969)

1. After the administration of large doses of androsterone, epiandrosterone, dehydroepiandrosterone and testosterone to mice, females excreted more of the dose conjugated with sulphuric acid than did males. 2. Liver slices from female mice conjugated androgens with sulphuric acid to a greater extent than did slices from males. 3. Sulphotransferase preparations from livers of female rats and mice catalysed the formation of dehydroepiandrosterone sulphate at a faster rate than preparations from livers of the male animals. 4. A possible explanation for the observed sex differences is discussed.

It has been known for some time that partially purified enzyme preparations from female rat and mouse livers are more active in conjugating dehydroepiandrosterone with sulphuric acid than are similar preparations from male animals (Roy, 1956*b*, 1958). These observations were shown to have some significance in the metabolism of whole animals when Lewis (1968*a*) administered large amounts of androsterone, epiandrosterone, dehydroepiandrosterone and testosterone separately to rats and found that the females excreted larger amounts of androgen sulphates in their urine than did the males. It was also shown that liver slices from the females conjugated the androgens with sulphuric acid to a greater extent than males.

The work described below is concerned with androgen sulphate formation by mice. It is also concerned with the enzymic synthesis of androgen sulphates in an attempt to provide an explanation for the observed sex differences in androgen sulphate formation in the whole animal. A preliminary report of this work has been given (Lewis, 1968*b*).

## MATERIALS AND METHODS

*Steroids.* Aetiocholanolone (3α-hydroxy-5β-androstan-17-one), androsterone (3α-hydroxy-5α-androstan-17-one), epiandrosterone (3β-hydroxy-5α-androstan-17-one), dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one), methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) and testosterone (17β-hydroxyandrost-4-en-3-one) were obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6. The steroids were recrystallized from aqueous ethanol before use. Dehydroepiandrosterone sulphate was prepared by the method of Paterson & Klyne (1948) as modified by Roy (1956*a*).

*Paper chromatography.* The techniques used were described by Lewis (1968*a*). The solvent systems used were: *A*, butan-1-ol–water–acetic acid–butyl acetate (24:10:5:2, by vol); *B*, butan-1-ol–ethanol–aq. $NH_3$ (sp.gr. 0·88)–water (10:10:1:4, by vol.); *C*, butan-1-ol–acetic acid–water (31:6:13, by vol.).

*Thin-layer chromatography.* This was carried out on aluminium oxide G (Stahl) by methods described by Lewis (1968*a*). The solvent systems used were: *D*, benzene–ethanol (49:1, v/v); *E*, ethyl acetate; *F*, *n*-butyl acetate.

*Gas–liquid chromatography.* The identification and determination of androgens were achieved by methods described by Lewis (1968*a*).

*Whole-animal experiments: animals and dosing.* Male and female mice of identical age (body wt. 30–50g.) were used. They were housed in metabolism cages designed to permit separate collection of urine and faeces. The mice were allowed food and water *ad libitum*. The steroids were finely ground in an agate mortar and then in a high-speed vibration mill (Research and Industrial Instruments Ltd., London S.E.22) for 10min. The steroids (6mg.) were then dispersed in 1% (w/v) starch solution and administered by stomach tube. In some experiments carrier-free $Na_2{}^{35}SO_4$ was administered by subcutaneous injection at the same time as the steroid. The urines were collected in 24hr. periods and stored frozen. The total amount of 17-oxo steroid sulphates in normal mouse urine was determined by the methods described by Lewis (1968*a*).

*Tissue experiments.* The formation of androgen sulphates by liver slices from male and female mice was carried out by methods identical with those used for rat liver slices (Lewis, 1968*a*).

*Preparation of enzyme fractions.* Partially purified steroid sulphotransferases (Roy, 1960*a*) and a PAPS*-generating system consisting of a mixture of sulphate adenylyltransferase (ATP–sulphate adenylyltransferase, EC 2.7.7.4) and adenylyl sulphate kinase (ATP–adenylyl sulphate 3′-phosphotransferase, EC 2.7.1.25) (De Meio, Wizerkaniuk & Schreibman, 1955) were prepared from particle-free supernatants of rat and mouse livers. PAPS was prepared biosynthetically by the method of Roy (1960*a*). The enzymic preparations were adjusted to a

* Abbreviation: PAPS, adenosine 3′-phosphate 5′-sulphatophosphate (3′-phosphoadenylyl sulphate).

concentration where 1 ml. of the solution corresponded to 400 mg. wet wt. of liver.

*Determination of androgen sulphate in urine.* (*a*) Rapid determination of sex differences in androgen sulphate formation. Sex differences in androgen sulphate formation were rapidly detected by the technique described by Lewis (1968*a*). The 24 hr. urine from male and female animals that had been dosed with both a steroid and $Na_2{}^{35}SO_4$ was examined by paper chromatography in solvent systems *A*, *B* and *C*, and the dried chromatograms were scanned by a radiochromatogram scanner. The sulphuric acid conjugates were detected by comparing the experimental patterns with control patterns. In addition, the areas of the chromatograms containing the metabolites were removed and eluted with methanol–water–ethyl acetate–acetic acid (75:5:20:3, by vol.) (Smith, 1960). The androgen sulphates in the eluates were hydrolysed by methods described by Lewis (1968*a*) and examined by paper chromatography and g.l.c. for the presence of steroids. Sex differences in androgen sulphate formation were detected by comparing the peak areas on the radiochromatogram-scanner chart of the metabolites formed by male and female animals.

(*b*) Determination of androgen sulphate. The amount of the principal androgen sulphate in each urine was determined by the method of Menini (1966), except that the final oxidation with *tert.*-butyl chromate was omitted. Portions of the extracts were mixed with an internal standard and examined by g.l.c.

*Formation of androgen sulphate by enzyme preparations.* The incubation medium consisted of 0·5 ml. of a sulphotransferase preparation, 0·5 ml. of a PAPS-generating system, 0·5 ml. of a 0·3 M-$KH_2PO_4$–30 mM-$K_2SO_4$–5 mM-$MgCl_2$–40 mM-ATP (3:3:3:1, by vol.) solution, and dehydroepiandrosterone dissolved in 30 $\mu$l. of propane-1,2-diol. The steroid was omitted from the controls. The reaction was carried out in 10 ml. stoppered tubes, which were incubated for 2 hr. at 37° in a water bath. The reaction was stopped by the addition of 5 ml. of methanol. The solutions were centrifuged and 5 ml. samples of the supernatants transferred to 10 ml. test tubes. The tubes were placed in a water bath at 75° and the temperature was slowly raised to boiling point. The solutions were then evaporated to dryness. The amount of dehydroepiandrosterone sulphate in each residue was determined by the Methylene Blue method of Roy (1956*a*) except that the extinction of the chloroform solution of the Methylene Blue salt was determined at 600 nm. Synthetic dehydroepiandrosterone sulphate was used to prepare a standard curve. In some experiments 0·5 ml. of the PAPS preparation was substituted for the PAPS-generating system and 0·5 ml. of 0·1 M-phosphate buffer, pH 6·8, was substituted in place of the buffered ATP solution. Preliminary experiments had shown that $Mg^{2+}$ is not required for the transferase reaction.

## RESULTS

*Sex differences in the amounts of androgen sulphates excreted by mice.* The relative amounts of the sulphates in the urines of the animals to which steroids and $Na_2{}^{35}SO_4$ had been administered are given in Table 1. The results show that the female mice excreted larger amounts of the dose conjugated with sulphuric acid than did the males. When the androgen sulphates were eluted from the chromatograms and hydrolysed it was found that the principal androgen present was the androgen administered. Androsterone as well as testosterone was detected in the hydrolysed eluates from chromatograms of the urine from mice that had been dosed with testosterone. The androgens were identified by t.l.c. and co-chromatography.

*Determination of androgen sulphate in urine.* The amounts of the sulphate metabolites determined by the Menini (1966) method in the urines of mice that had been dosed with androgens are given in Table 2. The determinations were made on the urines collected over the periods 0–24 hr., 24–48 hr. and 48–72 hr. after the dosing. The results show clearly that the female mice excreted larger amounts

Table 1. *Relative radioactivity of the androgen sulphate fractions of urines from male and female mice*

The chromatograms were scanned for radioactivity and the value:

$$\frac{\text{Androgen [}^{35}\text{S]sulphate area} \times 100}{\text{Total radioactive area}}$$

was calculated from the radiochromatogram-scanner chart. The experimental methods are given in the text. Values less than 1% are indicated by +. The urines were 24 hr. samples.

| | Relative radioactivity | | | | | |
|---|---|---|---|---|---|---|
| | Solvent *A* | | Solvent *B* | | Solvent *C* | |
| Steroid administered | Male | Female | Male | Female | Male | Female |
| Androsterone | 1 | 7 | 1 | 6 | 1 | 4 |
| Epiandrosterone | + | 6 | 1 | 9 | + | 9 |
| Dehydroepiandrosterone | 1 | 3 | 1 | 5 | 1 | 3 |
| Testosterone | 1 | 13 | 1 | 9 | 2 | 12 |
| Control | + | + | + | + | + | + |

Table 2. *Androgen sulphate excreted in urines, determined by the Menini (1966) method*

Each result was obtained from the urine of four mice (equivalent to 24mg. of steroid administered). The experimental details are given in the text.

| Steroid administered | Androgen measured in hydrolysate | Androgen determined (expressed as μg. of its sulphate/24hr.) Females Day 1 | Females Day 2 | Females Day 3 | Females Total | Males Day 1 | Males Day 2 | Males Day 3 | Males Total |
|---|---|---|---|---|---|---|---|---|---|
| Androsterone | Androsterone | 106 | 22 | 11 | 139 | 47 | 6 | 2 | 55 |
| Epiandrosterone | Epiandrosterone | 68 | 54 | 14 | 136 | 14 | 4 | 4 | 22 |
| Dehydroepiandrosterone | Dehydroepiandrosterone | 110 | 14 | 11 | 135 | 6 | 3 | 2 | 11 |
| Testosterone | Androsterone | 12 | 6 | 6 | 24 | 4 | 1 | 1 | 6 |
| Testosterone | Testosterone | 52 | — | — | 52 | 1 | — | — | 1 |

Table 3. *Formation of androgen sulphate by liver slices*

This experiment was carried out by methods described by Lewis (1968*a*). Each flask originally contained 150μg. of steroid in the presence of 1g. wet wt. of slices. The numbers of experiments are given in parentheses.

| Substrate | Conjugate formed (expressed as μg. of androgen sulphate ±S.D.) Female | Male |
|---|---|---|
| Androsterone | 82±2 (3) | 8±4 (3) |
| Epiandrosterone | 52±8 (3) | 10±7 (3) |
| Dehydroepiandrosterone | 29±4 (3) | 4±3 (3) |
| Methyltestosterone | 21±8 (3) | 4±3 (3) |
| Control | 2±2 (3) | 3±1 (3) |

of the dose as the sulphuric acid conjugates than did the males. The androgens present in the hydrolysates were identified by g.l.c., and this confirmed the observation that the main androgen present in the hydrolysed androgen sulphate fraction was the androgen administered. Minor metabolites were not identified. The urines from the control mice were collected over 3 days and the total 17-oxo steroid sulphate was determined in the pooled samples. The results showed that male mice excreted 3–5μg. of 17-oxo steroid sulphate over the 3 days and females 3–4μg. No correction has been made for endogenous 17-oxo steroid sulphate in Table 2.

*Formation of androgen sulphate by mouse liver slices.* The amount of androgen sulphate formed by liver slices is given in Table 3. These results follow the same pattern as those from the whole-animal experiments, in that the slices of female origin were more active in conjugating the steroid with sulphuric acid than were those from the males.

*Enzyme experiments.* The amounts of dehydroepiandrosterone sulphate formed by the enzymic coupling of dehydroepiandrosterone with sulphuric acid are given in Table 4. The results show that more dehydroepiandrosterone sulphate was formed when a sulphotransferase preparation of female origin was used instead of one of male origin. This suggests either that the enzyme is present in higher quantities in female rat liver, or that it possesses a greater activity due possibly to the absence of inhibitors or the presence of activators. No sex differences in the sulphate-activating system were observed, but it is possible that the steroid sulphotransferase concentrations were the limiting factor in the enzymic synthesis. Further evidence that differences in the amount, or activity, of steroid sulphotransferases may be responsible for the sex differences in androgen sulphate formation in the whole animal was obtained from the results of experiments with enzymes from rat and mouse where PAPS solution was added in place of a PAPS-generating system. These results (Table 5) show clearly that the sulphotransferase preparations from livers of female rats and mice were more active than those from the male animals.

## DISCUSSION

The results show that mice exhibit sex differences in androgen sulphate formation, in that females excrete a greater proportion of large doses of

Table 4. *Activities of the sulphate-activating enzymes and the steroid sulphotransferases from rat liver*

Rat liver preparations were incubated with dehydroepiandrosterone. The experimental details are given in the text. Duplicate results are given.

| Expt. no. | Sulphate-activating system (Sex of rat) | Steroid sulphotransferase (Sex of rat) | Conjugates formed (expressed as μg. of dehydroepiandrosterone sulphate): Amount of substrate (μg.) 0 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|---|---|
| 1 | Male | Male | 2, 2 | 9, 7 | 7, 7 | 9, 7 | 8, 8 |
| | Female | Male | 2, 1 | 5, 7 | 9, 7 | 8, 8 | 8, 9 |
| 2 | Male | Male | 2, 2 | 9, 10 | 7, 7 | 7, 7 | 9, 9 |
| | Male | Female | 2, 3 | 18, 19 | 22, 22 | 23, 24 | 25, 22 |
| 3 | Male | Female | 3, 1 | 15, 16 | 17, 21 | 20, 19 | 22, 21 |
| | Female | Female | 2, 2 | 12, 15 | 17, 17 | 19, 19 | 20, 19 |

Table 5. *Sulphotransferase activity in mouse and rat liver preparations*

Rat liver preparations were incubated with 100 μg. of dehydroepiandrosterone. The experimental details are given in the text.

| Species | Sex of animal | Conjugates formed (expressed as μg. of dehydroepiandrosterone sulphate) |
|---|---|---|
| Rat | Male | 7, 7, 7 |
| | Female | 30, 28, 28 |
| Mouse | Male | 13, 9 |
| | Female | 24, 20 |

administered androsterone, epiandrosterone, dehydroepiandrosterone and testosterone as sulphuric acid conjugates than do males. In addition, liver slices from females conjugate androgens with sulphuric acid to a greater extent than do slices from males. These results are similar to those obtained with rats (Lewis, 1968*a*). The enzymes concerned in the formation of androgen sulphates are the two enzymes of the PAPS-generating system mentioned above, and a steroid alcohol sulphotransferase. The sulphotransferase responsible for the formation of epiandrosterone sulphate and dehydroepiandrosterone sulphate is probably the 3-hydroxy steroid sulphotransferase (3′-phosphoadenylyl sulphate-3-hydroxy steroid sulphotransferase, EC 2.8.2.2) identified by Nose & Lipmann (1958). Whether this enzyme has any activity towards the 17β-hydroxyl group in testosterone or the 3α-hydroxyl group in androsterone is not certain (Roy, 1960*b*). It is possible that other steroid alcohol transferases are involved in the conjugations of these steroids.

A wide range of compounds is conjugated with sulphuric acid in animals, e.g. chondroitin (Davidson & Riley, 1960), various steroids (Schneider & Lewbart, 1956), arylamines (Roy, 1960*a*), phenols (Williams, 1959), cyclic hydrocarbon dihydrodiols (Sims, 1962; Lewis, 1966) and tyrosine (Tallan, Bella, Stein & Moore, 1955).

Some sex differences in ester sulphate formation have been reported. Weisburger, Grantham & Weisburger (1964) found that male rats excreted a larger amount of administered *N*-2-fluorenyl-*N*-hydroxyacetamide as a sulphuric acid conjugate than did female rats. Rao & Taylor (1965) found that liver homogenates from female rats formed more cold-acid-hydrolysable metabolites of progesterone than did homogenates of male livers. Roy (1958) found that partially purified enzyme preparations from female rats synthesized more dehydroepiandrosterone sulphate than did preparations from male rats. In contrast he found that the preparations did not show a sex difference in the synthesis of phenyl sulphate. He suggested that different amounts of the steroid alcohol sulphotransferase were responsible for the sex difference

observed in dehydroepiandrosterone sulphate synthesis. Quantitative differences in the amount of this enzyme in the preparations would not affect phenyl sulphate synthesis, since this sulphotransferase reaction is catalysed by aryl sulphotransferase (3′-phosphoadenylyl sulphate–phenol sulphotransferase, EC 2.8.2.1). The present results support this suggestion by Roy (1958). The present investigation shows that sulphate-conjugating preparations from the livers of female rats and mice have greater 3$\beta$-hydroxy steroid sulphotransferase activity than have preparations from male animals. It appears possible that the different activities of this liver enzyme are responsible for the sex differences observed in the amounts of 3$\beta$-hydroxy steroid sulphates excreted after the separate administration of the steroids to the animal. It also appears possible that the other sex differences in ester sulphate formation mentioned above are due to different concentrations or activities of the appropriate sulphotransferases in the animal livers.

Large amounts of androgens were used in the experiments, and since steroid metabolism is often dose-dependent the sex differences observed may have little relevance to the normal physiology of the animal. However, it has been shown that excess of androgen is able to stimulate androgen sulphate formation in the female animal to a greater extent than in the male. Since the sulphuric acid conjugates have a lower biological activity than the parent androgens it is possible that androgen sulphate formation is a mechanism by which the females are able to reduce an excess of androgen due to pathological or other causes.

The amounts of androgen sulphates detected in the urines were only a small part of the amounts of androgen administered. It is possible that oral administration was not an efficient method for administering large amounts of androgens and that only a portion of the dose was absorbed. Also it has been reported that rats and mice excrete a large proportion of administered testosterone as metabolites in the bile and faeces (Barry, Eidinoff, Dobriner & Gallagher, 1952). This observation suggests that a significant portion of the administered androgens was excreted as metabolites in the faeces.

The author acknowledges the award by The Royal Society of a grant for the purchase of liquid-scintillation counting equipment. He thanks Mrs E. Nelson for part-time technical assistance.

## REFERENCES

Barry, M. C., Eidinoff, M. L., Dobriner, W. & Gallagher, T. F. (1952). *Endocrinology*, **50**, 587.
Davidson, E. A. & Riley, J. G. (1960). *J. biol. Chem.* **235**, 3367.
De Meio, R. H., Wizerkaniuk, M. A. & Schreibman, I. (1955). *J. biol. Chem.* **213**, 439.
Lewis, D. A. (1966). *Biochem. J.* **99**, 694.
Lewis, D. A. (1968*a*). *Biochem. J.* **106**, 497.
Lewis, D. A. (1968*b*). *Biochem. J.* **109**, 12P.
Menini, E. (1966). *Biochem. J.* **99**, 747.
Nose, Y. & Lipmann, F. (1958). *J. biol. Chem.* **233**, 1348.
Paterson, J. Y. F. & Klyne, W. (1948). *Biochem. J.* **43**, 413.
Rao, C. G. S. & Taylor, W. (1965). *Biochem. J.* **96**, 172.
Roy, A. B. (1956*a*). *Biochem. J.* **62**, 41.
Roy, A. B. (1956*b*). *Biochem. J.* **63**, 294.
Roy, A. B. (1958). *Biochem. J.* **68**, 519.
Roy, A. B. (1960*a*). *Biochem. J.* **74**, 49.
Roy, A. B. (1960*b*). *Advanc. Enzymol.* **22**, 205.
Schneider, J. J. & Lewbart, M. L. (1956). *J. biol. Chem.* **222**, 787.
Sims, P. (1962). *Biochem. J.* **84**, 558.
Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 429. London: W. Heinemann (Medical Books) Ltd.
Tallan, H. H., Bella, S. T., Stein, W. H. & Moore, S. (1955). *J. biol. Chem.* **217**, 703.
Weisburger, E. K., Grantham, P. H. & Weisburger, J. H. (1964). *Biochemistry*, **3**, 308.
Williams, R. T. (1959). *Detoxication Mechanisms*, 2nd ed., p. 279. London: Chapman and Hall.