STUDIES ON THE METABOLISM OF CORTICOSTEROIDS C-21 YL SULFATES WITH PARTICULAR REFERENCE TO CORTISOL SULFATE

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SHORT TITLE

METABOLISM OF STEROID C-21 YL SULFATES

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Ph.D. ABSTRACT

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Investigations on biogenesis and metabolism of 17-hydroxy and 17-deoxysteroids of the pregn-4-ene C-21 yl sulfate series showed that (a) such conjugates are little if at all hydrolyzed by placental sulfatase and are poor substrates with respect to placental 11β-hydroxysteroid dehydrogenase compared to the corresponding steroid alcohols; (b) following injection to adult subjects, cortisol sulfate is in part metabolized to 11-oxo and 11β-hydroxy etiocholanolone glucuronides, but not to C-21 steroid glucuronides of the pregn-5-ene series; its rate of secretion represents only a fraction of that of cortisol; (c) when ^{3}H-cortisol and ^{14}C-cortisol sulfate or ^{3}H-corticosterone and ^{14}C-corticosterone sulfate are injected to human newborns, of the total amount of tritium excreted into the urine only small proportions are extractable - from 1.3 - 13% in the glucuronide and from 3 - 18% in the sulfate fraction. Almost negligible

amounts of ^{14}C appears in either the unconjugated or glucuronide fractions. The main components of the sulfate fraction are the sulfates of cortisol and corticosterone, which are devoid of tritium. From their specific activities secretion rate of cortisol and corticosterone sulfates are calculated to range between 0.12 - 0.35 and 1.8 - 5.4 $mg/m^2/day$ respectively. The findings indicate that cortisol and corticosterone are not sulfurylated prior to being metabolized; and that corticosterone sulfate may represent a significant secretory product of the adrenal in human newborn.

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LIST OF ABBREVIATIONS USED

ACTH	adrenocorticotrophic hormone
c.p.m.	count per minute
d.p.m.	disintegration per minute
µc	microcurie
mCi	millicurie
Sp.Act.	specific activity
cm	centimeter
m	meter
mm	millimeter
mµ	millimicron
g	gravitational force
gm	gram
mg	milligram
µg	microgram
ng	nanogram
mmole	millimole
µMole	micromole
mµMole	millimicromole
ml	milliliter
M	molar
ATP	adenosine-5'-triphosphate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

Δ^4 or Δ^5	double bond between C-4 and C-5 or C-5 and C-6
aldosterone	11β, 21-dihydroxypregn-4-ene-3,20-dion-18-al
Δ^4-androstenedione (Δ^4ASD)	androst-4-ene-3,17-dione
dehydroepiandrosterone (DHA)	3β-hydroxyandrost-5-en-17-one
16α-hydroxydehydro-epiandrosterone	3β,16α-dihydroxyandrost-5-en-17-one
11β-hydroxyetiocho-lanolone (11-OH-E)	3α,11β-dihydroxy-5β-androstan-17-one
11-ketoetiocholanolone (11-O-E)	3α-hydroxy-5β-androstane-11,17-dione
11β-hydroxyandrosterone (11-OH-A)	3α,11β-dihydroxy-5α-androstan-17-one
cholesterol	cholesta-5-en-3β-ol
estrone	3-hydroxyestra-1,3,5(10)-trien-17-one
estradiol-17β	estra-1,3,5(10)-triene-3,17β-diol
estriol	estra-1,3,5(10)-triene-3,16α,17β-triol
pregnenolone	3β-hydroxypregn-5-en-20-one
16α-hydroxypregnenolone	3β,16α-dihydroxypregn-5-en-20-one
17α-hydroxypregnenolone	3β,17α-dihydroxypregn-5-en-20-one
progesterone	pregn-4-ene-3,20-dione
11-deoxycorticosterone (DOC)	21-hydroxypregn-4-ene-3,20-dione
corticosterone (B)	11β,21-dihydroxypregn-4-ene-3,20-dione

6β-hydroxycorticosterone	6β,11β,21-trihydroxypregn-4-ene, 3,20-dione
11-dehydrocorticosterone (A)	21-hydroxypregn-4-ene-3,11,20-trione
11-deoxycortisol	17α,21-dihydroxypregn-4-ene-3,20-dione
cortisol (F)	11β,17α,21-trihydroxypregn-4-ene-3, 20-dione
2α-hydroxycortisol	2α,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione
6β-hydroxycortisol	6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione
cortisone (E)	17α,21-dihydroxypregn-4-ene-3,11, 20-trione
6β-hydroxycortisone	6β,17α,21-trihydroxypregn-4-ene, 3,11,20-trione
pregnenolone sulfate	20-ketopregn-5-ene-3β-yl sulfate
16α-hydroxypregnenolone sulfate	16α-hydroxy-20-ketopregn-5-ene-3β-yl sulfate
17α-hydroxypregnenolone sulfate	17α-hydroxy-20-ketopregn-5-ene-3β-yl sulfate
dehydroisoandrosterone sulfate (DHAS)	17-ketoandrost-5-ene-3β-yl sulfate
16α-hydroxydehydro-epiandrosterone sulfate	16α-hydroxy-17-ketoandrost-5-ene-3β-yl sulfate
11-deoxycorticosterone sulfate (DOCS)	3,20-diketopregn-4-ene-21-yl sulfate
corticosterone sulfate (BS)	11β-hydroxy-3,20-diketopregn-4-ene-21-yl sulfate
11-dehydrocorticosterone sulfate (AS)	3,11,20-triketopregn-4-ene-21-yl sulfate

cortisol sulfate (FS)	11β,17α-dihydroxy-3,20-diketopregn-4-ene-21-yl sulfate
6β-hydroxycortisol sulfate	6β,11β,17α-trihydroxy-3,20-diketopregn-4-ene-21-yl sulfate
cortisone sulfate (ES)	17α-hydroxy-3,11,20-triketopregn-4-ene-21-yl sulfate
tetrahydrocorticosterone sulfate	3α,11β-dihydroxy-20-keto-5β-pregane-21-yl sulfate
tetrahydrocortisol sulfate	3α,11β,17α-trihydroxy-20-keto-5β-pregnane-21-yl sulfate
tetrahydrocortisone sulfate	3α,17α-dihydroxy-11,20-diketo-5β-pregnane-21-yl sulfate
cortolone	3α,17α,20α,21-tetrahydroxy-5β-pregnan-11-one
β-cortolone	3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one
tetrahydrocortisol (THF)	3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one
tetrahydrocortisone (THE)	3α,17α,21-trihydroxy-5β-pregnane-11,20-dione
tetrahydrocorticosterone	3α,11β,21-trihydroxy-5β-pregnan-20-one
allo-tetrahydrocorticosterone	3α,11β,21-trihydroxy-5α-pregnane-20-one
3β,5α-tetrahydrocorticosterone	3β,11β,21-trihydroxy-5α-pregnan-20-one
tetrahydro-11-dehydrocorticosterone	3α,21-dihydroxy-5β-pregnane-11,20-dione
tetrahydrocortisol glucuronide	11β,17α,21-trihydroxy-20-keto-5β-pregnane-3α-yl-β-D-glucopyranosiduronic acid

tetrahydrocortisone glucuronide	17α,21-dihydroxy-11,20-diketo-5β-pregnane-3α-yl-β-D-glucopyranosiduronic acid
tetrahydrocorticosterone glucuronide	11β,21-dihydroxy-20-keto-5β-pregnane-3α-yl-β-D-glucopyranosiduronic acid
tetrahydro-11-dehydrocorticosterone glucuronide	21-hydroxy-11,20-diketo-5β-pregnane-3α-yl-β-D-glucopyranosiduronic acid

REVIEW OF LITERATURE

Our present knowledge concerning the metabolism of steroid hormones during human foetal life was established by studies performed either prior to or following therapeutic abortions at mid-pregnancy (1-5). The fundamental principles of these investigations involved the perfusion of previable foetuses with radioactive steroids, followed by analyzing the distribution of radioactivity in foetal organs and tissues and the subsequent identification and characterization of steroid metabolites from these sites (6-9). The results of these studies led to the recognition of an important metabolic inter-relationship or interplay between the foetus and the placenta and gave rise to the concept of the foeto-placental unit (2). Within the foetal and placental tissues exist complementary enzyme systems which are essential to the biosynthesis of a number of steroid hormones among which estrogens represent quantitatively the principal end products (2). This is exemplified by the biogenesis of estriol from pregnenolone of placental as well as of foetal origin. Within the foetal compartment, namely in the foetal adrenal and liver the latter steroid is hydroxylated on carbon 17 (6,10). Dehydroepiandrosterone is then formed by removal of the sidechain through the activity of a 17, 20-desmolase (6,10,11). The product of the reaction might or not undergo sulfurylation following which it is either

transferred to the placenta or hydroxylated on C-16 within the foetal adrenal and/or liver (9,13). Upon reaching the placenta the 3 sulfate of 16α-hydroxydehydroisoandrosterone is hydrolyzed by placental sulfatase (7,8,14-17) oxidized to 16α-hydroxyandrostenedione by 3β-hydroxysteroid dehydrogenase-isomerase (18) and finally converted to estriol by the microsomal aromatizing enzyme systems (18,19,20). This concept implies that (1) the placenta lacks 17, 20-desmolase as well as C-16 and C-17 hydroxylases activities (2); that the foetus lacks 3β-hydroxysteroid dehydrogenase-isomerase which is present in the placenta (3); that precursors are shuttled back and forth between the placenta and the foetus in the biosynthetic sequences necessary for estrogen production. In this context the role played by sulfo-conjugation of steroids versus hydrolysis of steroid sulfates should be emphasized i.e. a number of foetal tissues possess sulfokinase activity while steroid sulfatases are found in the placenta.

When considered in this perspective one is led to assume that the main function of the foetal adrenal is oriented towards the biosynthesis of dehydroisoandrosterone and its 3β-yl sulfate mainly from placental pregnenolone[1] thus supplying the placenta with its most important source of estrogen precursors. This is in keeping with the characteristic

[1] See page 12a.

decrease of maternal urinary estriol which is observed in instances of pregnancy associated with an anencephalic foetus, a condition characterized by marked hypoplasia of the foetal adrenal gland, or as a consequence of foetal distress or death (21-25). Inversely, the maternal adrenal may have an important role in steroidogenesis during late pregnancy since the excretion values for estrone, estradiol and estriol are consistently low in bilaterally adrenal-ectomized pregnant women (25-27), being in the range encountered in patients who give birth to infants with retarded growth and utero-placental insufficiency (28-32).

It is doubtful whether the foetal adrenal can utilize acetate (6,10,33-36) or cholesterol (6) for the biosynthesis of pregnenolone at a rate sufficient to meet the need of dehydroisoandrosterone biosynthesis.[2] The main origin of pregnenolone within the foeto-placental unit appears to be the placenta itself (37-41) which utilizes circulating maternal plasma cholesterol as the main precursor for the biosynthesis of this steroid (42). The validity of this statement is supported by the high concentrations of pregnenolone and pregnenolone sulfate in umbilical cord plasma (43) with an umbilical arteriovenous difference of about 60 µg for pregnenolone sulfate (44) as well as by perfusion of mid-term human foetuses with these steroids and the subsequent isolation and characterization of dehydro-

[2] See page 12a.

isoandrosterone and dehydroisoandrosterone sulfate from their adrenals (6,10). This is also in keeping with the observation that the concentration of dehydroisoandrosterone sulfate in umbilical arterial plasma is higher than in umbilical **venous** plasma (45-47). These observations strongly suggest that in the biosynthesis of C-19 steroids, the foetal adrenal operates within a relatively narrow biosynthetic sequence, namely from foetal plasma pregnenolone to dehydroisoandrosterone sulfate. As a consequence of the absence of 16α-hydroxylase the placenta is not capable of biosynthesizing estriol from C-19 steroid precursors such as dehydroisoandrosterone or dehydroisoandrosterone sulfate. On the other hand, the latter two steroids are both efficiently converted to estrone and estradiol-17β by placental aromatizing enzymes (7,17,20,48).

It appears, from experiments involving perfusion of previable human foetuses that there is a total absence or possibly an inhibition of 3β-hydroxysteroid dehydrogenase-isomerase activity with respect to pregnenolone within the foetal compartment at mid-gestation, preventing the formation of progesterone and the subsequent utilization of this steroid as precursor of cortisol (6). In contradistinction to this, some C-21 hydroxylated steroids of the pregn-5-ene series can be transformed by the foetal adrenal to the corresponding Δ^{5}- 3-ketosteroids (49,50). In vivo and in vitro

investigations show that the foetal adrenal possess the enzymes necessary for hydroxylating pregnenolone at carbons 11, 17, and 21 (6,51-53). These hydroxylated intermediates can in part be oxidized to 17-deoxy and 17-hydroxycorticosteroids such as corticosterone and cortisol. In fact, cortisol was isolated from the adrenal of previable foetuses perfused with 3β, 17α, 21-trihydroxy pregn-5-en-20-one and corticosterone from foetuses perfused with 3β, 21-dihydroxy pregn-5-en-20-one (49,50). Such compounds are present in the foetal circulation (43). It is therefore conceivable that they might in part be utilized for the biogenesis of cortisol and corticosterone during foetal life. In vitro studies with adrenals obtained either from full term or premature infants have also demonstrated that both pregnenolone and 17α-hydroxypregnenolone can be incorporated into cortisol without necessarily involving the formation of progesterone (53,54).

The placenta supplies the foetus with large amounts of progesterone (55,56). This is reflected in a much higher concentration of the steroid in umbilical venous blood compared to umbilical arterial blood (56). Progesterone circulating in the foetal compartment is thus accessible to the foetal adrenal where it may undergo a number of hydroxylation reactions (6,52). In support of this is the finding that following the administration of labelled

progesterone to foetuses, autoradiographic studies indicate a high accummulation of radioactivity in the adrenals as well as in the testicles and liver (57). Perfusion of previable foetuses with labelled progesterone have resulted in the isolation and characterization of 11-deoxycorticosterone, corticosterone and their respective sulfate esters from adrenal extracts (6,58,59). If adrenalectomized foetuses were perfused with the same precursor, the formation of these corticosteroids did not take place (6,60) clearly showing that their site of origin is the foetal adrenal. Such perfusion studies (6,58-61) supported by in vitro findings using foetal liver and adrenal preparations (36,52,62-64) indicate that the foetal adrenal is necessary for 11β and 21 hydroxylations, whereas 17α-hydroxylation can also occur in the liver i.e. on such steroids as progesterone.

Incubation of adrenal slices obtained from premature infants, with labelled progesterone led to the isolation of 11-deoxycorticosterone, corticosterone, 11-deoxycortisol and cortisol (36,63,65,66). Aldosterone was also characterized from the adrenal following perfusion of a previable mid-term foetus with radioactive corticosterone (67). Thus available evidence indicate that the foetus or rather the feto-placental unit appears capable of synthesizing practically all known biologically active C-21 corticosteroids.

Following birth the foetal zone of the adrenal begins

to involute, decreases in size and weight while the permanent zone, the adult cortex, significantly increases in size during the first month of life, (68-70). In spite of this however, processes of steroid biogenesis which originated in utero continue to manifest themselves during this period of life (43,71). This is illustrated by reports of high plasma concentration of steroids bearing a Δ^5-3β-hydroxy configuration, steroids which are primarily conjugated with sulfuric acid (43). Furthermore, estrogen precursors such as 16α-hydroxydehydroisoandrosterone and a number of other Δ^5-3β-hydroxysteroids are found in high concentrations in the urine of the newborn infant and mainly as sulfoconjugates (71-77). During the first month of life the urinary excretion of some of these Δ^5-3β-hydroxy-steroids increases (71). Two and a half months following birth, when their excretion is almost negligible, administration of ACTH induces their biosynthesis as reflected in a sharp increase in their urinary output (71,78).

Although sulfation of steroids of the C-18 and C-19 series represent an important aspect of the metabolism of these steroids in the foetal compartment (1,4,5), it is only lately that the occurence of sulfo-conjugation of C-21 steroid alcohols of the pregn-4-ene series has been recognized.

The earliest demonstration of this mode of conjugation was provided by the isolation from urine of corticosterone and cortisol sulfates, following the administration of pharmacological doses of the corresponding steroid alcohols to normal adults (79). Following incubation of an adrenal tumour with a number of steroids of the pregn-4-ene series, their respective sulfo-conjugates were characterized (80). 17α-hydroxysteroids, e.g. cortisol or cortisone were sulfated to a lesser degree than 17-deoxysteroids such as 11-deoxycorticosterone and corticosterone (80).

The capacity of the mid-term foetal adrenal and liver to form the sulfoconjugates of several phenolic steroids and steroids of the 3β-hydroxyandros-5-en, and of the 3β-hydroxypregn-5-ene series has been extensively documented in in-vitro systems (12). The same tissues were reported to also form the sulfates of corticosterone, 11-dehydro-corticosterone and 11-deoxycorticosterone but in low yield (81). However sulfation of steroids of the pregn-4-ene series (such as cortisol, corticosterone, 11-deoxycorticosterone and 11-dehydrocorticosterone) by the adrenal of the human premature or newborn infant was soon demonstrated beyond doubt (82). Once sulfation of a C-21 hydroxysteroid occurred, the conjugate does not appear to be capable of entering further biosynthetic pathways (82). In similar in vitro preparations, labelled progesterone was also shown to be

incorporated into corticosterone sulfate, 11-deoxycorticosterone sulfate and 11-deoxycortisol sulfate (65,53).

In themselves these results did not provide evidence that such conjugates were secreted by the foetal adrenal. More recently, however, corticosterone sulfate, 11-deoxycorticosterone sulfate, 11-dehydrocorticosterone sulfate, cortisone sulfate and cortisol sulfate were characterized from pools of umbilical cord plasma (83) and the concentrations of corticosterone and 11-deoxycorticosterone sulfates found to be several fold greater in the foetal than in the maternal circulation (84).

Following perfusion of the previable mid-term foetuses with ^{14}C-progesterone, corticosterone sulfate and 11-deoxycorticosterone sulfate were isolated from the adrenals (6,58,59). When the same precursor was injected into the umbilical vein, in addition to the aforementioned sulfo-conjugates, 11-dehydrocorticosterone sulfate and cortisol sulfate were also characterized from the same site (6).

In experiments involving the perfusion of corticosterone sulfate, analysis of the placenta showed that most of the radioactivity recovered from this tissue was in the sulfo-conjugated fraction, indicating that the conjugate had not been metabolized by placental sulfatase to any appreciable extent (85). Tetrahydrocorticosterone sulfate and its 5α-epimer were isolated in low yield from

the foetal liver, suggesting a limited metabolism of the steroid as long as the ester sulfate linkage remained intact (85).

Studies on the pattern of urinary corticosteroids in newborn infants showed that the excretion of cortisol sulfate plus cortisone sulfate is comparable to that of tetrahydrocortisone glucuronide and further that corticosterone sulfate is a predominant urinary 17-deoxysteroid (86) during the perinatal period. Similar results have lately been observed by other investigators who also reported the presence of large amounts of tetrahydrocortisol sulfate, tetrahydrocortisone sulfate and 6β-hydroxycortisol sulfate in the urine of newborn infants (87). These findings did not clarify to what extent steroid sulfates of the pregn-4-ene series represent products of adrenocortical secretion or are formed in part during the metabolism of the corresponding steroid alcohol by extra adrenal tissues.

Nevertheless, the presence of such steroid sulfates, in the foetal circulation and their urinary excretion by the newborn, for several weeks following birth, must reflect an aspect of corticosteroid biogenesis and/or metabolism the better understanding of which could further our knowledge of adrenocortical function during the neonatal period and early infancy.

One should mention here that a few observations

in human adults have indicated that corticosterone sulfate is secreted by the adrenal in amounts representing about 1/10 the secretion rate of corticosterone (88,89).

Numerous reports have been made on the secretion rate of cortisol by the newborn infant (90-92). When expressed per square meter of body surface area, the results obtained in newborn and adults are comparable (93). There are however marked differences between the newborn and the adult in their metabolism of corticosteroids (75,90,94-98). Thus, following administration of ^{14}C-cortisol to adult subjects up to 90% of the extractable radioactivity is accounted for in terms of characterized urinary metabolites (99). In the newborn the total radioactivity extractable from urine is much smaller (90,97); a greater proportion is excreted as unconjugated metabolites and lesser as glucuronides (97,98). A substantial amount is released by solvolysis and a large portion is unaccounted for (97, 98,100). In the newborn the major components of the unconjugated fraction appears to be hydroxylated metabolites of cortisol, such as 2α-hydroxycortisol, 6β-hydroxycortisone and 6β-hydroxycortisol among which the latter steroid is thought to be excreted in the largest amount (94,95). In the glucuronide fraction tetrahydrocortisone is probably the main metabolite of cortisol while only negligible amounts of tetrahydrocortisol are formed (86,101). As

mentioned previously, cortisol and cortisone together with corticosterone, are also major components of the sulfate fraction (86).

Systematic analysis of the pattern of corticosteroids extracted from the urine of newborn infants have been reported and compared to the corticosteroid excretion pattern of their mothers and of non-pregnant females (96). It was consistently shown that compounds which were not identified but, which, by chromatography and colour reactions, such as Zimmerman and blue tetrazolium behaved like "atypical steroids", were the dominant components of the neutral steroid excretion pattern (96). It is likely that these compounds are mainly products of secretion of "the foetal adrenal" such as 16α-hydroxypregnenolone sulfate and 16α-hydroxy-dehydroisoandrosterone sulfate and a number of other steroid sulfates of the pregn-5-ene and/or andros-5-ene series (71-75).

Thus it appears that a critical appraisal of adreno-cortical function and corticosteroid metabolism during the perinatal and postnatal periods should take into account the persistence of foetal processes of steroid biogenesis and metabolism and the evolution of autonomous processes. This is not an easy task, and all the more so since we still possess very scarce information on the extent to which such processes might be inter-related.

1 The statement that the biosynthesis of fetal dehydroisoandrosterone utilizes mainly placental pregnenolone as precursor is incorrect. It should be modified in view of the fact that pregnenolone concentration in cord plasma is low (2 µg/100 ml) with no arterio-venous differences (Hellig, H., Y. Gattereau, Y. Lefebvre, and E. Bolté, J. Clin. Endocr., 30, 624, 1970).

2 The statement that, "it is doubtful whether the fetal adrenal can utilize acetate or cholesterol for the biosynthesis of pregnenolone" should be modified because of the recent characterization of large quantities of pregnenolone and dehydroisoandrosterone from fetal perfusates, livers and adrenals, after perfusion of the isolated foetus with labelled acetate and cholesterol (Telegdy, G., J.W. Weeks, D.F. Archer, N. Wiqvist and E. Diczfalusy, Acta Endocr., 63, 119, 1970).

PURPOSE OF THE WORK

The studies which will be presented in the forthcoming sections reflect two different approaches to investigations of steroid biogenesis and metabolism during the perinatal period. The first relies upon in vitro systems, the second upon results obtained from human adult subjects and newborn infants. The authorization obtained by this laboratory to administer under precisely defined conditions, radio-labelled steroids to a limited number of infants represent a link, but we hope not the only apparent one, between these two experimental approaches. In fact, the characterization, in order of decreasing importance, of corticosterone, 11-deoxycorticosterone, 11-dehydrocorticosterone, cortisol and cortisone sulfates in human umbilical cord plasma (83) and the report that such conjugates are present in much lower (if not bearly detectable) amounts in maternal plasma (84) seemed to call for studies on the metabolic fate of such conjugates upon reaching the placenta. On the other hand the characterization of cortisol, cortisone and corticosterone sulfates in the urine of the human newborn (86,87) in amounts often comparable to or higher than that of other known metabolites of cortisol and corticosterone raised several questions: were these steroid conjugates products of adrenocortical secretion or were they formed during the metabolism of the corresponding

steroid alcohol in extra adrenal tissues or both? The simultaneous administration of labelled cortisol and cortisol sulfate on the one hand and of corticosterone and corticosterone sulfate on the other appeared to be the most direct approach to a possible solution of this problem. Studies were therefore undertaken first in human volunteers for both ethical reasons and the need of adult controls. Similar studies were then performed in newborn infants.

In the forthcoming chapters results of three main series of investigations will be presented under the following headings:-

1) Metabolism of C-21 Steroid Sulfates by Placental Tissue.
2) Simultaneous Metabolism of Cortisol Sulfate and Cortisol in Normal Human Adults.
3) Secretion Rate and Metabolism of the Sulfates of Cortisol and Corticosterone in Newborn Infants.

GENERAL METHODS

1. Reagents

All reagents used for extraction and chromatography were purified and redistilled according to the procedures recommended by Bush (102).

Ethanol was redistilled over 2-4 dinitrophenyl-hydrazine then redistilled twice.

Acetic anhydride, benzene and pyridine were distilled according to Kliman and Peterson (103) and stored at 5°C in a vacuum desiccator, over a layer of calcium chloride.

Phenylhydrazine hydrochloride was recrystallized three times from ethanol.

Toluene, was washed once with 1/10 vol. of reagent grade H_2SO_4, once with water, twice with 1/10 vol. of 10% Na_2CO_3 and with distilled water to neutrality; it was then dried with anhydrous sodium sulfate, filtered and redistilled.

Scintillation mixture: 4 gms. of 2,5-diphenyloxasole and 0.1 gm. of 1,4-bis-2- (5-phenyloxazolyl) benzene (Packard Instrument Co., La Grange, Ill., U.S.A.) were dissolved in 1 liter of toluene, purified as described above.

Co-factors: were obtained from Sigma Chemical Co. St. Louis, Mo.

2. Reference steroids

Non-radioactive

Steroid alcohols were obtained from Steraloids Inc. The monacetates of the steroid alcohols used as carriers for double isotope assays and the steroid acetate carriers used for crystallization were obtained from Steraloids Inc. Their purity was first assessed by chromatography and by obtaining a constant melting point on a Fisher-Johns apparatus - prior to and following recrystallization. 11-dehydrocorticosterone acetate was prepared by CrO_3 oxidation (103) of corticosterone acetate.

Radioactive

^{3}H or ^{14}C labelled steroid alcohols were purchased from New England Nuclear Corp. Their purity was verified by chromatography in appropriate systems, the chromatograms being scanned to ensure the presence of a single symmetrical peak of radioactivity of the expected R_f value. The specific activities of these steroids will be given in appropriate chapters.

Preparation of 1,2-^{3}H and 4-^{14}C-Steroid Sulfates

The sulfates of 4-^{14}C-cortisol, 4-^{14}C-cortisone, 4-^{14}C-11-deoxycorticosterone 4-^{14}C-corticosterone and ^{14}C-11-dehydrocorticosterone were prepared by one of two

methods. The first, a microscale adaptation of the method of Kornel (104) which involved the addition of chlorosulfonic acid (0.05 ml.) to pyridine (1 ml.), the reaction tube being kept in an ice bath. The steroid alcohol in pyridine (1.0 ml.) was then added to the mixture. The reaction was allowed to take place for 45 minutes at room temperature. Extraction and washings were as described in the original method.

The second method was an adaptation of that described by **Dusza** (105). The labelled steroid was dissolved in the minimum amount of pyridine and 10 mg. of triethylamine-sulfur trioxide was added. The tube was shaken for two hours. The sulfates were redissolved in water and extracted with methylene chloride.

The third method involved the biosynthesis of 1,2-^{3}H-cortisol and corticosterone sulfates by the soluble fraction of rat liver homogenate enriched with ATP (106). The sulfate was extracted from the aqueous media with n-butanol.

The radiochemical homogeneity of these steroid sulfates was assessed by thin layer and paper chromatography and following enzyme hydrolysis of an aliquot, by the recovery of one single radioactive product possessing the same chromatographic mobility as that of the corresponding ^{14}C-steroid alcohol (82).

3. ^{3}H-and ^{14}C-Acetic Anhydride

These reagents were purchased from New England Nuclear Corp. at a concentration of 15% in benzene. Their specific activity were verified as described by Kliman and Peterson (103) by acetylation of 0.5 mg. of cortisol and chromatographic purification of cortisol acetate and of its CrO_3 oxidation product to constant specific activity. The latter was established by counting the radioactivity and by the Porter-Silber reaction (107).

4. Chromatography

a) Paper Partition Chromatography and Detection of Steroids

Steroid extracts were applied and eluted from paper or from thin layer plate with ethanol. Whatman No. 1 paper was used for all impregnated paper chromatogram systems; Whatman No. 2 for all other systems. These papers were used unwashed after scanning under ultraviolet light (250mμ) in order to eliminate fluorescent or ultra violet absorbing impurities.

All systems were developed at room temperature. In the following chapters, paper chromatography systems will be referred to by the code number (Roman numerals) assigned to each in Table I.

Δ^4-3-ketosteroids were located on chromatograms by their **absorption** of ultra violet light (250 mμ). Δ^5-3β-hydroxysteroids, both sulfate esters and free alcohols as well as their acetates were located by spraying with phosphomolybdic acid a 10% solution in absolute ethanol (108), a reference strip on which appropriate reference steroids had been applied. The strip was subsequently heated for several minutes at 80-90°C. The steroid appeared as dark blue spots against a yellow background. Radioactive steroids were located by scanning chromatogram strips in a Packard Model 7200 Radiochromatogram scanner.

TABLE I

Paper Chromatography Systems

SYSTEM	SOLVENT COMPOSITION	REFERENCE
I	Benzene, methanol, water (10:5:5)	(109)
II	Benzene, n-butanol, methanol, water (100:10:30:30)	(110)
III	Benzene, ethyl acetate, methanol, water (100:20:50:50)	-
IV	Isooctane, t-butanol, water (50:25:45)	(111)
V	Benzene, light petroleum, methanol, water (33:66:80:20)	(109)
VI	Benzene, petroleum ether, methanol, water (7:3:5:5)	(112)
VII	Light petroleum, toluene, methanol, water (667:333:800:200)	(113)
VIII	Light petroleum, methanol, water (10:8:2)	(109)
IX	Methyl Cyclohexane/Propylene Glycol (25% in methanol)	(112)
X	Isooctane, t-butanol, methanol, water (50:25:22.5:5)	(111)
XI	Toluene/Propylene Glycol (30% in methanol)	(114)
XII	Isooctane, toluene, methanol, water (165:335:300:200)	(111)
XIII	Hexane/Propylene Glycol (50% in methanol)	(112)

TABLE I (Continued)

Paper Chromatography Systems

SYSTEM	SOLVENT COMPOSITION	REFEREMCE
XIV	Isooctane, methanol, water (50:45:5)	(111)
XV	Ethyl acetate, chloroform, methanol, water (25:75:50:50)	(115)
XVI	Isooctane, toluene, methanol, water (25:25:35:15)	(111)
XVII	Toluene, n-butyl acetate, n-butanol, methanol, 4N NH_4OH (30:60:10:50:50)	(116)
XVIII	Isoamyl alcohol, n-hexane, conc. NH_4OH, water (40:10:28:22)	(117)

b) Thin-Layer Chromatography (TLC)

Thin-layer chromatography equipment was purchased from Research Specialties Co., Richmond, Calif., U.S.A. Plates (20 x 20 cm.) were coated with Merck Silica gel G No. 8076, containing 1% of Lamp Phosphor (Sylvania Electric Products Inc., Towanda, Penn., U.S.A.) in order to provide a better detection of Δ^4-3-ketosteroids under ultraviolet light. Thickness of coating was 0.5 mm. The plates were dried overnight at room temperature and activated by heating under infrared lamps for 20 minutes before use. Chromatography plates were developed by ascending chromatography.

The composition of the solvent systems is indicated in Table II. These different systems will henceforth be referred to by the Arabic number assigned to each in this table.

TABLE II

Thin-Layer Chromatography Systems

SYSTEM	SOLVENT COMPOSITION	REFERENCE
1	Chloroform, methanol, water (140:57:3)	-
2	Chloroform, acetone (9:1)	(53)
3	Butyl acetate, cyclo-hexane (10:1)	-
4	Butyl acetate, benzene (1:19)	-
5	Chloroform, ethyl acetate (9:1)	(118)
6	Chloroform, butyl acetate (7:3)	(53)
7	Dichloromethane, butyl acetate (7:3)	(53)
8	Dichloromethane, acetone (9:1)	-
9	Chloroform, ethyl acetate, acetone (9:2:1)	-
10	Chloroform, ethanol (9:1)	-
11	Toluene, methanol (7:3)	-
12	Chloroform, ethanol (17:3)	-

5. Counting and Calculations

Either individual or simultaneous measurement of ^{3}H and/or ^{14}C counts were performed in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 4322, set at a single voltage of 1700 volts. Counts were obtained according to the discriminator method of Okita et al (119) as modified by Stachenko et al (120). Under these conditions the ^{3}H c.p.m. appearing in the ^{14}C channel was 0.4%; that of ^{14}C in the ^{3}H channel, 14%. The counting efficiencies for ^{3}H and ^{14}C were 16% and 60% respectively.

Samples were counted in glass vials. They were thoroughly dried under infrared lamps. Steroid sulfates were dissolved in 0.2 ml. of methanol prior to the addition of 15 ml. of scintillation solution. Each sample was counted for 20 minutes.

Correction for quenching was made by recounting samples following addition of known amounts of ^{3}H and/or ^{14}C toluene (88).

The secretion rate of steroid alcohols and of their respective sulfate esters (µg/day) were calculated according to equation previously published (88).

$$\text{Production rate per day} = \frac{S.W. \times R}{Cxd} - W$$

in which:

S = specific activity of steroid administered in cpm/μg.

W = μg amount of steroid administered.

$R = \frac{\text{M.Wt. of steroid administered}}{\text{M.Wt. of urinary metabolite}}$.

C = specific activity of urinary metabolite in cpm/μg.

d = number of days of urine collection.

When calculating the secretion rate of cortisol, C stands for the specific activity of either tetrahydrocortisol or tetrahydrocortisone. In the instance of corticosterone, C represents the specific activity of tetrahydro-11-dehydrocorticosterone diacetate. When calculating the secretion rate of cortisol sulfate or corticosterone sulfate, C stands for the specific activity of either cortisone acetate or 11-dehydrocorticosterone acetate following crystallization of these steroids to radiochemical homogeneity.

CHAPTER I

Metabolism of C-21 Steroid Sulfates by Placental Tissue

Summary

Sulfatase and 11β-hydroxysteroid dehydrogenase activities of human term placenta were studied with respect to several steroids of the pregn-4-ene C-21 yl sulfate series. Homogenate, as well as the microsomal fraction of placenta obtained either at mid-term or at term, possessed negligible sulfatase activity with respect to the sulfates of 11-dehydrocorticosterone and corticosterone, and low but consistent activity towards 11-deoxycorticosterone sulfate. Under the same conditions dehydroepiandrosterone sulfate was hydrolyzed almost quantitatively.

Placental homogenates readily converted cortisol to cortisone and corticosterone to 11-dehydrocorticosterone. The reaction was greatly enhanced by the addition of NADP. In contrast, the corresponding 11β-hydroxy-C-21-steroid sulfates were poor substrates with respect to placental 11β-hydroxysteroid dehydrogenase, as indicated by the low yields of the sulfates of cortisone and 11-dehydrocorticosterone even in the presence of NADP. These findings are consistent with the pattern of steroids of the pregn-4-ene series characterized in cord plasma, by Branchaud and Schweitzer (83,84).

Introduction

In 1965 Eberlein characterized a number of steroids in pools of human umbilical cord plasma, among which quantitatively the most important were: progesterone, and the sulfates of dehydroepiandrosterone, pregnenolone, 17α-hydroxypregnenolone and 16α-hydroxypregnenolone (43). It is well documented that among these steroids those of the 3β-yl sulfate series are extensively metabolized in the placenta, where they undergo hydrolysis to the corresponding steroid alcohols (7,8,14-17,121) following which they may be used as precursors of estrogens (in the instance of dehydroepiandrosterone (2) and progesterone in the instance of pregnenolone (121,122).

In this laboratory following the characterization of the 21 yl-sulfates of several steroids of the pregn-4-ene series such as 11-deoxycorticosterone and corticosterone sulfates in umbilical cord plasma (83), it appeared logical to raise the question as to whether such conjugates were metabolized upon reaching the placenta.

Experimental

Preparation of the tissue

Experimental conditions were similar to those previously used by French and Warren (16). Placentas were obtained at the time of normal delivery at term. They were collected in plastic bags containing 0.9% saline and packed in ice. In one instance following induced abortion, a placenta was obtained at mid-term. Upon arrival at the laboratory the placentas were freed from foetal membranes. Placental cotyledons were repeatedly washed in ice cold 0.9% saline. They were then shredded with forceps, and the larger blood vessels removed. The resulting mince was rinsed several times with saline, blotted on filter paper, weighed and homogenized.

Homogenates were prepared in either 0.25 M sucrose or in trishydrochloride buffer, pH 7.8 as will be indicated below. On occasions these homogenates were centrifuged: first in a Serval centrifuge at 700 g., in order to sediment nuclei and unbroken cells, then at 15,000 g to sediment the mitochondrial fraction. The supernatant of this second centrifugation was centrifuged at 105,000 g in a Spinco ultra-centrifuge for one hour. The sediment obtained will be referred to as the microsomal fraction. It was resuspended in an appropriate volume of buffer. During these manipulations care was taken to ensure that the

tissue preparations were kept at 5°C or below.

Incubation Conditions

In studies involving placental sulfatase activity 15% homogenates were prepared in 0.05M trishydrochloride buffer pH 7.8. The composition of the incubation media for each vial incubated was: tissue - wet weight equivalent: 200 mg; 0.05M trishydrochloride buffer pH 7.8: 0.7 ml; individual steroid substrate: 50 mμMole; the total volume of incubation media being 2 ml. The steroid substrates were: ^{3}H-dehydroepiandrosterone sulfate, ^{14}C-11-dehydrocorticosterone sulfate, ^{14}C-corticosterone sulfate and ^{14}C-11-deoxycorticosterone sulfates. Their specific activities were 1950 cpm/ mμMole; 1130 cpm/mμMole; 1160 cpm/ mμMole and 1100 cpm/mμMole respectively.

In the study where the hydrolysis of 11-deoxycorticosterone sulfate was further investigated, a 15% placental homogenate was prepared in 0.25M sucrose and incubated in different buffers namely: 0.05M trishydrochloride, pH 7.8; Krebs-Ringer Bicarbonate pH 7.4; 0.5M Acetate, pH 5.2; and 0.05M phosphate pH 7.4. The composition of the incubation media for each vial incubated was: tissue - wet weight equivalent, 500 mg; buffer: 1 ml; ^{3}H-deoxycorticosterone sulfate (Sp.Act. 1810 cpm/mμMole): 50 mμMole. The total volume of the incubation media was 4.3 ml. The time of incubation was one hour, in air.

In studies involving 11β-hydroxysteroid dehydrogenase activity an 11% homogenate was prepared in 0.25M sucrose. The composition of the incubation media was the following: tissue incubated - wet weight equivalent: - 500 mg; 0.05M phosphate buffer, pH 7.4: 0.5 ml. The steroid substrates were ^{14}C-cortisol (Sp.Act. 5,470 cpm/mμMole), ^{14}C-corticosterone (Sp.Act. 6,190 cpm/mμMole) and their respective sulfates (Sp.Act. 5,470 cpm/mμMole and 6,560 cpm/mμMole respectively) used at a concentration of 10 mμMole/flask. In certain instances NADP was added to the incubation at the dose of 2 μMole/flask. The final volume of the incubate was 5 ml. The time of incubation was one hour, in air.

At the end of the incubation, proteins were precipitated with ethanol-acetone (1:3 vol/vol). Tracer amounts of reference steroids bearing a different radioactive label from that of the steroid substrate incubated were then added (e.g. ^{3}H-corticosterone and ^{3}H-corticosterone sulfate in the study where the hydrolysis of ^{14}C-corticosterone sulfate was investigated). Steroid alcohols were extracted with dichloromethane and steroid sulfates with n-butanol.

Purification of Steroids by Double Isotope Assay

In all instances the n-butanol extracts were subjected to paper chromatography for preliminary purification

of steroid sulfates. When dealing with steroids possessing polarities ranging between that of cortisol sulfate and that of corticosterone sulfate, systems XVIII was used. For steroids possessing polarities ranging between that of 11-deoxycorticosterone sulfate and that of dehydroepiandrosterone sulfate, system XV or XVII was used. Following elution steroid sulfates were hydrolyzed enzymatically. Individual steroid fractions were redissolved in 5 ml. of 0.5 molar Acetate buffer, pH 5.2. Helicase Industrie Biologique Francaise was then added at the concentration of 500 units of sulfatase per ml. The mixture was incubated for 48 hrs. at 37°C, following which the hydrolyzed steroid was extracted with 5 volumes dichloromethane.

Unconjugated steroids as well as hydrolyzed steroid sulfates were subjected to paper chromatography prior to acetylation. The system used for steroids possessing polarities between that of cortisol and corticosterone was either system I or IV. 11-deoxycorticosterone was purified on system V, dehydroepiandrosterone and Δ^4-androstenedione on systems V and VIII. Individual steroids - with the exception of the last mentioned - were then acetylated with non-radioactive acetic anhydride in pyridine and their acetates - and on occasion their CrO_3 oxidation product - purified to radiochemical homogeneity by chromatography. The sequence of chromatography used is presented in Table III.

TABLE III

Sequence of Chromatography Systems After Acetylation

Product after acetylation	Sequence of Chromatography System #		
DHA-Ac	V;	VIII;	4
DOC-Ac	VIII;	2;	6
B-Ac *	2;	8;	7
A-Ac	2;	8;	7
F-Ac *	2;	9	
E-Ac	2;	9	
Δ^4ASD	V;	IX;	5

* Following the first chromatography, chromium trioxide oxidation was performed only in the instances of corticosterone acetate and cortisol acetate to yield 11-dehydrocorticosterone acetate and cortisone acetate respectively. For deciphering of code number of chromatography systems see Tables I and II.

Aliquots were taken after each chromatography and their content of radioactivity measured in terms of ^{3}H and ^{14}C. Radiochemical purity was considered established when at least two successive isotope ratios proved to be constant within an experimental error smaller than 10% (120). The yield of individual steroids is expressed as percent of either total radioactivity incubated or of the sum of the radioactivity extracted in the unconjugated plus sulfate fractions. All results are corrected for experimental losses from the recovery value of the reference steroids added prior to the extraction of the incubation media.

Results

Sulfatase activity of human placenta with respect to sulfates of the pregn-4-ene series

The sulfatase activity of human placental homogenate and microsomal fractions with respect to dehydroisoandrosterone sulfate - used here as control - and a number of steroids of the C-21 yl sulfate series is illustrated in Table IV. The results obtained with either placental homogenate or the microsomal fraction were comparable. In all instances of the total radioactivity extracted in the unconjugated and the sulfate fractions 68.4 - 98.0% was accounted for in terms of definite metabolites. Negligible sulfatase activity was observed

TABLE IV

Sulfatase Activity of Human Term Placenta Homogenate and Microsomal Fraction with Respect to Dehydroepiandrosterone Sulfate and Several Steroids of the Pregn-4-ene C-21 yl Sulfate Series*

Placenta	Substrate	Recovered as	% yield from extracted cpm
Homogenate	^{3}H-DHAS	DHA	80.0
		Δ^{4}ASD	18.0
		DHAS	0.6
	^{14}C-DOCS	DOC	7.6
		DOCS	79.0
	^{14}C-BS	B	0.2
		AS	1.5
		BS	87.1
	^{14}C-AS	A	0.2
		AS	68.2
Microsomal fraction	^{3}H-DHAS	DHA	94.5
		Δ^{4}ASD	1.5
		DHAS	2.3
	^{14}C-DOCS	DOC	2.9
		DOCS	85.8
	^{14}C-BS	B	0.1
		AS	0.8
		BS	92.5
	^{14}C-AS	A	0.2
		AS	70.0

* For conditions of incubation see text.

with respect to the sulfates of corticosterone and 11-dehydrocorticosterone which were recovered in the proportions of 68 to 92%. However an appreciable hydrolysis of 11-deoxycorticosterone sulfate was observed. The yield of the steroid alcohol being 3% in the instance of the microsomal fraction and 7.6% in the instance of the whole homogenate. Under the same experimental conditions dehydroepiandrosterone sulfate was hydrolyzed to the extent of 98 and 95%. Following hydrolysis, dehydroepiandrosterone sulfate was in part converted to androstenedione: to the extent of 18% by whole homogenate but only 1.5% by the microsomal fraction. This much lower figure might suggest a destruction of the 3β-hydroxysteroid dehydrogenase-isomerase system during the preparation of the microsomal fraction. The formation of androstenedione from dehydroepiandrosterone sulfate by whole placental homogenate was enhanced about twofold by the addition of NAD and fumarate at the dose level of 1 μmole of each, per incubation vial (this finding is not illustrated in Table IV).

Comparable results were obtained upon incubation of the ^{14}C-sulfates of 11-deoxycorticosterone, 11-dehydrocorticosterone and corticosterone with both the homogenate and the microsomal fraction of the mid-term placenta (Table V). The sulfates of corticosterone and 11-dehydrocorticosterone were recovered in the proportion of 74 to 97%, negligible amounts appearing as the corresponding steroid alcohol (less than 0.5%).

TABLE V

Sulfatase Activity of Human Mid-Term Placenta Homogenate and Microsomal Fraction with Respect to Various Steroids of the Pregn-4-ene C-21 yl Sulfate Series

Placenta	Substrate	Recovered as	% yield from substrate
Homogenate	^{14}C-DOCS	DOC	3.8
		DOCS	64.4
	^{14}C-BS	B	0.3
		AS	2.0
		BS	96.3
	^{14}C-AS	A	0.3
		AS	80.0
Microsomal fraction	^{3}H-DOCS	DOC	0.5
		DOCS	71.4
	^{14}C-BS	B	0.1
		AS	1.7
		BS	97.2
	^{14}C-AS	A	0.1
		AS	74.0

11-deoxycorticosterone sulfate was hydrolyzed to the extent of 3.8% by placental homogenate and 0.5% - a low figure - by the microsomal fraction.

In an attempt to establish whether this hydrolysis of 11-deoxycorticosterone sulfate was, or not an artifact of the incubation conditions, this conjugate was incubated in the presence of identical amounts of placental homogenate (500 mg. tissue equivalent) in different buffers. The results (Table VI) substantiates previous findings in showing that the conjugate was hydrolyzed to the extent of 4 to 16%, depending on the buffer used, the highest values being obtained for trishydrochloride and Krebs-Ringer Bicarbonate buffers.

In favour of the view that the nature of this hydrolysis was enzymatic, it was observed that it did not occur with homogenate previously denatured by boiling. Conversely, addition of Helix Pomatia sulfatase (Helicase) to the homogenate enhanced its capacity to hydrolyze this conjugate from 4% - control value - to 39%.

11β-hydroxysteroid dehydrogenase activity of human placenta with respect to the sulfates of cortisol and corticosterone

The activity of placental 11β-hydroxysteroid dehydrogenase has been documented previously by several authors (123,124). It is illustrated again (Table VII) by the results of control experiments, where the formation of

TABLE VI

Hydrolysis of 11-Deoxycorticosterone Sulfate* by Term Placenta Homogenate Incubated in Various Buffers

Buffer	% yield from extracted cpm as DOC	as DOCS
Krebs-Ringer Bicarbonate	15.3	67.4
Phosphate	7.0	78.0
Acetate	4.4	75.0
Acetate + Helicase	38.8	19.6
TRIS-HCl	16.7	69.2
TRIS-HCl (boiled tissue)	0.0	92.6

* In all instances 9.0×10^4 cpm of ^{3}H-11-deoxycorticosterone sulfate (50 mμMole) were incubated with 200 mg. of tissue (wet weight equivalent) diluted with 20% of the buffer listed in column I.

TABLE VII

11β-Hydroxysteroid Dehydrogenase Activity of Human Term Placenta Homogenates

Substrate	Co-factor	Recovered as	% yield from substrate
F	O	E	33.6
F	NADP	E	72.7
FS	O	ES	6.3
FS	NADP	ES	16.3
B	O	A	23.6
B	NADP	A	57.8
BS	O	AS	7.6
BS	NADP	AS	9.0

cortisone from cortisol and of 11-dehydrocorticosterone from corticosterone were observed in yields of 33% and 23% respectively. Addition of NADP at the dose of 2 µMole per incubation flask, enhanced this conversion two to threefold, as expected. In contrast when the sulfates of cortisol and corticosterone were used as substrates, the yield of the corresponding 11-oxo steroid sulfates were only of the order of 6 to 7%. The substrates cortisol and corticosterone sulfates were recovered unchanged to the extent of 86 and 83%. The addition of NADP increased the yield of cortisone sulfate by about 2.5 fold, but had only a questionable effect on the yield of 11-dehydrocorticosterone sulfate. In these instances the substrates cortisol sulfate and corticosterone sulfate were recovered in the proportion of 74% and 77% respectively.

Discussion

These results underline a striking difference between the placental metabolism of steroids of the 3β-yl and C-21-yl sulfate series. As documented by previous investigators (7,8,14-17,121) they clearly illustrate the activity of placental sulfatase with respect to dehydroepiandrosterone sulfate, under the experimental conditions used here. In contradistinction to this the hydrolysis of the sulfates of corticosterone and 11-dehydrocorticosterone was negligible. This finding is in general agreement with those previously reported by Pasqualini et al (85,125) following perfusion of either the isolated placenta or of the foeto-placental unit with labelled corticosterone sulfate. In both instances it was observed that the capacity of the placenta to hydrolyze this conjugate was limited, although the figures reported (1.0 - 3.5% hydrolysis) were higher than those obtained in the present study.

The substantial hydrolysis of 11-deoxycorticosterone sulfate observed in different buffers is of interest when one considers that this conjugate, quantitatively the most important among C-21 steroid sulfates of the pregn-4-ene series in human umbilical cord plasma (83,84) is not found in late pregnancy urine (126). This suggests an extensive metabolism of the conjugate prior to excretion, one aspect of which could involve its partial hydrolysis by the placenta.

The presence of 11β-hydroxysteroid dehydrogenase in the placenta has been previously documented by Osinski (124) who studied the kinetics of this enzyme using cortisol and corticosterone as substrates and by Sylbulski and Venning (123). Similar enzymatic activity is also present in human liver microsomes (127). During gestation this enzyme is most likely responsible for the presence of cortisone and 11-dehydrocorticosterone in the foetal circulation at concentrations substantially higher than in the maternal circulation (128-130). It is also reasonable to attribute to the activity of the same enzyme, the pre-eminence of 11-oxo-steroid metabolites of cortisol and corticosterone in the urine of the human newborn, as will be documented in a forthcoming section of this thesis.

The result obtained indicate further that the presence of the C-21 sulfate esters on 11β-hydroxysteroids considerably decreases the extent to which they are utilized as substrates by 11β-hydroxysteroid dehydrogenase, compared to the corresponding alcohols. Yet 11-dehydrocorticosterone sulfate and corticosterone sulfate concentrations in umbilical cord plasma are within the same range (84). This could be explained on the grounds that 11-dehydrocorticosterone sulfate is formed in part by the foetal adrenal as indicated by the isolation of the conjugate from adrenal extracts, following perfusion of the previable human foetus with labelled progesterone (6).

It is difficult to discuss the extent to which these findings are pertinent to the problem of maintenance of hormonal homeostasis within the foeto-placental unit. In this context, however, one should recall that the 11ß-hydroxy group is generally considered essential for the biological activity of gluco-corticoids and that, on the other hand, sulfation of corticosterone prevents this corticosteroid from promoting liver glycogen deposition in experimental animals (131).

CHAPTER II

Simultaneous Metabolism of Cortisol Sulfate and Cortisol in Normal Human Adults

Summary

Tracer doses of cortisol and of cortisol sulfate, each bearing a different radioactive label were administered intravenously to 4 subjects. Unconjugated steroids, steroids released following β-glucuronidase hydrolysis, and steroid sulfates were sequentially extracted from the urine voided within 48 hrs. after the injection. Following purification from the glucuronide fraction, 11β-hydroxy-and 11-ketoetiocholanolone contained greater or equal amounts of the isotope originally associated with the cortisol sulfate injected, while cortolone, tetrahydrocortisol and tetrahydrocortisone contained negligible amounts of the radioactivity originally associated with cortisol alcohol. From the specific activity of the latter 2 metabolites, cortisol secretion rates of 8-21 mg/day were calculated. Sulfation of cortisol did not occur to any appreciable extent. From the specific activity of cortisol sulfate, which was the main component of the sulfate fraction, secretion rates of this conjugate ranging from .04 to 0.1 mg/day were obtained. The data demonstrate that in adult subjects cortisol sulfate is in part metabolized through a pathway yielding 11β-hydroxy- and 11-oxo-17-ketosteroids, which are subsequently excreted as glucuronide esters, and that the secretion rate of cortisol

sulfate represents but a fraction of that of cortisol.

Introduction

Although cortisol sulfate was characterized in human urine following the administration of large amounts of the corresponding steroid alcohol (79), there is little evidence to suggest that this conjugate is a significant component of the urinary metabolites of cortisol in normal human adults (132). In contrast, cortisol sulfate is excreted in appreciable amounts during the third trimester of human pregnancy (133,134) as well as by the newborn infant (86). The present studies on the metabolism of cortisol and cortisol sulfate in human adults were undertaken in order to define the metabolism of cortisol sulfate and to establish the production rate of this conjugate compared to that of cortisol. The results of these investigations confirm and extend observations reported by Nakada et al (135) while these studies were in progress.

Methods

In the forthcoming report the secretion rates of cortisol and cortisol sulfate were calculated according to the equation published previously, in the instance of corticosterone and its sulfate (88).

Experimental Subjects

Four subjects were studied: 2 were males, JB and GH, aged 24 and 31, respectively, and 2 were females, BA, aged 18, and LL, 23. At the time of the study BA was under Ortho-Novum therapy, the daily dose being 1 mg norethindrone and 0.08 mg mestranol, in an attempt to re-establish a normal menstrual cycle. LL was under Ortho-Novum at the dose level of 2 mg of norethindrone and 0.1 mg of mestranol daily.

The labelled steroids, diluted in 5 ml of 10% ethanol in 0.9% NaCl, were injected into the antecubital vein over a period of 5 min. Following the injection the subjects were allowed to pursue their usual activities while collecting urine for 3 consecutive days. The specific activities in mCi/mmole of the injected steroids were: 4-^{14}C-cortisol and 4-^{14}C-cortisol sulfate: 52; 1,2-^{3}H-cortisol and 1,2-^{3}H-cortisol sulfate: 1.82×10^{4}. Subjects BA and GH both received ^{14}C-cortisol sulfate and ^{3}H-cortisol. The weight of the injected sulfate was 8 and 9 µg, that of the steroid alcohol 46 and 54 ng, respectively. Subjects LL and JB received ^{3}H-cortisol sulfate (31 and 40 ng, respectively) and ^{14}C-cortisol (6 and 7 µg, respectively). In all cases the isotope ratio of the injected steroids was one.

Extraction of Urine

Negligible amounts of radioactivity being present in the urine voided on the third day of collection, an aliquot equivalent to 36-hr. urine output of a first 48-hr. urine pool was processed. Unconjugated steroids were extracted with ethyl acetate (88). Steroid glucuronides were hydrolyzed with β-glucuronidase (Ketodase, Warner-Chilcott) at the concentration of 500 Fishman U/ml of urine, for 5 days, and subsequently extracted with ethyl acetate (glucuronide fraction). Ammonium sulfate (50 gm/100 ml) was then added to an aliquot equivalent to 24-hr. urine output and the steroid sulfate fraction extracted with ether-ethanol (136). Another aliquot equivalent to 3-hr. urine output was subjected to a second enzymatic hydrolysis with Glusulase (Endo Laboratories, Garden City, N.Y.), which possesses β-glucuronidase and sulfatase activities in the ratio of 3.8. This preparation was used at the concentration of 500 U of sulfatase activity per ml of urine (100). This extract will be referred to as the sulfate fraction. This is justified by the observation that during sequential hydrolysis of urine with Ketodase and Glusulase, cortisol sulfate is only hydrolyzed by the latter enzyme preparation (100).

Purification of Steroid Fractions

a) Unconjugated Fraction

This fraction was first chromatographed on system II (see, in Table I code number assigned to chromatographic systems). The main area of radioactivity possessed the polarity of 6β-hydroxycortisol and gave a positive Porter-Silber reaction. It was further purified on system III and on thin layer, in system 12.

b) Glucuronide Fraction

The glucuronide fraction (hydrolyzed with Ketodase) was chromatographed on system I for 24 hrs. Four radioactive areas, corresponding to the mobilities of cortol, cortolone, tetrahydrocortisol and tetrahydrocortisone, were detected on this chromatogram. The first 2 were further purified on system III, the last 2 on systems III and IV. The specific activities of tetrahydrocortisol and tetrahydrocortisone were then established by the Porter-Silber reaction and by estimating their content of radioactivity.

The overflow of system I, which contained both isotopes, was chromatographed in system V. The 2 main areas of radioactivity possessed the Rf of 11β-hydroxyetiocholanolone and of 11-ketoetiocholanolone, respectively. The former

fraction was further purified on system VI, the latter on system VII. Following acetylation with nonradioactive acetic anhydride, these 2 steroid acetates were rechromatographed in system V. After addition of 20 mg of the respective reference steroid acetate, they were crystallized to constant specific activity: 11β-hydroxy-etiocholanolone acetate from acetone-ethanol and 11-ketoetiocholanolone from ethanol.

In the instance of subject JB who received ^{14}C-cortisol and ^{3}H-cortisol sulfate only a negligible amount of tritium was detectable on the areas corresponding to the mobility of these two 17-ketosteroids judging from the 36-hr. aliquot of the 48-hr. pool processed. Therefore, these fractions were not further processed.

c) Sulfate Fraction

The preliminary purification of this ether-ethanol extract involved thin layer chromatography in system 1 (Table II). The chromatoplate was radioautographed for 48 hrs. revealing a single radioactive area (Rf:0.3).

Following this preliminary purification, the sulfate fraction was chromatographed on system XVIII. On this chromatogram, the main area of radioactivity possessed the same Rf as cortisol sulfate (0.45). The cortisol sulfate eluate was subjected to enzyme hydrolysis, using Helicase

(Industrie Biologique Francaise) (86). Cortisol was purified from the dichloromethane extract of this hydrolysate, using systems I and IV in sequence. Following acetylation with either ^{14}C or ^{3}H acetic anhydride, as applicable, and thin layer chromatography of cortisol acetate in a double dimensional system: (a) system 2, (b) system 3, 20 mg of cortisol acetate was added to this fraction, which was crystallized first as cortisol acetate from methanol-ether and, following CrO_3 oxidation (103), as cortisone acetate, from ethanol. The specific activity of the radiochemically pure cortisone acetate was used for calculating the production rate of cortisol sulfate.

Results

Table VIII presents the amounts of radioactivity expressed as percentage of the administered dose of either cortisol alcohol or cortisol sulfate which were extracted in the unconjugated and glucuronide fractions, and in the sulfate fraction hydrolyzed by Glusulase. It is readily apparent that substantial amounts of cortisol sulfate metabolites were extracted following hydrolysis of the urine with Ketodase. In the instance of subject JB, however, the figure amounts only to 1.2% of the injected dose, reflecting an overall low recovery of cortisol sulfate metabolites (25.5%). Cortisol alcohol metabolites appeared in the glucuronide

TABLE VIII

Amounts of Radioactivity Extracted from Urine in the Unconjugated, Glucuronide and Sulfate Fractions, as Percent of Injected Dose*

Subject	Unconjugated fraction		Glucuronide fraction		Sulfate fraction	
	1	2	1	2	1	2
BA	3.0	0.4	48.5	29.5	7.8	51.3
GH	2.2	0.2	49.7	18.5	9.1	57.5
LL	4.3	0.5	54.5	12.0	11.8	38.6
JB	3.9	0.3	51.2	1.2	9.1	24.0

* For each of the unconjugated, glucuronide and sulfate fractions the second column (#2) refers to percentages from the injected dose of cortisol sulfate, the first column (#1) to percentages from the injected dose of cortisol alcohol. Steroid glucuronides were hydrolyzed with Ketodase, steroid sulfates with Glusulase (see text).

fraction to the extent of 49 to 55%.

Following purification of these various fractions by chromatography and derivative formation, (Table IX to IXc) one observed that of the radioactivity originally associated with cortisol sulfate only small amounts were incorporated either into 6β-hydroxycortisol, or into cortolone, tetrahydrocortisone and tetrahydrocortisol glucuronides. In contrast to this, 11β-hydroxy- and 11-ketoetiocholanolone contained greater amounts of radioactivity originating from cortisol sulfate than from cortisol. These findings were further substantiated following crystallization of the acetates of these 17-ketosteroids to radiochemical homogeneity in the case of the first three subjects studied (Table X).

Cortisol sulfate, which contained negligible amounts of radioactivity originating from cortisol alcohol, was the main component of the sulfate fraction. After enzymatic hydrolysis and acetylation with either ^{3}H or ^{14}C acetic anhydride, as applicable, it was crystallized to constant isotope ratios prior to and following chromium trioxide oxidation to cortisone acetate. From the specific activity of the latter derivative, the secretion rate of cortisol sulfate was established. The values obtained represent but a fraction of the secretion rates of cortisol calculated from the specific activity of both tetrahydrocortisol and tetrahydrocortisone (Table XI).

TABLE IX

Isotope Content of 6β-Hydroxycortisol and of Several Metabolites Purified from the Glucuronide Fraction (Subject BA)*

Urinary metabolites	Total activity - in 10^2 cpm - originating from:		% of injected dose originating from:		Ratio
	F	FS	F	FS	F/FS
6β-OH-F	151	5	1.5	0.05	31.50
Cortolone	492	7	4.8	0.07	70.30
THF	396	21	3.8	0.22	19.30
THE	852	10	8.3	0.11	85.20
11-OH-E	108	792	1.1	8.40	0.13
11-O-E	228	540	2.2	5.70	0.42
11-OH-A	16	84	0.2	0.89	0.18

* For all steroids the total activity in terms of ^3H and ^{14}C was established following at least 3 chromatographies and the formation of a derivative by acetylation.

TABLE IXa

Isotope Content of 6β-Hydroxycortisol and of Several Metabolites Purified from the Glucuronide Fraction (Subject LL)

Urinary metabolites	Total activity - in 10^2cpm - originating from:		% of injected dose originating from:		Ratio
	F	FS	F	FS	F/FS
6β-OH-F	30	10	0.37	0.02	30.00
Cortolone	292	-	3.56	-	-
THF	240	-	2.93	-	-
THE	308	10	3.76	0.18	30.80
11-OH-E	60	98	0.73	1.77	0.62
11-O-E	100	102	1.22	1.85	0.98

TABLE IXb

Isotope Content of Several Metabolites Purified from the Glucuronide Fraction (Subject GH)

Urinary metabolites	Total activity - in 10^2 cpm - originating from:		% of injected dose originating from:		Ratio
	F	FS	F	FS	F/FS
Cortolone	265	520	2.89	0.05	101.90
THF	367	280	4.00	0.03	131.00
THE	344	-	3.75	-	-
11-OH-E	91	302	0.99	2.93	0.30
11-O-E	92	224	1.00	2.17	0.41

TABLE IXc

Isotope Content of 6β-Hydroxycortisol and of Several Metabolites Purified from the Glucuronide Fraction (Subject JB)*

Urinary metabolites	Total activity - in 10^2 cpm - originating from:		% of injected dose originating from:		Ratio
	F	FS	F	FS	F/FS
6β-OH-F	13	1.0	0.13	0.11	13.0
Cortolone	220	-	2.24	-	-
THF	340	-	3.45	-	-
THE	480	-	4.90	-	-

* Subject JB did not excrete any radioactivity associated with either 11-OH-E or 11-O-E.

TABLE X

Crystallization Data of 11β-Hydroxy and 11-Ketoetiocholanolone Purified from the Glucuronide Fraction*

Subject	Steroid	dpm/mg of crystal crop #			Isotope ratio of third crystal crop - cpm +
		1	2	3	
BA	11-O-E	680/470	620/410	660/480	0.39
	11-OH-E	490/1000	-	470/1000	0.13
GH	11-O-E	2500/1680	2480/1650	2570/1700	0.41
	11-OH-E	1650/1620	1910/1800	1740/1730	0.29
LL	11-O-E	730/2700	-	740/2670	0.99
	11-OH-E	420/2500	410/2700	420/2460	0.58

* In each column (1-3) the figures to the left represent ^{3}H dpm/mg, the figures to the right ^{14}C in the instance of subjects BA and GH. The reverse applies in the instance of subject LL.

+ Isotope ratio cortisol/cortisol sulfate injected - 1.

TABLE XI

Crystallization Data of Cortisol Sulfate and Secretion Rates of Cortisol Sulfate and of Cortisol

Subject	dpm/mg of crystal crop #		Secretion rate of		
	2*	3	cortisol sulfate: mg/day	cortisol mg/day+	
BA	100/1190	100/1150	.08	10.6	10.5
GH	790/12850	780/12550	.04	20.5	21.0
LL	1730/240	1730/240	.10	8.5	7.6
JB	1290/125	1260/120	.07	17.5	17.0

* Following CrO_3 oxidation of cortisol acetate to cortisone acetate. The figures to the left represent ^3H, the figures to the right ^{14}C in the instance of subjects BA and GH. The reverse applies in the instance of subjects LL and JB.

+ Established from the specific activity of THF and THE (last column).

Discussion

In normal adult subjects there is little evidence in favour of cortisol sulfate being a quantitatively significant metabolite of cortisol (79,132), although a relatively high proportion of urinary cortisol is excreted as the 21-yl-sulfate (132). This fraction, however, as in the case of cortisol itself, represents but a small percentage of the predominant urinary metabolites of the hormone such as tetrahydrocortisol and tetrahydrocortisone glucuronides (132). Since in the conditions of the present experiment the formation of cortisol sulfate from cortisol was not observed, the results presented show that urinary cortisol sulfate is a product, though minor, of adrenocortical biosynthesis and the main urinary metabolite of this conjugate.

This is in keeping with results obtained by others in showing that C-21 steroid sulfates of the pregn-4-ene series are not extensively metabolized in vivo as long as the ester linkage is not hydrolyzed (88,137). This appears to be the case both in adults and following perfusion of the human fetus at midpregnancy (138). The present results, however, as well as those of Nakada et al (135), show that a substantial amount of cortisol sulfate metabolites are excreted as 11-oxygenated, 17-ketosteroid glucuronides, mainly 11β-hydroxy-and 11-ketoetiocholanolone and, to a lesser extent, 11β-hydroxyandrosterone. This metabolic pathway does not appear to involve a significant formation of

steroid glucuronides bearing either a dihydroxyacetone or a glycerol side chain, since tetrahydrocortisone, tetrahydrocortisol, as well as cortolone, contained only small amounts of the isotope originally associated with the cortisol sulfate injected. The formation of a ring A reduced sulfo-conjugate intermediate cannot entirely be ruled out. It is unlikely, however, that such an intermediate be either tetrahydrocortisol or tetrahydrocortisone sulfates, since we were unable to detect their presence during the processing of the sulfate fraction. Nevertheless, it is difficult to conceive that if cortisol sulfate was hydrolyzed to cortisol only negligible amounts would be incorporated into tetrahydrocortisol and tetrahydrocortisone.

These considerations remain, however, of more biochemical than physiological interest, since the secretion rates of cortisol sulfate reported here for adults represent but a very small fraction of those of cortisol.

CHAPTER III

Secretion Rate and Metabolism of the Sulfates of Cortisol and Corticosterone in Newborn Infants

Summary

^{3}H-cortisol and ^{14}C-cortisol sulfate, and ^{3}H-corticosterone and ^{14}C-corticosterone sulfate, were injected intravenously to human newborns aged 2 days. From 49-79% of ^{3}H and from 62-100% of ^{14}C were accounted for in their urine. Of the total amount of tritium present in the urine only small proportions were extractable. The amounts extracted in both the sulfate (3-18%) and glucuronide (1.3-13%) fractions were substantially greater than in the unconjugated fraction. Almost negligible amounts of ^{14}C appeared in either the unconjugated or glucuronide fractions. The sulfate fraction consisted of two main components: the sulfates of cortisol and corticosterone, which were devoid of tritium; and a highly polar fraction which contained both ^{14}C and most of the tritium extractable in this fraction. From the specific activities of tetrahydrocortisone and tetrahydro-11-dehydrocorticosterone purified from the glucuronide fraction, and from those of the sulfates of cortisol and corticosterone the secretion rate of cortisol was calculated as ranging from 12.6 - 16.4 $mg/m^2/day$, that of corticosterone from 4.6 - 18.3; those of their respective

ester sulfates: 0.12 - 0.35 and 1.8 - 5.4. The findings indicate that cortisol and corticosterone are not sulfurylated prior to being metabolized; that a relatively substantial amount of their metabolites may be excreted as sulfate esters, and that corticosterone and its sulfate may represent very significant secretory products of the adrenal in the human newborn.

Introduction

Cortisol and corticosterone sulfates were previously characterized in the urine of the newborn infant (86,87,100) and their excretion rate found to be very significant compared to that of tetrahydrocortisone glucuronide, the predominant, known metabolite of cortisol during the perinatal period (101). These findings did not establish whether such sulfoconjugates were secreted by the adrenal of the newborn infant - which indeed is capable of forming them under appropriate in vitro conditions - (82) or whether they were formed during the metabolism of the corresponding steroid alcohols by extra-adrenal tissues. In an attempt to resolve this problem, ^{3}H-cortisol and ^{14}C-cortisol sulfate on the one hand, and ^{3}H-corticosterone and ^{14}C-corticosterone sulfate on the other, were administered simultaneously to newborn infants during the second day of life, in order to study their metabolism and their rate of formation.

Methods

Secretion rates of steroid alcohols and of their respective sulfate esters were calculated according to the equation previously published (88).

Experimental Subjects

All infants studied were full-term males. Studies were performed from the 2nd to the 4th day of life. One subject, however, (L-2) was also studied from the 7th to the 9th day. Only one infant was normal (L-14). All others were born with congenital abnormalities of the central nervous system characterized mainly by hydrocephaly and myelomeningocele of different degrees of severity.

Labelled steroids, diluted in 5 ml of 10% ethanol in 0.9% sodium chloride were injected into a scalp vein through the wall of a plastic tube connected to a bottle of 0.9% sodium chloride. The injection time was approximately five minutes, following which the intravenous set was flushed with saline for a period of ten minutes. Following the injection, urine was collected for three consecutive 24-hr. periods. Urine collection conditions were essentially similar to those described by Hepner and Lubchenco (139), with the exception that the urine was collected by means of a Hollister U-bag, connected by a tube to an ice-cooled bottle.

The nature and amounts of ^{3}H-and ^{14}C-steroids administered to individual infants are presented in Table XII.

Extraction of Urine

Negligible amounts of radioactivity was present in the urine voided during the 3rd day of collection. Therefore the 1st 48 hrs. urine excreted was pooled and extracted as described previously in the preceding chapter. Briefly, this method involved extensive ethyl acetate extraction of the urine prior to and following β-glucuronidase hydrolysis (Ketodase, Warner-Chilcott). Ammonium sulfate was then added to the urine, which was extracted with ether-ethanol according to the procedure of Edwards et al (136). The three fractions obtained by these procedures are referred to as the unconjugated, glucuronide and sulfate fractions.

Purification of Steroid Fractions

A) Studies with ^{3}H-cortisol and ^{14}C-cortisol sulfate

1) The unconjugated fraction was purified on system II. The main radioactive area possessed the polarity of 6β-hydroxycortisol, absorbed ultraviolet light and gave a positive Porter-Silber reaction. It contained

TABLE XII

Amounts of Steroids Administered to Individual Subjects*

Subject	^{3}H-Cortisol		^{14}C-Cortisol Sulfate	
	dpm x 10^{6}	ng	dpm x 10^{6}	µg
L-14	2.80	17	0.60	3.5
L-2	9.05	83	2.95	11.0
L-7	9.89	90	3.17	11.6
	^{3}H-Corticosterone		^{14}C-Corticosterone Sulfate	
	dpm x 10^{6}	ng	dpm x 10^{6}	µg
D	5.84	17	10.5	4.0
W	9.47	28	3.66	13.5
C	11.10	33	3.87	12.4

* Counting efficiency: ^{3}H: 19%; ^{14}C: 63%.

however, an amount of radioactivity too small to be further processed.

2) The glucuronide fraction was chromatographed on system I. The most polar radioactive area was further purified on system III where it had the same Rf as β-cortolone. Two additional radioactive areas were detected, corresponding to the mobilities of cortolone and tetrahydrocortisone. They did not contain detectable amounts of the isotope originally associated with the ^{14}C-cortisol sulfate injected. Cortolone was further purified on system III both prior to and following its acetylation with non-radioactive acetic anhydride. The acetylation product possessed the same mobility as authentic cortolone triacetate. Tetrahydrocortisone was chromatographed on systems IV and III, following which its specific activity was established on aliquots by the Porter-Silber reaction and by estimating their content of radioactivity. Another aliquot was acetylated with ^{14}C-acetic anhydride and the diacetate chromatographed in system X. Following elution, 20 mg of tetrahydrocortisone diacetate were added to this fraction, which was crystallized to constant specific activity from methanol. The specific activity obtained for tetrahydrocortisone by either method was used to calculate the secretion rate of cortisol.

3) The preliminary purification of the sulfate fraction which, in the instance of subject L-14, was extracted from the urine with ether-ethanol (136), involved chromatography on system XVIII. Two areas of radioactivity were detected; the first had an Rf of 0.16. The second, which contained negligible amounts of tritium, had the same Rf as cortisol sulfate (0.65). This latter fraction was further purified on system XVII, then hydrolyzed enzymatically with Helicase. Cortisol was purified from the dichloromethane extract of this hydrolysate on systems I and IV and then acetylated with ^{3}H-acetic anhydride. Cortisol acetate was subjected to double dimensional thin-layer chromatography using solvent systems 2 and 3 in sequence. Twenty milligrams of cortisol acetate were then added to this fraction, which was crystallized first as cortisol acetate from methanol-ether and, following chromium trioxide oxidation, as cortisone acetate from ethanol. The specific activity of the radiochemically pure cortisone acetate was used for calculating the secretion rate of cortisol sulfate.

In the case of subjects L-2 and L-7, following extraction of urine for the unconjugated and glucuronide fractions, the sulfate fraction was obtained by enzymatic hydrolysis of the urine with Glusulase (Endo Laboratories, Garden City, New York), used at the concentration of 500

units of sulfatase activity per ml of urine (100). Cortisol was purified from this fraction by chromatography on system I, IV, and XII and then by thin-layer chromatography in system 10. It was then acetylated with ^{3}H-acetic anhydride and crystallized as described above.

B) Studies with ^{3}H-corticosterone and ^{14}C-corticosterone sulfate

1) The methods used to purify the urinary metabolites of these two steroids follow in their broad outlines those applied when essentially similar investigations were performed in adult subjects (88). The unconjugated fraction was purified on system IV. The only major radioactive area had a polarity greater than cortisol. It was not further processed.

2) The glucuronide fraction was purified on system I. Two areas of radioactivity corresponding to allo-tetrahydrocorticosterone and to tetrahydro-11-dehydro-corticosterone were detected by scanning. The radioactivity associated with the allo-tetrahydrocorticosterone was too small to justify further purification. The tetrahydro-11-dehydrocorticosterone fraction, which did not contain detectable amounts of ^{14}C, was further purified on system XI, then acetylated with ^{14}C-acetic anhydride. The diacetate was chromatographed on system XIII and XIV, and twice on thin-layer in systems 7 and 11. Aliquots taken for counting

following each chromatography indicated that at this stage radiochemical purity had been achieved, as judged by the constancy of the $^{14}C/^{3}H$ ratios. From the final ratios the specific activity of tetrahydro-11-dehydro-corticosterone diacetate was calculated and used for the computation of the secretion rate of corticosterone (88).

3) The sulfate fraction was obtained by two different methodological approaches. The first involved the ether-ethanol extraction of urine according to the method of Edwards et al (136) (subject D); the second, enzyme hydrolysis with Glusulase (100) following extraction of the unconjugated and glucuronide fractions as described above (subjects W and C).

In the instance of subject D, the preliminary purification of the sulfate fraction (system XVIII) was followed by the enzymatic hydrolysis of corticosterone sulfate with Helicase. From this stage on the method used was the same for the three subjects studied. Corticosterone was purified on system IV and acetylated with ^{3}H-acetic anhydride. The acetate was chromatographed on thin-layer in the double dimensional solvent system 2 and system 3, then on system V and system X, following which it was oxidized to 11-dehydrocorticosterone acetate with chromium trioxide. Twenty milligrams of authentic 11-dehydro-corticosterone acetate were added to this fraction, which

was crystallized to constant specific activity from methanol. From the specific activity of 11-dehydro-corticosterone acetate the secretion rate of corticosterone sulfate was calculated.

Results

The amounts of radioactivity excreted into the urine within 48 hrs. following the administration of the ^{3}H-and ^{14}C-steroids are presented in Table XIII. All data are expressed as a percentage of the respective ^{3}H and ^{14}C doses injected.

With the exception of subjects L-2 and L-7, the urinary recovery of tritium is low (49-62%), while in all subjects that of ^{14}C is consistently higher (over 81% in all but one instance).

Of the total amount of ^{3}H excreted, only small proportions were accounted for in the unconjugated fraction, while substantially greater amounts were recovered in both the sulfate and glucuronide fractions. The total amount of ^{3}H extractable in the unconjugated, glucuronide and sulfate fractions represents 49, 17 and 21% of the amount present in the urine in the instance of ^{3}H-cortisol, and 24, 12 and 10% in the instance of ^{3}H-corticosterone.

Urinary ^{14}C was recovered in the sulfate fraction in the proportion of 59-83% in the studies involving cortisol

TABLE XIII

Excretion and Recovery of Radioactivity from Different Urine Fractions Following the Simultaneous Injection of 4-^{14}C-Cortisol Sulfate and 1,2-^{3}H-Cortisol; 4-^{14}C-Corticosterone Sulfate and 1,2-^{3}H-Corticosterone to Newborn Infants

*Radioactivity excreted in 1st 48 hrs. urine as percent injected cpm			Radioactivity extracted in urine fractions as percent injected dose					
Subject	^{3}H	^{14}C	Unconjugated		Glucuronide		Sulfate	
			^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
L-14	62.5	90	3.6	0.1	8.5	0.2	18.9	53.5
L-2	75.2	100.6	1.4	0.03	7.4	0.2	4.0	82.7
L-7	79.4	85.1	0.7	0.02	12.9	0.1	3.5	54.5
D	61.0	81.0	1.3	-	7.8	-	5.6	19.4
C	49.0	62.2	0.8	0.1	1.6	0.1	3.7	11.7
W	51.5	84.3	1.0	0.1	1.3	0.2	3.0	9.1

* The 1st 3 subjects received ^{3}H-cortisol and ^{14}C-cortisol sulfate; the last 3, ^{3}H-corticosterone and ^{14}C-corticosterone sulfate.

sulfate, and of 11-24% in the corticosterone sulfate studies. In all cases practically negligible amounts of ^{14}C radioactivity were extractable in either the unconjugated or glucuronide fractions.

The processing of these different fractions by chromatography revealed the following pattern of metabolites. In the instance of cortisol, the unconjugated fraction contained small amounts of radioactivity possessing the polarity of 6β-hydroxycortisol and cortisol. The glucuronide fraction was characterized by the presence of tetrahydrocortisone - consistently the major metabolite; of cortolone; and of a fraction tentatively characterized as β-cortolone. The absence of tetrahydrocortisol was noteworthy. The overflow of the glucuronide fraction contained only a small amount of radioactivity. It was nevertheless chromatographed on system V to investigate the possible presence of labelled metabolites such as 11β-hydroxy- or 11-oxo-17-ketosteroids. Such metabolites could not be detected. The sulfate fraction was characterized by the presence of two components: cortisol sulfate itself, and a polar fraction, in which most of the tritium originally associated with the steroid alcohol was detected. Its $^{3}H/^{14}C$ ratio varied from 0.11 to 0.44. In contrast, the $^{3}H/^{14}C$ ratio of the cortisol sulfate area ranged from 0.02 to 0.03.

The main metabolites of corticosterone in the glucuronide fraction were allo-tetrahydrocorticosterone and tetrahydro-11-dehydrocorticosterone, the latter being predominant. Here again the sulfate fraction consisted of two components: corticosterone sulfate, which did not contain detectable amount of ^{3}H, and a highly polar fraction - the $^{3}H/^{14}C$ ratio of which ranged between 0.36 and 0.58.

The secretion rates of cortisol and cortisol sulfate, expressed as $mg/m^2/day$ are presented in Table XIV together with the crystallization data of the respective urinary metabolites, from the specific activity of which these values were obtained. Table XV shows the corresponding findings for corticosterone and corticosterone sulfate.

The data show that the secretion rate of cortisol sulfate is of little quantitative significance compared to that of cortisol; while that of corticosterone sulfate represents 117, 18 and 11% of that of corticosterone; the secretion rate values for the latter steroid being 4.6, 18.3 and 16.3 $mg/m^2/day$.

Discussion

The results of these investigations do not lead to a clearcut interpretation, for reasons which one might attempt to appraise in the present discussion.

TABLE XIV

Crystallization Data and Secretion Rates of Cortisol and Cortisol Sulfate*

Subject	Urinary metabolite	dpm/mg of crystal crop No. 2	3	Secretion rate mg/m^2/24 hr. F	F-S
L-14	F-S	17,200/690	16,730/630		0.35
	THE	1170/2430	1160/2260	12.6	
L-2	F-S	6160/880	6250/880		0.23
	THE	1170/5780	1180/5940	16.4	
L-7	F-S	850/290	860/290		0.12
	THE	1790/12,470	1790/12,210	14.2	

* All patients received ^{3}H-F and ^{14}C-FS. Since urinary THE and F-S were devoid of ^{14}C and ^{3}H respectively, THE was acetylated with ^{14}C-acetic anhydride, cortisol sulfate (following hydrolysis) with ^{3}H-acetic anhydride. Therefore in columns 3 and 4, for F-S, the figure to the left represents ^{14}C dpm/mg, the figure to the right: ^{3}H. The reverse applies in the instance of THE.

TABLE XV

Crystallization Data of Corticosterone Sulfate and Secretion Rates of Corticosterone Sulfate and Corticosterone (1)

Subject	$^{3}H/^{14}C$ dpm/mg of crystal crop No.		Secretion rate	
	2	3	Corticosterone sulfate	Corticosterone(2)
			$mg/m^2/day$	$mg/m^2/day$
D	8400/660	8470/660	5.4	4.6
C*	800/410	810/420	3.3	18.3
W	230/240	240/260	1.8	16.3

(1) In columns 2 and 3 the figure to the left represents ^{3}H dpm/mg, the figure to the right: ^{14}C.

(2) The secretion rate of this steroid was calculated from the specific activity of tetrahydro-11-dehydrocorticosterone diacetate, established by double isotope assay (see text).

* This infant underwent neurosurgery 12 hours following injection of the labelled steroids.

Following administration of labelled cortisol to newborn infants, the amounts of radioactivity extractable from urine, using solvents of different polarity and different methods of hydrolysis, are invariably low (90, 97), representing only a fraction of those recovered from adult urine. This is mainly due to a much lower proportion of cortisol metabolites extractable following β-glucuronidase hydrolysis. The results presented here are in general agreement with these reports. Some investigators (91,97), however, obtained recoveries of ^{14}C-cortisol metabolites in the unconjugated fraction substantially greater than those reported here, in spite of the exhaustive ethyl acetate extraction of urine used in the present studies (88). The appearance of significant amounts of cortisol metabolites in the sulfate fraction is in keeping with values reported by Aarskog (97) following solvolysis. The design of the present experiment provides additional information in showing that cortisol sulfate metabolites were consistently extractable to the extent of 59-83%, and this almost exclusively in the sulfate fraction. This finding supports the view that during the neonatal period little hydrolysis of cortisol sulfate takes place in the course of metabolism of this conjugate (142). This is in sharp contrast to previous observations made on adult subjects (135,140) where very appreciable amounts of

^{14}C-cortisol sulfate metabolites appeared in the glucuronide fraction from which were identified 11β-hydroxy- and 11-keto-etiocholanolone.

In the studies with corticosterone and corticosterone sulfate, the amounts of corticosterone sulfate metabolites recovered in the sulfate fraction are very much lower than in the instance of cortisol sulfate, implying extensive metabolism of this conjugate to metabolites which remained unextractable.

The main metabolites of cortisol and corticosterone which were purified from the low amounts of radioactivity extractable in the unconjugated and glucuronide fractions appears to represent an almost oversimplified picture of cortisol metabolism, as documented for adult subjects (99). The present data do not appear to support the view generally entertained, since the recognition of 6β-hydroxycortisol in newborn urine by Ulstrom et al (95), that the formation of this steroid might represent an alternate pathway of cortisol metabolism. Indeed the amount of ^{3}H incorporated into the urinary fraction tentatively identified as 6β-hydroxycortisol was invariably very small. In this context one may recall the observation of Burstein et al (110), who established that following the simultaneous administration of ^{14}C-cortisol and ^{3}H-6β-hydroxycortisol to adult subjects, little 6β-hydroxycortisol is formed during the metabolism

of cortisol, while significant amounts are secreted by the adrenal. If such pathways were to operate during the postnatal period, our finding of a small incorporation of cortisol into 6β-hydroxycortisol would be readily explained.

In the instance of the glucuronide fraction the metabolites of cortisol, namely tetrahydrocortisone, cortolone and β-cortolone, are indicative of a pathway which predominantly involves the formation of metabolites of the 11-keto 5β-pregnane series. The predominance of this pathway is further indicated by the absence of tetrahydrocortisol, a finding which is in keeping with previous observations that during the postnatal period this metabolite is of little, if any significance (91,101). Along the same lines tetrahydro-11-dehydrocorticosterone was by far the main metabolite of corticosterone in the glucuronide fraction. Of particular interest was that fraction of both cortisol and corticosterone metabolites which became extractable in the sulfate fraction. In essentially similar experiments this fraction was also detected in the urine of adult subjects (88 , and unpublished observations). The systematic study of its components was not undertaken. In subjects L-2 and L-7 however, when the sulfate fraction was obtained by sulfatase hydrolysis of the urine, subsequent chromatography led to the detection of materials which contained both isotopes and possessed the polarity of

6β-hydroxycortisol and tetrahydrocortisol, respectively.

Although a systematic analysis of the sulfate fraction was not as yet undertaken the following observations appear to be warranted.

As indicated by the negligible incorporation of ^{3}H into urinary cortisol and corticosterone sulfates, sulfation of the corresponding steroid alcohols did not occur as a primary catabolic reaction. This in itself justified the use of urinary cortisol and corticosterone sulfates as a means of calculating the secretion rates of these conjugates.

The presence of both ^{3}H and ^{14}C in polar materials of the sulfate fraction probably reflect the presence of common metabolites of the steroid alcohol and the corresponding steroid sulfate. Such a pathway could involve the sulfation of metabolites of the steroid alcohol on the one hand, and the metabolism of cortisol sulfate through pathways which do not involve hydrolysis of the ester linkage on the other. The nature of these metabolites remains unknown, but with respect to corticosterone sulfate one may recall that Pasqualini (85) has characterized tetrahydrocorticosterone, 3β, 5α-tetrahydrocorticosterone and 6β-hydroxycorticosterone in the solvolyzed fraction of liver, gastrointestinal tract, placenta and residual foetal tissues following injection of corticosterone sulfate into the intact feto-placental circulation at mid-pregnancy.

In spite of its significance relative to the unconjugated and glucuronide fractions however, the sulfate fraction accounts for only a small additional amount of metabolites of either cortisol or corticosterone or their respective sulfate esters. That portion of radioactivity which is unaccounted for remains the most important.

The secretion rate values for cortisol are within the range of those previously reported for newborn infants (90,91). This is not the case for corticosterone, the values obtained here being comparable to those recently published for a small series of normal subjects following ACTH administration (141). The low cortisol sulfate secretion rate values practically rule out the hypothesis, once entertained from the measurement of this conjugate in the urine of the newborn (86), that its formation could represent an alternate pathway of cortisol biosynthesis and/or metabolism. In fact the secretion rate of cortisol sulfate appears to be closely related to its excretion rate, which is in the order of 300±40 (SE) $\mu g/m^2$/day during the 2nd day of life (86). As opposed to this compared to three observations previously made in adult subjects (88) (corticosterone sulfate secretion rate: 163, 321, and 472 $\mu g/m^2$/day), the secretion rate of this conjugate is much higher in the newborn. It is also much greater than can be accounted for by its excretion rate postnatally (330±55 (SE) $\mu g/m^2$/day) (86). This suggests that this sulfate undergoes extensive

metabolism, some products of which are likely to be found in the polar sulfate fraction. As in the instance of cortisol and corticosterone however, the largest proportion of corticosterone sulfate metabolites remains unaccounted for.

CLAIM OF ORIGINALITY

Data presented in the thesis are original as far as they provide further information or demonstrate for the first time several aspects of the biogenesis and metabolism of steroids of the pregn-4-ene C-21 yl sulfate series.

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