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GENERAL REVIEW

TESTOSTERONE SULPHATE, ITS BIOSYNTHESIS, METABOLISM, MEASUREMENT, FUNCTIONS AND PROPERTIES

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SUMMARY

TS has been identified and measured in human urine and blood. In normal males urinary excretion ranges from 1·9 to 17 μg/24 h and in women from 1 to 4 μg. Much larger amounts have been found in some patients with rare tumours of the endocrine glands. In peripheral plasma of normal men, values range from 49 to 175 ng/100 ml, and one-tenth of this value has been claimed to be present in women.

TS is secreted by the normal human testis; the concentration in spermatic vein plasma is 1 to 4 μg/100 ml, and does not rise after HCG administration. In peripheral plasma of patients with testicular feminization or adrenal tumours, and in women with masculinizing disorders TS was higher than in normals. In these cases the TS in the spermatic, adrenal and ovarian veins, respectively, was higher than in the peripheral circulation, suggesting that TS was secreted by these abnormal testes, adrenal tumours and ovaries. However, determination of TS in urine or plasma seems, for the present, to be of little help in clinical diagnosis.

Most evidence indicates that the human testis forms TS via the free steroid pathway. The low rate of conversion indicates that the testicular enzyme systems are oriented towards the preservation of T in free form, available for secretion, and it is generally supposed that TS does not serve as a reservoir for easy conversion into free T.

During pregnancy TS increases ten-fold (in maternal plasma). At delivery it is higher in the foetus than in the mother, suggesting foetal secretion of TS. T is readily sulphurylated by several foetal tissues, the adrenals especially having a much higher capacity in the foetus than in the adult. The foetal testis does not sulphurylate T, which suggests that T or some metabolite(s) remains in free form and is involved in the genital development of the human male foetus. The placenta does not hydrolyse or aromatize TS. Most TS is retained in the foeto-placental circulation, some being transferred unchanged across the placenta to the maternal compartment. Part of the TS formed in the foetus is further metabolized as the conjugate.

TS has low androgenic potency and also haemolytic properties. It binds to albumin and transcortin but not to S.B.P. and has a longer M.C.R. than T. It is not hydrolysed by several enzymes that split other steroid sulphates. Species and sex differences in TS biosynthesis and metabolism have been reported. Further information is needed on a number of points, such as whether production alters in connection with endocrine function tests, and whether there is any diurnal variation or change linked with the menstrual cycle or with increasing age. Most of the available evidence indicates that TS occurs as the 17-sulphate. But it is conceivable that the enolic 3-sulphate exists at least in the circulation. It is not yet known whether TS is present in bile. Finally, it seems that the physiological role of TS needs further investigation.

INTRODUCTION

Although steroid sulphates* are attracting ever-increasing interest, their biological role is still not well understood. Testosterone, the main androgen in the male, also occurs as the sulphoconjugate. However, most information concerning testosterone sulphate is scattered in the literature and no résumé encompassing all aspects of the subject has appeared. A review of the literature is expected to be useful in the light of some conflicting results, new methodology, and the number of questions still unanswered. Some physicochemical aspects and chromatographic data are also included, as they may be of use to investigators working with other steroid sulphates.

*Steroid sulphates are denoted with S, *e.g.* oestradiolS.

Biosynthesis and metabolism of testosterone sulphate

1. In vitro *studies with human tissue preparations.* Most *in vitro* studies on the metabolism of testosterone sulphate (TS) have involved incubation with preparations of steroid-producing or neoplastic tissues of varied origin.

Adrenals. In 1963, Lebeau and Baulieu[1] demonstrated the ability of an androgen-producing adrenal tumour homogenate to sulphoconjugate corticosteroids. When this homogenate was incubated with testosterone (T), conversion to TS was 80%. Adams

Table 1. Biosynthesis of TS. Incubation of T with tissue preparations

Test system	Species	References
Normal adrenal (adult, foetal)	Human	2,3,4,9,32,33,34
Adrenal tumours	Human	1,4,5+,9
Normal testis	Human	6++,7
Normal liver (adult, foetal)	Human	8,9,32,33
Cirrhotic liver	Human	9
Jejunum (adult, foetal)	Human	9,32,33
Liver	Rabbit	19,20,21,22
Liver	Guinea-pig	24
Liver	Rat	22,23
Liver	Bovine	25
Prostate	Canine	26,27

5+ Substrate: Progesterone.
6++ Substrate: Also DHAS.

[2, 3] showed that adrenal glands have the capacity to sulphate steroids. A wide range of 3α- and 3β-hydroxysteroids could be sulphated. When T was incubated with adrenal homogenate tissue at 37°C and pH 7·4, it was converted to TS with a "relative sulphation degree" of 0·39 (DHA = 1·0). However, pH 7·4 is not perhaps the optimum for this conversion [9]. When three normal adult adrenal tissues and one adrenal adenoma were incubated with 17 different steroids at pH 6·8, the sulphoconjugating ability was lower than for T with 17-hydroxy-DOC, cortisone, cortisol, aldosterone, and corticosterone, but higher with oestrone, DOC, oestriol, androsterone, pregnenolone, epiandrosterone, aetiocholanolone and DHA [4]. All these investigations, performed with the supernatant fraction in air, clearly demonstrate that the human adrenal, whether normal or diseased, is able to sulphate T. When incubating slices of a virilizing adrenal tumour with progesterone—at pH 7·4 in air—, Dixon *et al.*[5] obtained TS. As this product had not been anticipated, the yield was not calculated (see Note I).

Testis Incubation of [4-^{14}C]-T in air with slices of histologically normal testicular tissue from a 19-year-old man produced less than 1% of [4-^{14}C]-TS (pH 7·4) [6]. With [7α-^{3}H]-DHA ammonium sulphate as substrate, [7α-^{3}H]-TS was isolated [6]. Incubation of [4-^{14}C]-T and [7α-^{3}H]-5-androstene-3β,17β-diol-3-sulphate with histologically normal minced testicular tissue from a 62-year-old man gave [4-^{14}C]-TS in a 0·4% yield. 77% of the [^{3}H]-5-androstene-3β,17β-diol sulphate was recovered unchanged and no tritiated TS was detected. When DHA was used as precursor, no TS was detected [7].

Other tissues. Boström and Wengle [8] studied the synthesis of sulphates of T and nine other common steroids by adult *liver* supernatants from patients with cholecystitis or cholelithiasis, having normal liver function and structure. The synthesis of TS was lowest among them, approx. 100 nmol/g liver, that of DHAS being 450. A comprehensive study of the distribution of steroid sulphokinases in adult tissues has been presented by the same authors [9]. Healthy tissues studied (supernatant, at pH 6·8, in air) were: adrenal, liver, kidney, ovary, jejunal mucosa and spleen. Pathological specimens included: cirrhotic liver, adrenal adenoma, hypernephroma, nephritic renal tissue and thyroid adenoma. Steroid sulphokinases were present in liver, adrenal, jejunum. T-sulph/se was found only in liver and adrenal (g, 107). T was sulphated at a lower rate than the other steroids studied (DHA, androsterone, aetiocholanolone, oestrone, DOC, 3α- and 3β-hydroxypregnan-20-one). In the pathological tissues, the enzyme activity levels did not differ significantly from those of normal tissues. No effect of sex or age on the tissue level of sulphokinase was demonstrable. Sulphation of T by homogenates (conditions as in 2,3) from *primary mammary* carcinomata and their metastases in the *ovary* and from cystic mastopathy could not be demonstrated, although these were able to sulphate DHA [10].

2. In vivo *studies in the adult human organism in normal and pathological conditions.* Horton *et al.*[11] studied the urinary excretion pattern following injection of [1, 2-^{3}H]-T into six normal male and four normal female subjects between 20 and 30 yr of age. 90% or more of the injected radioactivity was recovered within 24 h of administration. Of the T administered, 0·1% was present as a fraction hydrolysable at pH 1. In four of the males, 0·03% of the dose was isolated in this fraction as a compound identical with T in mobility in two paper chromatographic systems. However, it was not possible to establish defi-

Table 2. Biosynthesis of TS. *In vivo* experiments

Precursor	Subjects	Route of administration	TS found in	References
T	Normal and diseased men and women	peripheral i.v.	urine	11,12,47,48
T	Normal man	portal vein	liver vein	13
TS	Normal men	peripheral i.v.	urine	14,15,17,18
TS	7-year-old boy with adrenal tumour	peripheral i.v.	urine	15
Androstenedione	Diseased man and woman	peripheral i.v.	urine	17,18
DHAS	Normal man	spermatic artery	spermatic vein	99
TS, 17-epiT, androstenedione, T	Male dog	perfusion into isolated liver	perfusate	18

nitely that this radioactivity was derived from TS or that it was not a result of solvolysis of testosterone glucuronide (TG). Identical results with regard to TS formation *in vivo* were obtained in four normal men and five women between the ages of 19 and 38 yr after administration of [4-^{14}C]-T [12].

[7α-^{3}H]-T injected into the portal vein of a 40-year-old man undergoing abdominal surgery was extensively and rapidly metabolized and sulphoconjugated [13]. Two-thirds of the sulphation occurred within 5 min. In samples from one of the liver veins 56% of the sulphate fraction consisted of TS, whereas only 0·03% of the total radioactivity excreted in the urine was associated with TS.

In seven female patients the mean excretion value of TS was the same as in normal subjects [12]. In four male patients (diagnoses: Klinefelter syndrome, eosinophilic adenoma of the pituitary, virilizing hepatoma, congenital adrenal hyperplasia) the mean value of TS was slightly lower, 0·02% of the injected dose. No definite proof that T is excreted as the sulphate conjugate was obtained, because minimal amounts of radioactivity corresponding to T were liberated after solvolysis [12]. Baulieu *et al.*[14] reported that after i.v. injection of 1 μCi of [^{14}C]-TS into a normal man, 3·5% of the dose was excreted in urine in 5 days. No [^{14}C]-androsterone or [^{14}C]-5β-androsterone glucuronide was found in the urine. Similar results were obtained after i.v. administration of [7-^{3}H]-TS to four normal adult males; 3–5% of the tritium injected was recovered in the urine as TS [15]. Calculations based on a number of assumptions suggested that about 1% of the plasma T is converted to plasma TS and 0·15% of plasma TS is converted to plasma T. Thus, in normal subjects the production and secretion of TS is likely to be insignificant. However, in a similar experiment in which [7-^{3}H]-TS was administered to a 7-yr-old boy with a virilizing adrenocortical tumour, the proportion of the injected radioactivity found in the urine as TS was 11% [15].

Knapstein *et al.*[16] simultaneously perfused [^{14}C]-T and [^{3}H]-T-[^{35}S]-sulphate through the liver of a 65-yr-old woman undergoing surgery for carcinoma of the rectum. They also administered [^{14}C]-T and [^{3}H]-TS simultaneously i.v. to a 60-yr-old woman with carcinoma of the cervix, and found that before metabolism of the steroid moiety most of the free [^{14}C]-T was rapidly esterified, mainly to sulphuric acid. Within 3 min after i.v. injection, more that 65% of the plasma T was sulphoconjugated. The endogenous [^{14}C]-T sulphoconjugate underwent extensive metabolism, hydrolysis and reconjugation. Part of the radioactive TS perfused through the liver underwent "direct" metabolism in rings A and D without splitting of the ester bond. Tamm *et al.*[17, 18] found that 0·6% of androstenedione (20 mg) administered i.v. to a 37-yr-old Addisonian male was excreted in the urine as TS, which rose from 10 μg (control) to 134 μg/24 h. In a similar experiment on a 44-yr-old adrenal-ovariectomized patient (disseminated mammary carcinoma) smaller concentrations (0·001%) of T were detected in the sulphate fraction. Infusion of 15 mg of TG into a 24-yr-old normal male was followed by a lower TS concentration in the urine (from 15 to 5 μg/24 h) with concomitant higher excretion of free T and TG. After infusion of 20 mg of TS to a 67-yr-old male the urinary TS on the second day was 13 μg/24 h (control: 6 μg/24 h). The above results [17, 18] were obtained after administration of comparatively high doses of steroids and may represent only metabolic transformations achieved under extreme conditions.

Lipsett *et al.*[47, 48] administered 10 μCi of [7α-^{3}H]-T i.v. to a patient with metastatic interstitial cell carcinoma. Urinary TS had an S.A. of 31 d.p.m./μg, whereas urinary TG had an S.A. of 146 d.p.m./μg, meaning that 80% of the urinary TS was not derived from plasma T. The urinary TS must have been diluted with TS that was not derived from plasma T. This TS probably originated from the tumour. Oertel *et al.*[99] injected [7α-^{3}H]-DHAS into the spermatic artery of a 52-yr-old patient undergoing a hernia operation, and detected 2% of the [^{3}H] activity in the spermatic vein blood in the form of sulphoconjugated T (see Note II).

3. *Studies in other species.* The biosynthesis and metabolism of TS has been studied in several other species besides man. However, these studies did not include sick animals and were conducted mainly *in vitro*.

Liver. Schneider and Lewbart[19] incubated 32 steroids in air with a rabbit liver microsome-free fraction. After incubation, 14 of the steroids, including T, were found to be conjugated with sulphuric acid. This experiment was one of the first in which steroid sulphoconjugation was reported at other than C_3. Nose and Lipmann [20] confirmed that male rabbit liver steroid sulphokinase (from the supernatant fraction) sulphates T with one-quarter the activity for DHA (11 nmol of TS formed per 1·7 mg of protein). Similarly, Roy[21] found that the enzyme in male rat liver conjugates T at the same relative rate. In addition, paper electrophoretic evidence has been obtained for the biosynthesis of TS from T [22] by microsome-free preparations of rat and rabbit liver. The ability of female rat liver supernatant to sulphurylate T and some of its synthetic derivatives has also been investigated [23]. Approximately the same ability to form sulphoconjugates was found for 19-nor-T, 17α-ethinyl-19-nor-T and T, but much lower for 17α-methyl-T and 17α-ethyl-19-nor-T, and almost double for 17-epiT. DHA and oestrone sulphotransferases of guinea-pig liver have been partially separated in the microsome-free fraction [24]. With T as sulphate acceptor, the situation was not clear. The rate of formation of TS was much lower than with the other steroids tested and the activity was associated with the DHA sulphotransferase [24, 110]. In an *in vivo* experiment [18] in which the isolated liver of a male dog was perfused with 300 mg of TS, 17-epiT, andro-

stenedione and T, TS was found in the perfusate in all cases, but only in trace amounts when T was perfused.

In a survey of the synthesis of DHAS, oestroneS and TS by twenty bovine adult tissues, only the liver supernatant exhibited the ability to transform T to TS [25].

Testis, prostate. Harper *et al.*[26, 27] have presented evidence indicating that TS is formed (but only 0·1%) in incubations of T with minced prostatic tissue of normal dogs. No conjugation was found in experiments with tissue from an old animal. Synthesis of TS from T could not be demonstrated in bovine testis or prostate [25]. Rat testis was found not to contain TS [96], nor was TS found in boar testis, despite a careful search [97] involving g.l.c.–m.s. In the last case, TS, if present, must be less than 0·5 μg/100 g wet tissue [97]. Earlier tentative identification of trace amounts of TS in boar testis [98] was based on chromatographic evidence without I.R. confirmation. Rat testis steroid sulphatase activity has been assessed by Notation and Ungar[28]. No significant cleavage of the 17β-sulphate group occurred in either saline- or HCG-treated animals when TS was used as substrate.

Fish. Only a few attempts to identify TS in blood plasma of fish have been reported, mainly by the Halifax Laboratory of the Canadian Fisheries Research Board. T was detected after solvolysis in a sample of salmon blood plasma [102]. Solvolysis of blood plasma from elasmobranchs (Raja species), after treatment with β-glucuronidase, released T [103]. If the T released by solvolysis was assumed to have been present in the blood originally as TS (this was not, however, established unequivocally), the amounts present in male specimens varied from 0·1 to 0·5 μg/100 ml plasma. It should be noted that free T was 3–20 μg/100 ml and TG 3–10 μg/100 ml in the plasma of these fish. For a synopsis see Tables 1 and 2.

Metabolism of testosterone sulphate. Wu and Mason[29] studied the hydrogenation of TS by the female rat liver microsomal 4-en-3-oxo-reductase. TS was converted predominantly to 17β-hydroxy-5α-androstan-3-one sulphate ester. Enzymatic activity was greater with TS than with T, especially at pH 5·5–7·0. TS incubations with supernatant and microsomal fractions of male rat liver demonstrated that TS is more readily metabolized by microsomal 4-ene-5α-reductase than by soluble 4-ene-5β-reductase and also that TS is not a good substrate for microsomal hydroxylases [30]. With female rat liver preparations TS also undergoes substantial metabolic transformations as the conjugate [31]. In incubations with 105,000 *g* supernatant in air, TS was a good substrate for 4-ene-reductases and 3α-hydroxysteroid oxidoreductase. After solvolysis, the metabolites identified were: hydroxylated polar steroids (4%), 5β-androstane-3α,17β-diol (14%), 5α-androstane-3α,17β-diol (41%) and unchanged T (16%). Formation of the 5α-steroid was not observed when TS was incubated with male rat liver supernatant. Incubation, in air, of TS with a microsomal fraction from female rat liver revealed metabolism by 4-ene-5α-reductase and 3α-hydroxysteroid oxidoreductase and hydroxylase. TS was converted to hydroxylated polar steroids (47%), 17β-hydroxyandrostan-3-one (16%) and 5α-androstane,3α,17β-diol (12%). TS incubation under a carbon monoxide atmosphere with the microsomal fraction of female rat liver produced 22% hydroxylated polar steroids, 40% 5α-androstane-3α,17β-diol and 13% 17β-hydroxy-5α-androstan-3-one (see Note III).

4. In vitro *studies in the foeto-placental unit.* Wengle [32, 33] screened the following foetal tissues (14–20 week) for steroid sulphokinases: adrenal, liver, kidney, cerebrum, jejunum, choroid plexus, skin, heart, lungs, skeletal muscle and thymus, but not testis. As the same author found in adult tissues [9], T-sulphokinase activity was present in the supernatant only in the adrenal, liver and jejunum. The activity was again the lowest of all the steroids tested [see 9]. Foetal tissues had a higher T-sulphokinase activity than the corresponding adult tissues. Neither 17α-methylT nor 17α-ethyl-19-nor-T was sulphurylated by foetal liver extract, although this did sulphurylate 17α-ethinyl-19-nor-T and 19-nor-T. Confirmation of some of these results was obtained by Jaffe and Payne[34]. Adrenal tissue homogenate from a male foetus (14th week) sulphated 38–80% of T; foetal testis tissue was unable to form TS [34]. Bovine placental tissue is also unable to synthesize TS from T [25].

French and Warren[35] found steroid sulphatase activity(ies) in the microsomal fraction of the placenta. However, the placenta is incapable of hydrolysing TS (the same is true of whole homogenate). Moreover, no evidence was found [36] of direct aromatization of TS to oestradiol-17β by fresh term placental microsomes. This is in accord with the observation that TS is not an oestrogen precursor in the pregnant female *in vivo* [14]. After injection of [^{14}C]-TS into a woman 9 months pregnant no radioactive oestrogens were found in the urine, as would have been the case if T had been liberated *in situ*. Thus, placental sulphatase is unable to split the 17β-hydroxysteroid sulphate.

Binding of testosterone sulphate to plasma proteins. Metabolic clearance rate (MCR). plasma half-life ($t_{\frac{1}{2}}$)

Wang and Bulbrook[37] have studied the binding of DHAS, TS, 17-acetoxypregnenoloneS and pregnenoloneS in human (women with benign breast tumours), rat and rabbit plasma. All showed a high capacity for TS binding. The percentage binding was higher in human than in rabbit or rat plasma. These studies also suggested that DHAS, TS and pregnenoloneS are bound to the same sites. On the basis of the displacement of DHAS by non-steroidal organic sulphates, they concluded that the binding sites involved are non-specific for steroid sulphates. TS binds to partially purified human corticosteroid-binding globulin at 4°C [38]. The order of decreasing affinity

of the other compounds studied was: cortisol, cortisolS, T, TS and TG. It was also stated that TS *does not bind* to sex steroid-binding plasma protein. Wang[39] investigated the half-life of TS in two women with advanced breast cancer. TS had a long $t_{\frac{1}{2}}$ (205 and 207 min) and a low miscible pool (4·4 and 5·2 l). The MCR values were 21 and 25 l/day. Orally administered, TS was better absorbed into the peripheral circulation than orally given T. In a similar study [40] on man, rat and rabbit, steroid sulphates were found to have a much lower MCR than the corresponding free steroids. This difference arises from the longer $t_{\frac{1}{2}}$ and lower distribution volumes of the sulphates. The MCR of TS in rabbits was 70–80 l/day and in rats 39 l/day (values for whole blood). As it is not known whether TS is a naturally occurring compound in rat and rabbit, some errors may be associated with the calculations for these species. The $t_{\frac{1}{2}}$ of TS in a woman with carcinoma of the cervix was more than 50 min, considerably longer than that of T [16], which was 8·5 min. Thus, the results of these studies were in good accord.

In four normal adult males, the MCR of plasma TS was 50–89 l/m^2/24 h and 203 l/m^2/24 h in a 7-yr-old boy with a virilizing adrenocortical tumour [15]. Direct comparison of the above results is difficult, because Wang *et al.* measured the whole blood MCR, whereas Saez *et al.* determined the plasma MCR. In Wang's work the disappearance curve of the radioactive TS could be represented by two exponentials ($a_{\frac{1}{2}}$ less than 8 min; $b_{\frac{1}{2}}$ approx. 210 min). In Saez's work the disappearance curve must be represented by at least three exponentials, therefore the metabolism and transport of TS must be described by a three-pool model.

Measurement of TS in both plasma and whole blood in one normal adult male indicated that TS is almost entirely in the plasma [15].

Identification and measurement of testosterone sulphate

1. *Urine of normal subjects.* Dulmanis *et al.*[41] measured urinary T by a double-isotope-derivative technique after incubation with HCl at 40°C and pH 1·0. The "pH 1 conjugate" had a range of 3–164 μg/24 h in 19 normal males and 3–62 μg/24 h in 19 normal females. They stated that the nature of the "pH 1 conjugate" was unknown. The values reported by them seem very high in all cases, in the light of subsequent reports. This suggests that separation of the steroids from interfering compounds was incomplete [see discussion in 47]. Lower values for a "pH 1 hydrolyzable T conjugate" were reported by van der Molen *et al.*[42]. Using g.l.c. with electron-capture detection and measuring the chloroacetate derivative, they detected "pH 1 hydrolyzable T" in urine from normal subjects. The amounts of T in this fraction were 3–12 μg/24 h for 7 males and 0·8–4·5 μg/24 h for 23 females. However, no further attempts were made to identify the conjugate. Owing to the high specificity of the technique used, the results must be regarded as accurate. Voigt *et al.*[43] found that in two instances, after hydrolysis with β-glucuronidase, additional dioxan solvolysis liberated further T from male urine in amounts of 15–17 μg/24 h. Although they did not identify the conjugate from which the additional T was liberated, they suggested that it was probably the sulphate conjugate of T and this soon proved correct. Stronger evidence for the presence of TS in the urine of normal males was presented in 1966 by Dessypris *et al.*[44] and in 1967 by Tamm *et al.*[45]. The technique of Dessypris *et al.* involved extraction of the free steroid, solvolysis of the sulphates by a modified Burstein-Lieberman procedure, t.l.c. and paper chromatography, followed by quantification by g.l.c. (flame-ionization detector). Positive identification of the steroid moiety was obtained with various physical methods (I.R. and U.V. spectroscopy) and an enzymatic technique (conversion of the unknown hydrolysed steroid to oestradiol-17β with a placental microsomal aromatizing preparation). Tamm *et al.* identified the urinary TS from a 51-yr-old male with a healthy endocrine system, on the basis of its identical R_f value with authentic TS in paper chromatography, positive methylene blue reaction, and U.V. contact photogram after paper chromatography. The urinary and reference TS were also shown to have identical absorption spectra in concentrated sulphuric acid.

With various techniques, the urinary TS excretion in normal males has been found to range from 1·9 to 17 μg/24 h (values in terms of T) [41–45].

2. *Urine of patients.* Saez *et al.*[15] reported a high value of TS (213 μg/24 h) in the urine of a 7-yr-old boy with a virilizing adrenocortical tumour and very high excretion of 17-KS. The highest TS excretion in disease was reported by Echt and Hadd[46] in a patient with a metastatic hilus cell tumour of the ovary (up to 4428 μg/day). The TG was only one-tenth of the TS. Owing to the large amount of substance excreted, U.V. spectroscopy was used for its quantification. Lipsett *et al.*[47, 48] found greatly elevated values of T in the sulphate fraction (approx. 700 μg/day) of the urine of a 67-yr-old patient with a metastatic interstitial cell tumour and presented evidence that the tumour secreted TS. In contrast, only negligible amounts of TS (less than 1 μg/24 h) have been found in three hirsute women with normal TG excretion, as well as in four female patients with elevated TG values [49], whereas in two women with Cushing's syndrome no T was detected in the sulphate fraction of the urine [49] (see Note IV).

3. *Peripheral plasma of normal subjects and patients.* Saez *et al.*[50] measured T (double-isotope-derivative technique) in the sulphate fraction of human plasma. The evidence for sulphoconjugation of T is based upon the behaviour of the compound as the sulphate on alumina column chromatography, on its R_f value in paper chromatography and as the sulphate during solvolysis. The concentration of TS in normal subjects and in several patients groups is shown in Table 3.

Table 3. Concentration of testosterone sulphate (as testosterone) in peripheral vein plasma of normal subjects and patients

Ref.	Diagnosis	No. of subjects	TS ng/100 ml Mean-range
50	Normal males, 23–50 yr	9	108 (49–175)
51	Normal males	Pool of 4	120
99	Normal male, 52 yr, (iliac vein)	1	less than 200
50	Normal females, 18–34 yr	4	18 (10–28)*
50	Adrenocortical tumour, Female infants, 5/6–3 1/4 yr	4	388 (143–558)
50	Adrenocortical tumour, male, 41 yr	1	169
15	Adrenocortical tumour, boy, 7 yr	1	1870–220**
50	Feminizing testis, 15–24 yr	4	165 (46–246)†
50	Stein-Leventhal syndrome	6	68 (10–170)
50	Ovarian-dermoid cyst	1	100
50	Benign ovarian tumour	1	180
50	Krukenberg ovarian tumour	1	1635; 607††
50	Precocious puberty due to a virilizing embryonal hepatoma. Boy, 2·5 yr	1	832

* Values doubtful owing to high blank.

** 8 months after operation. (Ref. 111) [See also Note V].

† In one case, ten days after gonadectomy the value fell from 191 to 24.

†† Two determinations, 10 and 30 days after delivery.

4. *Adrenal and ovarian vein plasma of patients, tumour tissues.* The TS in the adrenal vein plasma of a 7-yr-old boy with a virilizing adrenocortical tumour was 6800 ng/100 ml at the time of surgery [15, 52, 111]; in three female infants with the same diagnosis (ages 1–1·7 yr) it was 373, 744 and 1437 ng/100 ml [50]. A 24-yr-old woman with the same disease had 84 ng/100 ml in the adrenal vein plasma. In six cases of Stein-Leventhal syndrome the TS in the ovarian vein plasma varied from 15 to 446 ng/100 ml, and in the case of a subject with an ovarian dermoid cyst the value was 374 ng/100 ml [50]. The TS content of the right adrenal carcinoma removed from the above-mentioned 7-yr-old boy [15, 52] was 0·05 μg/g tissue, the total amount in the whole tumour being 110 μg. In the case of a 3·5-yr-old girl with progressive virilizing adenocarcinoma the TS concentration was 7·3 μg/g tissue, the total content being 109 μg.

5. *Spermatic vein plasma and testicular tissue.* Using modern analytical techniques for the separation and identification of steroids (Sephadex column chromatography, combined g.l.c.–MS), Vihko and his associates have measured the concentration of TS in spermatic vein plasma from eleven normal subjects (ages 30–88 yr) and found values ranging from less than 1 to 4 μg/100 ml of plasma [53]. Blood samples were obtained during operation for hernia under local anaesthesia. TS was not demonstrated in the peripheral plasma of these patients, in contrast to the results of others [15, 50, 51]. The difference is due to the lower sensitivity of the g.l.c.–MS used by Vihko and his group [53] (lower limit of detection 1 μg/100 ml). In a similar study [54], TS was determined in spermatic vein plasma after administration of HCG (Pregnyl, Organon, 5000 I.U. daily) for 5 days before the operation. In this study the respective TS concentrations in the three subjects studied (ages 25, 30 and 44 yr) were less than 1, 2·5 and 6·5 μg/100 ml. Again, peripheral TS could not be detected before or after HCG administration. These results compare reasonably well to the value reported earlier [99] for TS (2·3 μg/100 ml) in the spermatic vein blood of 52-yr-old patient undergoing a hernia operation and subjected to perfusion of the testis with tritiated DHAS.

The TS concentration in spermatic vein plasma has been reported [50] in five cases with feminizing testes (ages 15–24 yr). The range was 1219–3973 ng/100 ml (peripheral TS 46–246 ng/100 ml). Ruokonen *et al.*[55, 56] found TS in cadaver testis tissue (7 adults) in concentrations between 1·2 and 140 μg/100 g of wet tissue. In six cases orchidectomized for prostatic cancer the TS concentrations ranged from 3·1 to 10 μg/100 g wet tissue and did not differ significantly from those found in normal subjects. The values in these studies [53–56] were not corrected for methodological losses. Recently, T [103] and TS [104] have been quantified in human seminal plasma. The importance of these findings is as yet unknown (see Note VI).

6. *Maternal plasma and foeto-placental unit.* In seven cases in the 2nd and 3rd trimester of pregnancy, the mean value of TS in maternal plasma was 183 ± 104 (S.D.) ng/100 ml (range 63–423) [57]. A gradual rise of TS concentration during pregnancy was noted and high levels just before delivery. The levels during pregnancy were at least ten times as high as during non-pregnancy [see 50]. The sex of the foetus seems not to influence the TS values. The concentration in cord arterial blood exceeds that in the umbilical vein, both being higher than the concentration in maternal peripheral plasma. TS in amniotic fluid just before term (from mothers with Rhesus incompatibility) in two male foetal pools was 178; 198 ng/100 ml and in two female pools 67; 141 ng/100 ml [57]. Diczfalusy and co-workers have published several reports [58–64] dealing with the formation of TS in the foeto-placental unit and its concentration in foetal tissues. Their main findings are: little, if any, TS is formed in the foetal liver. TS found there is carried by the foeto-placental circulation. It has been identified there only after injection of T into the intact umbilical circulation but not in perfusions of isolated foetuses. The foetal adrenals represent an important source of TS. There is also evidence that other foetal tissues (*e.g.* gastro-intestinal tract) are capable of sulphurylating T. After perfusion of male and female foetuses at mid-gestation with T and androstenedione, TS was a major metabolite in the adrenals and gastrointestinal tract, but absent from the liver, carcass and lungs. The formation of TS was not influenced by the sex of the foetus. The physiological rôle of high TS in the foetal circulation is as yet unknown-one possibility [57] is that in this way the androgenic potential of T is lowered [69]. This might be a defence mechanism against excess T, reinforcing the rise of testosterone binding globulin in pregnancy [105].

The inability of the placenta to hydrolyse TS [see 35] was confirmed. In mid-gestation, TS is not hydrolysed. TS is metabolized as a conjugate in the foeto-placental unit, partly by the adrenals to 11β-hydroxy-

TS and partly by the liver to 5β-dihydro-TS and 5β-androstane-3α,17β-diol-S. Some as yet unidentified metabolites in foetal liver were also reported.

The above results coincide in principle with the results obtained in *in vitro* studies with foeto-placental tissues [see 32–36]. However, it remains to be clarified whether the results obtained at mid-gestation are also applicable to the situation near term, in view of the much higher steroid output from the foetal adrenals and the change in pattern of conjugation during the last trimester of pregnancy. Also, it is not known whether the same pattern is valid in other types of pregnancies (e.g. twin or anencephalic or in those accompanied by jaundice).

The TS content of a luteoma of pregnancy–an unusual tumour that apparently regresses with the termination of pregnancy–that caused virilization in the mother and female infant was 0·37 μg/g tissue [65].

Various biological functions and properties

Some biological effects of TS have been observed. Most of them were found in connection with studies not specifically dealing with TS. A note of caution on the haemolytic properties of water-soluble androgens [66] also deals with TS. In the course of therapy for advanced cancer, sodium-TS given i.v. led to severe haemoglobinuria. Further, *in vivo* and *in vitro* studies confirmed that TS is a potent haemolytic agent. In rats and dogs i.v. and subcutaneous injection of TS produced intravascular haemolysis leading to death [66]. The haemolytic activity of TS and other T esters was interpreted as a consequence of the entry of the steroids at the lipid-aqueous interface at the surface of the erythrocyte. A later study [67], not dealing with TS, has related the haemolytic activity of 13 other T esters to their lipophilic character.

Maternal and sexual behaviour in rats has been elicited by stimulation of separate brain loci with TS [68]. This maternal behaviour included nest building, and retrieval and grooming of litters of young. Whether this effect is due to TS or to liberated T was not investigated.

The androgenic potency of TS has been investigated by subcutaneous and oral administration of the compound to castrated rats [69]. TS exhibited a very low potency compared with T-propionate, but the effect in reducing adrenal weight was comparable. In intact rats TS, unlike T-propionate, does not cause reduction in testis weight, but its effect in reducing adrenal weight is comparable. Unpublished results of Marx and Williams[see 39] (see Note VII) confirm the very slight androgenic activity of TS when tested in the chick comb assay or in the prostate growth maintenance test in the castrate rat. *In vitro*, TS affects human, mouse, rabbit and rat uterus muscles [70]. It produces motor or inhibitory effects which apparently depend upon the hormonal environment of the uterus.

Sodium-TS inhibits the malic dehydrogenase and succinoxidase of rat liver homogenates [71]. The results suggest an irreversible combination of enzyme and inhibitor.

Chemistry

1. *Preparation of testosterone sulphate.* TS has been synthesized by a variety of methods that utilize different sulphating agents. The compound was prepared by Holden *et al.*[72], using chlorosulphonic acid in pyridine and chloroform. This or procedures that were similar in principle have been used by several authors [30, 67, 5, 29]. Roy[73], using pyridine-sulphur trioxide in benzene, claimed good yields even on a 15 mg scale and greater convenience than with chlorosulphonic acid directly. This technique has also been used by several investigators [8, 32, 33, 35, 36]. Bernstein and associates[74] prepared TS, using sulphamic acid in pyridine. They reported preparation of the sodium, potassium and ammonium salts, and obtained yields of 34, 40 and 20%, respectively. A novel and rapid technique introduced by the same group uses the triethylamine-sulphur trioxide complex [75]. No difficulty has arisen in the sulphation of the 17β-OH group. Pyridine is used both as a catalyst and as a solvent. The 17β-sulpho-oxy-4-androsten-3-one triethyl-ammonium salt is isolated in at least 80% yield (recrystallized analytical quality). The process of Mumma[76] is based on the reaction of sulphuric acid with an alcohol and dicyclohexylcarbodiimide in a polar solvent such as dimethylformamide. A good yield (over 70%) is claimed under mild conditions, with practically no side reactions and easy isolation of the product. The amorphous product obtained did not give a sharp melting point.

2. *Preparation of radioactive testosterone sulphate.* The synthetic approach of Mumma[76] can also be used for the radiolabelled compound, since $[^{35}SO_4]^=$ can be used directly without prior preparation of chlorosulphonic acid or pyridine-sulphur trioxide. The technique of Levitz[77] for the synthesis of [6,7-^{3}H]-oestrone-[^{35}S]-sulphate, in which pyridine sulphate is formed by treating sulphuric acid with pyridine and the steroid is subsequently treated with this in the presence of 10% molar excess anhydride, was adapted for TS by several authors. [4-^{14}C]-TS was synthesized [15] by a modification of Roy's technique [73].

Radioactive TS can be purchased from the usual commercial sources. In the Radiochemical Centre (Amersham, England), both [^{3}H]- and [^{35}S]-labelled TS are prepared essentially by the method of Joseph *et al.*[74], *i.e.* heating T with sulphamic acid and pyridine under a vacuum in a sealed tube at 95°C for 2 h. During the synthesis of the tritiated material there is a drop in specific activity of 30–40% (personal communication of Dr. A. E. Kilner). Storage of the aqueous solution of 4-^{14}C-TS-K salt −20°C was suggested by French and Warren[35].

3. *Physicochemical constants. Melting point, in °C.* Sodium TS: 215 [72]; 223–225 (uncorrected) [57]; 213–215 [74]; 210–213 [95]. Potassium TS: 260 [74]; 266 [78]. Ammonium TS: 201–203 [74]. Triethyl

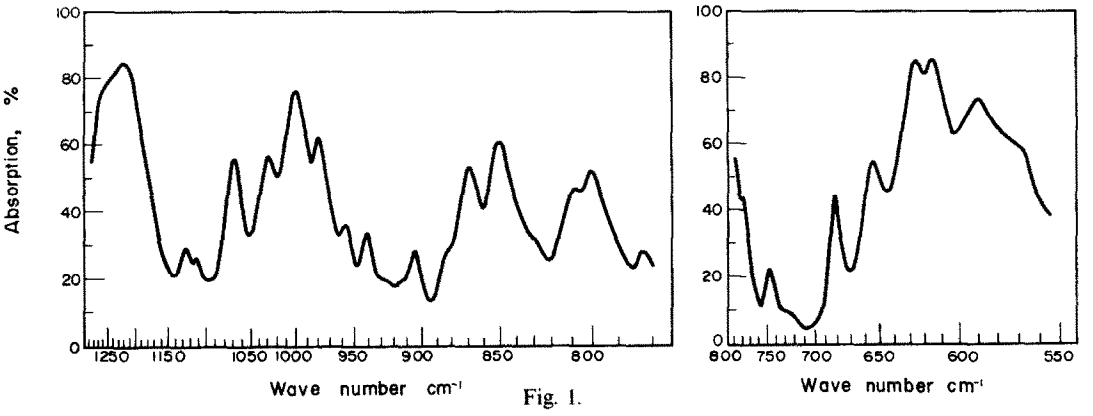

Fig. 1.

ammonium TS: 158–163 [75]. Sodium TS-3-semicarbazone [72].

$[\alpha]_D^{25}$. Sodium TS: +74·5° (1% ethanol) [72]; +74° [74]; +77·6° (1% ethanol) [57]; +83·5° (1% water) [57]. Potassium TS: +58° [74]; +54° (in water, 20°C) [40]. Ammonium TS: +70°C [74]. Triethyl ammonium TS: +64° (in chloroform) [75].

Absorption maximum (in nm)-Molar extinction coefficient. Sodium TS: (248–249) 20300 (in water) [72]; (240·5–241) 17700 (in ethanol) [72]; (240) 16300 (in methanol) [74]. Potassium TS: (240) 14800 (in methanol) [74]; (256) 15000 (in water) [40]. Ammonium TS: (240) 15000 (in methanol) [74]. Triethylammonium TS: (241) 16400 (in methanol) [75].

Infrared spectrum. The I.R. spectrum of the potassium salt of TS for the 550–1300 $cm.^{-1}$ region has been presented by Baulieu and Emiliozzi[78]. It is shown as recorded by M. F. Tayle (Fig. 1).

4. *Colour reactions.* The methylene blue reaction [79] is positive for TS [6, 7] and can be used for its location in chromatographic systems [20, 21, 25, 34].

The Zimmermann reagent gives the same bluish colour with TS as with T [78]. Other reagents used for the development of spots in chromatography are: 5% phosphomolybdic acid in ethanol (blue); 10% sulphuric acid in acetic acid (violet); Antimony trichloride (violet-rose) [for details of use see 95].

5. *Hydrolysis of testosterone sulphate. Chemical hydrolysis.* TS is quite stable to the hydrolytic action of barium chloride. After heating for 4 h on a steam-bath in a barium chloride-sodium acetate solution—the technique of Talbot *et al.*[80] for the hydrolysis of DHAS—only 6·5% of the theoretical weight was obtained [72]. Refluxing for 1 h with dilute HCl in the presence of toluene provided a 25% recovery [72].

Enzymatic hydrolysis. TS is not hydrolysed by the steroid sulphatase of the intestinal fluid of *Otala punctuata* (Mueller), a tropical terrestrial gastropod (African land snail) which hydrolyses DHAS [81]. Neither is it hydrolysed by ox liver steroid sulphatase [82] nor by the purified sulphatase of *Helix pomatia* [83]. In the last case, TS competitively inhibits the hydrolysis of DHAS, fixing on the enzyme at the same active site as the substrate [83] and having an affinity of the same order as the substrate. No hydrolysis is brought about by the enzymes of *Patella vulgata* [73, 83].

Solvolysis. The general procedure of solvolysis used for many steroid sulphates has been used for TS also and no special problems have been encountered. The original Burstein-Lieberman technique [84, 85] has been used by many authors [1, 5–7, 12, 15, 36, 44, 46, 48, 51, 52, 63]. Others [11, 13, 16, 17, 25, 29, 40, 45, 50] have used some of its modifications [86–89]. The potassium salt of TS is methanolysed in the presence of BF_3 (acting catalytically) at about 65°C in 2 h (90), and this time is shorter than in previously reported solvent systems. A milder reagent is dimethyl sulphoxide/water, requiring 6 h at 100°C and producing no detectable change in the structure or configuration of the parent steroid [90]. Direct solvolysis of TS eluted from chromatography paper was reported [6].

Pyrolysis on g.l.c. A single peak with a retention time matching that of the free compound was recorded when TS was chromatographed [25]. The elevated temperature of g.l.c. probably cleaves the sulphate ester bond.

Chromatographic techniques for the separation of testosterone sulphate from other steroid sulphates

1. *Paper chromatography.* A two-dimensional system (first direction: phenol–water (400:100) in an atmosphere of ammonia; second direction: butanol saturated with 2N NH_4OH) on Whatman No. 1 paper separates TS from other sulphates but not from DHAS, oestroneS or androsteroneS [10, 91, 3, 8].

The systems introduced by Schneider and Lewbart [19, 92] have been widely used [4–6, 8, 9, 32, 33, 35, 36, 40, 50, 52, 57]. The R_f values for a number of steroid sulphates in these systems are listed in Table 4. On account of the presence of large amounts of non-steroidal contaminants, the R_f values given are probably not absolute but rather indicate the relative positions, of the conjugates. Modifications of the above systems have been used in a number of the communications cited.

Partial separation of TS from other sulphates (Table 5) is also obtainable with the systems described in [78].

Table 4. R_f values of steroid sulphates in two paper-chromatographic systems, visualized with the phosphomolybdic acid reagent, (according to ref. 19)

Steroid	Alkaline system*	System II*
DHA	0·39	0·38
Androsterone	0·39	0·39
Epiandrosterone	—	0·41
Androstane-3α,17α-diol	0·39	0·39
5-Androstene-3β,17α-diol	0·36	0·36
17α-Methyl-5-androstene-3β,17β-diol	0·40	0·45
Androstane-3α,16β,17β-triol	0·045†	0·05
5-Androstene-3β,16β,17α-triol	0·045†	0·05
Testosterone	0·16†	0·20
Deoxycorticosterone	—	0·18
3β-Hydroxy-5,16-pregnadien-20-one	0·37	0·45
3β,21-Dihydroxy-5-pregnene-20-one	0·48	0·29
5α-Pregnane-3β,20,21-triol	0·27	0·24
5-Pregnene-3β,17α,20-triol	—	0·21
Pregnenolone	0·37	0·45

* The alkaline system consists of 0·2% aq. ammonium hydroxide-ethyl acetate-n-butanol (200:175:25 by vol.). System II consists of: n-butyl acetate-n-butanol-10% formic acid (80:20:100) by vol.).

† Visualized by the sulphate test.

2. *Glass fibre chromatography.* A system using two developments with chloroform–acetone–acetic acid (110:35:6) and silica gel impregnated glass fibre sheets separates TS from other steroid sulphates [93]. The free steroids move with the solvent front. The R_f of a number of sulphates is given in Table 6.

3. *Thin-layer chromatography.* t.l.c. on silica gel $HF_{254/366}$ (Merck) and a system of t-butanol-ethyl acetate-5N NH_4OH (41:50:20) gives R_f values of 0·32 for TS, DHAS, pregnenoloneS and 17α-hydroxypregnenoloneS (94). t.l.c. on an anion-exchange cellulose (MN -300 G -Ecteola, Macherey, Nagel) in a solvent system consisting of 4 M urea in 3 N NH_4OH gives R_f values for TS, DHAS, androstenediolS and 17α-hydroxy-pregnenoloneS of 0·68, 0·60, 0·45 and 0·45, respectively [94].

4. *High-voltage paper electrophoresis.* Matsui *et al.*[30, 31] used a solvent system of pyridine-acetic acid, at pH 6·4, and a 2 h run with 600 V. TS migrates closer to the cathode than TG, and T stays at the origin. Electrophoretic separation is also achieved [58] in veronal buffer, pH 8·6, on Whatman No. 3 MM paper with a running time of 19 h. Electrophoretic separation of 14 steroid sulphates on Whatman No. 1 paper with water–methanol and phosphate buffer of pH 7·4 is achieved in 6 h [95]. Other conditions of the system are 0·5 ma/cm. and 200 V. The R_f values obtained with 50 μg of material are given in Table 7. High-voltage paper electrophoresis (1500 V, 30 V/cm., 0·075 M sodium acetate–acetic acid buffer of pH 5·5, 1 h) was also used for the separation of TS from other steroid sulphates [4, 91].

5. *Counter-current distribution.* A system with 24 or 48 transfers using n-butanol-n-hexane-0·4 M NH_4OH (13:7:20) gives a partition coefficient for TS of 0·5 [58].

Table 5. R_f values of steroid sulphates in three systems (according to ref. 78)

Steroid	Systems* Isoamylic	B 5-10	B 23-9
7-Oxo-DHA	0·4	1	5
11-Oxo-aetiocholanolone	0·5	3	11
Testosterone	0·7	7	19
DHA	0·7	17	34
5-Androstene-3β,17β-diol	0·7	17	—
Epiandrosterone	0·7	22	—
Aetiocholanolone	0·7	24	38
Androsterone	0·7	29	42

* Description of the systems: Isoamylic [isoamyl alcohol-ammonia-water (55:27:18 by vol)], Whatman paper No. 1 at 24°C for 15 h. B 5-10 [ligroin-isopropyl oxide-t-butanol-ammonia-water (2:5:3:1:9 by vol.)] conditions the same as with isoamylique, but 36 h. B 23-9 [hexane-benzene-isopropyl oxide-t-butanol-ammonia-water (2:5:20:13:4:26 by vol.)], conditions the same as with isoamylic. For the last two systems the figures given in the table are not R_f values but cm/36 h.

Table 6. Mobility of steroid sulphates on silica gel glass fibre chromatography (according to ref. 93)

Steroid	R_C (Ref. OestroneS)	Mobility (cm)
Oestrone	1·00	11·6
Oestradiol (3-S)	0·80	9·3
Oestriol (3-S)	0·18	0·26
5-Androstene-3β,17β-diol (3-S)	0·70	8·1
DHA	0·89	10·3
5α-Androstane-3β,17β-diol (3-S)	0·72	8·3
Epiandrosterone (3-S)	0·91	10·6
17α-OH-Pregnenolone	0·73	8·5
Pregnenolone	0·96	11·1
Testosterone	0·72	8·4

System: chloroform-acetone-acetic acid (110:35:6 by vol.).

Table 7. R_f of steroid sulphates in the electrophoretic system of Grassman and Hannig (102) as reported by Cavina (95)

Steroid	R_f value
DHA	0·68
Androsterone	0·88
17α-Methyl-5-androstene-3β,17β-diol	0·88
5-Androstene-3β,17β-diol	0·77
DOC	0·79
Testosterone	0·80
Oestrone	0·78
Oestradiol	0·75
Cortisol	0·49

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NOTES ADDED IN PROOF

I. T-sulphokinase activity was observed in the cell-free supernatant of adrenal adenomata. It remained constant after storage of the frozen tissue for up to 6 months [106].

II. Hadd *et al.* [108] administered radioactive T and androstenedione to a patient with a metastatic cell ovarian tumour and found that when androstenedione was given the radioactivity of the urinary TS was 4–5 times as highas after T. This suggested that androstenedione is biogenetically closer than T to TS, or that sulphurylation of T was not the preferred route to TS.

III. Matsui *et al.*[109] confirmed their previous findings [30, 31]. Radioactive T and TS injected intraperitoneally into rats with bile fistulas were extensively metabolized and excreted predominantly in the bile. The metabolism of TS depended on sex, the biotransformation and conjugation of T/TS being different. T was excreted mainly as the disulphate. In the male, TS was metabolized mainly to 5α-androstane 3β,17β-diol, whereas in the female it was converted to 5α-androstane-3α.17β-diol and more polar steroids.

IV. A 9-year-old girl with a virilizing adrenal adenoma had a high excretion of TS, 93 μg/24 h [112].

V. Serial analyses of TS in two menstruating women (24-day and 28-day cycles) by g.l.c. [113], revealed a complex pattern of daily levels. The m.v. of TS in 10 women (20–35-year-old) was 18 ng/100 ml. [1–119].

VI. Slight sulphoconjugation of T by human semen, was demonstrated by incubation experiments [114]. It was measured by RIA in pooled human seminal plasma the concentration being 279 pg/ml [104]. Its physiological role remains to be ascertained. The large amounts of conjugated steroids in seminal plasma (including TS) may create artefacts in the measurement of free steroids, if the ejaculate contains steroid sulphatases [115].

VII. Since the activity of T is about 1000 times higher, this activity is almost certainly attributable to a very minor degree of hydrolysis of TS (see Wang-Bulbrook: *Advan. Reprod. Physiol.* **3** (1968) 113–146. Logos Press, London.)

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