The Extraction of Plasma 3-Hydroxy-17-oxo Steroid Sulphates and the Measurement of the Constituent Dehydroepiandrosterone Sulphate and Androsterone Sulphate

By JEAN MCKENNA* AND A. E. RIPPON†

Medical Research Council Pathology of Steroids Research Unit, Jessop Hospital for Women, Sheffield 3

(Received 19 June 1964)

1. A simple method for the extraction of 17-oxo steroid sulphates of plasma is described; glucosiduronates and orthophosphates are extracted, but to a smaller extent. 2. Four methods of analyses of the extracts are given and are relatively simple. Three of these are specific for steroid sulphates and two measure the sulphate conjugates directly. 3. Values for dehydroepiandrosterone sulphate and androsterone sulphate concentrations of normal and pathological plasmas are given.

There is considerable evidence that dehydroepiandrosterone sulphate is the major constituent of the known 3-hydroxy-17-oxo steroid conjugates of normal human plasma, and that androsterone sulphate and aetiocholanolone sulphate also occur (Baulieu, 1960; Conrad, Mahesh & Herrmann, 1961, and references therein). Free 3-hydroxy-17-oxo steroids and their conjugates with glucuronic acid are present at trace concentrations in normal plasma (Cohn, Bondy & Castiglione, 1961; Kellie & Smith, 1957). These concentrations are augmented under certain artificial and pathological conditions (Baulieu, 1960; Cohn *et al.* 1961). Evidence has also been presented for the existence in plasma of a phosphate complex of dehydroepiandrosterone (Oertel & Eik-Nes, 1959a; Oertel & Kaiser, 1962a).

It is with the extraction and measurement of the 17-oxo steroid sulphates that the present paper is mainly concerned.

Surface-active agents of type $C_nH_{2n+1}SO_4^-X^+$ have been estimated (Jones, 1945) by measurement of the methylene blue chromophore (λ_{max} 655 m μ) in chloroform after partitioning their methylene blue salts between water and chloroform in the presence of an excess of methylene blue chloride, acid and salt; this method was adapted by Roy (1956) for the estimation of the strongly anionic 3-hydroxy-17-oxo steroid sulphates. Partition coefficients favourable to the chloroform phase for the methylene blue salts of the alkyl sulphates are obtained only where the chain length exceeds six carbon atoms: methylene blue chloride is retained

in the aqueous phase. These findings prompted a study of partition coefficients in chloroform-aqueous systems of salts of relatively small cations with a variety of organic anions of biological interest.

The pyridinium salts of 3-hydroxy-17-oxo steroid sulphates had partition coefficients strongly in favour of the chloroform phase when partitioned between equal volumes of 0.3 M-pyridinium sulphate and chloroform (McKenna & Norymberski, 1960). In similar systems the small-anion sulphates (e.g. phenyl hydrogen sulphate) and the weak anion dehydroepiandrosterone glucosiduronate were retained substantially in the aqueous phase (J. McKenna & J. K. Norymberski, unpublished work). The utility of this extraction procedure has been demonstrated. Applied to small volumes of urine (20 ml.), it forms the basis of a method for the purification and estimation of urinary 17-oxo steroid sulphates (McKenna & Norymberski, 1960); applied to large volumes of urine, the isolation of oestrone sulphate from human pregnancy urine was accomplished (McKenna, Menini & Norymberski, 1961).

Application to plasma proved more difficult, and the present work deals with the modifications required and with the development of an analytical method for the estimation of the extracted plasma 17-oxo steroid sulphates in their conjugate form. This work has been briefly reported (McKenna, 1962).

MATERIALS AND METHODS

The term steroid sulphate refers to the half ester of sulphuric acid and the appropriate steroid alcohol.

Instruments. Hilger Uvispek and Unicam SP.600 spectrophotometers were used. Gas-liquid chromatography

* Present address: Department of Chemistry, University of Sheffield.

† Present address: Institut d'Endocrinologie, Université de Genève, Geneva, Switzerland.

was carried out on a Pye Argon Gas Chromatograph with a ^{90}Sr detector. The supporting material was Gas-Chrom P and the liquid phase S.E. 30. The column was maintained at 220°.

Reagents. Pyridine was refluxed and distilled over KOH. Dioxan and di-isopropyl ether were refluxed and distilled from CaH_2 . All other solvents except ethanol were AnalaR grade, and all except chloroform were redistilled. The methylene blue reagent (Roy, 1956) was diluted with 0.4 vol. of 50% (v/v) ethanol. Pyridinium sulphate was prepared by adding pyridine to 1 equiv. of aq. H_2SO_4 at the appropriate concentration.

Paper chromatography. The solvent systems B_3 [light petroleum–benzene–methanol–water (67:33:80:20, by vol.)] (Bush, 1952), E_4 [2,2,4-trimethylpentane–2-methylpropan-2-ol–methanol–water (20:9:9:2, by vol.)] (Eberlein & Bongiovanni, 1955), A [light petroleum–methanol–water (5:4:1, by vol.)] (Bush, 1952) and B_{23-9} [di-isopropyl ether–2-methylpropan-2-ol–*n*-hexane–benzene–aq. ammonia (sp.gr. 0.88)–water (25:16:37:62:50:45, by vol.)] (Baulieu, 1962) were used on Whatman no. 2 paper at 22°.

Reference compounds. Dipotassium dehydroepiandrosterone orthophosphate and sodium cortisone 21-sulphate were given respectively by Professor E. E. Baulieu and Dr J. R. Pasqualini of the Faculté de Médecine de Paris. The potassium and pyridinium salts of dehydroepiandrosterone sulphate, sodium androsterone sulphate and pyridinium oestrone sulphate were prepared by the general method of McKenna & Norymberski (1957).

Pyridinium 17 β -hydroxyandrost-5-en-3 β -yl sulphate was prepared as follows. Pyridinium dehydroepiandrosterone sulphate (41 mg.) was allowed to react in an aq. 33% (v/v) methanolic solution (15 ml.) with potassium borohydride (16 mg.) for 2.5 hr. at room temperature. (For the stereospecificity of this reduction, see Norymberski & Woods, 1955.) After neutralization with $\text{N-H}_2\text{SO}_4$ the reaction mixture was made 0.6 M with respect to pyridinium sulphate and extracted with chloroform (2 $\times$ 30 ml.). Evaporation of the chloroform, freed from water by freezing and filtration, gave pyridinium 17 β -hydroxyandrost-5-en-3 β -yl sulphate, m.p. 175–179°. The salt ran as a single methylene blue chromogen compound in solvent system B_{23-9} and was negative in the Zimmermann test. Proof of the sulphate moiety was obtained by elution of its methylene blue salt from paper by chloroform (Crepy & Judas, 1960), by a positive rhodizate test as described by Burma (1953) and by its specific solvolysis with dioxan in the absence of acid (the present paper). Androst-5-ene-3 β ,17 β -diol, released on dioxan solvolysis of the salt, was characterized by its negative Zimmermann reaction and by its conversion with *tert*.-butyl chromate reagent (Menini & Norymberski, 1962) into the expected products androst-4-ene-3,6,17-trione, 6 β -hydroxyandrost-4-ene-3,17-dione and 6 α -hydroxyandrost-4-ene-3,17-dione, identified by their R_F values (system B_3) and their characteristic properties in alkaline solution. The major product, the trione, was further characterized by its retention time on gas-liquid chromatography.

Pyridinium aetiocholanolone sulphate was prepared as follows. The 17-oxo steroid sulphates were extracted from normal female urine by the method of McKenna & Norymberski (1960) and resolved in solvent system B_{23-9} . Aetiocholanolone sulphate was eluted from paper as its methylene blue salt in chloroform (see the section on Determination of the 17-oxo steroid sulphates). The

methylene blue ion was transferred to the aqueous phase a methylene blue sulphate on shaking out the chloroform solution with 0.6 M-pyridinium sulphate (3 $\times$ 0.5 vol.) to yield pyridinium aetiocholanolone sulphate in chloroform. Dioxan solvolysis of the salt released a steroid having R values (solvent systems A and B_3) identical with those of an authentic specimen of aetiocholanolone: no other Zimmermann-staining spot was observed. Proof of the sulphate moiety was obtained as described for pyridinium 17 β -hydroxyandrost-5-en-3 β -yl sulphate.

Collection of plasma. Whole blood taken from normal subjects between 9 a.m. and 12 noon was treated with heparin and centrifuged. The plasma was withdrawn and stored at -5° . Blood-bank plasma was used in certain on the experiments.

Extraction of plasma. (a) Plasma (2 ml.) and 3 M-pyridinium sulphate (2 ml.) were shaken by hand (3 min.) with chloroform (25 ml.) and centrifuged, and the top layer was removed by suction. The thin solid layer was scraped aside and a sample of chloroform phase (20–23 ml.) evaporated to dryness to give residue A.

(b) The sample of chloroform phase (20–23 ml.) was shaken (1 min.) with 0.1 N-NaOH (5 ml.). A portion (4 ml.) of the alkaline phase was neutralized with 6 N- H_2SO_4 , made 0.6 M with respect to pyridinium sulphate and extracted with chloroform (6 ml.). A portion (5 ml.) of this extract was evaporated to dryness to give residue B.

Determination of the 17-oxo steroid sulphates. Whatman no. 2 paper was cut into lanes of 2.7 cm. each. The outside lanes were reserved for blank determinations and the central lane for a guide. Plasma extracts A or B were applied in chloroform to the remaining lanes. The extract for application to the guide strip was obtained from the appropriate processing of plasma containing added salts of androsterone sulphate and dehydroepiandrosterone sulphate. The papers were equilibrated overnight in solvent system B_{23-9} and run for 20–24 hr. On removal from the tanks, the papers were dried in air (for 30 min.) and the guide lane of each was cut out. This lane was treated with methylene blue reagent over rollers and immersed flat in a shallow dish of chloroform, when the ready elution of the formed methylene blue salts of the 17-oxo steroid sulphates showed their position as white bands on a blue background (Crepy & Judas, 1960). The corresponding areas were marked on the test and blank strips. Each strip was treated with methylene blue reagent, and the areas were cut out and immersed separately in chloroform (3 ml., for 3 hr.). The chloroform was decanted and washed with a saturated solution of Na_2SO_4 (0.25 ml.) and the intensity of the methylene blue chromophore measured (λ 655 m μ). Intensities were corrected for blank determinations and concentrations of the solutions were read from a calibration curve.

Calibration curve. Samples of pyridinium dehydroepiandrosterone sulphate (0.5–4.0 μg .) were partitioned in the chloroform–water–methylene blue reagent (3:1:1, by vol.) system and the intensity of the methylene blue chromophore in chloroform was read at 655 m μ against chloroform. Correction was made for reagent blanks determined concurrently.

Determination of released steroids. Residue A or B was dried in a vacuum desiccator overnight and incubated (at 38° for 6 hr.) with dioxan (2 ml.) (Cohen & Oneson, 1953; McKenna & Norymberski, 1957); chloroform (10 ml.) was added, the mixture was washed with 0.1 N-NaOH (1.5 ml.)

Table 1. *Transfer of dehydroepiandrosterone conjugates from aqueous phase into chloroform under various conditions*

The aqueous phase contained 10–15 $\mu\text{g.}$ of conjugate/ml. Experimental details are given in the text. All results are the mean of duplicates and are within the range $\pm 5\%$.

Aqueous phase	Aqueous phase/ chloroform ratio (v/v)	Percentage in chloroform		
		Sulphate ester	Phosphate ester	Glucosiduronate
Methylene blue reagent diluted with 3 vol. of water	1:5	100*	10*	10*
Saturated Na_2SO_4 in $\text{N-H}_2\text{SO}_4$	1:5	40	79†	55†
Saturated Na_2SO_4	1:1	< 10	< 10	< 10
1.5M-Pyridinium sulphate	1:1	100		85
	1:5	100	96	85
Methylene blue reagent without acid	1:5	100*	10†	10†

* Determined by methylene blue chromophore: all other values are obtained from Zimmermann estimations.

† These values are approximate: the small amount of methylene blue present in the chloroform contributes to the corrected estimation (λ 520 $\text{m}\mu$) in the Zimmermann reaction.

and water (1.5 ml.). The residue on evaporation of this solution was applied to paper as described for the sulphates and resolved in solvent system A. The method of development and elution was that of Brooks (1958). Determination of the eluted steroids was by the modified Zimmermann (1935) procedure (range 0.5–4.0 $\mu\text{g.}$) (Corker, Norymberski & Thow, 1962).

RESULTS

Transfer of steroid conjugates from aqueous solutions into chloroform (Table 1). An observation (E. Menini, unpublished work) that substantial amounts of 3-hydroxy-17-oxo steroid glucosiduronates were transferred into chloroform when urine was made 1M with respect to pyridinium sulphate, preferred molarity for transfer of steroid sulphates more polar than the group under consideration (J. McKenna & J. K. Norymberski, unpublished work), led to the investigation of the transfer of sodium dehydroepiandrosterone glucosiduronate in the present procedure. The transfer of dipotassium dehydroepiandrosterone orthophosphate was included in the investigation.

The sulphate, glucosiduronate and orthophosphate conjugates of dehydroepiandrosterone are transferred almost quantitatively to chloroform from 1.5M-pyridinium sulphate. That the sulphate conjugate is transferred as the pyridinium salt has been proved by isolation and identification of pyridinium dehydroepiandrosterone sulphate (J. McKenna & J. K. Norymberski, unpublished work); the form in which the other two conjugates is transferred was not known. In the system composed of chloroform and the acid methylene blue reagent (methylene blue chloride, sulphuric acid and sodium sulphate) all three conjugates were transferred to chloroform, although in varying

degrees. In the chloroform phase the sulphate conjugate (100%) can be accounted for in terms of methylene blue equivalents. In contrast, both glucosiduronate and orthophosphate conjugates are present in chloroform in much greater quantities (55 and 77% respectively by the Zimmermann chromophore) than their methylene blue equivalent would suggest (10%). The components of the methylene blue reagent were separately studied for their ability to effect the transfer. A saturated solution of sodium sulphate was ineffective in all three cases, but, when made 1N with respect to sulphuric acid, both glucosiduronate and orthophosphate conjugates were substantially transferred to chloroform and the sulphate conjugate was substantially retained in the aqueous phase. These results suggest that the steroid glucosiduronates and orthophosphates are transferred from water to chloroform in the form of the conjugates of the free acids, possibly in an un-ionized or dimerized state. By analogy, it is possible that this is also the form in which they are present in the chloroform phase of the pyridinium sulphate system.

In keeping with the experience that salt and acid were ineffective reagents in the transfer of the sulphate conjugate, it was found that methylene blue chloride alone or in the presence of salt gave a quantitative transfer of this conjugate to chloroform but in turn was ineffective for glucosiduronate and orthophosphate conjugates.

The observation that all three conjugates of a 17-oxo steroid can be transferred into chloroform from 1.5M-pyridinium sulphate solution, but only the sulphate conjugate from a methylene blue chloride-salt solution, forms the basis of a method (J. McKenna, unpublished work) for the isolation

of a pure 17-oxo steroid glucosiduronate fraction from urine (orthophosphates have not so far been detected in urine).

The alternative analytical procedures are summarized as follows:

Method A I. Plasma is made 1.5M with respect to pyridinium sulphate and extracted with chloroform. The concentrate of the pyridinium salts of the 17-oxo steroid sulphates so obtained is resolved on paper (solvent system B 23-9). The methylene blue salts of the individual steroid sulphates are formed *in situ*, eluted and measured colorimetrically.

Method A II. The concentrate of the pyridinium salts of the 17-oxo steroid sulphates obtained as described for method A I is solvolysed by dioxan. The released 17-oxo steroids together with any indigenous free steroids are resolved on paper (solvent system A), eluted with ethanol and estimated by the micro-Zimmermann reaction.

Method B I. The pyridinium 17-oxo steroid sulphate concentrate described for method A I is further purified by extracting its solution in chloroform with alkali, and the alkaline solution after acidification and addition of pyridinium sulphate again with chloroform. The concentrate from this second transfer procedure is then resolved and analysed as described for method A I.

Method B II. Dioxan solvolysis is carried out on the concentrate of pyridinium 17-oxo steroid sulphates from the second transfer procedure described for method B I. The released steroids, after resolution on paper (solvent system A), are eluted and estimated by the micro-Zimmermann reaction.

Plasma extracts and their purification. 17-Oxo-steroid sulphates can be transferred to chloroform from water or urine made 0.3M with respect to pyridinium sulphate (equal volumes); similar concentrations and volumes are ineffective with plasma systems. This may be consequent on or coincidental with the considerable precipitation of protein and emulsification that occurs; these obstacles are removed and transfer is accomplished by making the plasma 1.5M with respect to pyridinium sulphate and increasing the volume of chloroform for extraction (25:4, v/v). Our experiments were limited by availability of material to 2 ml. of plasma, and at this level all extracts of fresh plasma were suitable for direct application to paper (method A I) or for solvolysis (method A II). In a few cases of blood-bank plasma, material (presumed fatty) in the extract caused spreading on the chromatogram or incomplete solvolysis by dioxan. In these cases, back-extraction provided suitable samples for either analytical technique (method B I or B II). The latter extract contains considerably less methylene blue chromogen

material, although in some cases this is still in excess of the equivalent for the 17-oxo steroid sulphates present.

Dioxan solvolysis. The specificity of the dioxan solvolysis procedure for the acid salts of the steroid sulphates was tested by incubating with dioxan, dehydroepiandrosterone glucosiduronate and dehydroepiandrosterone dihydrogen phosphate obtained by extraction of solutions of their salts in 1.5M-pyridinium sulphate with chloroform (Table 1). No steroid was released in either case. Neutral steroid sulphates (e.g. sodium or potassium salts) were also stable to dioxan under the analytical conditions described in the Materials and Methods section. These neutral salts can be solvolysed at higher temperatures (McKenna & Norymbski, 1957).

Colorimetric measurements. The methylene blue estimation of steroid sulphates (Roy, 1956), modified as described in the Materials and Methods section, was found accurate for pyridinium dehydroepiandrosterone sulphate (0.5-4 μ g., Table 2). Under these conditions the extraction into chloroform of the methylene blue salts of dehydroepiandrosterone sulphate, androsterone sulphate and aetiocholanolone sulphate is quantitative and independent of the cation (Na^+ , K^+ , NH_4^+ , $\text{C}_5\text{H}_5\text{NH}^+$) in the aqueous phase [determined molecular extinction coefficients agreed with that for methylene blue chloride in chloroform ($83\,000 \pm 5\%$ at $655\text{m}\mu$)]. All of the above steroid sulphate salts can therefore be determined by the analytical procedure with reference to a single calibration graph (E/mole).

The spectra of the methylene blue steroid sulphates derived in the analytical procedures from biological sources are identical with those of the pure compounds.

Chromatography of 17-oxo steroid conjugates. Chromatographic mobility values in solvent system B 23-9 for sulphates of some C_{19} steroids with two

Table 2. *Proportionality and accuracy in the determination by methylene blue of pyridinium dehydroepiandrosterone sulphate*

Experimental details are given in the text. The results are given as means $\pm$ s.d., with the numbers of replicate determinations in parentheses. DHA, Dehydroepiandrosterone.

Amount of DHA sulphate (μ g.)	E_{655}/μ g. of DHA sulphate
0.5	0.060 $\pm$ 0.004 (5)
1.0	0.062 $\pm$ 0.005 (7)
2.0	0.062 $\pm$ 0.001 (7)
4.0	0.061 $\pm$ 0.001 (5)

oxygen functions are given in Table 3; the values for cortisone 21-sulphate and for the glucosiduronate and orthophosphate of dehydroepiandrosterone are included. The use of Whatman no. 2 paper and a lower temperature (22°) may account for the difference in running times reported by Baulieu (1962). The resolution of dehydroepiandrosterone sulphate and 17β-hydroxyandrost-5-en-3β-yl sulphate (a constituent of urine; Fotherby, 1958) is sufficient to ensure that, if the latter is present in plasma, it will not contribute to the dehydroepiandrosterone sulphate value (methods AI and BI).

Pyridinium dehydroepiandrosterone sulphate (5 μg.) and sodium androsterone sulphate (5 μg.) were chromatographed together, eluted as their methylene blue salts and estimated (see the Materials and Methods section). The mean recoveries in a series of six experiments were 98 ± 5% (s.d.) and 97 ± 4% (s.d.) respectively. Evidence that there is no overlap between these zones (important if aetiocholanolone sulphate is to be estimated) was obtained by applying the analysis to

the area between them. Values indistinguishable from blank values were obtained.

The possibility of a variable contribution to the methylene blue chromophore by materials in the paper or the solvent systems was tested as follows. After the usual treatment with methylene blue reagent over rollers, paper blanks equal in size to the steroid sulphate zones were cut randomly and divided into two groups (eight in each), which were eluted for 3 and 16 hr. respectively. The shorter elution time gave rise to a mean blank value and standard deviation in accord with those normally found in the methylene blue method. An increase of 15% in this mean value was obtained after 16 hr. elution.

Recovery of dehydroepiandrosterone sulphate from water by methods AI, AII, BI and BII. The analytical procedures described in the Materials and Methods section were applied to the recovery of pyridinium dehydroepiandrosterone sulphate (5 μg.) from water (2 ml.). The results are the mean of four experiments and are given with their standard deviations. Methods AI and BI led to recoveries of 96 ± 1% and 90 ± 1% respectively. Methods AII and BII gave recoveries of 87 ± 2% and 85 ± 2% respectively.

Recovery of dehydroepiandrosterone sulphate from plasma by methods AI, AII and BI (Table 4). The physiological concentration of dehydroepiandrosterone sulphate was determined by method AI for one sample of fresh plasma and one sample of blood-bank plasma. To the dry pyridinium dehydroepiandrosterone sulphate (at and above the physiological concentrations) pyridinium sulphate, plasma and chloroform were added in that order. Recoveries of the sulphate from fresh plasma approached the values obtained for water even at the lowest level of addition and were better (84–97%) than those obtained for blood-bank plasma (75–80%) or from bovine serum albumin (75–80%), which was included in the series.

Table 3. *Chromatographic mobility of some steroid conjugates in solvent system B23-9*

Experimental details are given in the text. Chromatographic mobility is independent of the cations of the salts.

Compound	Distance from origin after 24 hr. (cm.)
Cortisone sulphate	0
Oestrone sulphate	8
17β-Hydroxyandrost-5-en-3β-yl sulphate	13
Dehydroepiandrosterone sulphate	15.8
Aetiocholanolone sulphate	18.8
Androsterone sulphate	22.0
Dehydroepiandrosterone glucosiduronate	0
Dehydroepiandrosterone orthophosphate	0

Table 4. *Recovery of pyridinium dehydroepiandrosterone sulphate added to plasma*

The recoveries are given as the means of duplicate determinations, which agreed within ± 5%. Methods AI and AII are summarized in the Results section.

	Plasma concn. (μg./ml.)	Quantity assayed (μg./ml.)	Percentage recovery	Method
Fresh plasma	2	5	97	AI
		3.2	95	AI
		2.5	84	AI
Blood-bank plasma	1.5	5	72	AI
		3.75	72	AI
		2.5	77	AI
		1.25	84	AI
		0.625	80	AI
Aqueous 5% bovine serum albumin	0	2.5	80	AI
		2.5	75	AII
		0.8	75	AI

In addition, recoveries of various salts of steroid sulphates were carried out on a range of normal plasmas (Table 5). Here, with one exception, recoveries are in the range 61–104% (mean 70%). In general, recoveries of the pyridinium salts are greater than for the sodium or potassium salts. Since these latter salts are relatively insoluble in water, these results may arise from the mode of addition of the salt to the plasma.

Case 17 (Table 5) gave an atypical low recovery (45%) and was further investigated.

Effect of storage at -5° on plasma. Plasma from case 17 (Table 5), which gave a low recovery for added pyridinium dehydroepiandrosterone sulphate, was analysed before and after storage at -5° . The results are shown in Table 6. The three samples were withdrawn at intervals of several months.

The fall in values for dehydroepiandrosterone

occurring between the fresh and stored plasma (samples 17A and 17B) is of the same order (20%) and, coupled with the consistent values obtained on the stored plasmas irrespective of the length of storage (1–17 days), lead to the conclusions that the fall is significant, that it occurs immediately on storage and that further freezing and thawing have no effect. Other suitable cases have to be found for confirmation of these results. The androsterone fraction seems unaffected. It is also noted for this plasma after storage (sample 17C) that recovery of added steroid sulphate is affected by the level of addition (cf. Table 4). No comparable results are available for the unstored plasma.

Specificity of the analytical methods AI and BI. The methylene blue dehydroepiandrosterone sulphate (combined from 16 plasmas after analyses by method AI or BI) was converted into the

Table 5. *Plasma concentrations of dehydroepiandrosterone and androsterone ($\mu\text{g./100 ml.}$) for normal subjects and recoveries of added pyridinium dehydroepiandrosterone sulphate in terms of dehydroepiandrosterone ($\mu\text{g./ml.}$)*

Recoveries are given as the means of duplicate determinations (see Table 7). Methods AI, AII and BI are summarized in the Results section. DHA, Dehydroepiandrosterone; AND, androsterone.

	Case no.	Age (years)	DHA ($\mu\text{g./100 ml.}$)	AND ($\mu\text{g./100 ml.}$)	Method	Added DHA sulphate ($\mu\text{g. of DHA/ml.}$)	Percentage recovery of DHA
Females	1	21	100	15	AI	1.2	66
	2	17	120	66	AI		
	3	19	73	35	AI		
	4A	21	131	56	AI	3.2	97
	4B		170	70	AII	2.4	104
	5	29	81	20	BI	4.8	69
	6	24	209	111*	BI	4.8	80
	7	28	92	< 10	BI	2.4	70
	8	46	19	< 10	BI		
	9	58	< 10	< 10	BI		
	10	20	141	33	AII		
Range	...	...	...	...			66–104
Males	11	28	109	23	AI	3.6†	67
	12A	26	136	69	AI	2.4†	61
	12B		136	69	AI	2.3‡	65
	13A	48	27	< 10	AI	2.4†	70
	13B		27	< 10	AI	2.3‡	65
	14	24	110	< 10	BI	4	78
	15	30	101	15	BI	3.2	74
	16	22	38	< 10	BI	3.2	70
	17	30	167	68	AI	1.3	45§
Range	...	...	...	...			45–78
Pooled plasma	18A	22–48	121	48	AI	1.6	74
	18B		104	32	AII	1.6	71
Blood-bank plasma	19		58		AI	1.6	75

* Includes aetiocholanolone.

† Androsterone sulphate added as the sodium salt and expressed as $\mu\text{g.}$ of androsterone/ml.

‡ Dehydroepiandrosterone sulphate added as the potassium salt and expressed as $\mu\text{g.}$ of dehydroepiandrosterone/ml.

§ Subject of special study (see Table 6).

Table 6. *Effect of storage of a normal plasma at -5° on the concentrations of dehydroepiandrosterone sulphate and androsterone sulphate and on recoveries of added pyridinium dehydroepiandrosterone sulphate*

Values are given in $\mu\text{g. equiv.}$ of free dehydroepiandrosterone and androsterone. All results are the means of duplicate determinations. Methods AI, AII and BI are summarized in the Results section. DHA, Dehydroepiandrosterone; AND, androsterone.

Sample no.	Time of storage at -5° (days)	DHA ($\mu\text{g./100 ml.}$)	AND ($\mu\text{g./100 ml.}$)	Method	Added DHA sulphate ($\mu\text{g. of DHA/ml.}$)	Percentage recovery of DHA
17A	0	199	62	AI		
	14	167	68	AI	1.3	45
17B	0	230	71	AII	2.3	67
	5	181	70	AII	2.3	46
	17	178	78	AII	2.3	54
17C	1	145	63	BI	3.2	45
	12	143	63	BI	8.5	80

Table 7. *Precision of the methods AI and BI*

Methods AI and BI are summarized in the Results section. The standard deviation given is that between duplicate determinations. For $1 \mu\text{g. equiv.}$ of dehydroepiandrosterone, E is 0.095.

Operator		E determined		No. of experiments
		Range	Mean $\pm$ s.d.	
1	Blanks	0.016–0.039	0.029 $\pm$ 0.003	32
	Plasma	0.049–0.195	0.112 $\pm$ 0.004	22
		0.21–0.465	0.3 $\pm$ 0.008	20
2	Blanks	0.021–0.05	0.037 $\pm$ 0.006	40
	Plasma	0.067–0.205	0.171 $\pm$ 0.01	18

pyridinium salt (see the Materials and Methods section). In solvent system B23-9 it had R_F coincident with that of authentic pyridinium dehydroepiandrosterone sulphate and responded to tests for sulphate as described for pyridinium 17 β -hydroxyandrost-5-en-3 β -yl sulphate. The dry pyridinium salt was solvolysed by dioxan, and the released alcohol had R_F (solvent system A) identical with that of authentic dehydroepiandrosterone. Only one Zimmermann-staining area was observed. Similar evidence was obtained for the identity of the combined androsterone sulphate and aetiocholanolone sulphate. Androsterone (about $3 \mu\text{g.}$), aetiocholanolone (about $1 \mu\text{g.}$) and dehydroepiandrosterone (about $5 \mu\text{g.}$), released by dioxan solvolysis of the appropriate sulphate fractions, were oxidized by *tert.*-butyl chromate (Menini & Norymberski, 1962). The corresponding 5 α - and 5 β -androstanedione and androst-4-ene-3,6,17-trione showed the correct retention times and were pure by gas-liquid chromatography.

Precision of methods AI and BI. Standard deviations between duplicate determinations of the observed extinctions (methylene blue chromophore) were obtained from methods AI and BI and are given in Table 7. The limit of accurate

detection for method AI (2 ml. of plasma) lies at $25 \mu\text{g.}$ of 17-oxo steroid/100 ml. of plasma; for method BI, $35 \mu\text{g.}$ of 17-oxo steroid/100 ml. can be accurately determined. The ease of operation of the method is reflected in the degree of precision reached by operator 2 after a few weeks' training.

Normal plasma concentrations of dehydroepiandrosterone sulphate and androsterone sulphate. To obtain replicate analyses and replicate recoveries we were limited to 2 ml. of plasma for each analysis: values below $25 \mu\text{g.}$ of steroid/100 ml. of plasma (method AI) and $35 \mu\text{g./100 ml.}$ of plasma (method AII) are therefore approximate. Aetiocholanolone sulphate, whose presence in a number of plasmas was unequivocally demonstrated (see above), could not be estimated accurately.

The concentrations of dehydroepiandrosterone and androsterone for ten normal females and seven normal males are recorded in Table 5. Cases 8, 9 and 13 are normals over the age of 40 and show very low values for both steroids. The large spread for dehydroepiandrosterone (males, 38–167 $\mu\text{g./100 ml.}$; females 73–209 $\mu\text{g./100 ml.}$ of plasma) and for androsterone (males, < 10–69 $\mu\text{g./100 ml.}$; females, < 10–70 $\mu\text{g./100 ml.}$ of plasma) has been observed by other authors (Table 8).

Table 8. *Literature values for dehydroepiandrosterone sulphate, androsterone sulphate and aetiocholanolone sulphate concentrations of normal plasmas*

The age range of the subjects was 20–40 years. All values are expressed as $\mu\text{g. equiv.}$ of the free steroids, and are given as ranges, with the means in parentheses. The methods of Conrad *et al.* (1961) and Oertel & Kaiser (1962b) both require 10–15 ml. of plasma. DHA, Dehydroepiandrosterone; AND, androsterone; Aetio, aetiocholanolone.

Sex	DHA ($\mu\text{g./100 ml.}$)	AND ($\mu\text{g./100 ml.}$)	Aetio ($\mu\text{g./100 ml.}$)	No. of samples
Male*	121–232 (169)	22–72 (30)		7
Female	68–129 (94)	12–35 (19)		4
Male†	67–160 (101)	24–66 (45)	7–41 (28)	6
Female	44–106 (72)	24–46 (36)	16–34 (25)	6
Male‡	38–167 (110)	10–69 (32)		7
Female	73–209 (118)	10–70 (36)		7

* Conrad *et al.* (1961).

† Oertel & Kaiser (1962b).

‡ Present paper.

Table 9. *Diurnal variation of dehydroepiandrosterone sulphate and androsterone sulphate concentrations in plasma*

Values are given in $\mu\text{g. equiv.}$ of free dehydroepiandrosterone and androsterone. All results are the means of duplicate determinations by method AI, which is summarized in the Results section. DHA, Dehydroepiandrosterone; AND, androsterone.

	Time	DHA ($\mu\text{g./100 ml.}$)	AND ($\mu\text{g./100 ml.}$)
Case 1	5 p.m.	37	44
	9 p.m.	39	44
	1 p.m.	37	45
	5 a.m.	39	48
	9 a.m.	39	48
	1 p.m.	39	48
Case 2	4 a.m.	125	31
	8 a.m.	116	34
	12 p.m.	147	44
	4 p.m.	152	31
	8 p.m.	183	48

Case 1: female, simple hirsutism (confined to bed); case 2: normal male.

Diurnal variation of plasma concentrations of dehydroepiandrosterone sulphate and androsterone sulphate (Table 9). A female suffering from simple hirsutism and confined to bed during the 24 hr. period had plasma withdrawn at intervals of 4 hr. before meals. Samples were stored at -5° and analysed in one run in the next 24 hr. No diurnal variation was observed for either dehydroepiandrosterone or androsterone. Samples taken at 4 a.m. and 8 a.m. from a normal active male were stored at $0-5^{\circ}$ and analysed together with the 12 p.m. and 4 p.m. samples immediately on withdrawal of the 4 p.m. sample. Dehydroepiandrosterone in the two latter samples shows a slight

increase over the early-morning values. A further rise is observed in the dehydroepiandrosterone content of the plasma sample withdrawn at 8 p.m. and kept for 42 hr. at room temperature before analysis; the significance of this increase is difficult to assess in view of the different storage conditions of the samples.

Concentrations of dehydroepiandrosterone sulphate, androsterone sulphate and aetiocholanolone sulphate in pathological plasmas. In five cases of idiopathic hirsutism (ages 16–40) only two departed from the normal female steroid sulphate pattern. In one case the value ($114 \mu\text{g./100 ml.}$) for androsterone was above the normal range for either men or women; aetiocholanolone was high at $35 \mu\text{g./100 ml.}$ In this case the dehydroepiandrosterone value ($148 \mu\text{g./100 ml.}$) was normal, as it was in all five cases. One case had an atypical result of a reversed androsterone to dehydroepiandrosterone ratio (androsterone, $48 \mu\text{g./100 ml.}$; dehydroepiandrosterone, $38 \mu\text{g./100 ml.}$ of plasma). All values are given in free steroid equivalents.

A case of idiopathic fever was investigated, and the values for the plasma 17-oxo steroid sulphates before, during and after the pyrexial phase are recorded in Table 10, together with the corresponding values for the urinary 17-oxo steroid sulphates determined by the method (analogous to method A II for plasma) of McKenna & Norymberski (1960); these latter values are inclusive of free steroid. The values, however, are attributable to the sulphate fraction, since a corresponding run in the absence of pyridinium sulphate gave negligible values for the three 17-oxo steroids. Plasma determinations were by method AI, specific for sulphates.

The plasmas showed no gross variation in the constituent 17-oxo steroid sulphates, although there is a slight decrease in the dehydroepiandrosterone fraction during the pyrexial phase. Recoveries

Table 10. *Urinary and plasma 17-oxo steroid sulphates in a case of idiopathic periodic fever*

Plasma determinations were by method AI, which is summarized in the Results section. Recoveries (greater than 85%) of added pyridinium dehydroepiandrosterone sulphate were obtained for each plasma sample. DHA, Dehydroepiandrosterone; AND, androsterone; Aetio, aetiocholanolone.

	Time	DHA ($\mu\text{g./100 ml.}$)	AND ($\mu\text{g./100 ml.}$)	Aetio ($\mu\text{g./100 ml.}$)	$\frac{\text{AND}}{\text{Aetio}}$ ratio	$\frac{\text{AND}}{\text{DHA}}$ ratio
Plasma	4 days before pyrexial phase	137	52	15*		0.4
	Pyrexial phase	108	42	15*		0.4
	9 days after pyrexial phase	131	Not determined			
Urine	4 days before pyrexial phase	(mg./24 hr.) 2.5	(mg./24 hr.) 1.3	(mg./24 hr.) 0.8	1.5	0.5
	Pyrexial phase	6.4	1.8	1.1	1.6	0.3
	6 days after pyrexial phase	1.5	1.0	0.7	1.4	0.7

* Approximate results.

from all plasma samples of added pyridinium dehydroepiandrosterone sulphate (even at physiological concentration) were excellent (greater than 85%). In the urine, during the pyrexial phase, gross changes in the 17-oxo steroid sulphates occurred in an upward direction: the largest increase was in the dehydroepiandrosterone fraction (over twice the apyrexial value). The androsterone to aetiocholanolone ratio remained the same, but the ratio of androsterone to dehydroepiandrosterone fell to half the value of the pre- and post-pyrexial phases.

Evidence for the presence of more polar steroid sulphates in plasma. Examination of the developed guide strips (method AI) reveals the regular occurrence of sulphate bands in the more polar region of the chromatogram; less often the polar region of the guide strip (method BII) reveals a Zimmermann-staining area (less sensitive qualitative test). In one of these cases (simple hirsutism), extract equivalent to 1 ml. of plasma was solvolysed by dioxan and the released steroids were treated with Girard T reagent. The ketonic fraction was analysed by gas-liquid chromatography. Compounds with retention times the same as those of dehydroepiandrosterone, androsterone aetiocholanolone and 11β -hydroxyaetiocholanolone were observed.

DISCUSSION

A recent monograph (Oertel, 1962) presents in detail the methods so far available for the estimation of the individual 3-hydroxy-17-oxo steroids in plasma. In general, these methods are based on precipitation of the plasma protein and defatting of the supernatant liquid followed by acid and/or

solvolytic procedures for release of the 3-hydroxy-17-oxo steroids from their partially purified sulphate esters. After resolution on paper the steroids are estimated by the Zimmermann (1935) reaction. In the special case of dehydroepiandrosterone, estimation without resolution can be achieved by an ethanol-sulphuric acid reaction (Oertel & Eik-Nes, 1959b). Conrad *et al.* (1961) estimate the intact 3-hydroxy-17-oxo steroid sulphates as Zimmermann chromogens after purification on a Fluorosil column and resolution on paper. The number of manipulations and time involved in the extraction and purification procedures in these methods makes their employment on a routine basis difficult.

Applied to small (2 ml.) samples, method AI (the extraction of 17-oxo steroid sulphates into chloroform from plasma made 1.5 M with respect to pyridinium sulphate, followed by chromatography and estimation of the conjugates) provides a simple reproducible method for dehydroepiandrosterone sulphate and androsterone sulphate. Larger samples are required for the estimation of aetiocholanolone sulphate, and for dehydroepiandrosterone sulphate and androsterone sulphate at less than $25 \mu\text{g./100 ml.}$ of plasma. No technical difficulties arise in extracting larger volumes of plasma: any increase in the concentration of impurities in the extract can be dealt with by the back-extraction of the sulphates into alkali and re-extraction into chloroform (method BI). The specificity of these methods rests, first, on the separation of the conjugates in solvent system B23-9, and, secondly, on the specificity of the methylene blue estimation.

The extraction steps for methods AII and BII parallel those of methods AI and BI, but the

steroids released on dioxan solvolysis of the sulphates are estimated as Zimmermann chromogens after chromatography. Method AII gives an inclusive value for sulphates and free steroids; phosphates and glucosiduronates are excluded by their stability to dioxan. Exclusion of free steroids is obtained by the double-extraction procedure (method BII).

The degree of specificity involved in the methods described corroborates the partial identification of dehydroepiandrosterone, androsterone and aetiocholanolone as sulphate esters in normal human plasma (Baulieu, 1960). Recently 11β -hydroxy-aetiocholanolone was indicated as a constituent of normal plasma (Oertel & Eik-Nes, 1961). Some evidence is now offered that it occurs as a sulphate conjugate; the reported concentrations (Oertel & Kaiser, 1962b) are too low to be recorded by the present method with 2 ml. of plasma.

Mills & Wilkinson (1962) observed that refrigeration causes gross changes in the lipoprotein fraction in a small number of plasmas only. It seemed possible that on storage at low temperature other selective changes either associated with or independent of this observation might occur. The decrease in the concentration of dehydroepiandrosterone sulphate on the storage of one plasma (case 17 in Table 6) at -5° may be one such case; it is therefore recommended that plasma be extracted immediately on withdrawal and the chloroform extract stored for analysis.

Migeon & Plager (1954) recorded 25–60 $\mu\text{g.}/100\text{ml.}$ for plasma dehydroepiandrosterone; with the refinement of methods, the upper limit has gradually been extended, and a comparison of the present results with some recently determined is presented in Table 8. In every case (male and female) the concentration of dehydroepiandrosterone is greater than that for androsterone, although values for both compounds show a very wide spread. The mean values (present work) for dehydroepiandrosterone and for androsterone in females is greater than the mean values for these two compounds in males: this is contrary to published data and points the need for more extensive observations.

A diurnal rhythm in concentrations of plasma and urinary 17-hydroxycorticosteroids has been observed (Bliss & Samuels, 1956), and a similar pattern for urinary 17-oxo steroids has been established (Pincus, 1943). Moreover, the work of MacDonald, Vande Wiele & Lieberman (1962) points to a diurnal variation in the secretion of dehydroepiandrosterone. However, in the resting female subject examined, we found no diurnal variation in plasma dehydroepiandrosterone concentration, whereas in the active male, subject to a very rushed day, the concentrations of dehydro-

epiandrosterone rise towards the period of peak activity, perhaps due to stress, a factor influencing the output of urinary dehydroepiandrosterone (Pincus, Romanoff & Carlo, 1948, and references therein).

The present findings of a relatively high concentration of the sulphate conjugates of 17-oxo steroids in plasma and urine during the febrile phase of the periodic-fever case are in sharp contrast with those reported by Bondy, Cohn, Herrmann & Crispell (1958), who failed to detect any conjugated 17-oxo steroids in plasma during the febrile phase of their periodic-fever cases nor any androsterone or dehydroepiandrosterone in the urine: in our case the urinary dehydroepiandrosterone sulphate concentration was substantially increased in the febrile phase.

In each of the two hirsutism cases with the abnormal 17-oxo steroid sulphate pattern, the concentration of plasma dehydroepiandrosterone sulphate is in itself unremarkable but becomes so in its relationship to its metabolites androsterone sulphate and aetiocholanolone sulphate; abnormal values for plasma androsterone sulphate concentration in hirsutism cases have been previously noted and ascribed to adrenal or ovarian dysfunction (Mahesh & Greenblatt, 1962, and references therein).

In conclusion, we would like to underline the potentialities of applying on a large scale the analytical methods AI and BI to the isolation and identification of minor constituents of the plasma steroid sulphate fraction.

We are indebted to Professor Stuart Harris for samples from the periodic-fever case and to Dr Ann Walker for providing normal and pathological samples. Our thanks are due to Dr J. K. Norymberski for helpful discussions.

REFERENCES

- Baulieu, E. E. (1960). *J. clin. Endocrin.* **20**, 900.
- Baulieu, E. E. (1962). *J. clin. Endocrin.* **22**, 501.
- Bliss, E. L. & Samuels, L. T. (1956). *J. clin. Endocrin.* **16**, 622.
- Bondy, P. K., Cohn, G. L., Herrmann, W. & Crispell, K. R. (1958). *Yale J. Biol. Med.* **30**, 395.
- Brooks, R. V. (1958). *Biochem. J.* **63**, 50.
- Burma, D. P. (1953). *Analyt. clin. Acta*, **9**, 513.
- Bush, I. E. (1952). *Biochem. J.* **50**, 370.
- Cohen, S. L. & Oneson, I. B. (1953). *J. biol. Chem.* **204**, 245.
- Cohn, G. L., Bondy, P. K. & Castiglione, C. (1961). *J. clin. Invest.* **40**, 400.
- Conrad, S., Mahesh, V. & Herrmann, W. (1961). *J. clin. Invest.* **40**, 947.
- Corker, C., Norymberski, J. K. & Thow, R. (1962). *Biochem. J.* **83**, 583.
- Crepy, O. & Judas, O. (1960). *Rev. franç. Ét. clin. biol.* **5**, 284.

- Eberlein, W. R. & Bongiovanni, A. M. (1955). *Arch. Biochem. Biophys.* **59**, 90.
- Fotherby, K. (1958). *Biochem. J.* **69**, 596.
- Jones, J. (1945). *J. Ass. off. agric. Chem.* **28**, 398.
- Kellie, A. E. & Smith, E. R. (1957). *Biochem. J.* **66**, 490.
- MacDonald, P. C., Vande Wiele, R. L. & Lieberman, S. (1962). *J. clin. Endocrin.* **22**, 1229.
- McKenna, J. (1962). *Excerpta med. Milan: int. Congr. Ser.* no. 51, p. 174.
- McKenna, J., Menini, E. & Norymberski, J. K. (1961). *Biochem. J.* **79**, 11 p.
- McKenna, J. & Norymberski, J. K. (1957). *J. chem. Soc.* p. 3889.
- McKenna, J. & Norymberski, J. K. (1960). *Biochem. J.* **76**, 60 p.
- Mahesh, V. B. & Greenblatt, R. B. (1962). *J. clin. Endocrin.* **22**, 441.
- Menini, E. & Norymberski, J. K. (1962). *Biochem. J.* **84**, 195.
- Migeon, G. J. & Plager, J. E. (1954). *Recent Progr. Hormone Res.* **9**, 235.
- Mills, C. L. & Wilkinson, P. A. (1962). *Clin. chim. Acta*, **7**, 685.
- Norymberski, J. K. & Woods, G. (1955). *J. chem. Soc.* p. 3426.
- Oertel, G. W. (1962). *Chemische Bestimmung von Steroiden in menschlichen Plasma*, p. 19. Berlin, Göttingen and Heidelberg: Springer-Verlag.
- Oertel, G. W. & Eik-Nes, K. B. (1959a). *Acta endocr., Kbh.*, **30**, 93.
- Oertel, G. W. & Eik-Nes, K. B. (1959b). *Analyt. Chem.* **31**, 98.
- Oertel, G. W. & Eik-Nes, K. B. (1961). *Arch. Biochem. Biophys.* **92**, 150.
- Oertel, G. W. & Kaiser, E. (1962a). *Excerpta med., Milan: int. Congr. Ser.* no. 51, p. 173.
- Oertel, G. W. & Kaiser, E. (1962b). *Clin. chim. Acta*, **7**, 221.
- Pincus, G. (1943). *J. clin. Endocrin.* **3**, 195.
- Pincus, G., Romanoff, L. P. & Carlo, J. (1948). *J. clin. Endocrin.* **8**, 221.
- Roy, A. B. (1956). *Biochem. J.* **62**, 41.
- Zimmermann, W. (1935). *Hoppe-Seyl. Z.* **233**, 257.