Biochem. J. (1968) **106**, 497
Printed in Great Britain

Androgen Sulphate Formation in Male and Female Rats

By D. A. LEWIS*
Department of Physiological Chemistry, University of Birmingham

(*Received* 3 *August* 1967)

1. After large doses of androsterone, epiandrosterone, dehydroepiandrosterone and testosterone, female rats excreted more of the dose conjugated with sulphuric acid than did the males. 2. Androgens were also incubated with liver slices from male and female rats. Slices from females conjugated androgens with sulphuric acid to a greater extent than did slices from males. 3. The amount of unchanged androgen present in the faeces of orally dosed animals was 4–35% of the dose.

Physiologically active steroids and their metabolites are often excreted in the urine of animals conjugated with either glucuronic acid or sulphuric acid. Formerly these conjugates were regarded as metabolic end products, but recent work has suggested that the sulphates may be important intermediates in steroid metabolism. Raggatt & Whitehouse (1966) have shown that cholesteryl sulphate is an alternative substrate in the oxidation of cholesterol. As pregnenolone sulphate is oxidized to dehydroepiandrosterone sulphate without hydrolysis of the sulphate group (Calvin, Vande Wiele & Lieberman, 1963; Calvin & Lieberman, 1964), Raggatt & Whitehouse (1966) suggested that there may be a 'sulphate pathway' of steroid hormone formation in which the steroids are sulphated at C-3 and undergo oxidation or hydroxylation elsewhere in the molecule. Dehydroepiandrosterone sulphate has been shown to be metabolized to androstenediol sulphate, androstenetriol sulphate and 16α-hydroxydehydroepiandrosterone sulphate without cleavage of the sulphate group (Baulieu *et al.* 1965). Raggatt & Whitehouse (1966) suggest that the 'sulphate pathway' may be of especial importance in the biosynthesis and metabolism of androgens.

MATERIALS AND METHODS

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) and testosterone (17β-hydroxyandrost-4-en-3-one) were obtained from Sigma (London) Chemical Co. Ltd., London, S.W. 6. The steroids were recrystallized from aqueous ethanol before use.

The reference steroids 16α,17β-dihydroxyandrost-4-en-3-one, 5α-androstane-3α,17β-diol, 5α-androstane-3α,17α-diol, 5α-androstane-3β,17β-diol, 5α-androstane-3β,17α-diol, 3β,16α-dihydroxyandrost-5-en-17-one (16α-hydroxydehydroepiandrosterone) and androst-5-ene-3β,17β-diol were from the Medical Research Council Steroid Reference Collection.

A solution of $Na_2{}^{35}SO_4$ was obtained from The Radiochemical Centre, Amersham, Bucks.

Animals and dosing. Male and female rats of identical ages (body wt. 150–250g.) of the Wistar strain were used. They were housed in metabolism cages designed to permit separate collection of urine and faeces. The rats were allowed water *ad libitum*, and access to rat cakes for 1hr. each day. The steroids were finely ground in an agate mortar and then in a high-speed vibration mill (Research and Industrial Instruments Co., London, S.E. 22) for 10min. For administration by stomach tube, 80mg. of steroid was suspended in 1ml. of 1% starch solution. For injection, 25mg. of the steroid was suspended in warm sunflower oil and injected subcutaneously in the lumbar region. Carrier-free $Na_2{}^{35}SO_4$ was administered by subcutaneous injection in the lumbar region at the same time as the administration of the steroid.

The urines and faeces were collected separately in 24hr. periods. The urines were stored frozen at −20° and the faeces over P_2O_5 in a desiccator.

Paper chromatography. The descending technique on Whatman 3MM paper was used. 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.); *D*, butan-1-ol saturated with 2N-NH_3. The radioactive androgen sulphates were detected by 'scanning' the dried chromatograms on a Packard model 7200 radiochromatogram scanner, and the areas of the various peaks on the chart were determined by triangulation. Radioautographs were also prepared on Kodak X-ray paper.

Thin-layer chromatography. This was carried out on aluminium oxide G (Stahl) supplied by the Shandon Scientific Co. Ltd., London, N.W. 10. The layer was 250μ thick. The freshly prepared plates were dried and activated at 90° for 2hr. and stored in a desiccator until required. Separations of androgens were obtained in: solvent *E*, benzene–ethanol (49:1, v/v); solvent *F*, ethyl acetate; solvent *G*, *n*-butyl acetate. Steroids were detected by

* Present address: School of Pharmacy, Bath University of Technology.

spraying the dried plate with methanol–sulphuric acid (1:1, v/v) (Randerath, 1963) or with the modified alkaline *m*-dinitrobenzene reagent described by Smith (1960).

Gas–liquid chromatography. This was carried out in a Pye series 104 chromatograph (W. G. Pye and Co. Ltd., Cambridge) with a flame ionization detector. Glass columns (152cm. long, int. diam. 4·0mm.) were packed with silanized Gas-Chrom W (80–100 mesh) coated with 1% (w/w) QF1 [Wilkens Instrument and Research (U.K.) Ltd., Manchester, 22]. The carrier gas was argon (flow rate 78ml./min.) and the operating temperature was 200°. Steroid solutions (1–10μl.) were injected on to the column by a microsyringe (Hamilton Company Inc., Whittier, Calif., U.S.A.). Standard solutions of synthetic androgens were used as internal standards.

Paper electrophoresis. Radioactive androgen sulphates were separated on 5cm. Whatman 3MM paper strips impregnated with 0·1M-sodium acetate–acetic acid buffer, pH4·5. A current of 0·5mA/cm. was applied for 1–2hr. The strips were then dried at 80°. The volume of urine applied was 100–200μl.

Identification of the androgens in hydrolysed androgen sulphate fractions. Pairs of rats were dosed orally with androgen and at the same time injected with $Na_2{}^{35}SO_4$ (200μc). The urines from each pair were made up to 60ml. and 1ml. of each was streaked on to chromatography-paper strips (5cm. wide). The papers were developed in solvent system *A*. In this solvent system the free androgens ran with the solvent front, well clear of the androgen sulphate fraction. The areas of the chromatogram containing the androgen sulphates were cut out and extracted exhaustively with methanol–water–ethyl acetate–acetic acid (150:10:40:1, by vol.) (Smith, 1960). After filtration, the extract was evaporated to dryness under reduced pressure, the androgen sulphates were hydrolysed by cold HCl, and the hydrolysates were extracted with ether (Kellie & Wade, 1957). The ether was removed by evaporation under reduced pressure, and the residue was dissolved in $CHCl_3$ (1ml.). The androgens were identified by thin-layer chromatography and co-chromatography.

Determination of androgen sulphate in urine. The amount of the principal 17-oxosteroid sulphate of each androgen 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 submitted to gas–liquid chromatography. Experiments in which radioactive androgen sulphate fractions were added to normal urines showed that the androgens recovered corresponded to at least 90% of the original radioactivity. The total amount of 17-oxo steroid sulphate in urine was determined by applying the modified Zimmermann reaction (Corker, Norymberski & Thow, 1962) to portions of the hydrolysed 17-oxo steroid sulphate fraction (Menini, 1966).

Determination of unchanged androgens in faeces. The faeces were ground to a fine powder and the powder was extracted exhaustively with $CHCl_3$. The filtered extracts were evaporated to dryness under reduced pressure, the residue was dissolved in a known volume of $CHCl_3$ (5–25ml.) and the androgens were determined by gas–liquid chromatography.

Formation of androgen sulphate by liver slices. Twenty flasks, each containing 6mg. of ATP, 30μl. of $Na_2{}^{35}SO_4$ (approx. 0·1μc), 4mg. of sodium succinate and 6ml. of oxygenated Krebs–Ringer phosphate, pH7·4 (Krebs & Henseleit, 1932), were divided into four sets of five, and androgen dissolved in 0·03ml. of propylene glycol was added to four flasks in each set. The fifth flasks (controls) received 0·03ml. of propylene glycol alone. The amounts of androgen added were 150μg., 300μg., 600μg. and 750μg. Slices (approx. 0·5mm. thick) of liver from freshly killed male and female rats were cut on a machine giving uniform thickness; 1g. wet wt. of slices from male rats was added to each flask in two sets, and 1g. wet wt. of slices from female rats added to each flask in the remaining two sets. (There was no significant difference in the nitrogen content of the liver slices from the two sexes.) The flasks were incubated under O_2 for 4hr. at 37° in a water bath. The reaction was stopped by the addition of 10ml. of methanol to each flask. The solutions were centrifuged and 5ml. portions of the supernatants were evaporated to dryness under reduced pressure. The residues were each dissolved in 1ml. of methanol and the solutions were streaked on to 5cm.-wide chromatography-paper strips. The papers were developed in solvent system *A*, and the areas containing the radioactive metabolites were cut out and eluted with the methanol–water–ethyl acetate–acetic acid solvent. The eluates were made up to 100ml., and 1ml. portions were evaporated to dryness as infinitely thin films on aluminium planchets. The radioactivity on each planchet was determined by a lead-shielded thin-end-window Geiger–Müller tube connected to an Ekco automatic scaler type N5306 unit (efficiency approx. 8%). The remainder of the eluate from each flask was evaporated to dryness, and the androgen sulphates were hydrolysed with dioxan. The hydrolysates were examined for androgens by thin-layer chromatography. The total $Na_2{}^{35}SO_4$ added to each flask was determined by evaporating 30μl. portions of the $Na_2{}^{35}SO_4$ solution to dryness as infinitely thin films on planchets. These 'blank' planchets were counted at the same efficiency as the experimental ones.

RESULTS

Identification of the androgen in hydrolysed androgen sulphate fractions. The R_F and M_S values of the androgen sulphate fractions of the urines of dosed male and female rats are given in Table 1. The relative amounts of the sulphates in the urines of the male and female rats to which steroids were administered by stomach tube are recorded in Table 2. The results show clearly that the dosed females excreted considerably more androgen sulphate than the males. Similar results were obtained when the steroid was administered by injection.

The androgens liberated by the hydrolysis of 17-oxo steroid sulphates in the eluates of paper chromatograms were identified by thin-layer chromatography and co-chromatography in solvent systems *E*, *F* and *G*. The suggested identity of the main metabolites in the urines and in the tissue experiments is given in Table 3. The R_F values of the androgen, the androgen run with the reference compound and the reference compound alone did not vary by more than 0·02 R_F, and the R_F value

Table 1. *Paper chromatography and paper electrophoresis of androgen sulphate fractions from urine*

The experimental details and solvent systems used are given in the text. In the paper-electrophoresis experiments:

$$M_s = \frac{\text{Distance moved by the metabolite}}{\text{Distance moved by inorganic sulphate}}$$

Steroid administered	R_F value: Solvent *A*	Solvent *B*	Solvent *C*	Solvent *D*	M_s value
Androsterone	0·84	0·68	0·69	0·64	0·36
Epiandrosterone	0·79	0·68	0·66	0·63	0·36
Dehydroepiandrosterone	0·81	0·69	0·68	0·68	0·39
Testosterone*	0·83	0·70	0·69	0·67	0·35

* The sulphate in urine is mainly that of androsterone (see Table 3).

Table 2. *Relative radioactivity of the androgen sulphate fractions of urine*

The chromatograms were 'scanned' for radioactivity and the value:

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

calculated from the radiochromatogram scanner chart. Experimental details are described in the text. Values less than 1% are indicated by +.

Steroid administered	Urine sample (day)	Solvent *A* Male	Solvent *A* Female	Solvent *B* Male	Solvent *B* Female	Solvent *C* Male	Solvent *C* Female	Solvent *D* Male	Solvent *D* Female
Androsterone	1	1	19	1	20	1	18	1	20
	2	1	2	1	2	1	2	1	2
Epiandrosterone	1	1	25	1	26	1	22	1	26
	2	1	3	1	3	1	3	1	4
Dehydroepiandrosterone	1	1	37	1	33	1	35	1	32
	2	1	5	1	5	1	7	1	4
Testosterone	1	1	32	1	31	1	30	2	33
	2	1	6	1	5	1	5	1	7
Control	1	1	1	1	+	+	+	1	1
	2	1	+	1	1	1	1	+	1

Table 3. *Thin-layer chromatography of androgens*

Chloroform or ether extracts of hydrolysed androgen sulphate fractions were examined by thin-layer chromatography. The experimental details are described in the text. Unless stated otherwise, results were obtained from both urines and liver slices.

Original androgen	Compound detected in hydrolysate	R_F value: Solvent *E*	Solvent *F*	Solvent *G*
Androsterone	Androsterone	0·68	0·89	0·56
Epiandrosterone	Epiandrosterone	0·48	0·63	0·55
Dehydroepiandrosterone	Dehydroepiandrosterone	0·53	0·69	0·59
	16α-Hydroxydehydroepiandrosterone*	0·27	0·69	0·49
Testosterone	Androsterone	0·69	0·89	0·56
	Testosterone†	0·59	0·84	—

* Identified in urine only.
† Identified in liver slice incubations only.

recorded in Table 3 is that of the reference compound. Only metabolites that gave strong spots on the chromatograms were identified. Minor metabolites, with the exception of 16α-hydroxydehydroepiandrosterone detected in urine after the administration of dehydroepiandrosterone, were

Table 4. *Androgen sulphate excreted in urines, determined by the Menini method*

The experimental details are given in the text. Each result was obtained from the urine of a pair of rats (i.e. equivalent to 160mg. of steroid dosed). + Indicates amounts of 100μg./24hr. Less than 100μg. was excreted during the third day.

		Androgen determined (expressed as mg. of its sulphate/24hr.)					
		Females			Males		
Steroid administered	Main androgen in hydrolysate	Day 1	Day 2	Total	Day 1	Day 2	Total
Androsterone	Androsterone	10·3	4·0	14·3	0·7	0·6	1·3
		4·5	1·7	6·2	0·5	+	0·5
Epiandrosterone	Epiandrosterone	7·4	0·5	7·9	0·5	+	0·5
		4·8	0·1	4·9	0·1	+	0·1
Dehydroepiandrosterone	Dehydroepiandrosterone	21·8	3·6	25·4	0·2	+	0·2
		16·2	1·9	18·1	1·9	0·2	2·1
Testosterone	Androsterone	11·7	2·0	13·7	0·7	0·3	1·0
		9·5	1·2	10·7	0·9	0·1	1·0
		7·3	0·5	7·8	0·2	+	0·2
		5·9	2·0	7·9	0·1	+	0·1

Table 5. *Formation of androgen sulphate by liver slices*

The experimental details are described in the text. Results in each line were from two animals.

		Conjugates formed (expressed as μg. of androgen sulphate)				
Amount of substrate (μg.)		0	150	300	600	750
Substrate	Sex of rat					
Androsterone	Male	3, 3	5, 17	4, 3	30, 59	20, 60
	Female	7, 11	63, 127	108, 167	190, 221	67, 88
Epiandrosterone	Male	3, 4	10, 12	14, 23	11, 13	23, 23
	Female	2, 5	17, 38	71, 129	304, 334	289, 335
Dehydroepiandrosterone	Male	9, 9	42, 41	52, 73	20, 48	8, 18
	Female	6, 12	45, 59	146, 194	201, 293	252, 313
Testosterone	Male	18, 26	108, 112	69, 101	28, 36	38, 52
	Female	22, 30	114, 145	127, 160	245, 288	94, 176

not identified. Most androgens were conjugated without other changes, in both the whole-animal and the tissue experiments.

Testosterone sulphate was not detected in the urine of animals dosed with testosterone. There was, however, a sulphate that on hydrolysis yielded a substance that was not a 17-oxo steroid, as judged by its failure to react with *m*-dinitrobenzene reagent. Its R_F values in solvent systems *E* and *F* were 0·45 and 0·41 respectively. Testosterone sulphate was detected in the tissue experiments, as was expected from the results of Schneider & Lewbart (1956). The major metabolite of testosterone in the sulphate fractions obtained from both the liver slices and urine was androsterone sulphate.

Determination of androgen sulphate in urine. The sulphate metabolites determined by the Menini method in the urines of rats to which androgens were administered by stomach tube are recorded in Table 4. No correction has been made for endogenous 17-oxo steroid sulphate.

The amounts of the total 17-oxo steroid sulphates of control urines from male and female rats were determined, and individual values were found to range from 4 to 8μg./24hr. for female rats and from 8 to 10μg./24hr. for males. Although it is possible that the administration of large amounts of androgens alters the pathways of excretion of endogenous steroids, this factor is unlikely to have contributed significantly to the sex differences reported. The results paralleled those obtained from radiochromatogram 'scans' in that the female rats excreted more androgen sulphate than did the males. The percentages of the dose of androgen found unchanged in the faeces of the rats used in the experiments of Table 4 were 13–35% for females and 4–25% for males.

Formation of androgen sulphate by liver slices. The

amount of androgen sulphate formed by liver slices is given in Table 5. The results resembled those obtained with whole animals in that slices from females formed greater quantities of androgen sulphates than did those from males.

DISCUSSION

The present investigation has shown that female rats excrete larger amounts of administered androsterone, epiandrosterone, dehydroepiandrosterone and testosterone as sulphuric acid conjugates than do male rats. It has also been shown that liver slices of female rats can conjugate androgens with sulphuric acid to a greater extent than liver slices of male rats. These results are in agreement with those of Roy (1956), who investigated the conjugation of dehydroepiandrosterone with sulphuric acid, and reported that crude enzyme preparations from the livers of female rats were consistently more active than were those from males. They also support the results of Rao & Taylor (1964, 1965), who, in an investigation of progesterone metabolism with liver homogenates, found that livers of female rats formed more cold-acid-hydrolysable metabolites than livers of male rats. The reason for the difference is not clear, although Roy (1958) has suggested that the sex difference in the ability to synthesize dehydroepiandrosterone sulphate (Roy, 1956) is due to differing amounts of the appropriate sulphotransferase rather than in the enzymes required to synthesize adenosine 3′-phosphate 5′-sulphatophosphate. Roy (1958) has shown that there is no difference in the synthesis of phenyl sulphate by preparations from livers from male and female rats. These results are in accord with the suggestion of Gregory & Nose (1957) that different sulphotransferases are involved in the synthesis of aryl and steroid sulphates.

A considerable number of sex differences in steroid metabolism of liver have been reported. Colas (1962) found that livers of male rats were more efficient in 16α-hydroxylation of dehydroepiandrosterone *in vitro*. Rubin (1957) reported on species and sex differences in the reduction of the 3-oxo group in androgens to 3α- and 3β-hydroxylated products in homogenates of rat, rabbit, mouse, hamster, guinea-pig and turtle liver. Sex differences in the reduction of the A ring (Leybold & Staudinger, 1959; Yates, Herbst & Urquhart, 1958; Forchielli, Brown-Grant & Dorfman, 1958), 3-oxo group (Bell, Popoola & Taylor, 1962; Rubin, 1957; Rubin & Strecker, 1961), 11-oxo group (Hubener & Amelung, 1953) and 20-oxo group (Hagen & Troop, 1960) have also been reported. Sex differences in glucuronide conjugation have also been reported by Rao & Taylor (1965).

The author thanks Professor W. Klyne for the gift of reference steroids from the M.R.C. collection, and Miss G. Hall for technical assistance.

REFERENCES

Baulieu, E. E., Corpechot, C., Dray, F., Emiliozzi, R., Lebeau, M. C., Mauvais-Jarvis, P. & Robel, P. (1965). *Recent Progr. Hormone Res.* **21**, 411.

Bell, E. T., Popoola, A. O. & Taylor, W. (1962). *Biochem. J.* **82**, 47P.

Calvin, H. I. & Lieberman, S. (1964). *Biochemistry*, **3**, 259.

Calvin, H. I., Vande Wiele, R. L. & Lieberman, S. (1963). *Biochemistry*, **2**, 648.

Colas, A. (1962). *Biochem. J.* **82**, 390.

Corker, C., Norymberski, J. K. & Thow, R. (1962). *Biochem. J.* **83**, 583.

Forchielli, E., Brown-Grant, K. & Dorfman, R. I. (1958). *Proc. Soc. exp. Biol., N.Y.*, **99**, 594.

Gregory, J. D. & Nose, Y. (1957). *Fed. Proc.* **16**, 189.

Hagen, A. A. & Troop, R. C. (1960). *Endocrinology*, **67**, 194.

Hubener, H. J. & Amelung, J. (1953). *Hoppe-Seyl. Z.* **293**, 137.

Kellie, A. E. & Wade, A. P. (1957). *Biochem. J.* **66**, 196.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

Leybold, K. & Staudinger, H. (1959). *Biochem. Z.* **331**, 389.

Menini, E. (1966). *Biochem. J.* **99**, 747.

Raggatt, P. R. & Whitehouse, M. W. (1966). *Biochem. J.* **101**, 819.

Randerath, K. (1963). In *Thin Layer Chromatography*, p. 111. Translated by Libmann, D. D. London and New York: Academic Press Inc.

Rao, L. G. S. & Taylor, W. (1964). *Biochem. J.* **90**, 30P.

Rao, L. G. S. & Taylor, W. (1965). *Biochem. J.* **96**, 172.

Roy, A. B. (1956). *Biochem. J.* **63**, 294.

Roy, A. B. (1958). *Biochem. J.* **68**, 519.

Rubin, B. L. (1957). *J. biol. Chem.* **227**, 917.

Rubin, B. L. & Strecker, H. J. (1961). *Endocrinology*, **69**, 257.

Schneider, J. J. & Lewbart, M. L. (1956). *J. biol. Chem.* **222**, 787.

Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 429. London: W. Heinemann (Medical Books) Ltd.

Yates, E. F., Herbst, A. L. & Urquhart, J. (1958). *Endocrinology*, **63**, 887.